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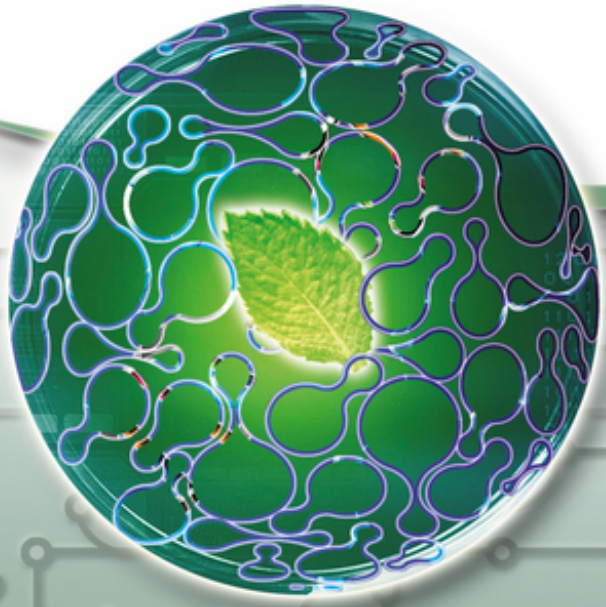

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1
Introduction

Toshiomi Yoshida

1.1
Introduction

The European Federation of Biotechnology proposed a definition of biotechnology as "The integration of natural science and organisms, cells, parts thereof and molecular analogs for products and services." The Concise Oxford English Dictionary states "biotechnology is the exploitation of biological processes for industrial and other purposes especially the genetic manipulation of microorganisms for the production of antibiotics, hormone, and so on" [1].

Biochemical engineering has developed as a branch of chemical engineering, and deals with the design and construction of unit processes that involve biological molecules or organisms. Biochemical engineering is often taught as a supplementary option to students of chemical engineering or biological engineering courses because of the overlap in the curriculum and similarities in problem-solving techniques used in both professions. Its contribution is widely found in the food, feed, pharmaceutical, and biotechnological industries, and in water treatment plants.

Biological engineering or bioengineering is the application of the concepts, principles, and methods of biology to solve real-world problems using engineering methodologies and also its traditional sensitivity to the cost advantage and practicality. In this context, while traditional engineering applies physical and mathematical sciences to analyze, design, and manufacture inanimate tools, structures, and processes, biological engineering primarily utilizes knowledge of molecular biology to study, investigate, and develop applications of living organisms. In summary, biological engineers principally focus on applying engineering principles and the knowledge of molecular biology to study and enhance biological systems for varied applications.

Referring to the above review and brief discussion, it is proposed to have a section titled "Applied Bioengineering" be included in the Wiley Biotechnology Series. This section will deal with recent progress in all subjects closely related to "engineering and technologies" in the field of biotechnology; widening the coverage beyond conventional biochemical engineering and bioprocess engineering

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to include other biology-based engineering disciplines. The topics involved were selected specifically from the perspective of practical applications.

The volume "Applied Bioengineering" comprises five topics: enzyme technology, microbial process engineering, plant cell culture, animal cell culture, and environmental bioengineering. Each topic is figured in several chapters, though with more chapters pertaining to environmental bioengineering. This field has seen an increase in active research as mentioned below because of growing awareness and concern about conservation, remediation, and improvement of the environment.

The later part of this chapter provides a brief overview on the developments in bioengineering, referring to recent highly cited research.

1.2 Enzyme Technology

Recently, several attempts have been made to screen organic-solvent-tolerant enzymes from various microorganisms [2]. The ligninolytic oxidoreductases are being improved utilizing protein engineering by the application of different "omics" technologies. Enzymatic delignification will soon come into practical use in pulp mills [3]. Enzyme stabilization has been attempted using various approaches such as protein engineering, chemical modification, and immobilization [4].

Microbial glucose oxidase has garnered considerable interest because of its wide applications in chemical, pharmaceutical, food, beverage, clinical chemistry, biotechnology, and other industries. Novel applications of glucose oxidase in biosensors have further increased its demand [5]. Numerous oxidative biotransformation studies have demonstrated that enzymes have diverse characteristics and wide range of potential, and established applications [6]. Multienzymatic cascade reactions used in the asymmetric synthesis of chiral alcohols, amines, and amino acids, as well as for C–C bond formation, have been extensively studied [7].

1.3 Microbial Process Engineering

1.3.1 Bioreactor Development

Stirred-tank bioreactors are used in a large variety of bioprocesses because of their high rates of mass and heat transfer and excellent mixing. Theoretical predictions of the volumetric mass transfer coefficient have been recently proposed, and different criteria for bioreactor scale-up have been reported [8].

Miniaturized bioreactor (MBR) systems have made great advances both in function and in performance. The dissolved oxygen transfer performance of submilliliter microbioreactors and 1–10 ml mini-bioreactors has been well examined. MBRs have achieved considerably high $k_L a$ values and offer flexible instrumentation and functionality comparable to that of production systems at high-throughput screening volumes; furthermore, the superior integration of these bioreactors with automated fluid handling systems demonstrates that they allow efficient scale-up [9].

The pharmaceutical and biotechnology industries face constant pressure to reduce development costs and accelerate process development. A small scale bioreactor system enabling multiple reactions in parallel ($n \geq 20$) with automated sampling would provide significant improvement in development timelines. State-of-the-art equipment that facilitates high-throughput process developments includes shake flasks, microfluidic reactors, microtiter plates, and small-scale stirred reactors [10].

An expert panel organized by the M^3C Working Group of the European Section of Biochemical Engineering Science (ESBES) reviewed the prevailing methods of monitoring of MBRs and identified the need for further development [11]. Their recommendations includes combining online analytics such as chromatography or mass spectrometry with bioreactors, preferably using noninvasive sensors such as optical or electronic ones. The sensors to be used online in these bioreactors should be selected on the basis of three criteria: (i) detection limits in relation to analytes, (ii) stability in relation to the testing period, and (iii) the possibility for miniaturization to the volume ranges and dimensions of the microfluidic system applied in the bioreactors. In addition, mathematical models based on soft sensor principles should be exploited to reduce the number of sensors.

1.3.2 Measurement and Monitoring

Biosensors for detection of cellobiose, lactose, and glucose based on various cellobiose dehydrogenases from different fungal producers, which differ with respect to their substrate specificity, optimum pH, electron transfer efficiency, and surface-binding affinity; therefore, promising a wide range of new applications [12].

Infrared sensors are ideal tools for bioprocess monitoring, because they are noninvasive, of no-time-delay, and harmless on the bioprocess itself, and furthermore, simultaneous analyses of several components are possible. Therefore, directly monitoring of substrates, products, metabolites, and the biomass itself is possible [13]. The panel of the M^3C Working Group of ESBES recommended the use of soft sensors in bioprocess engineering [14]. In the Food and Drug Administration's (FDA) proposed and promoted process analytical technology (PAT) initiative, intending to collaborate with industry to promote the integration of new manufacturing technologies with pharmaceutical production [15]. The

program aimed to design, develop, and operate processes consistently ensuring a predefined quality at the end of the manufacturing process [15]. An advanced monitoring and control system has been developed, based on different inline, online and at-line measurements for substrates and products. Observation of cell viability by inline measurement of radio frequency impedance and online determination of intracellular recombinant target protein using the reporter protein T-sapphire green fluorescent protein (GFP) could allow real-time monitoring of critical process states [16].

1.3.3 Modeling and Control

Stoichiometric models of cell metabolism have been developed with the use of information about reaction stoichiometry embedded in metabolic networks and the assumption of a pseudo-steady state. Stoichiometric models have been used to estimate the metabolic flux distribution under given circumstances in the cell at some given moment (metabolic flux analysis) and to predict it on the basis of some optimality hypothesis (flux balance analysis). Mechanistic models based on deterministic principles, recently, have been interested in substantially. Gernaey *et al*. [17] highlighted the utility of models with respect to the selection of variables required for the measurement, control, and process design. In the near future, mechanistic models will play key roles in the development of next-generation fermentation, especially in the frame of multiobjective decision making. One of the key issues in the process engineering of microbial production processes is the control of culture conditions to maximize production. In a repeated batch or fed-batch fermentation, optimizing the trajectory to maximize productivity and yield is desired. For example, temperature profiles for a temperature induction system, based on optimal control theory [18] or on past industrial experience, should be monitored, and then a model predictive control (MPC) system should be designed. Among the methodologies and practical application of bioprocess controls, the online optimized control for continuous culture, cascade control for mixed cultures, and supervision and fault detection have been developed [19].

1.3.4 Solid-State Fermentation

Solid-state fermentation (SSF), which has long been used in fermented foods production, ethanol fermentation, fungi cultivation, etc, is currently considered superior to submerged fermentation for use in modern bioprocessing because of the recent improvements in the design. Mathematical models based on mechanistic equations give insight into how microscale processes like the interaction of growth with intraparticle diffusion of enzymes, hydrolysis products, and oxygen can potentially limit the overall performance of a bioreactor [20]. Bioremediation,

bioleaching, biopulping, and so on, are the major applications of SSF in new bioprocesses. Utilization of agroindustrial residues as substrates in SSF processes open a way for efficient use of under- or unutilized residues. In future, SSF technology will steadily develop if rationalization and standardization continue as per the current trend [21].

1.4 Plant Cell Culture

Switchgrass is a promising natural feedstock for the production of biofuels and other value-added materials from biomass due to its high productivity, low requirements for agricultural inputs, and positive environmental impacts. Pretreatment of switchgrass is required to improve the yields of fermentable sugars. Depending on the type of pretreatment, glucose yields range from 70% to 90% and xylose yields from 70% to 100% after hydrolysis. Following pretreatment and hydrolysis, ethanol yields range from 72% to 92% of the theoretical maximum [22].

Plant cell factories constitute an alternative source of high-value-added phytochemicals such as the anticancer drug taxol (generic name paclitaxel), biosynthesized in *Taxus* spp. The production of taxol and related taxanes in *Taxus baccata*, (European yew), was investigated using cell suspension culture to develop mass production technology [23]. Antioxidants are an important group of preventive medicinal compounds as well as food additives that prevent the loss of easily oxidizable nutrients. The efficiency of *in vitro* production of antioxidants has been improved by media optimization, biotransformation, elicitation, *Agrobacterium* transformation, and scale-up [24].

1.5 Animal Cell Culture

The gel-like endothelial glycocalyx layer (EGL) that coats the luminal surface of blood vessels has garnered great interest recently. Among its, interesting functions, EGL modulate oncotic forces that regulate the exchange of water in microvessels [25].

Stem cells have emerged as the starting material of choice for bioprocesses to produce cells and tissues to treat degenerative, genetic, and immunological diseases. Fundamentals of bioprocess engineering, including bioreactor design and process control, need to be combined with principles of cellular systems biology to guide the development of next-generation technologies capable of producing cell-based products in a safe, robust, and cost-effective manner [26].

The insect cell baculovirus expression vector system (IC-BEVS) has been shown to be a powerful and convenient system for rapid and easy production of a virus-like protein. A rotavirus-like particle was produced by culturing the IC-BEVS using bioprocess engineering devices [27].

1.6 Environmental Bioengineering

Adsorption techniques are widely used to remove certain classes of pollutants from waters, especially those that are not easily biodegradable (e.g., biosorption of Cr(III) and Cr(VI) onto the cell surface of *Pseudomonas aeruginosa* [28], removal of Gryfalan Black RL metal complex dye by fungi [29], and methylene blue remediation by use of agricultural waste [30]). Cyanide removal using biological methods is more cost-effective than that using chemical and physical methods. Several microbial species can effectively degrade cyanide into less toxic products. Biological treatment of cyanide is possible under anaerobic and aerobic conditions [31].

A combined solar photo-Fenton and biological treatment was proposed for the decontamination of surface waters contaminated with pesticides and pharmaceutical wastewater [32, 33]. Currently there are global efforts towards development of water reuse technologies. Advanced oxidation processes (AOPs) with other bioremediation technologies has been developed for the removal of organic pollutants with high chemical stability and/or low biodegradability. Special emphasis is also placed on large-scale combination schemes developed in Mediterranean countries for treatment and reuse of nonbiodegradable wastewater [34]. "Produced water" is the largest waste stream generated in the oil and gas industries. The effect of discharging produced water in the environment has become a serious issue of environmental concern. Major research efforts in the future could focus on optimizing current technologies and combining physicochemical and/or biological methods of treatment [31]. Sewage contains various organic compounds, which should be recycled. The approach involves concentration of municipal effluents on its arrival at the water treatment plant, followed by anaerobic digestion of organics and maximum reuse of its mineral contents as nutrients. Because of the increasing economic and ecological value of the recovered nutrients, this new conceptual design for the treatment of "used water" will become a reality in the next decade [35]. The feasibility of using biological hydrolysis and the acidification for the treatment of different types of municipal sludge, from six major treatment plants located in Denmark, was investigated by batch and semicontinuous experiments. The results showed that fermentation of primary sludge produced greater amount of volatile fatty acids (VFAs) and generated significantly higher yield of COD- and VFAs than fermentation of other sludge types [36].

Bioaugmentation-assisted phytoextraction is a promising method for decontaminating soil containing metals. The system is composed of bacteria mainly plant-growth-promoting rhizobacteria, and fungi, mainly arbuscular mycorrhizal fungi, associated with hyperaccumulating or non-hyperaccumulating plants. This association was analyzed using a bioprocess engineering approach, and, in general, bioaugmentation increased metal accumulation by shoots [37].

1.7
Composition of the Volume

Chapters in the subsequent part of the volume of "Applied Bioengineering" provide reviews on the selected topics, outlining the progress of current researches supplemented with unique perspectives and meaningful discussions. The volume consists of five sections after the introduction. The first section, on enzyme technology, presents an overview of the history and current trends, followed by detailed discussion on the topics: molecular engineering of enzymes, development of biocatalytic processes, and development of enzymatic reactions in miniaturized reactors. The second section, microbial process engineering, presents an overviews on bioreactor development and process analytical technology, omics-integrated approach for metabolic state analysis of microbial processes, and control of microbial processes. The third section, on plant culture and engineering, comprises three articles: one on contained molecular farming using plant cell and tissue cultures, one on bioprocess engineering of plant cell culture, and one on the role of bacteria in phytoremediation. The fourth section, on animal cell culture, contains three articles one on cell line development for biomanufacturing processes; medium design, culture management and the PAT initiative; and advanced bioprocess engineering: fed-batch and perfusion processes. Finally, the fifth section, on environmental bioengineering, contains five articles: treatment of industrial and municipal wastewater, treatment of solid waste, energy recovery from organic waste, microbial removal and recovery of metal resources from wastewater, and sustainable use of phosphorus through bio-based recycling.

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Part I
Enzyme Technology

Applied Bioengineering: Innovations and Future Directions, First Edition. Edited by Toshiomi Yoshida.

2
Enzyme Technology: History and Current Trends

Klaus Buchholz and Uwe T. Bornscheuer

2.1
The Early Period up to 1890

2.1.1
Observations and Empirical Results

Applied biocatalysis has its roots in ancient Mesopotamia, China, and Japan, in the manufacture of food and alcoholic drinks. Thus, about 3500 BC, brewers in Mesopotamia manufactured beer following established recipes. Without knowing, humans utilized microbial amylases and proteases, in particular for the production of soy-derived foods. In Europe too, applied biocatalysis has a long history. Cheese making has always involved the use of enzymes. Much material on the scientific and technological development has been summarized in articles by Neidleman [1], Sumner and Myrbäck [2], Sumner and Somers [3], Tauber [4], and Buchholz and Poulson [5]. The history of enzyme technology is, of course, an essential part of the history of biotechnology [6–9].

First scientific observations on enzyme activities in the late eighteenth century were by Spallanzani and Scheele (Table 2.1). In 1814, Kirchhoff found that a glutinous component of wheat is capable of converting starch into sugar and dextrin. Then, in 1833, diastase was found to perform this hydrolysis [10]. Its isolation is described, following Payen and Persoz, by Knapp [13]: it is precipitated from a malt extract and can be further purified by repeated dissolution in water and precipitation by the addition of alcohol. Diastase was the dominating object of research throughout the century, with ∼10–20% of the publications dealing with it during decades, because of its economic importance (see below).

Claude Bernard was the first to show lipolytic activity in pancreas in 1856 [4], and Dobell [12] found that an extract from pancreas hydrolyzed both fat and starch. Further enzymatic conversions (ferment actions) observed in that early period, summarized in Frankland's list of soluble ferments (1885) and by Sumner and Somers [3], are presented in Table 2.1.

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Table 2.1 Ferments (enzyme activities) known until 1880.

Enzyme	Ferment source	Catalyzed reaction	References
Protease	Gastric juice	Meat liquefaction	Spallanzani (1783)[a]
Tannase	Gall nut	Hydrolysis of tannin	Scheele (1786)[b]
Cyanogen	Plant roots	Substrate: guaiacum	Planche (1810)[a]
Glutin comp.	Wheat	Starch hydrolysis	Kirchhoff (1814)[a]
Emulsin	Bitter almonds	Amygdalin hydrolysis	Robiquet and Boutron (1830)[a]; Chalard, Liebig and Wöhler (1837)[a]
Diastatic activity	Ptyalin	Starch hydrolysis	Leuchs (1831)[a]
Amylase	Malt	Starch hydrolysis	Payen and Persoz [10]
Sinigrinase			Faure (1835)[a]
Pepsin		Protein hydrolysis	Schwann (1836)[a]
Trypsin		Protein hydrolysis	Corvisart (1856)[a]
Saccharase	Yeast	Sucrose hydrolysis	Berthelot (1860)[a]
Pectase	Plants	Pectin hydrolysis	Payen [11]
Pancreas		Lipolytic activity	Bernard (1856)[b]
Pancreas extract		Fatand starch hydrolysis	Dobell [12]
Pancreatic ferment	Pancreas	Fat hydrolysis	Frankland (1885)

a) From: Sumner and Somers [3].
b) From: Tauber [4].

Technical application of diastase was a major issue in the 1840s and onwards. The treatment of starch by acids or diastase yielded a gummy syrup, essentially dextrins. Notably, French products seemed to have been produced enzymatically, as was obvious by their smell of malt [13]. Sugar-containing dextrin was produced following a process of Payen and Heuzé, by treating 100 parts of starch with 5–8 parts of malt in water at 60–70 °C (Figure 2.1) [11, 14]. This product was used mainly in France in bakeries, where it served as an additive in baking, and for the production of beer, wines, and alcoholic beverages from fruits, all applications with a big market. Lab products were used to produce cheese [13]. Berzelius is cited with details stating that 1 part of lab ferment preparation coagulates 1800 parts of milk.

A remarkable process was developed by Schützenbach in 1823: an acetic acid fermentation process known as "fast acetic acid manufacture" ("Schnellessigfabrikation"). It worked with active acetic acid bacteria (of course, not recognized at that time) immobilized spontaneously on beechwood chips. The process was carried out in vessels made of wood, some 1–2 m wide and 2–4 m high, and was aerated to oxidize the substrate alcohol (Figure 2.2).

2.1.2 Theoretical Approaches

A *theoretical concept* that is valid even today was developed by Berzelius as early as 1835, who acknowledged the first enzymatic actions, notably starch hydrolysis

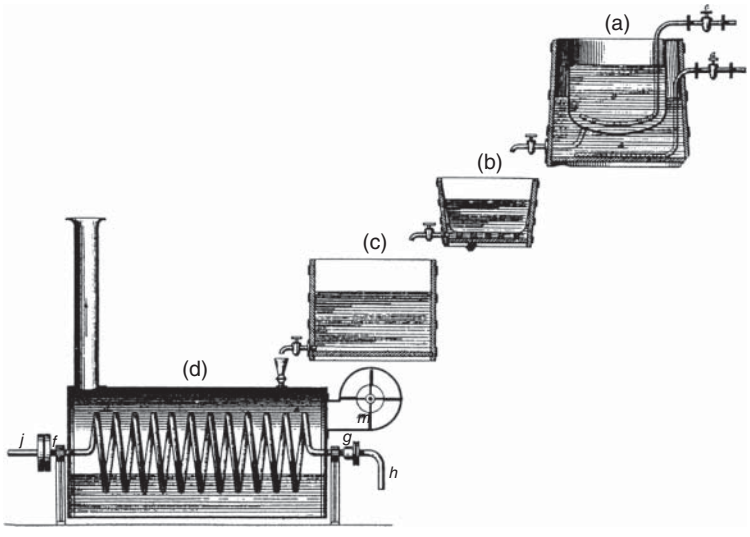


Figure 2.1 Process for dextrin production, with reaction vessel for starch hydrolysis (a), filtration unit (b), reservoir for intermediate storage (c), concentration unit where water is evaporated to give a concentrated syrup of dextrin solution (d) [11].

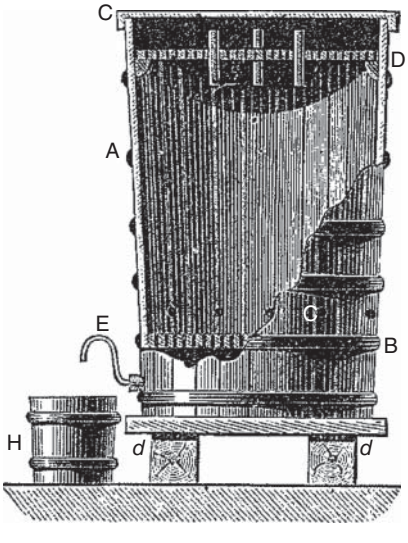


Figure 2.2 Acetic acid fermentation using immobilized bacteria. The vessel was equipped with sieve plates in positions D and B. Space A was filled with beech wood chips (on which the bacteria were immobilized). A 6–10% alcohol solution was added from the top to a solution containing 20% acetic acid and beer (containing nutrients). Air for oxidation was introduced through holes in a position above B, and the temperature was maintained at 20–25 °C. The product containing 4–10% acetic acid was continuously removed via position E [15].

by diastase, as a catalytic effect. He interpreted also fermentation as caused by a catalytic force. He postulated that a body by its mere presence could, by affinity to the fermentable substance, cause its rearrangement to the products. About a decade later, the book by Knapp [13] presented, when summarizing approaches published by several authors, a rather distinct and even mysterious concept of the action of ferments (mainly referring to diastase and the work by Payen and Persoz)

speaking of "mighty chemical forces" and the ability, that one part (of the ferment) can transform 2000 parts of starch into sugar, and that "even if one does not know anything of the chemical composition," one might assume a "hypothetical body," a "symbol," and the most strange energy, by which the transformation (of starch) occurs; he assumed that the nitrogen-containing parts (the "Kleber") of the seed are being transformed to a ferment, and that diastase is not a certain substance, but rather a state or form ("Zustand").

Again, a decade later the distinction of *organized* and *unorganized* ferments was developed. Wagner [14] describes two types of ferments that can cause fermentation; one is an organized (obviously living) body, like yeast, and the other a protein-like body, which is in the state of decomposition (a term coined by Liebig; see [8], pp. 20, 21). That segmentation is expressed more precisely by Payen [11]. Fermentation is seen as a contact (catalytic) process of a degradation ("Spaltungs-") or addition process (with water). It can be performed by two substances or bodies:

- A nitrogen-containing organic (*unorganized*) substance, such as protein material undergoing degradation.
- An *organized* body, a lower class plant, or an *infusorium*, such as with alcoholic fermentation.

Probably, the type of effect is the same insofar as the ferment of the second class produces a body of the first class, possibly a big number of singular ferments. In 1877, Kühne termed the first class of substances *enzymes* (in yeast) [16].

2.2 The Period from 1890 to 1940

2.2.1 Scientific Progress

From about 1894 onwards, Emil Fischer investigated in a series of experiments the action of different enzymes using several glycosides and oligosaccharides; the results revealed specificity as one of the key characteristics of enzymes. In 1894, he compared invertin and emulsin. He extracted invertin from yeast, a usual procedure, and showed that it hydrolyzed α-, but not β-methyl-D-glucoside. In contrast, emulsin, a commercial preparation from Merck, hydrolyzed β-, but not α-methyl-D-glucoside [17]. Among a series of tests with different saccharides, he observed that invertin hydrolyzed sucrose and maltose, but not lactose. An extract from a "lactose-yeast," however, was able to hydrolyze lactose but not maltose. These observations are the most essential from a major range of which Fischer derived his famous theory on specificity (see below) (summarized by Fischer [18]).

A few years after Fischer's investigations, Eduard Buchner published a series of papers (Refs [19, 20]; see also review by Florkin [21]), which signaled a breakthrough in fermentation and enzymology. The experiments began in 1893.

In his first paper on alcoholic fermentation without yeast cells (1897), Buchner described, in a remarkably short and precise manner, the separation of the (alcoholic) fermentation from the living yeast cells by an extract. He published the experimental details for the preparation of a cell-free press juice from yeast cells, with disruption, filtration under high pressure, and further filtration. He then described the formation of carbon dioxide and alcohol from carbohydrates, sucrose, glucose, fructose, and maltose. No microscopic organisms were present. At elevated temperature, protein was precipitated, the activity reduced, and finally destroyed. From these and further results, Buchner derived essential new insights, both into the nature of alcoholic fermentation and enzymatic activity governing the transformations observed (see Section 2.2.2). In subsequent papers, he communicated further important experiments [22], which also led to immediate objections of other scientists active in the field (see e.g., [23]). These findings (with many more details published by Buchner *et al*.) deserve special attention since they represent the demarcation of a breakthrough, which reduced all reactions in physiological (or bio-) chemistry to chemistry.

Further findings relevant for the establishment of the chemical nature of enzymatic catalysis and technical application followed shortly thereafter. Croft and Hill performed the first enzymatic synthesis, that of isomaltose, in 1898, allowing a yeast extract (α-glycosidase) to act on a 40% glucose solution [3]. In 1900, Kastle and Loevenhart found that the hydrolysis of fat and other esters by lipases is a reversible reaction and that enzymatic synthesis can occur in a dilute mixture of alcohol and acid [2]. This principle was utilized for the synthesis of numerous glycosides by Fischer and coworkers in 1902.

Bertrand, in 1897, observed that certain enzymes required dialyzable substances to exert catalytic activity. He named these substances "coenzymes." Sörensen pointed out the dependence of enzyme activity on pH in 1909 [3]. An important step entering physico-chemistry, and thereby extending the theoretical basis of enzymology, was the kinetic investigations and their interpretation by Michaelis and Menten. They postulated that enzymatic action is due to the formation of an intermediate compound between the enzyme and the substrate, and they presented a mathematical form that is still used today [2].

The definite establishment of the chemical paradigm was the crystallization of urease by Sumner in 1926, and further enzymes (trypsin, etc.) by Northrup and Kunitz in 1930/1931. In every known case, the pure enzyme turned out to be a protein [2].

2.2.2
Theoretical Developments

Fischer [18] in his work elaborated the essential aspects of enzyme catalysis during the 1890s. The first aspect is specificity. The agents of the living cell (enzymes) are optically active, and therefore one might assume that the yeast cells with their asymmetric agents can utilize only those sugars whose geometry is not too far from that of glucose. From there, Fischer deduced the famous

picture of lock and key; he assumed that the "geometrical form of the (enzyme) molecule concerning its asymmetry, corresponds to that of the natural hexoses" (sugars).

The second aspect refers to the protein nature of enzymes. In 1894, Fischer [18] stated that among the agents that serve the living cell, the proteins are the most important. He was convinced that enzymes are proteins. Controversies on the nature of enzymes and proteins continued for long [5]. Proteins, such as albumin and casein, were included in the group of colloids, which were attested a dynamic state of matter. "…The colloid possesses ENERGIA, …the probably primary source of the force … of vitality." Protoplasm was given mystical and even magical properties." To the contrary, Béchamp, also referring to his former work in 1853–1857, had demonstrated that soluble ferments are a basic or original principle (*pure principei immediat*) [24]. But Willstätter, still in 1927, denied that enzymes were proteins [25].

Buchner initiated a new paradigm, which, in strict contrast to that of Pasteur, stated that enzyme catalysis, including complex phenomena like that of alcoholic fermentation, was a chemical process not necessarily linked to the presence and action of living cells. In his first paper, he wrote that he presented the proof that (alcoholic) fermentation does not require the presence of "such a complex apparatus as is the yeast cell." The agent is a soluble substance, without doubt a protein body, which he called *zymase* [19]. Buchner's findings marked a new – biochemical – paradigm leading research and theory on enzymes. It displaced an established paradigm which taught that processes in living organisms – alcoholic fermentation being the most important example – were not of pure chemical nature but required a *vis vitalis*, a vital force. Now, the chemical paradigm, which reduced all reactions in physiology (or bio-)chemistry to the laws of chemistry without further hidden forces, began to play the dominant role. Technical development also got a new scientific basis on which to proceed in a rational way.

2.2.3 Technological Developments

The industrial development of enzymes was very slow initially during the late nineteenth century. An exception was the work of Christian Hansen, who started a company in Copenhagen, Denmark, in 1874, the Chr. Hansen A/S to this day, the first in the industrial market with a standardized enzyme preparation, rennet, for cheese making. Takamine began isolating bacterial amylases in the 1890s, based on "koji" mold cultures. In 1894, Takamine applied for, and was granted, a patent entitled "Process of making diastatic enzyme" (U.S. Patent No. 525,823) on his method of growing mold on bran and using aqueous alcohol to extract amylase, the first patent on a microbial enzyme in the United States, for example, for saccharifying starch for application in distilleries. Takamine licensed his enzyme preparation under the brand name "Taka-diastase." He subsequently founded the International Ferment Company of New York, which later was sold

to Miles Laboratories. Early applications and patents on enzymes in the food industry have been collected by Neidleman [1]. Together with Effront, working on enzymes for alcohol production since 1900, Boidin founded the SociétéRapidase (later a part of DSM-Gist-Brocades) in 1920 ([26, p. 6]).

Plant lipases were utilized for the production of fatty acids from oils and fats. It was also found that the reaction is reversible, and an enzymatic synthesis of fat from glycerol and fatty acid was described by Welter in 1911 [27]. For the chill-proofing of beer, proteolytic enzymes have been used successfully since 1911 in the United States [4]. Lintner, as early as 1890, observed that wheat diastase interacts in dough-making. This effect was extensively studied, the addition of malt extract came into practice, and American bakers in 1922 used 30 million lb (13 500 tons) of malt extract valued at US$2.5 million [4].

In 1907, Röhm patented the application of a mixture of pancreatic extract and ammonium salts as a bating agent [4]. His motivation as a chemist was to find an alternative to the unpleasant bating practice using dung. First experiments with aqueous ammonia were a failure. Since he knew Buchner's work on enzymes, he came to assume that enzymes could be the active component in dung, and looked for sources that were technically feasible. Tests with pancreas were successful, when he compared the results with those obtained with dung, whereas amylase did not work. With this perspective, he founded his company in 1907, which successfully entered the market; also a company in the United States, which later became the Rohm and Haas company, was founded. In 1908, 10 tons of the product – pancreas extract – with the trade name Oropon were sold, followed by 53 and 150 tons in the subsequent years. In 1913, the company worked with 22 chemists, 30 other employees, and 48 workers [28]. Later enzyme preparations were produced by fermentation using *Bacillus* or *Aspergillus* sp. [4].The history of the Röhm company makes obvious that the market for a new product providing technical progress was an important factor, but that the background of scientific knowledge on the principles of enzyme action was equally important, leading experiments to a technically feasible solution. It was, however, only around 1955 that the development of enzyme production gained speed by the growing sales of bacterial amylases and proteases.

2.3
A New Biocatalyst Concept – Immobilized Enzymes

2.3.1
Fundamental Research

One question – which is as old as industrial enzymes – was: "Can enzymes be re-used?" Immobilization represents a key for the economic application of many enzymes. It offers all advantages of classical heterogeneous catalysis. The most obvious reason is the need to reuse enzymes in order to make their application in industrial processes economical. Solutions critically depend on knowledge of

the protein structure. Further aspects are convenient separation for reuse after the reaction by filtration, centrifugation, and so on; and application in continuous processes, in fixed-bed, or fluidized-bed and stirred-tank reactors provided with a filter system for retention. Continuous processes combine the generally simpler technical equipment with the potential for convenient process control, automation, and coordination with upstream and downstream processing, including product recovery and purification.

Around 1950, several groups started to immobilize enzymes on solid supports. After the early work by Micheel and Ewers [29] and Grubhofer and Schleith [30], Manecke [31] recognized the potential of the method for industrial application, and applied for a patent that was granted on his method; however, he could not convince industry, notably, and ironically, the company Bayer, of the importance of his invention. Subsequently, work on enzyme immobilization became an important topic in research, reflected by international conferences, notably the Enzyme Engineering Conferences by the Engineering Foundation, New York [32]; however, the consideration of practical aspects was not yet established, and research results were mostly not appropriate for application in technical processes – such as low active enzyme yield and stability, high costs, poor mechanical stability, poor sedimentation and filtration properties, low yields of product, and so on. This is reflected in a major research project organized by DECHEMA (Frankfurt), with a number of academic research groups as well as scientists of companies participating, which aimed at solutions to aspects of application. The results were published [33, 34], as well as monographs collecting most of the relevant research results both by academic and industrial research (e.g., [35]). Among the pioneer groups providing insight into fundamental aspects and phenomena, including mass transfer and efficiency, was that of Katchalski (see e.g., [36]).

The idea and concept of immobilizing proteins, and notably enzymes, was born in academia, but the breakthrough, however, came with industrial development and application. Thus the first industrial application of immobilized enzymes was in the production of amino acids. Most important, however, until today, are two processes, the hydrolysis of penicillin and the isomerization of glucose [37, Chapter 8].

2.3.2 Examples of Industrial Development: The Case of Penicillin Amidase (PA) – Penicillin Hydrolysis and Derivatives

Enzymatic penicillin hydrolysis by penicillin amidase (also named penicillin acylase, PA) represents a landmark. After Chibata's first modest scale process (amino acid synthesis) [38], it was the first industrially and economically most important process using immobilized biocatalysts.

One priority item in pharmaceutical research was the development of a process for 6-aminopenicillanic acid (6-APA) production, serving for the synthesis

of penicillin derivatives escaping resistance (Scheme 2.1).[1] Early in the 1950s, an enzyme had been found that elegantly did the job; it could accomplish the hydrolysis of penicillin in one step without requiring chemicals (other than water and buffer). PAs from different organisms, for example, *Escherichia coli*, were found at different institutions during the mid-1950s [39], including Bayer AG, Germany [40], and early processes were applied in Germany and the United States, however with poor yields [37(pp. 523–531), 41, 42]. However, no solution was obvious to accomplish the reaction on a technical scale, to produce the enzyme, to stabilize it, and to apply it several times, or continuously, and an engineering concept for a process to achieve high yields of 6-APA required for an economic process.

In the 1950s, the development of enzyme-catalyzed processes started in two companies; first, at Beecham Pharma (UK), which had missed out on penicillin but aimed at the development of new penicillin derivatives; and also at Hoechst AG (Germany), a producer of penicillin, who, however, discontinued research on PA and sold the know-how to Bayer (Germany). Screening for PA-producing strains had begun around 1960 at Beecham and Bayer; strain development was performed by conventional mutation of strains and subsequent screening; they applied for patents in 1959, and published their work on the hydrolysis of penicillin G using PA from *E. coli* in 1960 [40, 43].

At that time, all the main producers of penicillin had started to develop enzymatic methods to produce 6-APA from penicillin G and V, using PA from microorganisms other than *E. coli* [37(pp. 523–531), 41, 44]. The enzyme however was very expensive, and thus initially not an economical solution; immobilization was expected to solve the problem.

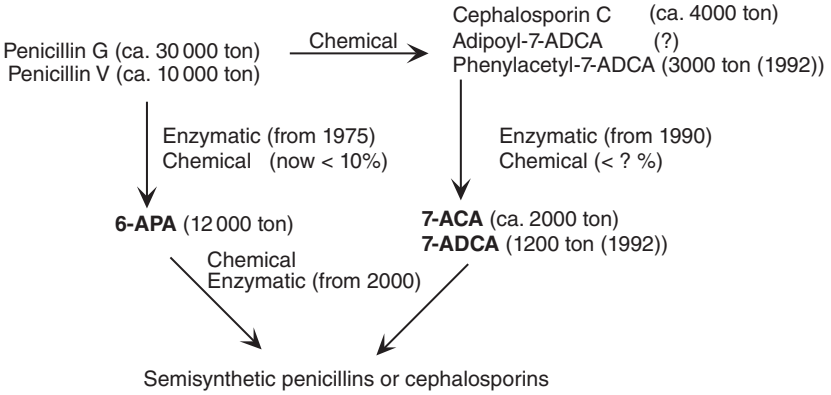
Among the first groups to develop PA immobilization processes for industrial application by the end of the 1960s and early 1970s were those of G. Schmidt-Kastner, at Bayer AG (Germany), and of M. D. Lilly, University College, London, in close cooperation with Beecham Pharmaceuticals (UK). 6-APA, up to that time, was produced chemically in a tedious process and used to synthesize different semisynthetic penicillins of high added value, such as ampicillin. The straight enzymatic process promised to be much easier and environmentally favorable (as compared to the chemical manufacture).

Here, one example of this development, at Bayer, will be presented in more detail, since it illustrates the achievement of solutions to problems of industrial application of such a process, notably development of an immobilization method for PA suitable for scale-up. This achievement includes the work required to solve

1 Resistance of pathogens to antibiotics, notably to penicillin, had become a major topic in pharmaceutical research and development, thus the search for derivatives escaping resistance was a priority item. The development of penicillin derivatives, such as amoxicillin – by laborious and complicated chemical steps – enabled medical treatment of resistant strains and a wider range of pathogenic microorganisms. These chemical methods, besides their complexity, required large amounts of toxic and expensive reagents, including solvents to be regenerated, notably for the first step, penicillin hydrolysis to give 6-APA. This step furthermore was cost intensive. Nevertheless, the decision at Bayer for the industrial production of 6-APA was in favor of a chemical process, not the enzyme process, probably due to high cost of the enzyme.

a series of bottlenecks and technical problems of large-scale application, as well as problems of economy. Günter Schmidt-Kastner decided, as head of the biochemical engineering department (Biotechnikum), to develop the enzyme process in preference to the established chemical process. He decided to devote the personal and technical capacities (a new biotechnical pilot plant) required for this task. His decision to develop an enzymatic route implied struggling against the "chemical hierarchy" – the management that relied on chemical synthesis as a superior tool for many decades (Schmidt-Kastner, personal communication). His motives might have been success and lead in experience and R&D performance of the new field. Schmidt-Kastner knew about the scientific work of the Katchalski group, with whom he started a collaboration; he knew Manecke, who had offered his patent on immobilization to Bayer earlier, personally, but not his work and publications (Schmidt-Kastner, personal communication).

The most relevant aspects for the industrial success of the process were (according to Schmidt-Kastner) a PA-producing strain with high efficiency and productivity obtained by classical mutation and screening methods, and an interdisciplinary group including biochemists, microbiologists, polymer chemists, and chemical engineers to establish the know-how in order to solve the problems arising from the previous state of the art. A support (a functional copolymer) had to be designed specifically for covalent binding of proteins, with appropriate properties providing for high binding yields of active enzyme, functional groups, avoiding aggressive reactions but with sufficiently high density and mechanical stability, high internal surface, particle density, and hydrophilic character.



R	*R*-6-APA	*R*-7-ADCA	*R*-7-ACA
(*R*)-Phenylglycyl	Ampicillin	Cephalexin	Cephaloglycin
(*R*)-Hydroxy-phenylglycyl	Amoxicillin	Cephadroxil	

Scheme 2.1 Enzymatic and chemical production of semisynthetic penicillins and cephalosporins from the hydrolysis products (6-APA, 7-ACA, 7-ADCA) of β-lactam antibiotics. The by-products phenylacetate and adipate can be recycled in the fermentations. The amounts produced are estimated from literature data [45].

Patents by Bayer (which filed some nine patents in 1972 and 1973 on immobilized PA) refer to known literature highlighting the shortcomings – common in academic development [46, 47]. Catalyst design is given in detail, including excellent binding yields (up to 92% of active enzyme), with high specific activities, high stability (producing more than 30 batches), and yield of reaction product (91% of PA) – much better than the data from the literature at that time. The immobilized enzyme system allowed scale-up over several dimensions, up to industrial reactor scale (several cubic meters), exhibiting low abrasion in such stirred-tank reactors.

The immobilization procedure is given in the patents, along with the ratio of protein and the polymer carrier, temperature, pH, buffer, ionic strength, and reaction time [47]. However, several specific results were kept secret, such as the distribution of the enzyme inside the carrier, providing for enhanced efficiency of the biocatalyst (compare for parallel developments published by the group of Lilly and Buchholz [48, 49]). The results reflect the performance required in a successful industrial process. Most importantly, the costs for the biocatalyst finally were marginal compared to the overall cost of the process (Scheme 2.1).

Production of 6-APA by PA began in 1972 in the Biotechnikum (pilot plant) of Bayer, in a scale of 1000 l up to 3000 l. Production was transferred subsequently to the plant with production in three reactors, in the 10 m^3 scale, with several batches per day of 6-APA. The product was also manufactured for another antibiotics producer (Schmidt-Kastner, personal communication).

The development represents, even today, high-grade state of the art. The second important development was by Lilly and his group (at the University College London), working with PA from *E. coli* and developing an immobilized biocatalyst as well as process technology (see e.g., [49]), in close cooperation with Beecham; process scale-up was undertaken in the late 1970s.[2] Today, the production of 6-APA is over 10 000 tpa (tons per annum) and that of 7-ACA some 4000 tpa, and semisynthetic penicillins and cephalosporins represent a market value of over US$20 billion per annum [37, p. 525].[3]

2.3.3 Examples of Industrial Development: The Case of Sugar Isomerization

The largest process with immobilized enzymes with respect to volume is that with glucose isomerase. The first commercial enzymatic production of high-fructose corn syrups (HFCS, with 53% glucose, 42% fructose, by isomerization of a

2 Development with immobilized PA comprised stirred-tank cascades, but application was restricted to batch stirred tanks. Many graduates (including Carleysmith, who contributed to catalyst design) went to Beecham to continue work on the industrial process. The group of Lilly represented, unconventional in academia at the time, an integrated group (biochemistry, biochemical engineering, including downstream processing) in the early 1970s. The idea was possibly stimulated by an MIT group (Cooney, Cambridge, MA, USA) (Fish, personal communication).

3 The development of the PA process also shows how much industrial interest has stimulated and encouraged fundamental work, including recombinant technology, as is obvious from an increasing number of papers (over 600) published subsequently on the subject. Remarkably, work on recombinant PA did not meet the interest of the first companies active in the field (see Section 2.5).

glucose syrup, with enhanced sweetness) took place in Japan in 1969 (Takasaki), however with limited success. The sky-rocking sucrose price in 1973–1975 contributed to the interest in HFCS, and consequently immobilized glucose isomerase, dramatically. Companies like Novo Industri (now Novozymes, AS, DK) and Gist-Brocades (NL; now DSM) developed more stable enzyme products, which were cheaper and easy to use. Resources were spent on optimizing the production of glucose isomerase, its immobilization, and the engineering of the processes [50]. As a result, productivities of the commercial process increased from ~500 kg HFCS kg^{-1} immobilized enzyme product (1975) to ~20 000 kg kg^{-1} (2003). Currently about 12 million tpa of HFCS is produced worldwide [37, Section 8.4].

2.4 Expanding Enzyme Application after the 1950s

During the late 1960s, with the development of detergent proteases, the use of enzymes increased dramatically. At the same time, an enzyme process to produce dextrose using glucoamylase was introduced for starch processing.

Non-immobilized (soluble) enzymes continue to dominate technical applications in economic terms. The areas are

- food manufacturing, with starch hydrolysis (Figure 2.3), and further processing (over 15 million tpa), bread, cheese, fruit juice manufacture, and so on (estimated at 40–45% of enzyme sales)

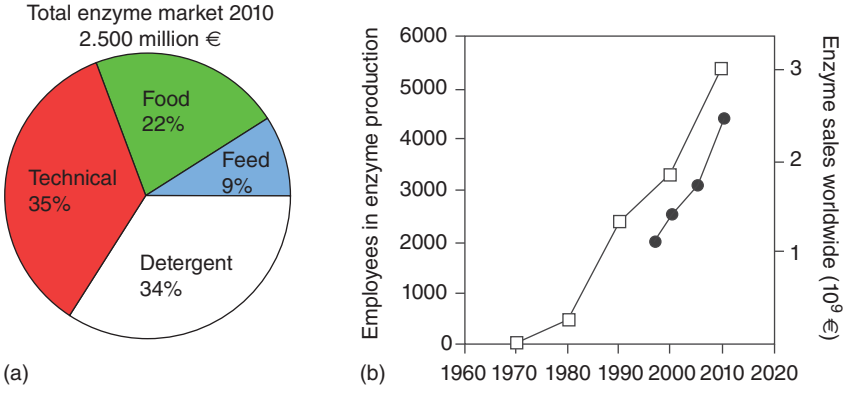


Figure 2.3 Market for enzymes used as biocatalysts for different purposes 2010 (a), and the increase in the application of enzymes reflected in the number of employees in the industry producing enzymes for biocatalytic purposes and their worldwide sales since 1970 (b). Number of Novozymes employees that has about 50% of the world market for such enzymes (squares) and value of their worldwide sales (filled circles) are shown (Novozymes yearly reports, last one from 2010). The value of the world production of technical enzymes is much larger than shown in (a), as many companies that use enzymes as biocatalysts produce them in-house in order to have a safe and stable enzyme supply and/or protect their proprietary knowledge.

- detergent formulations with proteases, lipases, and cellulases (35–40%)
- bioethanol production based on starch (beverage and fuel ethanol ~42 million m^3 per year) [37, Chapter 12].

They represent the most important areas with respect to volume and turnover. Application has extended into the areas of paper and pulp, textile manufacture, degumming of oil, and many others. One-step reactions and hydrolytic enzymes still are dominating. They are often offered at low price (5–20 €/kg or €/l of concentrate, respectively). The hydrolysis of high-molecular-weight, insoluble, or adsorbed substrates proceeds efficiently with soluble enzymes only.

- Immobilized biocatalysts play an eminent role, with two of very large volume and value, as has been described previously: glucose isomerase, and penicillin amidase. They have been established even on a large scale for the manufacture of basic chemicals and acrylamide by immobilized nitrilase (400 000 tpa) [37, Chapter 8, 51].

Today, more than 15 processes of major importance are on stream, and several hundred processes exist for further special applications. Selected examples of industrial application are shown in Table 2.2, ranging from a production scale of several million to some hundred tons per year, however with high added value.

The growth of applied biocatalysis has been remarkable in recent years. Thus the turnover of the enzyme division of Novo Industri (DK), the leading enzyme manufacturer, did not exceed $1 million annually until 1965. By 1969, within only 4 years, Novo's enzyme turnover exceeded US$50 million annually, and in 1997 Novo Nordisk's enzyme division (today Novozymes AS) had a turnover of ~US$650 million. Data on enzyme sales from different sources are illustrated in Figure 2.3. A nearly exponential increase over three orders of magnitude in 35 years is evident [37, Chapter 1]. Total sales were €2.5 billion in 2010; however, the value of the world production of technical enzymes is considerably larger, since many companies that use enzymes as biocatalysts produce them in-house in order to have a safe and stable enzyme supply and/or to protect their proprietary knowledge.

One development, with fashions following up and decrease of energy prices, were *Cellulases* – a career beginning in the 1950s, with several boom cycles since the 1970s following the first oil crisis. They represent the key to alcohol from lignocellulosic material, which offers a huge potential for biofuel manufacture, providing new raw material sources, without competing with food production, and the option for considerably reduced greenhouse gas emissions. Research on enzymatic cellulose degradation has been pursued with varying intensity during recent decades. The pioneer was Elwin T. Reese, who investigated during the early 1950s the deterioration of cotton tents at the U.S. Army Laboratories during World War II. He identified fungi of the genus *Trichoderma* that produced major amounts of cellulases, potent in hydrolyzing cellulose to glucose, including one, which was then named *Trichoderma reesei*. Reese was joined in 1956 by Mary Mandels, and the focus of cellulase research changed from preventing hydrolysis to enhancing

Table 2.2 Industrial applications of enzymes: major selected areas [37, Chapter 7] – Additional sector: diagnostic enzymes[a].

Market share[b]	Enzyme	Purpose, application in solution	Membrane systems	Immobilized systems
	Hydrolases			
Detergents: about 30%	Proteases	Detergents		
	Cellulases	Detergents		
	Lipases	Textile, oil		
Food (total): 40–45%				
	Rennin, chymosin	Cheese manufacture		
	Glycosidases			
(Starch: 11–15%)[c]	Amylases, amyloglucosidases	Starch hydrolysis, biofuel production, detergents, baking, brewing		(Dextrin hydrolysis)
	Cellulases	Pulp and paper manufacture, textiles, biofuels		
	Xylanases, ß-glucanases, pectinases[d]	Juice manufacture, feed processing		
	Lactase	Milk products		Lactose hydrolysis
	Esterases			
	Acylases		Amino acid synthesis	Amino acid synthesis
	Penicillin amidase			Penicillin hydrolysis/synthesis
	Oxidoreductases			
	Glucose oxidase	Drinks manufacture Analytics		Glucose analyzer
	Lipoxygenase	Baking		
	Transferases			
	Aminotransferase			Amino acid manufacture
	Cyclodextrin transferase			Cyclodextrin manufacture
	Isomerases			
(12%)[c]	Glucose isomerase			Manufacture of high fructose corn syrup (HFCS)
	Total turnover			
2500 million €[e]	Worldwide			

a) Diagnostic enzymes; purpose, application: analysis of metabolites in medical care. Market estimated at over 10 billion €.
b) Technical enzymes, worldwide, estimates.
c) Included in food.
d) Including other activities.
e) 2010; however, the value of the world production of technical enzymes is much larger, since many companies that use enzymes as biocatalysts produce them in house in order to have a safe and stable enzyme supply and/or protect their proprietary knowledge.

it for the production of glucose. Systematic classification and characterization, as well as early research on reaction and synergism of cellulases, was pioneered by the group of Henrissat since 1983 [52]. Among the early reviews and papers concerning the kinetics and processing are those of Ladisch *et al*. [53], Ghose [54], and Buchholz *et al.* [55]. Early advanced concepts were to be forgotten, such as simultaneous cellulose hydrolysis and ethanol formation [56], and engineering concepts, for example, a counter-current trickle bed tubular reactor [57]. Subsequent insights made new approaches possible, and necessary, and provided more advanced concepts for better efficiency and much lower cost [52]. An overview of the recent development is given by Bornscheuer *et al*. [52]. With respect to application, a range of pilot studies, demonstration, and/or commercial production facilities have been announced in recent years; however, only a few have been in operation on a relevant scale, for example, by POET (USA) and Iogen Corporation (Canada) which has been operating the first demonstration facility in Ottawa (Canada) since 2004, by Renmatrix, Inbicon, in Denmark, and Abengoa in Spain. Among the first commercial plants are Chemtex's plant in Italy, and the Beta Renewables biorefinery in Crescentino, with a capacity of 75 000 m^3 per year, using enzymes from Novozymes. However, despite these recent innovations, "There is an old joke in the energy business that advanced biofuels are the fuel of the future, and always will be." (The New York Times International/SZ SüddeutscheZeitung, May 2, 2014, p. 8).

2.5
Recombinant Technology – A New Era in Biocatalysis and Enzyme Technology

Most relevant for enzyme research and technology were two basic accomplishments: exploring and understanding the protein structure, and the establishment of nucleic acids as hereditary units, the resolution of their structure and function as carriers of biological information. Knowledge of the protein structure is critical for application in industrial processes, notably immobilization. Genetic engineering furthermore provides the key tool to improve the properties required for enzymes in industrial catalysis, such as appropriate stability.

2.5.1
New Enzymes – A Key to Genetic Engineering

Genetic engineering, along with recombinant techniques, has played a major role in extending the field of biocatalysis since the 1980s. It was the discovery of restriction enzymes in the second half of the 1970s, as well as the synthesis of oligonucleotides, in about 1970, that promoted dynamics in the field, including technological development, which in turn stimulated basic research. Restriction enzymes made it possible to cut genes at defined positions and transfer them, by appropriate methods, into other organisms, for example, for production of the respective proteins in large-scale fermenters, or for the construction

of recombinant or newly synthesized genes exhibiting modified properties expressed in the proteins, notably enzymes, which they encode for (see [8, Section 7]; [58, Sections 1.4 and 1.5]).

The discovery of DNA polymerases, restriction endonucleases, and further enzymes that replicate, break, repair, and recombine DNA formed the basis of recombinant DNA technologies. Kornberg, in 1955, discovered an enzyme of *E. coli* that could replicate DNA from any microbial, plant, or animal source. He later found further replication enzymes and characterized them. The splicing techniques for generating recombinant DNA use enzymes that cut, fill, and seal breaks in DNA [59, pp. 10, 25, 27, 180].

Arber, and Smith and Wilcox, during the late 1960s, reported that restriction enzymes degraded DNA at particular nucleotide sequences. In 1971, Boyer discovered *Eco*RI, which cleaves DNA site-specifically [60]. In subsequent experiments, it was found that cleavage of duplex DNA by *Eco*RI generates fragments that have complementary cohesive termini, "sticky" DNA ends, to allow for enzymatic joining of DNA molecules, a finding published by three groups in 1972. Boyer had seen at once that it would be a much more precise operating tool for use of plasmid construction, notably for Cohen's early plasmids. Boyer offered it to Berg and Cohen, with whom he immediately began to cooperate, and to many others. By March 1973, Boyer and Cohen had demonstrated the feasibility of the DNA cloning approach they had outlined a few months earlier [61, 62, p. 649]. Subsequently, many different restriction enzymes were found that cut DNA molecules at different places.

A prerequisite for cloning was the availability of such pure, well-characterized enzymes (collected by Collins, [58, Section 1.5]). Important were restriction endonucleases (notably *Eco*RI), further DNA ligases (for joining DNA fragments with paired cohesive ends), DNA polymerase I (for efficient DNA synthesis on a template (by Kornberg *et al*. [63]), and reverse transcriptase (for synthesis of DNA from mRNA [64, 65]. A considerable number of these and further enzymes have become available commercially since the 1980s (see following section).

A new tool, CRISPR, for genome editing of remarkable precision has been invented (CRISPR means clustered regularly interspaced short palindromic repeats and the associated Cas9 enzyme, Cas9 for CRISPR-associated protein9). The technique is based on the research and development of two scientists, Jennifer Doudna, University of California, Berkeley (USA), and Emmanuelle Charpentier, at that time at the Umea University, Uppsala (S), who in 2012 developed CRISPR/Cas9.

"The result has been an explosion in research and commercial use." The essentials of this discovery are twofold, the precise direction of the enzyme complex to the position to be split, by short guide RNA which can be readily synthesized, and the general ability to hydrolyze DNA at any selected position. The guide RNA can be paired with locations in any genomic DNA for use of CRISPR as an editing tool, including animal and human DNA. The latest developments in this new enzymatic tool for *in vivo* genetic engineering represent a great increase in the ability to accurately manipulate genetic material by DNA replacement (recombination) directly

in living cells or whole organisms, efficiently and with little or no unwanted genetic side effects. If the cells edited are stem cells, the edit will persist, and if the cell is a somatic, the edit will last only as long as that cell survives. As an example, one company aims at creating anticancer cell therapies [66].

This technology is expected to revolutionize much of modern medicine and biotechnology in the near future. One innovative example can be seen in the work of, for example, George Church's group, in which this new method was used to destroy 69 retroviral copies in the genome of a pig in a single experiment. This is the first step to generate pigs, as potential organ donors, which are free of retroviruses that might be harmful to humans [58].

Based on this development, recently a remarkable series of new companies, four within 4 years, have been founded in order to explore this new technology for genome editing tools of remarkable precision, two with the inventors Doudna and Charpentier. These companies created an investment of $345 million within that short time. However, some have raised ethical concerns with respect to germline alterations (as opposed to somatic tissue alterations) [66, 67, 68].

2.5.2 Analytical and Diagnostic Enzymes

Basic findings for clinical enzymology at the beginning of the twentieth century were the detection of activities of serum amylase in patients with pancreas diseases and phosphatases and cholinesterase in patients with prostate cancer and liver diseases, respectively. During 1954–1956, the clinical significance of alanine and aspartate aminotransferases and lactate dehydrogenase for liver deficiencies and heart attack were reported, followed by others, such as creatine kinase and γ-glutamyltransferase a few years later. The measurement of enzyme activities as well as the development of standardized test methods and biochemical reagents and their use in medical laboratories became increasingly important in those years [69, pp. 209–211].

In Germany, gene technology was difficult to establish in the 1970s and early 1980s due to political limitations and the conservative strategies of the big chemical companies (the case of insulin in Hoechst). In contrast to the conservative strategies of the big chemical companies, Boehringer Mannheim GmbH (Germany) established biomolecular and recombinant technologies in Tutzing, from 1977 onward. Galactose dehydrogenase for food analysis and α-galactosidase for application in the sugar industry (hydrolysis of the trisaccharide raffinose in molasses) were cloned from 1979 onward, resulting in higher yields, as well as higher purity and quality, and in 1982 a series of recombinant enzymes for food analysis were introduced [70]. When a leading scientist asked Herb Boyer in 1979, at a meeting in Berlin, how to clone bacterial enzymes, his answer was to use the – at that time – "super high tech" way using the protein sequence, oligonucleotide synthesis, and hybridization for fishing of the gene – which is routine today. In 1980, cloning of creatinase, the most important parameter for kidney analysis, was started, which had been obtained in low amounts and at

high price from *Pseudomonas*; it was then obtained in *E. coli* with 50% yield of the total protein, and introduced in 1985 [71, 72].

The t-PA (tissue plasminogen activator) – the product expected to give a new endogene human protein, a much more efficient thrombolytic for treatment of cardial attacks) – story resembles a thriller: in 1983 Genentech had announced the successful cloning of the gene, followed by a race for the best expression yields – with Wall street ups and downs, with failure of approval by the FDA first, then approval in 1987, great initial sales, followed by disillusion due to unrealistic prices. Starting 3 years later than Genentech, Boehringer Mannheim succeeded, with others, in a better product with superior quality and much lower cost. By 1988, Boehringer Mannheim had introduced some 90 commercial recombinant biochemical products, including analytical and restriction enzymes and antibodies [71]. In 1995, Roche Diagnostics offered 200 recombinant products (Hoffman-La Roche AG – short: Roche – had taken over Boehringer Mannheim GmbH) [69]. The market for diagnostics is currently dominated by Roche and Abbot, the sales by Roche in this sector were €8.6 billion in 2013 (CHEManager 7–8/2014, p. 3).

Polymerase chain reaction (PCR) has been called *the* revolutionary molecular biology method of the twentieth century, enabling the amplification of tiny amounts of DNA by as much as a billion-fold within a short or reasonable time [59, pp. 236–241, 69, p. 280]. The principle of PCR was conceived in 1983 by Kary Mullis, while working for Cetus Corporation, California, "during a late evening, when driving, together with his girl friend, on a California Highway through the mountains, by combining conventional, but independent ideas to that process which he called later the PCR, all when a tropical flavor was in the air" [73]. In 1985, a patent for PCR was granted to Mullis and assigned to Cetus Corporation. Despite initial problems, for example, with contaminations, and initially high expenditure of time, the first commercial test was introduced in 1990, and tests for the HIV-1-provirus and *Chlamydia trachomatis*, a bacterium transferred by sexual contact, were introduced in 1992 (by Roche with the trade name Amplicor). For immediate application of the technique to the diagnosis of the disease, a license was granted by Cetus to Roche AG [69]. The breakthrough was achieved in subsequent years, with different and multiple applications; among them are tests for hepatitis C virus, hepatitis B, *Mycobacterium tuberculosis*, and numerous others. Most important were tests for the confirmation of fatherhood, and for elucidation in criminal cases, by testing for DNA in preserved traces of blood, or in human history from a fossilized prehistoric creature. PCR is moreover well established in genetics, basic studies on evolution, on inherited disease, and other biological sciences, as well as in biotechnology [59, pp. 236–241, 69, pp. 280–287, 337].

An additional hot story reported by Kornberg was discussed at a trial in the U.S. District Court in San Francisco where Du Pont challenged the Cetus patent. The group of Kornberg had discovered and characterized DNA polymerase over the preceding decade; this and other prior findings were argued against the invention of PCR. The case involved a billion-dollar market at stake, where Cetus finally

was the winner. Chiron subsequently purchased Cetus and sold the PCR patent portfolio to Roche for $300 million [59, pp. 236–241].

2.5.3 Expanding Market of Industrial Enzymes

With recombinant technologies, a boost for enzymes occurred, from the 1980s onward, extending the range of enzymes available. The new techniques considerably improved yields, lowered prices, and markedly extended applications, with modified and/or improved selectivity, effectiveness, and stability (both at elevated temperatures and pH). Boehringer Mannheim GmbH (Germany) had produced the first recombinant enzyme, α-galactosidase, in the industrial scale (10 m^3 fermenter) as early as 1982 [71, 74, 75]. In 1980, genetic engineering of amylase production was a test case at Novo for food enzymes, going to the approval process, and finally marketing in 1984. Over 50 % of market share for recombinant enzymes was reached in 1992 (Poulsen, personal communication).

Work on recombinant penicillin G amidase (PA), one of the most important industrial enzymes (see above), was initiated by F. Wagner in cooperation with H. Mayer and J. Collins. The group cloned an *E. coli* strain that overexpressed PA at a level much higher than publicly known strains [76], but not much exceeding that of industrial strains. The enzyme was produced constitutively, in contrast to conventional strains. Unexpected results were insights in the autocatalytic processing of the enzyme after expression ([37, Chapter 12.3, 77]). Results were first presented at the Enzyme Engineering Conference, Henniker (New Hampshire, USA) 1979 [76]. Discussions with several companies had taken place in order to check their interest, thus with Genentech and Pfizer before 1979. Remarkably, most of the companies with established projects on PA were not interested. Industrial development with the new strain would have required new scale-up with unknown results, notably with respect to stability and product yield (Schmidt-Kastner, personal communication). A cooperation was, however, established with BiochemieKundl (Austria), which utilized the strain for the hydrolysis of penicillin G, which was a by-product (with 10%) in their established process with penicillin V as the main component (hydrolysis of both penicillins yields the same product, penicillanic acid) (Mayer, personal communication).

Important factors for the success of recombinant enzymes were the following: the productivities and/or yields improved by factors of up to 100, and prices for recombinant enzymes now typically lower by a factor of 10 as compared to traditional (non-recombinant) enzymes. Thus the ratio of the amount of recombinant and wild-type microorganism, respectively (*E. coli* or yeast), required for the production of an enzyme is in the range of 1 : 25. The time required for the scale-up procedure for recombinant detergent enzymes is in the range of 1 year (traditionally 4 years) and for food enzymes 1.5 years (traditionally 6–8 years; Poulsen, personal communication). Examples for recombinant enzymes used in major industrial processes comprise amylases, glucoamylases, and glucose isomerases, the protease subtilisin used in washing powders, and glutarylamidase and PA

for the hydrolysis and synthesis of ß-lactam antibiotics. A range of successful modifications have been summarized by Bommarius and Riebel [78] and by Buchholz *et al*. [37]. Thus thermal stability could be improved significantly by site-directed and random mutagenesis as well as by directed evolution (see below) in amylases and glucose isomerase. Thermostable amylases from hyperthermophilic microorganisms are active even up to 130 °C and are applied industrially in the range 105–110 °C during starch processing. Other important improvements concern the shift in the pH application range, which is relevant in starch processing by amylases and glucoamylases.

2.6 Current Strategies for Biocatalyst Search and Tailor Design

2.6.1 Enzyme Discovery from the Metagenome or Protein Databases

Extending the range of enzymes for application as well as the search for new solutions in synthesis, notably of chiral compounds, has been a continuing challenge. The traditional method to identify new enzymes for such purposes is based on screening of, for example, soil samples or strain collections by enrichment culture, many impressive examples of which can be found in the literature [79]. Unfortunately, only a tiny fraction of the biodiversity can be accessed by this means using common cultivation technology. Indeed, the number of culturable microorganisms from a sample has been estimated to 0.001–1% depending on their origin [80]. In turn, more than 99% of the biodiversity escaped our efforts to identify them for biocatalytic applications. After the 1990s, two new strategies have been developed to include the plethora of "non-culturable" biodiversity in biocatalysis: (i) the metagenome approach [81, 82] and (ii) sequence-based discovery. Here, the enormous progress in sequencing technology has led to an exponential increase in the number of sequence data (currently ~40 million sequences in the UniProt database). For instance, the metagenome sequencing of just the biological diversity found in the Sargasso sea [83] and in the Global Ocean Survey project [84] identified 7.3 million new gene sequences, including 1700 new protein families. This rich source of information can be considered as a gold mine, as these genes encode for a plethora of novel and mostly unexplored enzymes useful for biocatalysis for the production of fine chemicals and pharmaceutical building blocks or metabolic engineering of new pathways to produce bulk chemicals or biofuels. In the metagenome approach, the entire genomic DNA from uncultivated microbial consortia (i.e., soil samples) is directly extracted, cloned, and expressed. Next, distinct enzymatic activities are identified by suitable assay methods [85, 86]. The major advantage of this approach is that a large number of new biocatalysts can be found. Phylogenetic analyses have revealed that new subclasses of enzymes can be identified that show a very broad evolutionary diversity, and thus the chance to identify biocatalysts with unique properties is substantially

increased. In addition, the enzymes identified are already expressed by recombinant methods and thus in principle available at a large scale. One impressive example was the discovery of >130 novel nitrilases from more than 600 biotope-specific environmental DNA libraries [87] by Diversa Inc. (San Diego, USA), as compared to less than 20 nitrilases known so far, which were isolated by classical cultivation methods. The application of these novel nitrilases in biocatalysis revealed that 27 enzymes afforded mandelic acid in >90% ee in a dynamic kinetic resolution and one nitrilase afforded (*R*)-mandelic acid in 86% yield and 98% ee. In another example, a nitrilase from this discovery was used to make (*R*)-4-cyano-3-hydroxybutyric acid from 3-hydroxyglutaronitrile, which served as building block for atorvastatin, a major drug sold as Lipitor [88]. The wild-type enzyme was then improved by directed evolution [89], and the process using 3 M substrate (300 g l^{-1}) was up-scaled by researchers from Dow Chemicals to afford the chiral building block in 98.5% ee in 82% isolated yield [90]. In a more recent contribution, the company BRAINAG (Zwingenberg, Germany) identified a surprisingly large molecular diversity for the well-known serine protease from *Bacillus subtilis* in Carlsberg [91]. In only four soil samples they were able to find 94 sequences of this protease bearing 38 mutations, equivalent to 2–8 amino acid exchanges per variant. Fifty-one unique protein variants could be functionally expressed in *Bacillus subtilis*, which also differed in their functionality. Hence, their discovery of coexisting gene variants is a potent source for novel enzyme variants.

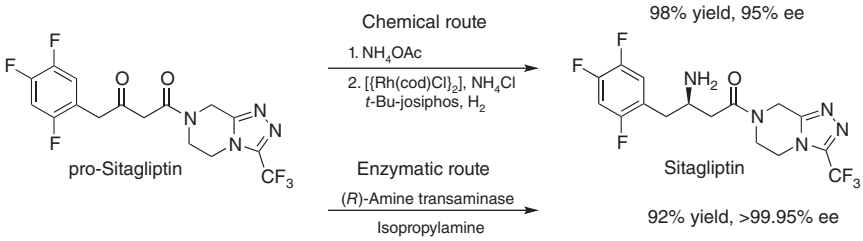
In case a novel protein sequence is discovered by the metagenome approach or from genome sequencing, the annotation of a protein function is performed automatically and consequently can lead to misleading interpretations. In a recent example, we took advantage of these errors while trying to identify (*R*)-selective amine transaminases (ATA) for which no protein sequence was reported in the literature at the time of our study. ATA are pyridoxal-5′-phosphate (PLP)-dependent enzymes catalyzing the synthesis of chiral amines from ketones (in contrast to amino acid transaminases that convert α-keto acid to α-amino acids), and only (*S*)-selective ATA protein sequences were known at that time. Using a sophisticated search algorithm – based on distinct amino acid motifs of known amino acid transaminases – an alignment of >5000 protein sequences from public database of PLP-dependent transaminases identified 21 new protein sequences (equivalent to a hit rate of 0.5%) [92]. For 17 proteins, it could be confirmed that they all were true ATAs having the predicted (*R*)-enantio preference and these were then used in the asymmetric synthesis of a set of 12 chiral (*R*)-amines [93]. Another (still only scarcely explored) source for novel enzymes is the Brookhaven protein structure database (www.pdb.org), which contains numerous proteins whose the 3D structure has been deposited but where the proteins were never biochemically characterized. The advantage of this strategy is that the protein structure is known and hence subsequent protein engineering by rational design is substantially facilitated; the disadvantage is that neither the substrates nor suitable reaction conditions are known in such a case and need to be elucidated. Using this approach, we recently identified four (*S*)-selective ATA in the Protein Database (PDB) and could functionally assign them by biochemical characterization using

a cocktail of potential amino donors and acceptors to identify suitable substrate pairs and by performing the enzymatic reactions at different pH values [94, 95].

2.6.2 Protein Engineering of Enzymes

Once the enzymes are identified for a given application, they usually need to be optimized to meet the requirements of a given process. Protein engineering targets include improved stability at high temperature, at extreme pH, as well as in unconventional media such as organic solvents and at high substrate loadings. Further aims are altered specific activity, selectivity, and broadened substrate scope. For this, two major strategies for the improvement of an enzyme on the amino acid sequence level can be followed: (i) rational protein design, which requires the availability of the three-dimensional structure (or a homology model) necessary to identify type and position for the introduction of appropriate amino acid changes by site-directed mutagenesis, or (ii) directed evolution [96]. Directed evolution emerged in the mid-1990s (also called *in vitro* or molecular evolution) and essentially comprises two steps: first, random mutagenesis of the gene(s) encoding the enzyme(s), and second identification of desired biocatalyst variants within these mutant libraries by screening or selection. Prerequisites for *in vitro* evolution are the availability of the gene(s) encoding the enzyme(s) of interest, a suitable (usually microbial) expression system, an effective method to create mutant libraries, and a suitable screening or selection system. Many detailed protocols for this are available from books [85, 97, 98], and updates are given in reviews [99, 100]. These modern tools for protein engineering helped solve the various issues related to the application of enzymes, which have been summarized in a recent review [101] covering more than 40 examples in which enzymes were improved by protein engineering to make pharmaceutical intermediates on a large scale. As stated in that review, one can summarize the major technological achievements made in the last decade: "in the past, an enzyme-based process was designed around the limitations of the enzyme; today, the enzyme is engineered to fit the process specifications."

Probably the most impressive recent example of successful protein engineering is the expansion of the substrate scope of the transaminase ATA-117 to accept ketones with two bulky substituents, as shown in a joint project by Codexis Inc. (Redwood City, USA) and Merck & Co (Rahway, USA) to make the antidiabetic drug sitagliptin (Scheme 2.2). Starting from ATA-117, a close homologue of the wild-type enzyme, which had no detectable activity on the substrate, the first variant provided very low activity (0.2% conversion of $2\,g\,l^{-1}$ substrate using $10\,g\,l^{-1}$ enzyme) towards prositagliptin; the final variant having a ~40 000-fold increased activity converts $200\,g\,l^{-1}$ ketone to sitagliptin with 99.95% ee at 92% yield [102]. The biocatalytic process not only reduced the total wastage and eliminated all transition metals but also increased the overall yield and the productivity (kilogram per liter per day) by 53% compared to the metal-catalyzed process [103]. This chemical process for asymmetric hydrogenation using a transition-metal catalyst



Scheme 2.2 An engineered amine transaminase could replace the earlier chemical process in the large-scale production of the drug sitagliptin [102, 103].

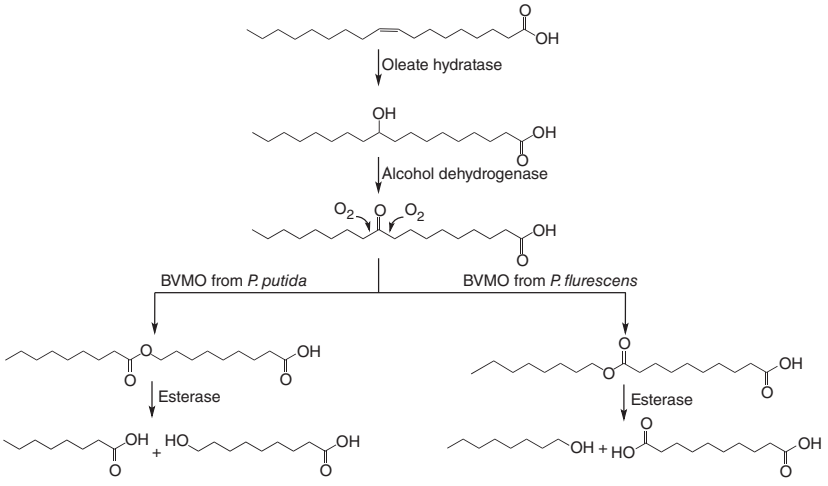
was developed earlier at Merck & Co. The enzyme engineering started with a small ketone substrate, created more space in the active site, and used increasingly larger ketones.

2.6.3 Enzyme Cascade Reactions

Enzymatic cascade reactions are comprised of several consecutive biocatalytic steps toward the final product. Advantages are that no intermittent product isolation is required, unstable intermediates are directly further converted, reversible reactions can be driven to completion, inhibition is reduced or eliminated, investment costs can be lower, wastage is considerably reduced, and higher overall yields can be achieved. This requires, however, the availability of suitable enzymes having similar pH and temperature profiles and preferentially also similar specific activities and stabilities. Usually, the enzymes are recombinantly expressed and used either as whole cell systems or as crude cell extracts for which it must be ensured that no undesired enzyme activities are present from the host's background. Recent reviews provide excellent overviews about the status of this field [104–108], so only a few selected examples are covered below.

One important area is the synthesis of α,ω-dicarboxylic acids, ω-hydroxycarboxylic acids, or ω-aminocarboxylic acids, as these are important starting materials for the synthesis of a variety of chemical products and intermediates such as nylons and other polyamides, polyesters, resins, hot-melt adhesives, powder coatings, corrosion inhibitors, lubricants, plasticizers, greases, and perfumes. For instance, several tens of thousands of tons of sebacic acid (1,10-decanedioic acid), which is produced from ricinoleic acid, is used annually for the preparation of Nylon-6,10. The majority of dicarboxylic acids are made from petrochemical feedstock or fatty acids via chemical routes, although whole-cell biotransformation routes for the preparation of dicarboxylic acids and their precursors ω-hydroxycarboxylic acids have been also reported [109]. For instance, sebacic and azelaic acids can be produced via fermentation of *Candida tropicalis* [110]. However, petrochemical feedstock such as decane and nonane, respectively, are used here as starting materials. Further, the diversity of the products is limited by the availability of the reactants used and the range of

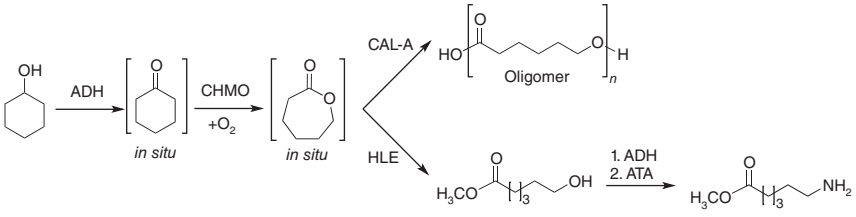
intermediates accepted by the metabolic pathways present in the microorganism *C. tropicalis*. Recently, a biocatalytic cascade to produce α,ω-dicarboxylic acids and ω-hydroxycarboxylic acids from renewable fatty acids obtained from vegetable oils (e.g., oleic acid, ricinoleic acid) [111, 112] was described. The cascade starts by hydration of the internal double bond of the fatty acid by oleate hydratase followed by enzymatic oxidation of the hydroxyl group to the ketone by an alcohol dehydrogenase (ADH). Based on our earlier discovery that certain Baeyer–Villiger monooxygenases (BVMO) show distinct regioselectivity in the formation of esters from ketones, two different BVMOs could be used for the oxidation of the ketone into the two regioisomeric esters: the enzyme from *Pseudomonas putida* provides access to ω-hydroxy fatty acids, whereas the BVMO from *Pseudomonas fluorescens* leads to the formation of an ester. Finally, esterase-catalyzed hydrolysis yields the α,ω-dicarboxylic acid (Scheme 2.3). This made the cascade reaction much more versatile, as two different target products can be easily produced as demonstrated for a variety of fatty acids as starting materials. However, this process has not yet been commercialized. All enzymes could be functionally expressed in *E. coli*, and the biocatalysis was performed using crude cell extracts [111].



Scheme 2.3 An enzyme cascade reaction to convert unsaturated fatty acids such as oleic acid into ω-hydroxycarboxylic acids or dicarboxylic acids. Cofactors are not shown for clarity [111, 112].

More recently, this enzyme cascade was further extended to yield ω-amino carboxylic acids serving as precursors for polyamide synthesis. For this, the ω-hydroxy carboxylic acid is first oxidized to the corresponding aldehyde using an ADH followed by the formation of the ω-amino carboxylic acid using an ATA [112]. Related concepts have been proposed by other groups: Schrewe *et al*. started directly from fatty acids and used an alkane monooxygenase in a recombinant *E. coli* strain to introduce the terminal aldehyde function,

which is then converted to the amine by an ATA [113]. This process is currently performed on pilot scale to make ω-aminolauric acid. As an alternative, Otte *et al*. described the use of a lipoxygenase and a hydroperoxide lyase to convert linoleic acid into 9-oxononanoic acid or the corresponding ω-aminocarboxylic acid [114]. However, best results were attained when the two enzymes were used in a successive rather than in a one-pot manner. Very recently, we have described a cascade reaction in which cyclohexanol is oxidized to cyclohexanone and then transformed into ε-caprolactone using a mutant of the cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* (expressed in *E. coli*). To overcome severe product inhibition, the lactone could be directly oligomerized in an aqueous phase owing to the unique acyltransferase activity of lipase A from *Candida antarctica*. This allowed the conversion of 200 mM substrate quantitatively into the easy-to-separate oligomer [115] (Scheme 2.4), which then can be used to make the polymer. In parallel, the Kroutil group reported the synthesis of 6-amino-hexanoic acid, a precursor for Nylon 6, also using ε-caprolactone as key intermediate. Here, the lactone was first converted into 6-hydroxy-hexanoic acid methylester using horse liver esterase (HLE) followed by oxidation with a dehydrogenase to the aldehyde and finally transformation into the ω-amine using a transaminase [116] (Scheme 2.4).



Scheme 2.4 Enzyme cascade reactions to afford ε-caprolactone oligomers (top right, [115]) or 6-amino-hexanoic acid (bottom right, [116]), a Nylon 6 precursor, starting from cyclohexanol. In all cases, the required enzymes were recombinantly expressed in *E. coli* and whole cell extracts or lyophilized cells were used. ADH: alcohol dehydrogenase, CHMO: cyclohexanone monooxygenase, CAL-A: lipase A from *C. antarctica*, HLE: horse liver esterase, ATA: amine transaminase.

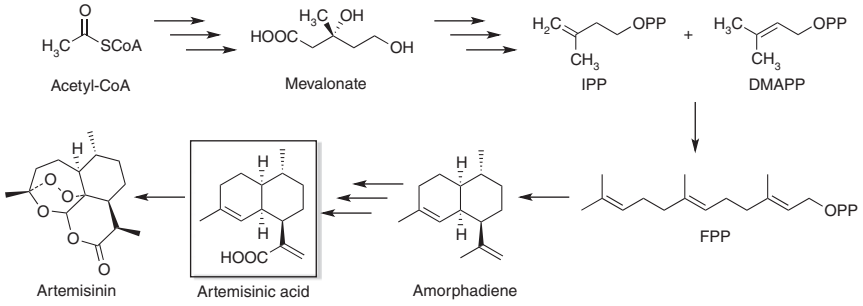
2.6.4
Metabolic Engineering

Although there is a long history of the use of certain yeasts or fungi to produce triglycerides as the so-called single cell oils (SCO or microbial lipids), the strains studied often accumulated high amounts of lipids only in the presence of a large excess of the carbon source and under nitrogen limitation [109, 117]. Nevertheless, microbial biotransformations are especially useful for multistep conversion of triglycerides, fatty acids, or alkanes and the *de novo* synthesis of lipid products. Modern metabolic engineering and synthetic biology tools now allow the creation of recombinant microorganisms designed to synthesize a

broad set of useful compounds [118]. As pointed out previously, these concepts especially take advantage of the huge number of genome and protein sequences available in public databases, and this vast source of information enables the identification of novel enzymes with distinct function and selectivity required for tailoring biosynthetic pathways. Numerous examples for successful metabolic engineering can be found in literature, with the microbial synthesis of 1,3-propane diol as one of the first successful large-scale productions [119]. In addition to this diol for polymer synthesis, also the metabolic engineering of *E. coli* to produce 1,4-butane diol at up to 18 g l^{-1} has been reported [120]. Also, the improvement of the production of amino acids such as Glu, Lys, or Ser using engineered *Corynebacterium glutamicum* strains has been reported [121].

More recently, scientists at DuPont (Wilmington, USA) have established a pathway to produce eicosapentaenoic acid (EPA) in the oleaginous yeast *Yarrowia lipolytica* [122]. This provides an alternative supply of this important ω3-PUFA (poly unsaturated fatty acid) simply by fermentation instead of extraction from fish oil or other less abundant sources. The engineered strain produces EPA at 15% cell dry weight, and within the lipid fraction EPA accounts to 56.6% of the total fatty acids, which contained only 5% saturated fatty acids. Key to success was the introduction of nine different chimeric genes into the yeast and, of these, a Δ17-desaturase showing high activity in the conversion of arachidonic acid into EPA was the most important. Only a mutation of the peroxisome biogenesis gene *PEX10* resulted, however, in the extremely high EPA titers observed with the final production strain [122].

Artemisinin is believed to be the most potent drug against malaria. This natural compound is produced by the plant *Artemisia annua*, but this natural resource does not provide sufficient amounts of the drug to treat the many million people affected by malaria. The chemical total synthesis is highly complicated, requires numerous steps, and results in low overall yields. Researchers of the Keasling group have thus developed a highly impressive metabolic engineering approach to establish the biosynthetic pathway to the advanced intermediate artemisinic acid in the yeast *Saccharomyces cerevisiae* (Scheme 2.5). The current fermentation



Scheme 2.5 Artemisinic acid (box) production pathway in an engineered *S. cerevisiae* strain [123]. The last step to the final product artemisinin is performed chemically. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate.

process yields titers of 25 g l^{-1} of artemisinic acid, which is then converted in an efficient and scalable chemical process to artemisinin [123, 124].

These examples demonstrate that metabolic engineering has become mature and enables cost-competitive production of compounds, which had been so far only accessible from natural sources.

2.7 Summary and Conclusions

In early scientific approaches enzymes long remained mysterious things; nevertheless, Berzelius introduced the rational concept of catalysis including enzyme reactions. In early pioneering work, Payen and Persoz isolated and characterized diastase, and established the first industrial enzyme process for starch hydrolysis to produce dextrins in the 1830s. Despite the unknown chemical nature, and even mysterious properties assigned to enzymes and proteins, further practical applications were invented and introduced, for example, Hansen's company producing rennet for cheese making and Takamine being assigned a patent for microbial amylases, applied for starch hydrolysis during the late nineteenth century.

Emil Fischer, from the 1890s onward, investigated in highly sophisticated experiments the stereospecificity of enzymes, and he promoted the assumption of their protein nature. Nevertheless, their chemical and structural nature were subjects of controversial debates until Buchner (during the 1890s) ended the mysteries of "vital forces" and laid the foundations of biochemistry. But even after Sumner's crystallization of urease in 1926, it took years for the acceptance of the protein nature of enzymes.

Even so, technical application expanded, notably for food and beer manufacture. Röhm, who was aware of Buchner's work, pioneered the application of enzymes as a bating agent in 1907, based on a strong market pull in Germany and the United States, and later introduced enzymes for detergent preparations.

The final breakthrough for understanding mechanisms and structure was the X-ray diffraction pattern of crystalline pepsin in 1934, followed by the structural analysis of several enzymes in the 1950s. It provided the basis for a new breakthrough for extended enzyme application, namely immobilization, which was invented at the universities around 1950. Some 20 years later, around 1970, the Bayer and Beecham companies developed the immobilization of PA for the production of 6-APA, and semisynthetic penicillins; it was followed by other highly important processes, such as glucose isomerization.

The continuous interplay between scientific insight, practical application, and economics is obvious with the development of recombinant technologies. They were established using restriction enzymes, which were critical to this technology. In turn, it was these methods that opened the way to the much improved and extended production of enzymes at significantly lower prices. Further, they allowed the design of enzymes by rational approaches or by directed evolution for scientific insight at the molecular level, and for improved properties, such

as process stability and stereoselectivity. This further boosted the application of biocatalysts into new markets, such as the synthesis of advanced pharmaceutical intermediates and many other compounds. Modern biotechnological tools such as metabolic engineering and synthetic biology go a step further and successfully combine many enzymes to make non-natural compounds in a microbial host organism. The significant achievements made here in the last two decades not only allow us to tailor design entire pathways but also, especially, reduce development times substantially.

The role of scientific progress, pioneering technical developments, and economics is obvious from early processes to the remarkable expansion of modern enzyme technology, and the exponential growth of their application since 1950.

Acknowledgment

We are grateful to Ulrich Behrendt (former Roche) for information concerning diagnostic enzymes.

Abbreviations

6-APA	6-aminopenicillanic acid
GBF	Gesellschaft für Biotechnologische Forschung, Braunschweig (Germany) (today HZI, Helmholz-Zentrum für Infektionsforschung)
PA	penicillin amidase (also penicillin acylase)
PCR	polymerase chain reaction
t-PA	tissue plasminogen activator

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3
Molecular Engineering of Enzymes

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3.1
Introduction

More than a half-century has passed by since the tertiary structure of the first protein was unveiled. In 1958, Kendrew *et al.* obtained the three-dimensional (3D) model of the sperm whale myoglobin by X-ray diffraction [1]: the extraordinary journey into the protein's structure–function relationship had begun.

Up to now, more than 600 genomes of cellular organisms have been fully sequenced, which represents more than 5 million protein sequences added to public databases [2], and structural genomics projects accumulate an increasing number of protein structures. At the time of writing this chapter, more than 100 000 protein structures have been deposited in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and ∼53% correspond to proteins with enzymatic activity. Proteins' 3D structures become relevant since they provide fundamental insights not only into protein functions but also into their evolutionary relationships, improving in this way our understanding of how structure and function relate to each other. Unfortunately, only the structure of less than 1% of known sequences has been elucidated either by X-ray crystallography or nuclear magnetic resonance (NMR) techniques [2], and protein structure determination is still considered as a challenging, expensive, and time-consuming task. Therefore, over the last decades several disciplines have converged attempting to improve the predictive tools that may finally lead to the finding of the Holy Grail of structural chemical biology, namely the accurate prediction of structure and function from an amino acid sequence, although such goal may still be far from being met [3].

Understanding the molecular bases that govern protein structure and how structure associates with function is of highest importance for the design of better and novel proteins that are required to expand the range of enzyme-based biotechnological and biocatalytic processes [4, 5].

Enzymes are currently applied in paper and textile manufacturing, food and beverage production [6], diagnosis, cleaning [7], and the synthesis of biological

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active compounds and fine chemicals [8]. However, the scope for potential applications is enormous, and engineered enzymes may find application in fields such as functional biohybrid nanomaterials and the catalysis of non-natural reactions [9, 10].

The advantages offered by enzymes over their chemical counterparts are well known; however, the widespread use of enzymes in the synthesis of organic compounds is still prevented by insufficient substrate specificity, a limited substrate range, lack of enantio-selectivity, low catalytic activity, low thermal stability, and limited tolerance to organic solvents [11]. While the performance of an enzyme under mild or harsh conditions is dependent on the particular case, natural biocatalysts are often not suitable for chemical transformations that are interesting for biotechnological applications without undergoing redesign at different levels. Furthermore, there are several reactions for which no enzymatic synthesis has yet been reported. Engineered enzymes are then relevant for enzyme-based synthetic processes aiming to obtain faster and more stable biocatalysts either to compete with already established chemical transformations or to achieve efficient synthesis of molecules that are difficult to produce by other means. Molecular evolution has shown that modifying the amino acid sequence of a protein through mutation or/and recombination may result in new folds and/or functions [12]. Thus, over the last years a number of methods have been developed to generate protein variability, along with improved computational tools focused on protein design and analysis. This chapter aims to provide an overview of the different approaches for the design, redesign, and identification of enzymes with improved or new functionalities. Among the vast number of enzymes that have been improved over the last years, we chose industrially relevant biocatalysts such as lipases, cellulases, and the cytochrome P450 to demonstrate the applicability of different protein engineering approaches.

3.2 Protein Engineering: An Expanding Toolbox

Enzymes are attractive biocatalysts because of their inherent properties such as reaction, substrate, and regio and stereo specificity. Although enzymes are highly selective regarding their catalyzed reaction and the type of substrate they recognize, secondary (often inefficient) or barely related reactions may also take place because of the reactive nature of their catalytic sites [13, 14]. Secondary reactions are frequently sought as the starting point to develop enzymes with improved activity toward non-native substrates and are considered a key feature for evolvability [14–16], as exemplified by the evolution of a weak, promiscuous lactonase into an extremely efficient organophosphate hydrolase [17] and the 200-fold improved chlorohydrolase activity of a melamine deaminase [16, 18].

Catalytic activity may also emerge from noncatalytic protein scaffolds, and new functions have been created combining *in silico* design and laboratory evolution. The synthesis of sitagliptin (an antihyperglycemic drug) in the gram

scale involving a transaminase bearing 27 mutations is a remarkable example of such a strategy. Under optimal conditions, 200 g l^{-1} prositagliptin ketone is converted to sitagliptin of >99.95% ee, which exceeds by 10–13% the overall yield and by 53% the productivity of the rhodium-catalyzed process, with a concomitant 19% reduction in the total generated waste [19]. Over the last years, a number of methods have been developed aiming to expand the enzyme's functional range, along with computational tools focused on protein design and analysis. Some of these approaches will be described in the next sections.

3.2.1
From Sequence to Fold and Function

Assigning function to proteins from their sequence is still a challenge in the post-genomic era. Genomic and metagenomics projects deliver a vast amount of information as a resut of the fast development of high-throughput sequencing technologies; however, biochemical characterization of proteins and elucidation of their 3D structure generally proceed slower than the release of new protein sequences. Bioinformatics tools are frequently used in parallel to experimental methods for the prediction of protein structure, function, and target positions for mutagenesis ("hotspots"), the most common approaches being those based on sequence and structural similarity.

Bioinformatics tools aim to annotate function to genes and their products based on sequence homology and non-homology methods. FASTA [20] and the Basic Local Alignment and Search Tool (BLAST) [21], for instance, identify local similarity between homologous sequences from nucleotide and protein databases. However, assigning function to newly sequenced proteins that do not share apparent sequence or structural similarity with already characterized ones requires methods that use local structure similarity or distantly related proteins [22–24].

Structure prediction methods provide valuable information for proteins whose structures are not available. Homology modeling is currently the most accurate approach for obtaining all-atom models of proteins with unknown 3D structure. According to the last biannual Critical Assessment of Protein Structure Prediction (CASP) evaluation, some of the platforms that generate reliable protein models are Iterative Threading ASSEmbly Refinement (I-TASSER) [25], Protein Homology/AnalogY Recognition Engine (PHYRE2) [26], Rosetta [27], IntFOLD [28], and SWISS-MODEL [29]. Rosetta was originally developed for *de novo* structure prediction and, along with other programs, can be used to predict the structure of proteins that do not have a known structural homologue or when determination of their 3D structure is challenging, which is the case of transmembrane proteins, for instance [30].

3.2.2
Improving Enzyme Properties by Rational Design and Directed Evolution

Protein engineering has proven successful in altering the properties of enzymes either through rational design or directed evolution. Rational design relies on

structure- and usually mechanistic-guided predictions about the mutations that may be needed to modify a given enzyme property. In the absence of structural information, such analyses are based on homology models. Although changes at specific positions are easily introduced at the DNA level by site-directed mutagenesis, they may result in protein variants that exhibit the sought property but also show deleterious effects on the enzyme activity or stability. In those cases, the change at one position may require additional fine-tuning at other position(s) to compensate for the loss in activity, substrate affinity, or stability derived from the first mutation. Unfortunately, prediction of additive and/or cooperative effects resulting from simultaneous changes at two or more positions is still difficult. Despite these limitations, the successful design of an impressive number of enzymes through rational approaches is well documented, and some computational tools that allow prediction of, for instance, stabilizing mutations are continuously being developed, evaluated, and improved [31–34].

It may take more than a few changes either to significantly enhance an enzyme's performance or to evolve an enzyme into a non-native function. In this context, directed evolution emerges as a robust method to create enzymes with improved or novel functionalities by mimicking natural selection. In directed evolution experiments, DNA libraries of variants are produced by iterative cycles of diversity generation. The gene of the most improved variant (best hit) of one round is then used as a template for subsequent rounds of mutagenesis, expression, and screening for the sought properties [35, 36], that is, specificity [37–41], activity [42–44], and stability [45–50]. To date, rational and semirational approaches have been followed in directed evolution experiments making use of methods such as error-prone polymerase chain reaction (epPCR), DNA recombination, saturation mutagenesis, and variations of these techniques [51].

Random mutagenesis generates variability by randomly introducing changes over the whole gene, and does not require any prior structural knowledge of the target protein. It has been traditionally used to generate large libraries by methods involving epPCR [52–55] and the use of mutator strains and chemical mutagens [56]. However, random mutagenesis has significant drawbacks, some of them being the mutational and codon bias affecting the composition of the library, the number of deleterious/neutral mutations that are introduced by this technique, and the screening workload. The frequency of beneficial mutations introduced by random mutagenesis is in the order of 10^{-3}, whereas deleterious mutations account about 30% of all mutations [3].

It is imperative to navigate the protein sequence space (the number of variations of that sequence that can possibly exist) efficiently, as the huge number of possible variations that can be generated is often impossible to sample thoroughly. For instance, for a rather small protein of 300 amino acids (considering it only contains the 20 canonical amino acids), the number of combinations that can possibly exist is $\sim 16 \times 10^6$ for only two substitutions [57]. When high-throughput screening is not possible, random approaches may be inefficient.

The number of variants to be screened grows exponentially with the number of modified positions, and very often only a small fraction of the theoretical protein

sequence can be sampled in directed evolution experiments [36]. To circumvent this drawback, different strategies have been proposed to generate "smart" or "focused" libraries over the last years. Semirational and rational approaches aim to improve the libraries' quality (a high frequency of improved enzyme variants and a high degree of improvement of the sought parameter) and variability and to minimize the screening efforts [51]. Semirational or rational design is often assisted by *in silico* analyses to identify a restricted number of "hotspots" to be modified. Algorithms integrating evolutionary, structural, thermodynamic, and functional data have been developed aiming to localize potential "hotspots" and to predict the effects of such changes on the enzyme parameters. Computational toolboxes and molecular methods developed to create focused libraries will be described in the next sections.

3.2.3 Designing Smart Libraries

Saturation mutagenesis is a method that allows the creation of focused libraries by randomizing amino acids at one position or simultaneously at two or more positions in a protein, reducing by this means the sequence space to be explored and the screening efforts [35].

Iterative saturation mutagenesis (ISM) is an approach that combines rational design and combinatorial randomization by focusing on regions or positions that may have an effect on the sought catalytic property. Libraries are designed by randomizing each chosen region (multi-residue sites) or position independently from the other sites. Residues may be mutated by all the standard amino acids (NNK codon degeneracy that involves 32 codons, N: adenine/cytosine/guanine/thymine; K: guanine/thymine) or by a defined amino acid set with desired characteristics (i.e., NDT codon degeneracy that involves 12 codons, D: adenine/guanine/thymine; T: thymine) [51, 58]. The best hit of a given library is then used as a template for another round of saturation mutagenesis at the other chosen sites, and so on. The iterative process continues until the variant with the improved property arises [35, 59]. This approach maximizes the probability of finding improved mutants due to the additive and/or cooperative effects that can be introduced by new mutations in a defined region [26]. SwiftLib is an algorithm reported recently for codon degeneracy optimization while keeping the size of the library within a diversity limit [60].

Selection of the residues to be randomized will depend on the aimed catalytic property. The B-factor iterative test (B-FIT), for instance, can be applied to improve thermostability, thermoresistance to undesired aggregation, or robustness toward hostile organic solvents by targeting highly flexible regions. The approach is based on the B-factor values from a crystallographic structure: the more flexible a region is, the larger will be the B-factor values. Residues characterized by the highest B-factors are chosen for mutagenesis, and the enzyme is then subjected to several rounds of ISM [61, 62].

Framework for Rapid Enzyme Stabilization by Computational Libraries (FRESCO) is another computational-aided approach that predicts a large number of single stabilizing mutations. FRESCO uses the software packages FoldX [33] and Rosettaddg [63] to predict the relative changes in folding free energy $\Delta\Delta G^{\text{Fold}}$ resulting from point mutations and the 3D structures of the corresponding enzyme variants. The Dynamic Disulfide Discovery tool designs disulfide bonds, and the process is followed by *in silico* screening to remove chemically unreasonable mutations and to select the best variants. The best hits are expressed and characterized, and the most stabilizing mutations are combined to obtain highly thermostable enzymes [64]. This process considerably reduces the number of mutants to be experimentally screened, as demonstrated in the stabilization of a limonene epoxide hydrolase [64] and a haloalkane dehalogenase [65], which exhibit >250-fold and 200-fold longer half-lives than the parental enzymes, respectively.

Prediction of Protein Mutant Stability Changes (PoPMuSiC) [31] and the Cologne University Protein Stability Analysis Tool (CUPSAT) [34] are *in silico* methods that allow predictions of the stability free energy changes resulting from single point mutations in proteins on the basis of their 3D structures. PoPMuSiC predicts stability in globular proteins by using a linear combination of statistical potentials whose coefficients depend on the solvent accessibility of the mutated residues. It has been used to identify mutations that stabilize enzymes such as pyruvate formate lyase and feruloyl esterase, as well as other proteins without enzymatic function [31, 66, 67]. CUPSAT uses structural-environment-specific atom potentials and torsion angle potentials to predict the difference in free energy of unfolding between the wild type and the variants and provides information about changes in protein stability derived from 19 possible amino acid substitutions at specific positions [37].

Another powerful and widely used method to enhance the stability of biocatalysts is the data-driven construction of synthetic consensus genes, which does not require any knowledge of structural information [68]. The so-called consensus approach is based on statistical information from homologous sequence alignments and identifies and substitutes specific amino acids at given positions by the most prevalent ones in the consensus. It is thought that consensus mutations are a reversion to ancestral amino acid residues [69], and the rationale behind the method is the assumption that a high frequency of some amino acids at a given position in a multiple alignment correlates with a higher stability, so that a synthetic protein having the most frequent residue at each position should exhibit maximum stability [70]. This method has been successfully applied to improve the stability of several proteins either alone or in combination with other strategies, and its success depends on the phylogenetic diversity of the analyzed sequence data [70–73].

Methods combining computational design and bioinformatics have been applied to control enzyme selectivity and to modify substrate scope, that is, CAST [74], HotSpot Wizard [75], CASCO [76] (Figure 3.1), IPRO [77], and 3DM [78]. The Combinatorial Active-Site Saturation Test (CAST) requires

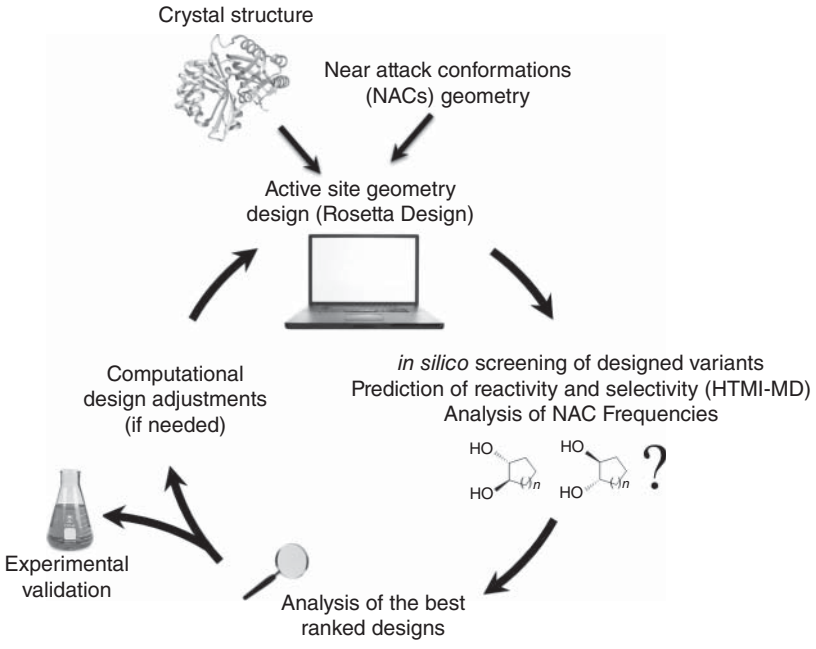


Figure 3.1 The CASCO approach for designing enantioselective enzymes. Two rounds of *in silico* screening HTMI-MD simulations are included to reduce the occurrence of variants with low-energy structures that allow undesired substrate orientations (see also [76]).

structural 3D information of the target enzyme and can be applied to change stereo/regioselectivity or to modify the substrate scope. It searches for synergistic effects by simultaneously randomizing sets of two or three spatially close amino acids at the catalytic center simultaneously, which generates variants with different combinations of amino acids [74, 79–82].

HotSpot Wizard [75] is a user-friendly bioinformatics tool developed to predict key positions that may influence substrate specificity, activity, or enantioselectivity of enzymes. It is based on structural and evolutionary information obtained from different enzyme, protein, and genome databases, and uses some bioinformatics tools such as CASTp, CAVER (a software tool used for the identification of tunnels and channels in biomolecules [83, 84]), BLAST, and Rate4Site. HotSpot Wizard targets the residues within the active site and those shaping the access tunnels, providing a list of hotspot residues ranked by mutability [75, 85, 86].

CAtalytic Selectivity by COmputational design (CASCO) is a computational-based strategy to design small mutant libraries for enantioselective enzymes, and was recently published [76]. The method uses the RosettaDesign software and high-throughput molecular dynamic simulations to identify and evaluate the mutations needed to create an active site in which the substrate may bind in the correct orientation to selectively produce only one enantiomer. High-throughput–multiple independent MD (HTMI-MD) simulations screen *in silico* for variants with low-energy structures that allow undesired substrate

orientations and reduce their occurrence. The reactivity and selectivity of the pro-*RR* and pro-*SS* attack are predicted and quantified by using geometric criteria for conformations that approach the transition state structure (near attack conformations, NACs) (Figure 3.1). When applied to identify enantioselective epoxide hydrolases, only 37 variants were experimentally analyzed due to the previous *in silico* screening, which accelerated the discovery of improved variants.

The Iterative Protein Redesign and Optimization (IPRO) approach uses energy-based scoring functions to identify mutations in the parental sequences for the redesign of combinatorial protein libraries [77]. Several computational tools made use of the basic IPRO workflow, thus the IPRO suite was developed to share a common modular core of code that allows full integration of such protein engineering methods under a single interface [87]. The suite contains computational tools for the design of binding sites into existing scaffolds [88], novel regions for antigen-binding affinity [89], and improved enzymatic activity [90], as well as a database for antibody structure prediction and a Mutator program for predicting the properties of specific protein mutants [87].

The amount of data submitted to protein, enzyme, genome, and other databases is continuously growing, so software packages that help to handle and analyze such large amounts of information are needed. The bioinformatics tool 3DM (Bio-Prodict), for instance, integrates, and organizes information retrieved from heterogeneous databases allowing pin-point conservation and correlated mutations associated to specific subgroups within a superfamily. 3DM superfamilies group structures with a common structural fold. Since 3DM performs structure-based sequence alignments, it allows the comparison of proteins with sequence identity as low as ~8% [91]. Within this platform, the correlated mutation tool analyzes the coevolution of residues at different positions and creates networks of functionally related residues to be mutated in a coordinated manner [78]. The analysis and use of data retrieved from structure-based alignments restricts the protein sequence space and facilitates the design of short and smart libraries. 3DM has been used to enhance activity, enantioselectivity, and thermostability of different enzymes [92] such as sucrose phosphorylases [93], transaminases [94], and esterases [95–98]. An overview of computational tools that can be utilized for identification and prediction of hotspots is provided elsewhere [99].

3.2.4 *In Vivo* Continuous Directed Evolution

Continuous direct evolution allows a broader exploration of the sequence space in considerably less time than traditional evolution techniques [100]. A few years ago, Liu's group developed a Phage-Assisted Continuous Evolution (PACE) system that enables the evolution of the T7 RNA polymerase in *Escherichia coli* with minimal human intervention [101]. This system integrates replication, mutation, translation, and selection in a continuous and uninterrupted cycle [100, 102]. The method has also been applied for evolution of proteases [102] and has been modified to allow negative selection [103]. Although PACE results in

more than 100 theoretical rounds of evolution per week [101], its application is restricted to proteins related to expression. A review by Badran and Liu covering *in vivo* continuous directed evolution in different systems such as bacterial and eukaryotic cells discusses the advantages, drawbacks, and applicability of these methods [100].

3.2.5 Diversification of Enzyme Functionalities by Recombination

In contrast to site-directed and random mutagenesis, genome rearrangements by recombination and fusion generate hybrid proteins by combining secondary structural elements, which produce enzymes with a range of properties that may or may not be shared by the parents [104]. Engineering chimeric proteins in the laboratory is possible by mimicking natural evolution either by homologous [105, 106] or *illegitimate* recombination [12]. While the former involves closely related sequences, the latter includes nonhomologous or very distant sequences and is supported by model-building and computational design methodologies. An example of how recombination can be used to synthesize custom molecules is provided by modular polyketide synthases (PKS) [107]. A recent paper by Chemler *et al*. [106] describes the generation of functional chimeric forms of large multifunctional PKS enzymes, which were applied in the synthesis of tailor-made macrolactones and macrolides. Although some of the chimeric modular constructs were unstable, there are currently some available approaches that could be applied to improve further designs.

The number of unfolded and nonfunctional chimeras in a library may be reduced by minimizing the level of structural disruption, which is possible by choosing the crossover sections (recombination sites) [108]. Computational programs, such as SCHEMA [109–113] and the recently published Mutagenic Organized Recombination Process by Homologous IN vivo Grouping (MORPHING) [114], can be used to improve the design of functional protein chimeras.

MORPHING is a random domain mutagenesis/recombination method for segments of less than 30 amino acids. While *E. coli* is the most common host for molecular biology experiments, MORPHING takes advantage of the high frequency of homologous recombination in *Saccharomyces cerevisiae*, allowing a one-pot construction of mutant libraries. Using this method, libraries can be prepared with different mutational loads and can be assembled into the remaining unaltered DNA regions *in vivo* with high fidelity. This strategy has been used for evolution of peroxidases, peroxygenases, and an aryl-alcohol oxidase [114–116].

Another strategy to alter functional properties is circular permutation in which the amino and carboxyl termini of a protein are covalently linked by a peptide linker and new termini are relocated in the protein structure by disruption of a peptide bond [117–120].

A different approach for the rational design of protein chimeras was proposed by the Höcker lab, which is based on a combinatorial assembly of small, intrinsically

stable structural subunits (the smallest stable blocks domains are built of) [121]. The group has developed several folded and functional proteins, which highlights the potential of recombining natural protein subunits with unique properties to create new scaffolds for enzyme design [121–123].

3.3 High-Throughput Screening Systems

Having an appropriate screening methodology is still the bottleneck of direct evolution experiments, although novel and improved methods that explore a higher number of variants have been reported over the last years, for example, microfluidic and flow cytometry screening formats. With such approaches, it is possible to analyze up to 64 million variants (from six fully randomized amino acids) generated by targeted mutagenesis, which is sufficient to considerably change enzyme properties such as activity and selectivity by reshaping the catalytic pocket [124]. Methods based on flow cytometry, such as fluorescence-activated droplet sorting (FADS) [125] and fluorescence-activated cell sorting (FACS), have been extensively used for screening in directed evolution experiments. They rely on the transduction of a given biological property into quantifiable fluorescent signals by using different proteins, dyes, or probes. Hence, mixtures of samples can thus be sorted according their fluorescence output signal.

Two libraries of mutants of the horseradish peroxidase (HRP) were generated by epPCR and active-site-targeted saturation mutagenesis starting from the wild-type *HRP* gene that was C-terminally fused to the *Aga2* gene. The fusion allowed surface display of the HRP in yeast. Upon transformation of the libraries, single yeast cells and a nonfluorescent substrate were co-encapsulated in emulsion droplets and screened by FADS at the rate of thousands per second. The most active clones from the first generation were mutated again to create a new library of $\sim 10^7$ variants, finding a mutant 12 times faster than the wild type. In general, $\sim 10^8$ individual HRP reactions were screened in only 10 h, using <150 µl of total reagent volume, which was 1000-fold faster than robotic based-screening systems and 1 million times cheaper [126].

Recently, a screening method based on the detection of improved phosphotriesterase mutants from a library containing 5×10^5 epPCR generated clones was reported. Single cell compartmentalization (gel-shell beads, GSBs) was followed by FACS-based screening and selection of $\sim 10^7$ GSBs beads per hour [127]. Briefly, single *E. coli* cells bearing the expressed phosphotriesterase and its encoding plasmid are trapped into water-in-oil emulsion droplets (Figure 3.2a,b). The aqueous solution contains agarose and polyanion alginate, so that a solid bead forms inside the droplet when the temperature is dropped from 30 °C to 4 °C. Droplets also contain the phosphotriesterase substrate and a lysis agent. Once the cells are lysed, the released enzyme reacts with the substrate at 30 °C and subsequently the enzyme is heat-inactivated at 90 °C (Figure 3.2c). After de-emulsification of the system in the presence of the polycation PAH, the

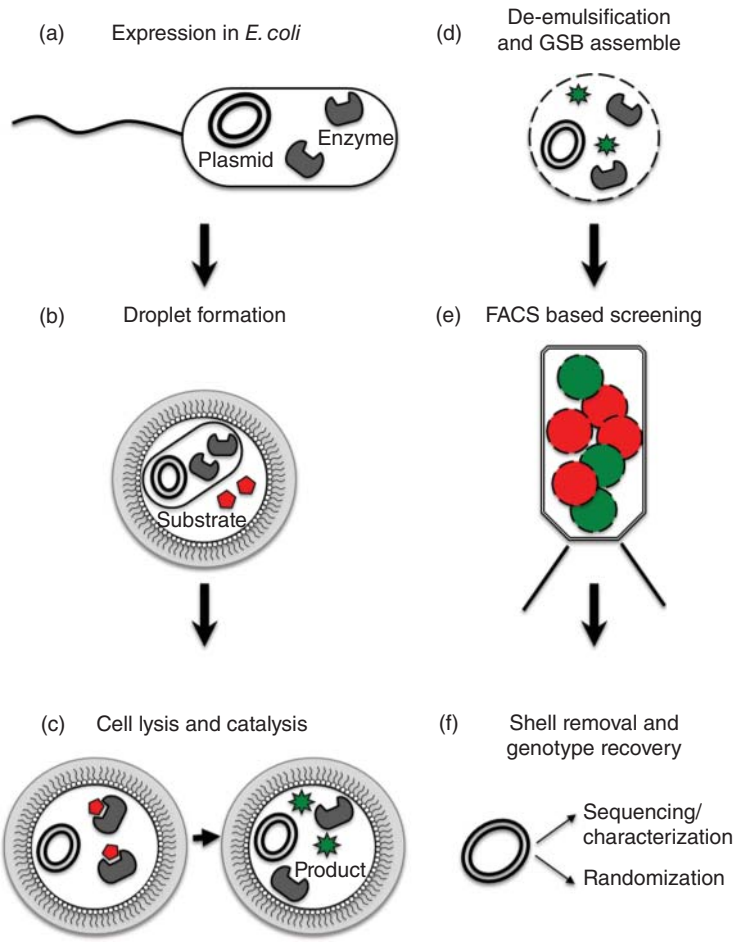


Figure 3.2 Directed evolution in biomimetic gel-shell beads (GSBs). Single *E. coli* cells expressing the phosphotriesterase (a) are trapped into water-in-oil emulsion droplets that contain the phosphotriesterase substrate and a lysis agent (b). The enzyme reacts (if active) with the substrate at 30 °C, releasing a fluorescent product (c). After de-emulsification, the GBSs are formed retaining the enzyme, its encoding plasmid, and the reaction product (d). GBSs are sorted by fluorescence based on the variants' activity (e), and the phosphotriesterase encoding plasmid is recovered after removal of the polyelectrolyte shell by raising the pH (f). Isolated variants are characterized or subjected to further rounds of evolution.

polyanion alginate diffuses at the opposite direction to PAH and a polyelectrolyte complex surrounding the agarose beads is formed. GSBs retain the enzyme, its encoding plasmid, and the reaction fluorescent product, which allows enzyme variants to be selected by FACS based on their activity (Figure 3.2d–f) [127].

A protein-function-independent screening system (not based on activity or specific binding) named Hot-CoFi was applied to 10 proteins having different melting

temperatures and functions, aiming at the identification of more thermostable variants [128]. Hot-CoFi identifies temperature-induced aggregation and unfolding of overexpressed proteins inside the cell. Transformed libraries are plated, and after growth the colonies are transferred to a filter membrane and incubated onto plates containing the inducer for protein expression. Following expression, the plates are incubated at different temperatures (20–80 °C) and the colonies lysed afterwards. The filter membrane allows diffusion of soluble protein onto a nitrocellulose membrane, enabling its detection using a HisProbe-HRP, which specifically detects his-tagged proteins, with strong signals correlating with high solubility. Ninety five percent of the selected clones showed improved stability after one round of mutagenesis, with median stabilization of 9 °C (thermostability was improved by 26.6 °C for the best mutant). Hot-CoFi allows evolution in parallel of several targets with a throughput of up to 100 000 clones per experiment and may be applicable to any protein expressed in colony-forming microorganisms.

Many high-throughput screening methods for catalytic asymmetric synthesis have also been published, such as solid-phase assays [129] and mass spectrometry-based methods [130].

3.4 Engineered Enzymes for Improved Stability and Asymmetric Catalysis

Molecular biology has been successfully applied in the case of a number of enzymes aiming for activity, specificity, and stability improvements with remarkable results in some cases. The thermal and solvent stability of the carbonic anhydrase from *Desulfovibrio vulgaris*, for instance, was dramatically improved by directed evolution, rendering variants that exhibit 4 000 000-fold stability improvement over the parental enzyme, finding thus potential application in industrial carbon capture processes [131]. Among the vast number of enzymes that have been improved over the last years, we chose industrially relevant biocatalysts such as lipases, cellulases, and the monooxygenase cytochrome P450 to demonstrate the applicability of the protein engineering approaches mentioned above.

3.4.1 Stability

Enzyme stability and resistance to organic solvents are two of the most significant parameters to take into consideration when tailoring an enzyme for industrial application or bioremediation. Furthermore, thermostability is usually a desirable parameter in directed evolution experiments since it correlates with mutational robustness [132]. Operating enzymes at high temperatures offers many advantages in bioconversion processes, such as faster reaction rates, higher substrate solubility, increased solvent miscibility, lower solution viscosity, and reduced risk of system contamination [133]. Methods for stabilization include insertion

of protein domains into stable scaffolds [134], stabilization of flexible regions, consensus-based approaches, introduction of stabilizing mutations in regions that initiate unfolding, and identification and modification of the active site access tunnels [84].

Cellulases and lipases are two of the most widely used groups of enzymes in biotechnological transformations for which thermal stability is often a requirement. In this section, some examples involving these enzymes are presented.

3.4.1.1 Cellulases

Lignocellulosic materials have a huge potential for the production of biofuels and other biomolecules from nonedible renewable sources. Despite current drawbacks related to the costs, high energy input, and harsh pretreatments required to break down the complex structure of the cell-wall polymers before enzymatic conversion of the substrate into fermentable sugars [135], ethanol production from lignocellulosic materials offers an opportunity to reduce greenhouse gas emissions by 90% compared to gasoline [136]. Enzymatic hydrolysis of cellulose involves the synergistic action of three main activities: chain-end-cleaving cellobiohydrolases (CBH), internal chain cleaving endoglucanases, and β-glucosidases, which hydrolyze soluble short-chain glucooligosaccharides to glucose [136]. To be applied under non-natural industrial environments, these enzymes have to be modified such that their activity and stability meet the process requirements [137]. Thermostability of Cel8A, an endoglucanase produced by the anaerobic thermophilic bacterium *Clostridium thermocellum*, was enhanced via the consensus approach. A library combining multiple consensus mutations in different combinations was designed following the alignment of sequences from mesophilic bacteria sharing 30%–60% identity. The variant G283P showed an increase in melting temperature (T_m) of 3.5 °C [71]. Introducing this change to a previously reported triple mutant [68] increased the half-life of the enzyme by 14-fold at 85 °C.

In a different approach, the regions that initiate the partial unfolding ("weak spots") of the endoglucanase Cel7B from *Trichoderma reesei* were identified by molecular dynamics (MD) simulation [138]. Local melting temperature was assigned to individual residue pairs, and a total of eight disulfide bonds were designed in the selected regions (lower T_m), all of them enhancing the endoglucanase thermostability. Disulfide bonds enhance the kinetic stability of a protein by increasing the activation energy barrier of unfolding and can also enhance stability by decreasing the entropy of the unfolded state [133]. The best variant showed a T_{50} (the temperature at which 50% of the activity is lost) of 62.8 °C, compared to 54.6 °C of the wild type [138].

Recently, Arnold's group created a highly thermostable endoglucanase derived from a class II endoglucanase Cel5A by combining 16 stabilizing mutations that were identified by using the consensus approach, chimeragenesis (homologous recombination), and various structure-based computational methods. The variant has an optimal temperature 17 °C higher than that of the wild-type enzyme [72]. A synergistic mixture of this enzyme and the engineered thermostable variants of

the fungal CBHs Cel6A and Cel7A [72, 73] hydrolyzes cellulose at 70 °C to produce 3 times more total sugar than the best mixture of the wild-type enzymes at its optimum temperature (60 °C).

FoldX and the consensus approach were used to identify individual mutations present in five homologous fungal CBH I (Cel7A) cellulases that were used as parents to generate stable chimeras by SCHEMA structure guided recombination [73]. FoldX evaluates the effect of a mutation on protein stability ($\Delta\Delta G_{\mathrm{FoldX}}$) based on its high-resolution 3D structure using an atomic force field with empirically determined coefficients [33]. The most thermostable chimera has a T_{m} that is 9.28 °C higher than that of the most stable parent (*Talaromyces emersonii*), which resulted in a 10.8 °C increased optimal temperature and a 50% increased total sugar production from crystalline cellulose.

The β-glucosidase A from *Bacillus polymyxa* was stabilized by introducing ion pairs that were computationally and experimentally designed. The best variant has a T_{m} 15.7 °C higher than that of the mesophilic wild-type enzyme [139].

Xylanases also contribute to the biodegradation of lignocellulosic biomass and are used in biofuels production, as well as in other industrial processes such as pulp bleaching and baking. Based on a 3D model, PoPMuSic was used to identify potential hotspots to stabilize a xylanase from *Aspergillus niger*, and the most flexible region (the first 87 residues of the protein) was randomized by epPCR. Five single variants exhibited higher thermostability, thus these positions were targeted for stepwise ISM evolution. A quintuple mutant showed a 30-fold increase in half-life at 60 °C, and its T_{m} increased by 17.4 °C due to the mutations' synergistic effects [140]. The thermal stability of other xylanases has also been improved up to 10 times by using a combination of computational tools [141].

3.4.1.2 Lipases

Lipases are members of the α/β-hydrolase fold superfamily and constitute one of the most important groups of enzymes for industrial applications. They catalyze the hydrolysis of long-chain triglycerides at the interface between substrate and water and are used in the food, pharmaceutical, chemical, and detergent industries. *Rhizopus* lipases are widely used in the food industry but they are generally produced by mesophilic organisms and exhibit low thermal stability. The stability of *Rhizopus chinensis* lipase (RCL) was improved after two rounds of epPCR followed by two rounds of DNA shuffling. The mutant library was constructed in *Pichia pastoris* based on the formation of a recombination cassette *in vivo*. The most thermostable variant has a T_{m} that is 22 °C higher than the parental enzyme and 46 and 23 times longer half-lives at 60 °C and 65 °C, respectively [142]. In a different approach, RCL was stabilized via disulfide bond design at the hinge region of the lid domain based on predictions supported by the "Disulfide by Design" algorithm and a 3D structural model. The variant's half-life increased 11-fold at 60 °C and its T_{m} by 7 °C [143].

Two hyperthermophilic lipases were designed by recombination of distant sequences from three enzymes that belong to different families within the same superfamily (α/β-hydrolase). The strategy focused on structural modules instead

of flexible loops between domains. The hyperthermophilic carboxylesterase AFEST from *Archaeoglobus fulgidus*, the hyperthermophilic peptidase apAPH from *Aeropyrum pernix* K1, and the mesophilic lipase Lip1 from *Candida rugosa*, which share sequence identities of less than 21%, were used as parental enzymes to create LAf and LAp, the chimeric versions of a lipase/esterase and a lipase/peptidase, respectively. The crossover sections were identified by using the MEME server and the PROMOTIF program. Both chimeras exhibited more than 100-fold increased thermostability at 50 °C when compared to the mesophilic lipase [144].

Candida antarctica lipase B (CalB) is one of the most widely used enzymes in biocatalytic processes. CalB saturation mutagenesis libraries were created for six residues located within 10 Å of its catalytic serine (Figure 3.3) that show high B-factor values and screened for thermostability. L278M and D223G showed higher thermal stability, and therefore the variant L278M was used as a template for ISM at the other five positions. The double mutant L278M/D223G showed the highest thermostability, and ISM at the other four positions did not result in further stabilization. Seven additional residues with the highest B-factor values were also chosen for iterative ISM without any apparent thermostability improvement (Figure 3.3). Analysis of the 3D structure of the double mutant and molecular dynamic MD simulations showed that mutations D223G/L278M increased the active site rigidity, which resulted in a 13-fold increase in half-life at 48 °C and a 12 °C higher T_{50} (after 15 min heat treatment) without affecting the enzyme catalytic efficiency. L278M, D223G, and D223G/L278M also showed better chemical stability than the wild type against urea inactivation. Interestingly, the variants A281F and I285F showed a fivefold activity increase compared to wild-type CalB [145].

Directed evolution has been also used to improve enzymes' stability against pH and solvents. For instance, lipases are promising biocatalysts for biodiesel production by transesterification of triacylglyerol with methanol, but show low tolerance

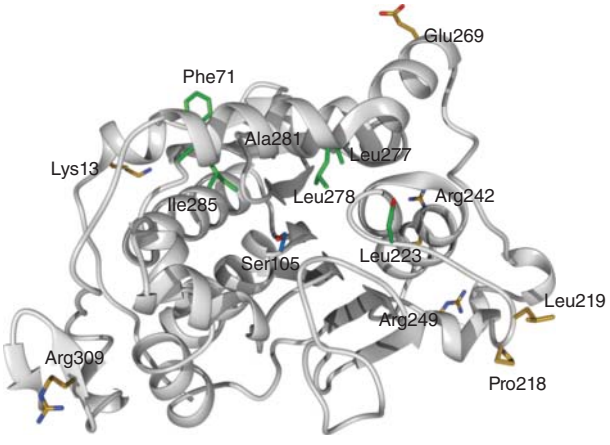


Figure 3.3 CalB residues selected for iterative saturation mutagenesis (ISM).

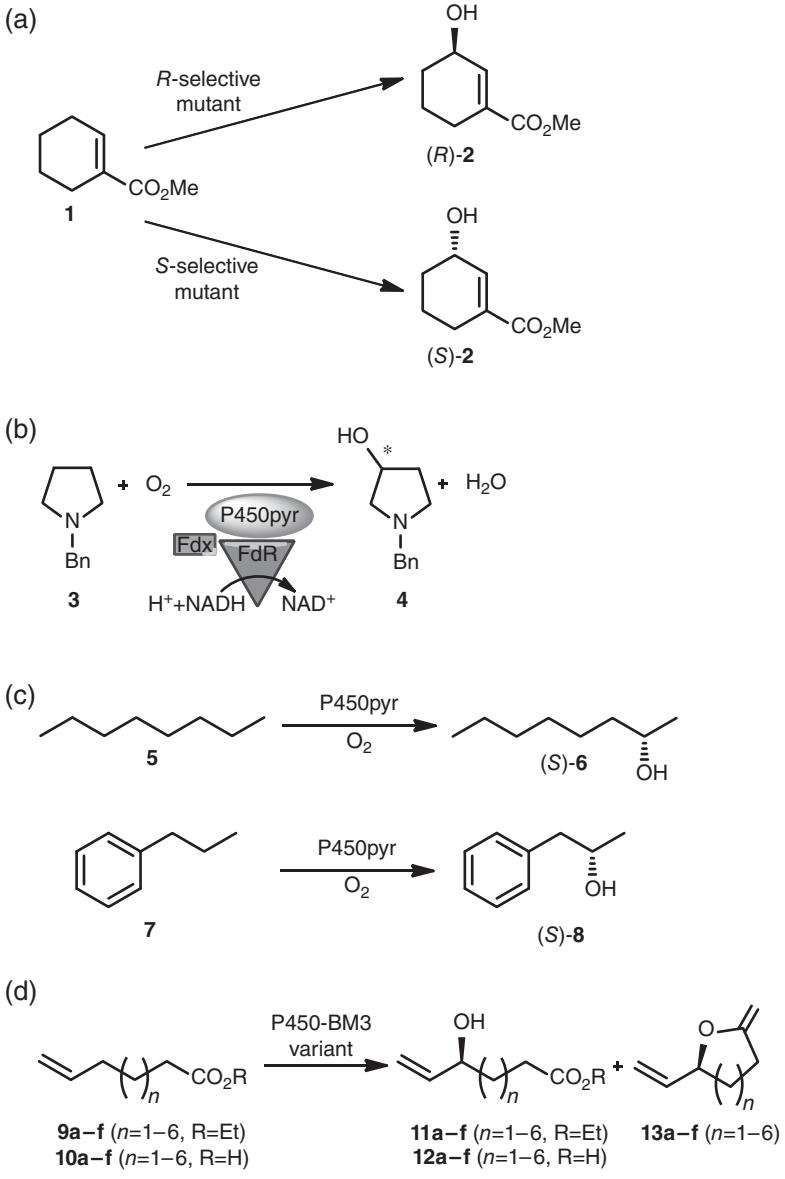
to high methanol concentration. A variant of the lipase from *Proteus mirabilis* with a newly introduced disulfide bond was used as a template to create a library by epPCR. After three rounds of random mutagenesis combined with site-directed recombination, a variant that exhibited 30-fold increased thermal stability was identified. When immobilized, the variant showed improved longevity over several cycles of diesel production when compared to both the parental enzyme and the industrially used *Brukholderia cepacia* lipase [146].

3.4.2 Asymmetric Biocatalysis

The conversion of an achiral substrate into a chiral product favoring the formation of one of the stereoisomers (asymmetric catalysis) [147] has a preponderant role in synthetic organic chemistry for the production of fine chemicals and chiral pharmaceuticals intermediates.

Aiming to obtain maximum benefit from the use of enzymes in organic synthesis, Turner and O'Reilly proposed the concept of "biocatalytic retrosynthesis," in which the target molecules are disconnected into smaller building blocks based on (i) the availability of biocatalysts, chemical catalysts, and reagents for the bonding forming steps and (ii) the feasibility of enzymatic synthesis of the identified building blocks [148]. The chemoenzymatic synthesis of perindopril (angiotensin 1-conversion enzyme inhibitor) and atorvastatin (Lipitor, a cholesterol-lowering drug with annual sales exceeding $10 billion), which includes engineered enzymes, illustrates this concept. Therefore, engineered biocatalysts are ideal candidates both for the production of final products and the synthesis of individual synthons to be later incorporated into new enzymatic or chemoenzymatic synthetic routes. Modified enzymes have been applied for asymmetric synthesis, kinetic resolution of racemates, and desymmetrization of prostereogenic compounds [149].

Regio- and stereoselective hydroxylation at a non-activated carbon atom still remains a challenge in classical chemistry, and there are numerous reactions in organic synthesis that cannot be performed by natural enzymes, that is, the isoelectronic carbine transfer to olefins (C–C bond forming reaction) or intramolecular C–H amination [150–153]. Engineered monooxygenases such as cytochrome P450 are now able to perform such reactions, while the native enzymes often show a limited substrate range and poor regio- and stereoselectivity [150]. Cytochrome P450 enzymes catalyze oxidative transformations such as hydroxylation, epoxidation, oxidative ring coupling, heteroatom release, and heteroatom oxygenation [152], and are one of the most engineered systems for asymmetric synthesis [151]. P450-BM3 was modified by CASTing/ISM to achieve regio- and enantioselectivity in the oxidative hydroxylation of cyclohexene-1-carboxylic acid methyl ester **1** (Scheme 3.1a) [154]. Twenty-four residues at and near the active site were chosen for saturation mutagenesis, and the best variant was used as a template in ISM experiments randomizing 3 of the 24 selected residues. While wild-type



Scheme 3.1 (a) P450-BM3 hydroxylation of cyclohexene-1-carboxylic acid methyl ester. (b) P450pyr hydroxylation of *N*-benzyl pyrrolidine. (c) P450pyr regio- and enantioselective subterminal hydroxylation of alkanes. (d) P450-BM3 (A74G/L188Q) llylic hydroxylation of ω-alkenoic acids and esters.

P450-BM3 is 84% regioselective for the allylic 3-position with 34% enantioselectivity in favor of the *R* alcohol, several variants showed >95% ee (*R* or *S*, **2**).

P450pyr hydroxylase from *Sphingomonas* sp. HXN-200, which requires ferredoxin (Fdx) and ferredoxin reductase (FdR) for electron transfer, was modified by following a similar strategy. Twenty residues were subjected to saturation mutagenesis and the best mutant was used as template for the next round of evolution by randomizing the other positions. A triple variant reached ≥98% ee in the hydroxylation of *N*-benzyl pyrrolidine **3** to produce (*S*)-*N*-benzyl 3-hydroxypyrrolidine **4** using molecular oxygen as a green oxidant (Scheme 3.1b) and showed nearly the same catalytic efficiency as wild-type P450pyr [151]. The native enzyme produces **4**, which is a useful pharmaceutical intermediate, with only 53% ee.

ISM was also applied to evolve P450pyr for the sub-terminal hydroxylation of *n*-octane **5** and propylbenzene **6** (Scheme 3.1c) [150]. The products (*S*)-2-octanol (**7**) and (*S*)-1-phenyl-2-propanol **8** are useful as inhibitors of a lipoprotein-associated phospholipase (treatment of heart disease) and as an intermediate in the preparation of amphetamine. A variant bearing six mutations, which was created after six rounds of evolution, showed >99% sub-terminal selectivity and synthesized (*S*)-2-octanol with 98% ee. This was the first enzyme reported to show highly selective alkane hydroxylation. Another multiple mutant exhibited 98% sub-terminal selectivity for the synthesis of **8** with 95% ee.

In another remarkable example, libraries of P450-BM3 carrying mutations at five positions within the active site were constructed. Three of these positions were subjected to saturation mutagenesis [155]. A double mutant catalyzes allylic hydroxylation of *ω*-alkenoic acids and their esters (**9** and **10**) to synthesize *S*-*ω*-2-hydroxy-*ω*-alkenoic products (**11** and **12**) (Scheme 3.1d), which are building blocks for the synthesis of biologically active compounds, with unprecedented enantioselectivity of up to >99% ee.

P450 catalysts have also been successfully evolved for the hydroxylation of molecules such as artemisinin [156], testosterone [157, 158], 1-tetralones [159], and erythromycin intermediates [160]. In two excellent pieces of work, P450 was modified to catalyze the enantioselective intermolecular nitrene transfer to olefines to produce aziridines [153] and the diastereo- and enantioselective cyclopropanation of styrenes from diazoester reagents via putative carbine transfer [152, 161]; both are synthetically important reactions with no natural biological counterpart. A comprehensive look at P450 evolution and engineering for catalysis of natural and non-natural chemistry is provided elsewhere [10, 162].

Besides P450 catalysts, a number of other engineered enzymes have been applied in the production of enantiomerically pure compounds with impressive results, for example, epoxide hydrolases [163], amine dehydrogenases [81], monooxygenases [164], transketolases [82], lactonases [17], phosphotriesterases [41] phenylalanine ammonia lyases [165], imine reductases [166], lipases[40], and transaminases [94]. Variants of the amine monooxidases have been used in the synthesis of pharmaceutical building blocks and alkaloid natural products on a preparative scale [167–169]. Transaminases are an important biocatalytic tool in the production of chiral amines precursors such as those involved in the synthesis

of sitagliptin (antihyperglycemic drug for the treatment of diabetes), dilevalol (antihypertensive), and (*S*)-rivastigmine (used for the treatment of Alzheimer's disease) [19, 94]. A recent review by O'Reilly and Turner provides an overview of this topic [170].

3.5 *De Novo* Design of Catalysts: Novel Activities within Common Scaffolds

There are several reactions for which no enzymatic synthesis has yet been reported. Efficient design of tailored activities remains a challenging test for the current understanding of how proteins fold and function and defies current knowledge of enzyme catalysis [171, 172].

Over the last two decades, different protein structural elements were designed with increasing accuracy owing to the development of better molecular and computational tools [173–175]. The next challenge was the design of enzymes that were able to catalyze new reactions. Enzymatic catalysis requires protein binding sites able to accommodate the substrates in productive orientation, stabilize the transition state, and finally release the products, enabling in this way the enzyme for a new catalytic cycle [176]. Rosetta is a set of computational tools that has been successfully used for protein–structure and protein–protein complex prediction as well as for protein design [177] (Figure 3.4). Experimentally generated data (often from protein engineering and structural experiments) provides feedback to computational protein design and contributes to the identification of sequence features that may have not been covered by computational approaches alone.

Kemp elimination, a model reaction for proton transfer from carbon (Scheme 3.2), is a well-studied reaction for which several attempts to generate an enzyme-like catalyzed reaction have been reported. Quantum mechanics and the Rosetta software were used to generate the first computationally designed enzymes for Kemp elimination and retro-aldolase activity (Scheme 3.3) [172, 178]. Later on, other enzymes able to catalyze Kemp elimination as well as other reactions were created and their performance improved by directed evolution. Turnover numbers of the Kemp-elimination-catalyzing enzymes using 5-nitrobenzisoxazole **14** as substrate were enhanced from <1 to 700 s^{-1}, a value higher than that of some natural enzymes and the highest Kemp elimination activity reported for a computationally designed enzyme so far [180–182].

The catalytic efficiency of the artificial retro-aldolase, which was reported to be the most challenging *de novo* designed enzyme from the mechanistic point of view, was improved from 0.02 to 850 μ^{-1} s^{-1} for the synthesis of acetone **17** from 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone **16**. The catalytic efficiency of the most active variant is close to that of some natural enzymes, and the turnover number is higher than that of the commercially available aldolase antibody 38C2 (170×10^3 and 12×10^3 s^{-1} respectively) [42, 178, 183].

The cycloaddition between a conjugated diene **18** and an alkene **19** to form a substituted cyclohexene system **20** is known as the Diels–Alder reaction

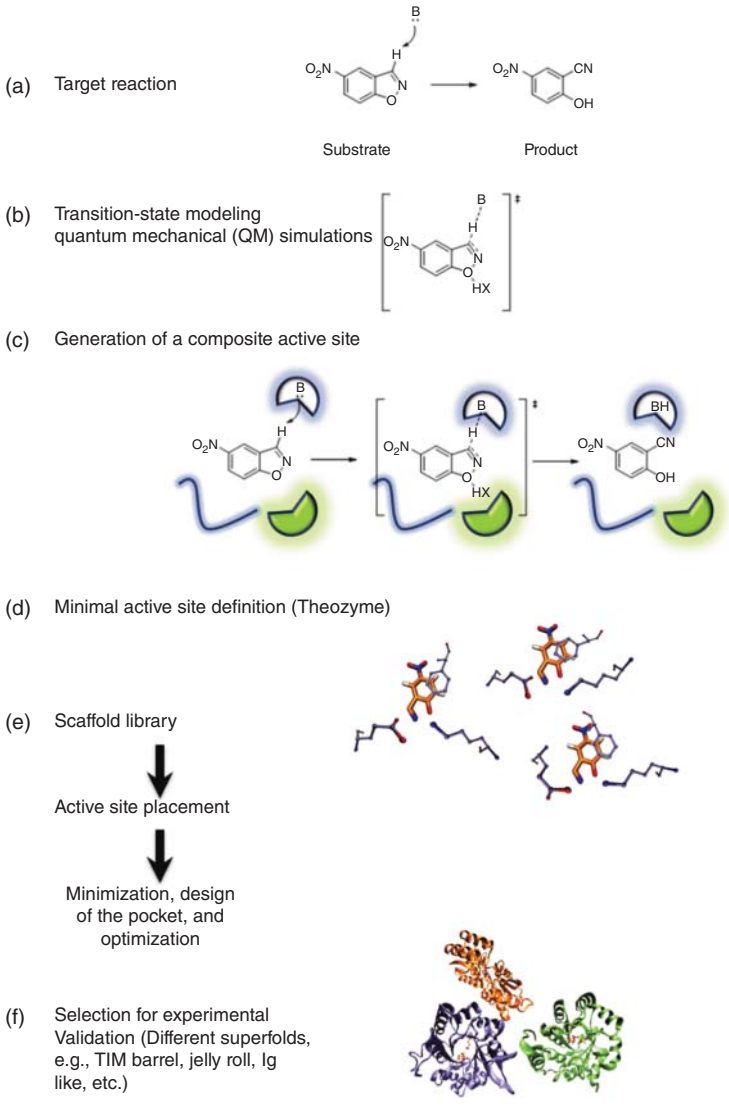
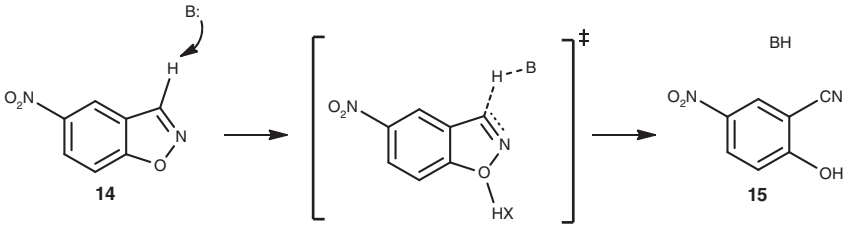
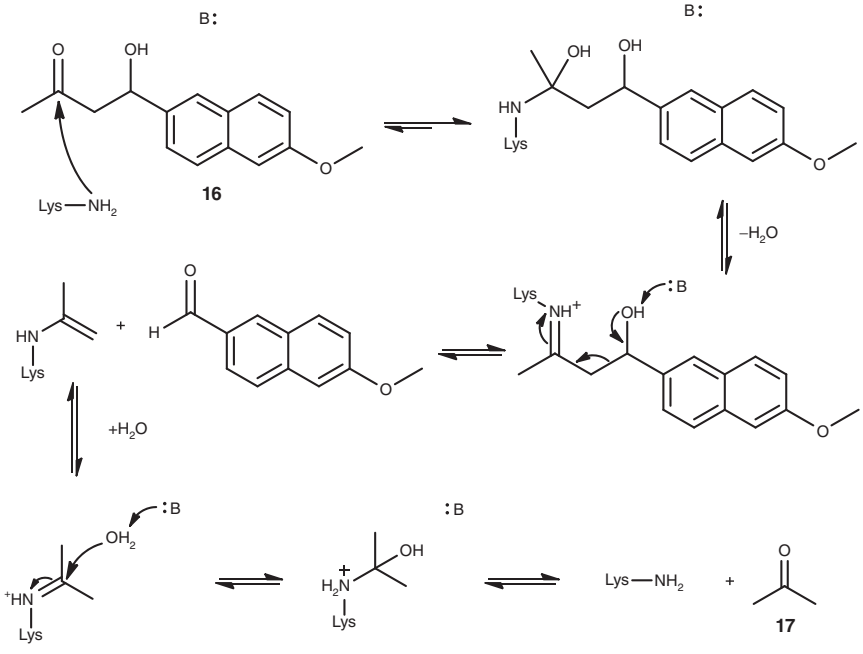


Figure 3.4 General scheme for computational enzyme design using Rosetta. (a) A target reaction and its reaction mechanism are chosen; (b) key intermediates and the transition state (TS) are modeled in the context of a given binding pocket; (c) models are overlaid based on the protein functional group positions to create an idealized active site that can accommodate each state (namely substrates, TS and product); (d) active site models (theozymes) are computed with catalytic residues placed around the optimal geometry for the composite TS. Large ensembles of different conformations of these composite active sites are generated by varying the degrees of freedom of the composite TS, the orientation of the catalytic side chains regarding the composite TS, and the internal conformation of the catalytic side chains; (e) protein backbone positions able to hold such an idealized active site are searched among high-resolution crystal structures with ligand-binding pockets. Matches are optimized, including neighbor residues shaping the binding pocket; (f) best ranked designs are chosen for experimental validation [171, 172, 178, 179].

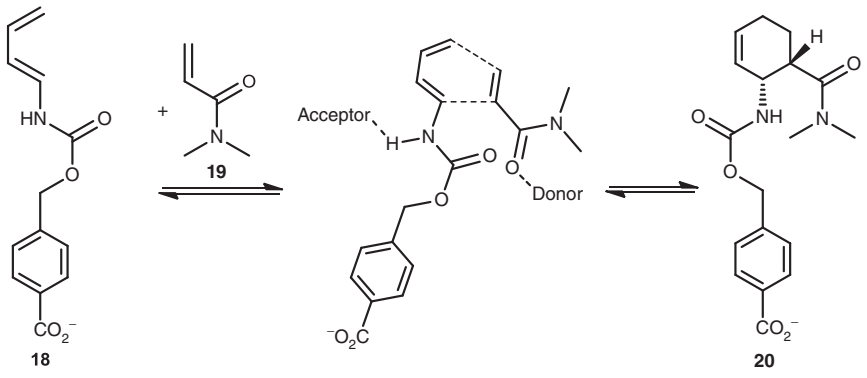


Scheme 3.2 Kemp elimination.



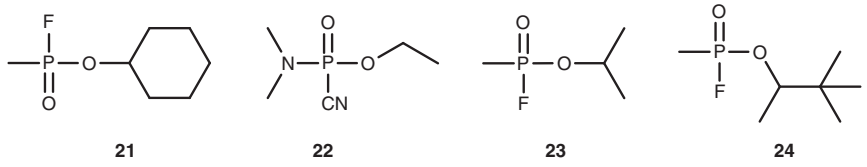
Scheme 3.3 Retro-aldol reaction. The retro-aldol reaction is initiated by a nucleophilic lysine, which forms with the substrate **16** a covalent enzyme–substrate imine complex. Fragmentation is followed by deprotonation of the hydroxyl group with a base, and the imine is then hydrolyzed to yield **17**.

(Scheme 3.4) and is considered one of the most versatile organic transformations for the synthesis of a range of products such as therapeutic agents and several kinds of synthetic materials [184]. Although several modified antibodies [184] and RNA [185] were reported to catalyze Diels–Alder reactions, natural enzymes able to catalyze such transformations are unknown. Following computational refinement and laboratory evolution, the catalytic efficiency of the best artificial Diels–Alderase was improved by ~9000-fold compared to the first designed enzyme for the reaction between *E*-4-carboxybenzyl-1,3-butadiene-1-carbamate and *N*,*N*-dimethylacrylamide [179, 186, 187].



Scheme 3.4 Diels–Alder reaction. Diene (**18**) and dienophile (**19**) undergo a pericyclic [4 + 2] cycloaddition to form a chiral cyclohexene ring (**20**).

Some enzymes with potential medical and therapeutic application were also computationally designed. Organophosphates (OPs) are nerve agents that continue to concern military personnel and civilians as potential battlefield and terrorist threats [188]. OPs, which exert biological action by covalently binding a serine residue in the catalytic site and inactivating acetylcholinesterase (AChE), are used in agriculture and have been associated with several cases of intentional and accidental misuse. The OPs known as G-agents (cyclosarin **21**, tabun **22**, sarin **23**, and soman **24**) (Scheme 3.5) are among the most toxic chemical warfare agents [189]. An enzyme for OP hydrolysis was designed within the zinc-containing mouse adenosine deaminase scaffold using the Rosetta software. After directed evolution, the best variant showed a 2440-fold increase in activity compared to the originally designed enzyme.



Scheme 3.5 Chemical structures of the G-type nerve agents.

Although some computationally designed enzymes exhibit real catalytic efficiencies that are close to those of natural enzymes, such activities are usually the result of additional adjustments and refinements at the active sites by laboratory evolution, since a precise design is still beyond the current capabilities of computational methods [182]. Continuous feedback between theoretical and experimental work is imperative to improve the prediction capacities in the future [176, 178]. The reader is referred elsewhere for detailed reviews on computational protein design [27, 190].

3.6 Conclusions

Altering substrate specificity as well as enantioselectivity and regioselectivity is a long-standing goal in protein engineering.

Here we reviewed different approaches for the design, redesign, and identification of enzymes with improved or new functionalities, which include molecular, computational, and combinatorial methods as well as wet-lab screening. Laboratory evolution has shown to be the most successful approach to access better biocatalysts, although many lessons remain to be learned from natural evolution.

The production of molecules for which no biological catalyst is known or those that are produced inefficiently by naturally occurring enzymes represents even a bigger challenge. A combination of computational design and directed evolution has given place to a new generation of enzymes able to catalyze reactions for which no enzymatic synthesis had been reported. These artificial enzymes exhibit better catalytic efficiencies than those from previous designs, which opens the door to a more amenable synthesis of difficult compounds. Although structural biology is still far from getting the final answer to some critical interrogations and faces computational and experimental challenges, the design of new folds and functions from scratch opens new ways to explore functions that have not yet been observed in nature.

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4
Biocatalytic Process Development

John M. Woodley

Biocatalysis uses the power of enzymes to catalyze chemical conversions. The biocatalyst itself can potentially be used in several different formats, including isolated enzyme, immobilized enzyme, or contained within microbial cells. The enzymes can be used individually or as part of a network or pathway, in parallel or sequentially. Despite the many options, the process concept itself is, nevertheless, relatively simple, as shown in Figure 4.1.

Today, several hundred application examples of biocatalysis can be found in numerous industrial chemical sectors, and the rationale used for its introduction depends on the product value in a given sector (Table 4.1). The majority (by number) of processes implemented to date have focused on the pharmaceutical sector, in large part due to the high turnover of target molecules, as a result of high attrition rates and patent-limited lifetimes, as well as the complex nature of the synthetic routes and the target molecules [1]. Many of the target molecules contain multiple chiral centers, where biocatalytic conversion is particularly well suited. Nevertheless, today biocatalytic synthesis has developed to such an extent that many new opportunities are forthcoming [2], and examples can increasingly be found in the fine chemicals sector (in particular flavors and fragrances).

The transfer of a given biocatalytic reaction from a synthetically valuable laboratory tool into a commercially viable industrial process requires process development, scale-up, and process implementation to be carried out in an effective and systematic manner. Compared to chemical processes, there has, to date, been relatively little attention given to this aspect of biocatalysis [3]. Additionally, the required process development and scale-up need to be carried out within a regulatory and economic context appropriate for the given product and market. In this chapter, a systematic approach to biocatalytic process development will be discussed, and two examples are used to illustrate the methods and tools employed.

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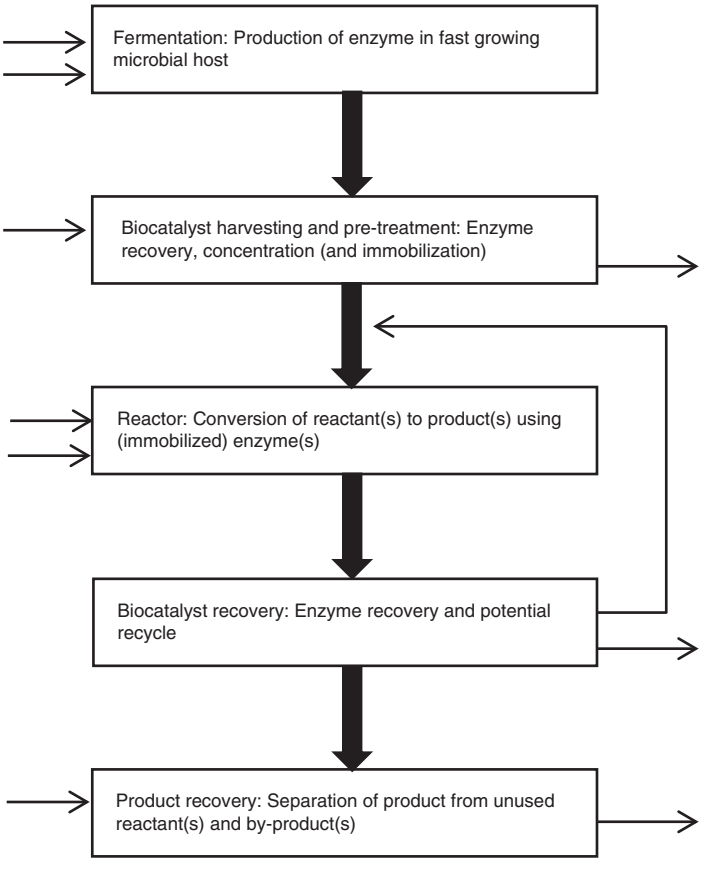


Figure 4.1 General biocatalytic process using (immobilized) enzymes for the production of chemicals.

Table 4.1 Rationale for introduction of biocatalytic processes.

Industry sector	Rationale for introduction of biocatalysis
Pharmaceuticals	• Eliminate protection and deprotection steps in syntheses • Synthesis of optically pure molecules containing multiple chiral centers
Fine chemicals	• Replacement of hazardous and difficult syntheses • "Natural" syntheses
Bulk chemicals	• Use of alternative "greener" feedstock • Add value to existing syntheses by forward and backward integration

4.1 A Structured Approach to Biocatalytic Process Development

The implementation of any new process in industry is, of course, first and foremost dependent upon a suitable economic return on investment. This means that even at an early stage of process development it is essential to consider the costs of potential manufacturing. Clearly, this is also necessary to consider when implementing a new biocatalytic process and can be used to establish a first basis for scalability. However, for biocatalytic processes, the conditions used in a manufacturing plant (e.g., high concentrations of reagents in the molar range) are far from those found in nature (e.g., low concentrations of reagents in the micromolar range). This is not surprising since enzymes and microbial cells have evolved to operate very efficiently at low concentrations. The consequence is that for any new biocatalytic process it is necessary to develop the biocatalyst and adapt the process such that they are matched to the requirements of the relevant industry sector. This means that not only must the potential manufacturing cost of a product be considered but also the cost of biocatalyst and process development must be taken into account. Indeed, it is highly advantageous at an early stage to consider whether such extensive development can even be justified. In some industry sectors, the timeline required to deliver the product may also determine the outcome of such a decision. One of the challenges of assessing potential development and manufacturing costs at an early stage is that an estimate of the potential for process improvement needs to be made, and for biocatalytic processes this is particularly difficult because not only are process technologies available to improve the process (and overcome biocatalyst limitations) but also a vast array of biological techniques for improvement of the biocatalyst exist. Improvements to the biocatalyst can be very substantial, making an estimate of the potential improvement difficult. For example, genetic engineering (involving improved expression of the enzyme of interest or changing the host organism) as well as protein engineering (involving improved properties to better match application conditions) offers the opportunity to improve the biocatalyst and its associated production process. This implies also that an assessment of the feasibility of a potential biocatalytic route is more about assessing the feasibility of development than manufacturing itself. In addition to the particular focus on biocatalyst modification and improvement, there are some basic economic requirements that also need to be met to enable implementation.

4.2 Process Metrics

The economic requirements are best described in terms of process metrics, and can very helpfully be used to provide targets to meet given economic demands. In this way, the metrics can be used either to exclude a route from further consideration or help define a suitable development strategy. A potential development

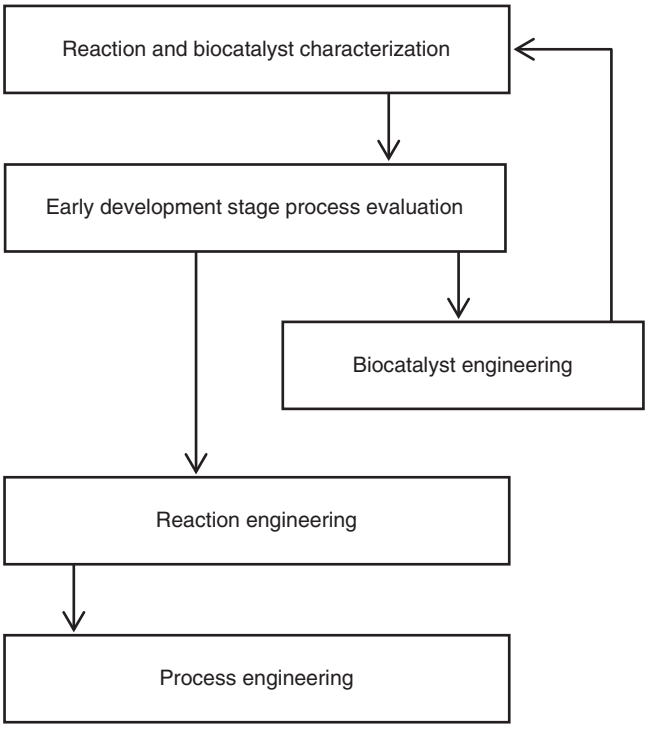


Figure 4.2 Proposed methodology for the systematic development of biocatalytic processes.

methodology is outlined in Figure 4.2. The metrics are determined by the main economic and cost drivers and will be described in the following sections.

4.2.1 Reaction Yield

In order that the process itself is economically feasible, it is a primary requirement that the reaction step itself adds value to the starting material. The simplest way to calculate this is to ensure that the value of the product exceeds the value of the feedstock and other raw materials and reagents. However, while this is a necessary condition, it is not sufficient because the value added by the reaction is also dependent of the reaction yield (mass product/mass reactant). This implies that the first process metric should be the reaction yield. For lower value products, where the cost of a process is often driven by the cost of the reactant (up to 70% of the production cost of the product), the yield needs to be high enough to achieve an economical process. For higher value products (e.g., pharmaceuticals), the required value of the reaction yield will be determined by the downstream product recovery requirements. In some cases, the reaction yield will be determined by the thermodynamic equilibrium, emphasizing also the importance of

determining this, especially under the conditions to be used in the process. The value added by the reaction will ultimately determine the minimum value for the contribution of the biocatalyst, reactor, and downstream processing.

4.2.2 Productivity

The space–time yield (gram of product/liter of the reactor/hour of operation), often referred to as the "productivity," in combination with knowledge of the required demand for a given product (per time) will give information about the required plant capacity. Frequently it is necessary to place biocatalytic processes in an existing plant, since building a dedicated purpose-built plant is hard to justify. Nevertheless, knowledge about the productivity is of vital importance in assessing a given conversion, since it will form a first basis for knowledge of plant sizing. For reactions not using growing cells, the space–time yield can always be increased by adding more biocatalyst. Although reactor capacity may be a limiting factor (especially with immobilized enzymes), more important is that the biocatalyst yield (see below) will often be compromised when using very high biocatalyst concentrations. In such cases, calculation of the space–time yield can give a target for specific activity. For biocatalytic conversions using growing whole cells, the space–time yield cannot be increased by adding more biocatalyst. For this reason, it is a primary measure of the effectiveness of such a system and an essential metric (typically values over $2\,g\,l^{-1}\,h^{-1}$ are required).

4.2.3 Biocatalyst Yield

An essential issue in the evaluation of a new biocatalytic process concerns the cost of the biocatalyst [4]. Despite the enormous improvements in fermentation technology, coupled with improved expression as a result of recombinant DNA technology, it is nevertheless essential to assess the cost contribution of the biocatalyst to the product. Measuring the biocatalyst yield over the whole operational lifetime of the catalyst will give the biocatalyst yield (gram of product produced/gram of biocatalyst added). The necessity to measure this value over the whole lifetime of the biocatalyst clearly makes practical measurement and estimation difficult. However, this is important because expensive biocatalysts (i.e., enzymes requiring recovery and isolation following fermentation, linked with immobilization) will require recycling in order to make a low enough contribution to the overall cost of making a given product. No matter what the format of the biocatalyst, development is required in order to express sufficient activity for a given reaction.

Larger markets will justify more development, reducing the cost of biocatalyst production. Since the biocatalyst is contributing as a catalytic entity alone, its cost should be evaluated in relation to the value of the product (and more accurately the cost difference between the product and the reactant). Hence, knowledge about the specific activity of a biocatalyst is insufficient. Stability (the dynamic change of

Table 4.2 Examples of appropriate biocatalyst yield and product concentration for scalable processes in different industrial sectors.

Industrial sector	Product value ($\$ kg^{-1}$)	Biocatalyst yield[a] ($g g^{-1}$)	Product concentration[b] ($g l^{-1}$)
High value (e.g., pharmaceuticals)	1 000	50	100
Medium value (e.g., flavors)	100	500	200
Low value (e.g., biofuels)	1	10 000	400

a) Values here are given for immobilized enzymes.
b) Precise values will depend upon the ease of product separation from other compounds leaving the reactor.

rate with respect to time on account of denaturation) must also be known. Determination of specific activity and stability in a single batch can give the amount of product that can be produced for a given charge of biocatalyst. Clearly, it is also necessary to account for enzyme recovery or immobilization, depending upon the biocatalyst format. Based upon acceptable contributions to the final product cost, it is possible to calculate a reasonable biocatalyst yield (gram of product/gram of biocatalyst) for a given product class. Some examples are given in Table 4.2. Such information is invaluable to obtain since it gives a target for biocatalyst specific activity and stability.

This information also allows an estimation of the total amount of biocatalyst required for a given application, and also, even more critically, the fraction of total enzyme (required to achieve the necessary biocatalyst yield) for a single batch. Interestingly, if the cost of biocatalyst in a single batch is very high (implying multiple recycles to give a low enough biocatalyst yield for the overall process), it becomes important to consider the risks that might be entailed by loss of that batch, due to a processing problem. For example, if there is a risk of mistakenly overfeeding an inhibitory reactant to the biocatalyst in the first batch, it could make it hard to justify the use of an immobilized enzyme, where multiple reuses (recycles) would be required.

4.2.4 Product Concentration

The product concentration leaving the reactor will determine the scale of the downstream process, which will be sized according to the recovery of a given mass of product from a given reactor volume. In general, since biocatalytic reactions are carried out at ambient temperature, the energy required to evaporate water is significant. Hence a high concentration leaving the reactor is required, depending

upon the value of the product. As discussed previously, such conditions are far from the natural environment of biocatalysts, making this a particularly important development target. The precise values are, of course, dependent upon the ease of separation of the product from the other compounds (unreacted reactant(s) and by-products) leaving the reactor. See Table 4.2 for example values.

4.3 Technologies for Implementation of Biocatalytic Processes

The development of a biocatalytic process is an essential stage in implementation and scale-up, and the main aim is to obtain the target values of the process metrics described earlier in this chapter. A number of technologies are available to achieve this objective. The investment each requires, and the gains in terms of productivity, have still not been fully benchmarked, but the technologies mentioned have all been implemented in large-scale processes using biocatalysis.

4.3.1 Biocatalyst Engineering

Biocatalyst engineering is the part of process development, which is different from conventional chemical process development. For this reason, it has often been left as an afterthought when in reality it can be on the rate-limiting path to process implementation and should therefore be implemented as early in process development as it is known to be useful.

4.3.1.1 Protein and Genetic Engineering

Biocatalyst engineering involving either protein engineering or modification to the host organism containing the biocatalyst is enormously powerful. Indeed, many excellent reviews cover the field [5–14]. Although most are focused on alteration of selectivity, reaction rate, and substrate scope, this is of course the first step in implementing a new process. The field is developing a whole range of technologies including the introduction of more computer-based and semirational approaches [15]. In the context of this chapter, it is interesting also to see that a start has been made to biocatalyst modifications focused on process needs [16, 17].

4.3.1.2 Biocatalyst Immobilization

While an isolated enzyme-catalyzed reaction is easier to implement than a whole-cell-catalyzed reaction due to its simplicity, the trade-off is higher upstream cost, and therefore reuse of the enzyme is often necessary (potentially via immobilization) to keep costs down. As a rule of thumb, the crudest possible form of the enzyme acceptable to maintain product quality should be used [18, 19].

Immobilization is often the key to improving the operational performance of an enzyme [20, 21]. Especially for use in a dry medium, such as an organic solvent,

it is difficult to use biocatalysts without immobilization or cross-linking since the enzymes are prone to aggregation. Other potential benefits of immobilization are enhanced stability, the possibility of use in a packed-bed (or even fluidized-bed) reactor, and prevention of protein contamination in the product stream [22]. Fast and easy separation of the biocatalyst from the reaction medium is sometimes a key factor for enzymatic resolutions where the reaction has to be stopped at a certain conversion in order to preserve high enantiomeric excess (ee) of the product. Simpler downstream processing and easy biocatalyst recovery for immobilized enzymes also lead to an improved process economy, which may be essential for developing a competitive process. Enzyme immobilization is a particularly important intensification method for obtaining adequate biocatalyst yield. In principle, improvements in activity, stability, and selectivity are potentially possible [23, 24], although improved stability and separation are the usual arguments for immobilization. A general method of immobilization, that can be applied to any enzyme, is not available, and therefore the typical approach is by trial and error. Efficient immobilization protocols should take into account the physicochemical properties of the carrier (or matrix) as well as the enzyme to obtain the best compromise between stability, activity, handling, and cost (see Refs [25, 26] for comprehensive reviews). For large-scale production of the biocatalyst, the procedures should be quick, robust, scalable, and reproducible and the enzyme should be stable during each step.

4.3.2 Reaction Engineering

Reaction engineering refers to the ability to alter conditions in the reaction such that optimal use can be made of the biocatalyst and reactant, which in many cases help achieve the required metrics to enable implementation. The concentration of biocatalyst and reactants to be used is of course dependent upon the kinetics and thermodynamics of a given reaction. For many reactions, the choice of media to be used also makes a very important difference to the reaction performance. It has become well established that addition of an organic solvent to an otherwise aqueous medium can have a great effect in biocatalytic reactions. While an aqueous solution is highly compatible with the natural environment of most enzymes, poorly water soluble compounds will not be converted effectively – meaning limited product concentration (and expensive downstream processing). Many attempts have been made to overcome the solubility limitation by the addition of water-miscible organic solvents, but these tend to strip essential water from the enzyme, resulting in a loss of activity. For reactions that are thermodynamically unfavorable in water (such as esterifications), it can be advantageous to run the reactions in neat water-immiscible organic solvents. This also enables enzymes to be immobilized simply by adsorption. Nevertheless, without immobilization, aggregation is frequently a problem, as mentioned previously. Finally, aqueous–organic biphasic mixtures can be used, which from an industrial perspective are particularly interesting, although a balance is required between

the necessary mixing to afford good mass transfer and subsequent separation to recover the product from one or the other phase and allow recycle of the organic solvent. Potentially, enzyme denaturation at the liquid–liquid interface can be a challenge also, although it can be overcome by immobilization. In all cases, a rational basis for organic solvent selection remains a significant challenge. Today, other suggested and successfully tested biocatalytic reaction media such a supercritical (sc)CO_2, ionic liquids, and all gas-phase reactions remain primarily for application in the laboratory, rather than at a large scale.

A variety of technologies can be introduced with the general objective of controlling the reactant and product concentration. All involve changes to the reactor operation, to ensure the local conditions for the biocatalyst are kept as optimal as possible, while the overall process fulfills the necessary process metrics, as previously discussed.

4.3.2.1 Reactant Supply

In cases where the reactant is inhibitory or toxic to the biocatalyst, it is necessary either to engineer tolerance to the reactant into the biocatalyst or to avoid the issue. The latter approach is the most common and can be achieved simply by feeding the reactant to the reactor to limit its concentration in solution. The maximum feed concentration will determine the maximum achievable product concentration. The maximum feed concentration will be limited by the water solubility of the compound, meaning more water-soluble compounds can always be fed. Those with limited water solubility can be "fed" via a second phase, either an organic (water immiscible) solvent or alternatively a porous resin.

4.3.2.2 Product Removal

Throughout the development of a process, it is essential to examine not only the enzymatic reaction but also the preceding and following reaction steps as well as the necessary separation steps. Changes to pH, temperature, pressure, and solvent should be minimized. However, the need to address the problems of inhibitory or toxic products is so common that it deserves special mention here. The common approach is to use *in situ* product removal (ISPR) (see e.g., [27–30]), where the product is removed from the reaction mixture during conversion. ISPR has been widely reported and studied, although more in-depth engineering studies are still required to provide guidelines for implementation. The basic concept as implemented in industrial processes uses a recycle loop in which biocatalyst is retained in the reactor (Figure 4.3).

The loop passes through an ISPR unit in which product is selectively removed. Common operations for the removal include the use of water-immiscible organic solvents and also resins, and some details are given in the following. The system can also be linked to chromatography for products that justify the cost [31]. In principle, ISPR can also be used to shift the equilibrium of a thermodynamically unfavorable reaction by means of selectively removing product over the substrate. Using carbonyl reductases [32] this has proved very effective, but clearly it will not work in all cases.

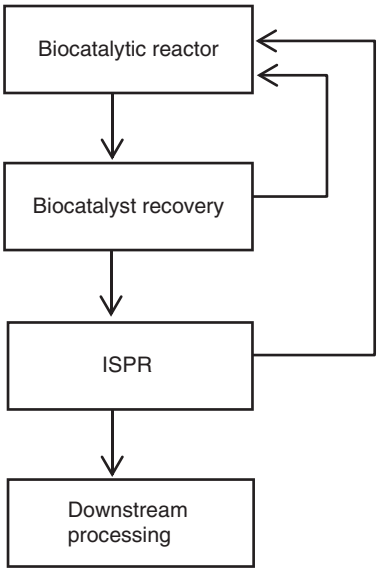


Figure 4.3 Concept for ISPR in a biocatalytic process with recycle stream.

4.3.2.3 **Two-Phase Systems**

As discussed previously, the addition of an organic water-miscible solvent can help solubilize poorly water soluble compounds. However, the solubility increases are limited, and the water-miscible solvents are often harmful to the enzymes. In other cases, two-liquid phase biocatalysis (TLPB) may be used to enhance solubility. In addition, an established way to run some batch processes with biocatalysts is to avoid the toxic and inhibitory concentrations by supplying the reactant via a second (water-immiscible) liquid phase. In this case, the solvent acts as a reservoir. Using such technology, order of magnitude improvements in product concentration can be achieved. In general, conventional equipment can be used, although the extent of mixing and subsequent phase separation is critical. Extra parameters to be evaluated are the phase ratio of the two liquid phases, mass transfer, and the partition of the substrate and product between the phases. It remains difficult to select the organic solvent – and this is a subject of ongoing research – although much has been learnt for whole-cell biotransformation studies [33]. Several processes have been scaled up using this technology, and indeed the use of two-liquid phase systems is a common method of ISPR [34], although kinetics and selectivity need to be evaluated in each case [35]. Emulsification can also be a problem, although the use of membrane technology is an interesting potential solution [36].

An alternative two-phase system to addition of water-immiscible organic solvents is to introduce a porous resin to supply the reactant or to remove the product [37]. Such an approach has several advantages because it avoids the difficulties of organic solvent selection (both from an environmental perspective and the problems frequently faced by enzyme in contact with organic solvents). Similar parameters need to be investigated to organic solvent TLPB methods, but it is clear that the capacity of the resin is particularly important. Indeed, the option

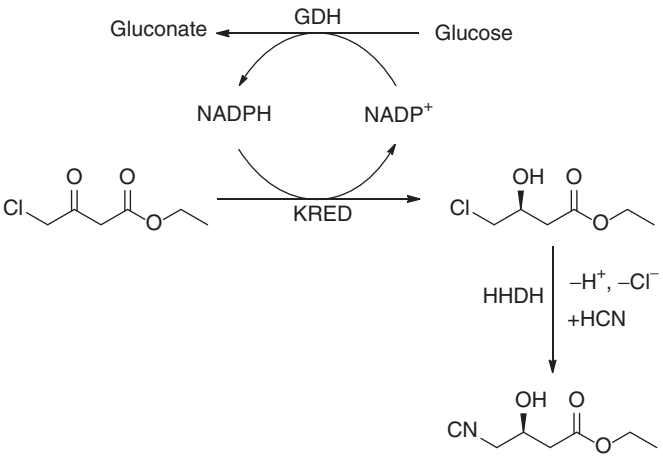
of a recycle stream containing the resin to simultaneously supply the reactant and remove the product looks particularly attractive. A few processes have been scaled up using this technology [38].

4.4 Industrial Development Examples

In the following section, two examples of process development for industrial biocatalytic processes are presented, both in the pharmaceutical sector. They have been chosen to illustrate the integration required between process and biocatalyst development. The examples are presented in order of increasing complexity.

4.4.1 Development of a Biocatalytic Route to Atorvastatin (Developed by Codexis Inc., USA)

- **Background**
  Atorvastatin is the active ingredient in the drug Lipitor®, which is a cholesterol-lowering drug. It is one of the largest selling drugs of all time; for example, in 2010 the annual sales exceeded USD10 billion. The molecule contains two chiral centers and therefore biocatalytic routes have always been of interest to help synthesize the product in an efficient way. This, coupled with the high demand for the drug, makes it a particularly interesting process development case. Early work to synthesize Atorvastatin by biocatalytic methods focused on kinetic resolutions using whole cells as well as chemoenzymatic routes using nitrilases and lipases. Nevertheless, these routes failed to meet the requirements for a scalable, commercial process, and were therefore not implemented. At Codexis Inc. (Redwood City, CA, USA), scientists began work on another route, using a ketoreductase (KRED), linked with glucose dehydrogenase (GDH) to recycle the NADPH, followed in a second enzymatic step, by use of an halohydrin dehalogenase (HHDH).
- **Step 1: Engineer improvements in enzyme activity (GDH, KRED)**
  Having identified a suitable route (see Scheme 4.1; details can be found in [39] and two patents to Codexis: US 7,125,693 and US 7,132,267), initial experiments were carried out with the two reactions separately.
  In the first reaction, while the selectivity was found to be suitably high (ee > 99.5%), the reaction rate was very low, implying a large amount of enzyme was required. Both the KRED and GDH protein were improved using several rounds of DNA shuffling. GDH activity was improved 13-fold, and KRED activity 7-fold. This meant that less enzyme could be used, resulting not only in a better biocatalyst yield (gram of product/gram of biocatalyst), but also in improvements for the downstream product recovery. The GDH recycle reaction produced gluconate, implying the pH would drop unless neutralized, and this therefore demanded the use of a stirred-tank reactor with pH measurement and control.



Scheme 4.1 Biocatalytic synthesis of atorvastatin.

Table 4.3 Process metrics for biocatalytic atorvastatin process.

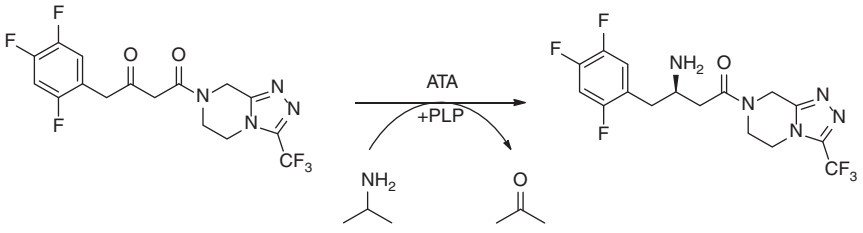
Metric	Laboratory	Process
Space–time yield ($g\,l^{-1}\,h^{-1}$)	3.3	20
Biocatalyst yield ($g\,g^{-1}$)	9	178
Product concentration ($g\,l^{-1}$)	85	152

Data derived from Ma *et al.* [39].

- **Step 2: Engineer improvement in enzyme activity and stability (HHDH)**
  In the subsequent step, HHDH was used. In initial experiments, the activity and stability were found to be low, and this was improved via DNA shuffling.
- **Step 3: Engineer out product inhibition (HHDH)**
  A particular feature of the kinetics of the HHDH reaction is strong product inhibition. Although attempts were made to overcome this by ISPR, ultimately a better solution in this case was found by improving the enzyme. The activity was increased 2500-fold over the wild type.
- **Step 4: Implementation**
  Today, the process is run at a large scale with process metrics as indicated in Table 4.3.

4.4.2 Development of a Biocatalytic Route to Sitagliptin (Developed by Codexis Inc., USA and Merck and Co., USA)

- **Background**
  Sitagliptin is an anti-diabetic compound, and as the phosphate salt it is the active ingredient in a pharmaceutical product marketed as Januvia™



Scheme 4.2 Biocatalytic synthesis of sitagliptin.

by Merck and Co. (Rahway, NJ, USA). It is a highly complicated molecule and for some time was manufactured using asymmetric hydrogenation of an enamine. The reaction takes place at high pressure (17 bar) using a rhodium-based chiral catalyst [40]. However, the application of pressure and the use of the metal-based catalyst, potentially contaminating the product, are not ideal. Likewise, the stereoselectivity is limited. For these reasons, scientists at Merck and Co. (Rahway, NJ, USA) considered using an aminotransferase (ω-transaminase) to asymmetrically synthesize the chiral center with a suitable amine donor (see Scheme 4.2).

- **Step 1: Engineer enzyme activity for a new substrate**
  Although in the last decade there has been significant research focus on the application of ω-transaminases [41–44], the molecule to be converted to synthesize sitagliptin was without precedent, given the size of the large substituent adjacent to the ketone. Hence, initially *in silico* design techniques were applied to provide a suitable starting point to engineer the enzyme. Using substrate walking (in which the substrate is altered in steps, so as to allow the enzyme to be altered and screened in stages), the large binding pocket of the enzyme was altered and subsequently evolved for activity. This enabled a 75-fold improvement of activity on the first mutated enzyme. This provided the starting point for fine-tuning the enzyme and is the basis of the data given in Table 4.4.

Table 4.4 Example process metrics for biocatalytic sitagliptin process.

Metric	Laboratory[a)]	Process[b)]
Space–time yield ($g\,l^{-1}\,h^{-1}$)	0.033	12
Biocatalyst yield ($g\,g^{-1}$)	0.08	5.3–6.6
Product concentration ($g\,l^{-1}$)	0.8	160

Derived from Refs [45, 46].

a) Data for the laboratory benchmark were taken from the point at which activity was detected and tuning began – meaning the variant from the Round 2 as detailed in Table 1 of Savile *et al*. [45].

b) Process with immobilized enzyme.

- **Step 2: Engineer the enzyme for industrial reaction conditions**
  Although an active enzyme was created, it could only work at concentrations of 2 g l^{-1}, clearly too low for commercial operation of a reactor. The problem was made worse since the water solubility of the compound was low, and therefore various solvents were assessed. Both methanol and DMSO proved effective solvents. In addition, the equilibrium of the reaction needed to be pushed toward the product, meaning an excess of the amine donor was required [47]. Indeed, despite the reasonable thermodynamic favorability of this reaction, still 1 molar concentration (fourfold excess) of amine donor was required. Since Isopropylamine (IPA) is converted to acetone during the reaction, clearly the enzyme must also be tolerant to high concentrations of acetone. In an evolution program, the enzyme tolerance was improved to convert substrate concentrations from 2 to 100 g l^{-1}, IPA from 0.5 to 1 M, DMSO from 5% to 50%, pH from 7.5 to 8.5, and temperature from 22 to 45 °C. A selectivity of >99.99% was achieved. Simultaneous optimization included improving the stability and expression levels in *Escherichia coli* so as to reduce the biocatalyst cost. The optimal process at this point converted 200 g l^{-1} with >99.5% conversion at 92% yield with 6 g l^{-1} enzyme in 50% DMSO. Compared to the rhodium-based process, this gives at 10–13% increase in yield, 53% increase in space–time yield (g l^{-1} h^{-1}), and 19% reduction in total waste. Additionally, the process can operate in a nonspecialized plant at ambient pressure.
- **Step 3: Introduction of immobilized enzyme process**
  In a further development [46], a second generation of biocatalytic process has been developed to operate in a neat organic solvent using immobilized ω-transaminase, to afford further benefits to the product recovery and potentially allow continuous operation. Indeed, operation could be now achieved for 200 h, which means that the biocatalyst yield was improved to manageable levels. This is important for the final implemented industrial process.
- **Step 4: Process implementation**
  Today, the process is implemented at a large scale. Examples of the process metrics are given in Table 4.4. In this rather special case, it is hard to give a starting point, but a defined point has been identified based on the point at which tuning of the enzyme was started (Step 2 above). Likewise, an example of the process metrics is given to illustrate what is achievable. This still requires fine-tuning for the final implementation to balance the individual metrics based on process economics. It is perhaps worth mentioning that space–time yields based on initial rates alone are not accurate because the real space–time yield should be determined over the entire reaction time, since this is the time the reactor is occupied. Likewise, a direct comparison of the metrics for the laboratory and process should be treated with care, since a different format of biocatalyst was used whereas for true comparison immobilized enzyme loading data would also be required.

4.5 Future Outlook

The metrics discussed earlier are not often reported in the scientific literature, and it requires effort to develop fed-batch, continuous, or recycle-based processes, all of which require significant experimental work. Hence, achieving the metrics requires process intensification prior to scaling up. This is a basic philosophy to help develop the process with greater reliability. The philosophy of implementation proposed in this chapter is that intensification of the process should take priority over scaling up. Nevertheless, the subsequent scaling up needs to address issues such a mass transfer (liquid–liquid or gas–liquid) as well as mixing. The increased mixing time due to the increase in scale can bring limitations for addition of reagents and control of pH (via acid or alkali for neutralization) in many systems. Mixing requires attention, and in recent years alternative mixing concepts such as the rotary jet head system have been applied with success [48]. Reactants will most normally be added in the batch mode at the start of the reaction. However, in cases where the solubility is limited, alternative methods must be used. Reactants can also be used above saturation concentration in the form of a slurry reactor or a two-liquid phase reactor. In other cases, if the reactants are toxic or inhibitory, it may be essential to feed the reactant to the reactor, which can be done directly (if the solubility in the reaction medium is high) or via another phase (water-immiscible organic solvent or porous resin).

Clearly, for many reactions it is essential to intensify a reaction or process in order that it can be applied in an industrial context. This is not surprising given that the conditions required in a biocatalytic reactor are very different to those found in nature. Protein engineering offers the engineer involved in scale-up and implementation of a biocatalytic process fantastic opportunities [17]. Although we may search for the ideal biocatalyst [49], in reality it is a combined effort of protein and process engineering that is required to achieve successful industrial implementation. The approach discussed here can be complemented by protein and enzyme engineering efforts. Indeed, in reality it is the integration of these (process and biological) technologies together that leads to the most effective solution for development. Several studies have already shown the integration of multiple technologies to achieve the required degree of intensification [50]. For instance, in the synthesis of optically pure chiral amines using ω-transaminases, protein engineering, reaction engineering, and process engineering were all used to significant effect in the final industrial process for the synthesis of sitagliptin [45, 46], and likewise in other industrial work using ω-transaminase for the synthesis of a JAK2 kinase inhibitor [51].

The methodology proposed here is to initially set targets, characterize, then compare the difference to develop a strategy for different technologies involving biocatalyst engineering, reaction engineering, and finally process engineering. In a recent review, the approach was further outlined and the integration emphasized [52].

4.6 Concluding Remarks

The implementation and development of new biocatalytic processes require considerable investment in a variety of technologies from ISPR to protein engineering. Nevertheless, it is clear that as knowledge is built in each of these areas (in particular to identify limits for application) and a suitable methodology for implementation is built, the necessary tools will become available. This will be important not only to implement single-step biocatalytic reactions but, in the future, also to address multistep biocatalytic reactions (both multi-enzymatic and chemoenzymatic) such that processes can be designed (both by engineers and chemists) in such a way as to capitalize upon the best features of both conventional and biological catalysis.

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5
Development of Enzymatic Reactions in Miniaturized Reactors

Takeshi Honda, Hiroshi Yamaguchi, and Masaya Miyazaki

5.1
Introduction

Enzymatic reactions proceed with high regio and stereoselectivity. In general, enzymatic processes generate less waste than conventional chemical synthetic processes and provide products of high purity. Therefore, during the last two decades, the application of enzymes has been continuously increasing across various industries. For industrial use, the catalytic and biophysical properties of enzymes, such as catalytic efficiency, substrate specificity, and stability, need to be improved. A variety of approaches, such as screening of enzymes from natural sources or random mutations, have been attempted for these purposes [1].

Modern protein engineering techniques have improved the catalytic and biophysical properties of some enzymes. However, many enzymes that have a great deal of potential in industry require a higher level of improvement for industrial use. In addition, enzymes often lack operational and storage stability. Because enzymes are proteins with unique conformations (three-dimensional structure), which are required for their catalytic activity, they can easily be denatured at high temperature and in organic solvents. The stability of enzymes in organic solvents and enhancing the thermostability are usually considered in an industrial process. Moreover, the enzymes require to be efficiently separated from other molecules such as the products in the reaction system. The ability to recover and reuse enzymes is also important in industry.

To overcome these obstacles, the immobilization of enzymes has been studied [2]. Immobilized enzymes provide several advantages for the use as catalysts in industry. A high concentration of enzymes can be immobilized on the substrates, resulting in a high enzyme to substrate ratio, thus allowing the immobilized enzyme reactor to perform rapid catalytic reactions. Furthermore, enzymes that are immobilized on the substrate walls or surfaces of microparticles can be isolated and removed easily from the reaction systems. Moreover, in comparison to free enzymes in solution, immobilized enzymes are reportedly stable and highly

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resistant to environmental changes such as high temperatures or organic solvents. The enzymes are also reusable.

Recently, a variety of enzyme-immobilized reactors have been developed for highly efficient catalytic reactions [3]. The immobilized enzyme can be packed into microchannels in a microfluidic device. The combination of immobilized enzymes with microfluidics provides several advantages, such as the realization of automated and high-throughput catalytic systems. Enzymatic reactors can be classified into two types: chip-type reactors and microtube-type reactors. Chip-type reactors offer several advantages, including easy control of microfluidic reactions and the integration of many processes into one reaction device. The manufacturing processes of chip-type reactors are adaptations mainly from the microelectronics industry. Dry- or wet-etching processes have been used for creating microchannels on silicon or glass plates. Polymer-based materials can be used for the preparation of enzyme reactors because enzyme reactions are often performed in aqueous solutions. Poly(dimethyl siloxane) (PDMS), poly(methyl methacrylate) (PMMA), polycarbonate, polystyrene, poly(ethylene terephthalate), poly(ether ether ketone), and polytetrafluoroethylene (known by the name of Teflon®) have been used for preparation of the devices. Various microchannel shapes have been developed in chip-type reactors, which could be processed by photolithography, soft lithography, injection molding, embossing and micromachining with laser, or microdrilling. A combined process of lithography, electrochemical technology, and molding has also been used for the production of reactors.

The key processes in enzymatic reactions are molecular mixing and separation. The large liquid interface-to-volume ratio in microscale reactors generates rapid mass transfer, which enables efficient molecular mixing and separation in enzymatic microreactors [4]. Miniaturized structures in microreactors provide excellent controllability in the formation of turbulent and laminar flows; as such, they are utilized for rapid mixing and *in situ* product removal/extraction in enzymatic reactions. A detailed discussion is beyond the scope of this chapter, but we refer readers to some excellent reviews [5–10].

In this chapter, we will introduce the immobilization of enzymes for application in a variety of fields, including industrial applications. The following section focuses on the fundamental techniques of immobilized enzymes, novel and interesting immobilization techniques, and their applications, including miniaturization of reactors. As the field of immobilized enzyme systems is extensive, each section presents an introductory overview of ongoing research.

5.2
Fundamental Techniques for Enzyme Immobilization

Enzyme immobilization technologies have been applied to various enzymatic transformation systems. The immobilized catalyst in the reaction systems allows

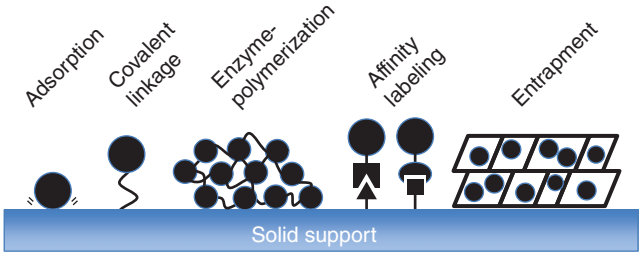


Figure 5.1 Enzyme immobilization techniques.

easy separation of the catalyst and product, resulting in simplified microfluidic handling. Therefore, enormous effort has been directed toward the development of enzyme immobilization methods and utilization of enzymatic reactors.

Enzyme immobilization can be achieved by covalent linking, affinity labeling, physical adsorption, enzyme polymerization, and entrapment (Figure 5.1). Enzyme polymerization, which covalently connects enzymes to each other with a cross-linker, can be partly categorized as covalent linking. It enables the formation of miniature structures made of the polymerized enzymes in a reactor. To prepare immobilized enzymes, they are attached to solid materials, such as microspheres/beads, membranes, fibers, monoliths, or microchannel/capillary walls. In this section, recent trends in the development of enzyme immobilizing methods are discussed comprehensively.

5.2.1
Enzyme Immobilization by Adsorption

Adsorption is the simplest method for enzyme immobilization, involving reversible surface interactions between the enzyme and the support material. Adsorption is achieved by incubating enzymes with support materials under suitable conditions, including pH and ionic strength. Enzyme immobilization using the adsorption technique has a number of disadvantages, such as steric hindrance by the support, nonspecific bonding, overloading on the support, contamination of the product, and leakage of the enzymes from the carriers owing to the weak interaction between the enzyme and the carrier, which can be destroyed by desorption forces such as high ionic strength and pH. Therefore, a number of variations to counter these drawbacks have been developed, including adsorption–cross-linking, modification–adsorption, adsorption–coating, and selective adsorption–covalent attachment. A detailed discussion of these variations is presented in the book by Cao and Schmid [11]. Enzyme adsorption is carried out on a solid support, including the channel wall, monolith, particle, and membrane. Table 5.1 presents a summary of enzyme immobilization methods using the adsorption technique.

Table 5.1 Adsorption techniques for enzyme-immobilized microreactor preparation.

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
Borosilicate tube with micro-bores	Lipase	Channel wall	TEOS	A microreactor, which is a borosilicate tube containing six bores 200 μm in inner diameter, was coated with mesoporous silica film (a thickness of less than 100 nm) prepared by TEOS sol–gel technique on the inner wall. The enzyme was immobilized on the film by passive adsorption	[12]
Fused-silica capillary	Trypsin	Channel wall	GPTES, iminodiacetic acid, copper ion	The inner surface of capillary was functionalized with chelator-silane that was prepared by covalent bonding between the epoxy group of GPTES and iminodiacetic acid as the chelator. The enzyme was adsorbed on the capillary surface through the chelating copper ion	[13]
Fused-silica capillary	Trypsin	Channel wall	MAPTS, TMOS, poly(diallyldimethyl-ammonium chloride) (PDDA)	TMOS sol–gel polymerization in the presence of PDDA was performed in the capillary whose surface was functionalized with MAPTS, followed by assembly of trypsin onto the positively charged surface through electrostatic adsorption	[14]

PET-microchip	Trypsin	Channel wall	β-Zeolite nanocrystal, poly(diallyldimethyl-ammonium chloride) (PDDA)	The nano-zeolite microstructure was prepared by electrostatical assembly based on a layer-by-layer technique using PDDA and β-zeolite nanocrystals on the channel walls of PET-microchip. The enzyme was adsorbed to the nano-zeolite network	[15]
Fused-silica capillary	Tyrosinase	Channel wall	Hexadimethrine	The preparation of the enzyme-immobilized capillary includes the following steps: coating with positively charged hexadimethrine on the capillary inner surface, immobilization of the enzyme to the capillary surface through electrostatic interaction, and subsequent coating with hexadimethrine to cover and stabilize the immobilized enzyme with an additional hexadimethrine layer	[16]
PET microchip	Trypsin	Channel wall	Chitosan, hyaluronic acid	The enzyme was adsorbed in the multilayer membrane assembled with positively charged chitosan and negatively charged hyaluronic acid on the microchannel surface of a PET microchip	[17]

(*continued*)

Table 5.1 (*Continued*)

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
Glass microchip	Glucose oxidase, choline oxidase	Monolith	TMOS, MTES, polyethylenimine (PEI)	The silica monoliths were prepared from TMOS and MTES using a sol–gel method in a microchannel, followed by adsorption of PEI to the monolith. Enzymes were immobilized on the PEI-coated monolith through electrostatic adsorption between electronegative enzymes and electropositive PEI polymers	[18]
Glass microchip	Glucose oxidase	Monolith	MAPTES, *N*-isopropylacrylamide (IPAA), *N,N*-methylene-bis-arylamide (MBA), 2-hydroxy-2-methylpropiophenone (HMP)	The microchannel wall was coated with MAPTES, followed by photopolymerization of IPAA (monomer), MBA (cross-linker), and HMP (photoinitiator) in the microchannels to form poly-*N*-isopropylacrylamide (PNIPAAm)-monolith with 3D macroporous framework. PNIPAAm-integrated glass microchip was heated at 37 °C to maintain the hydrophobic status of PNIPAAm, followed by adsorption of enzyme to the monolith via hydrophobic interaction	[19]

Glass/PDMS microchip	β-Galactosidase	Membrane	Nitrocellulose (NC), octadecyltrichlorosilane	An NC membrane dissolved in acetone/propanol was spotted on the surface of a glass slide modified with octadecyltrichlorosilane, followed by reconstitution of the NC membrane by air-drying. The enzyme was adsorbed on the membrane, and PDMS chip with a microchannel was manually assembled on the glass slide using the NC membrane	[20]
PDMS microchip	Trypsin	Membrane	Poly(vinylidine fluoride) (PVDF)	A porous PVDF membrane was sandwiched between two PDMS-based chips with the microchannels facing the membrane. The enzyme was adsorbed onto the surface of the membrane by continuous flow	[21]
PE microchip	Trypsin, chymotrypsin	Membrane	Poly(vinylidine fluoride) (PVDF), ion-exchange-membranes	A PE-based plate with a microchannel was sandwiched between two PVDF-membranes. Cationic and anionic exchange membranes were contiguously located outside of the PVDF membranes. The bound membranes were sandwiched between polymer layers with buffer-flow channels. Enzymes were immobilized on the surface of PVDF membranes by electric field orientation in a continuous flow-through chamber	[22]

(*continued*)

Table 5.1 (*Continued*)

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
Fused-silica capillary	Trypsin	Beads	HILIC microsphere	An enzymatic microreactor was prepared by an enzyme dynamically immobilized on a HILIC (hydrophilic interaction liquid chromatography) matrix, followed by packing the enzyme–HILIC microspheres in a fused-silica capillary	[23]
PDMS microchip	Acetylcholines-terase	Magnetic particle	Graphene oxide (GO)–Fe_3O_4 magnetic nanocomposites (MNCs)	The enzyme was immobilized to GO/Fe_3O_4-MNCs through passive adsorption, and then enzyme-immobilized GO/Fe_3O_4-MNCs were packed into the channel of PDMS microchip by permanent magnets	[24]
PDMS microchip	Lipase	Nanoparticle	Folded-sheet mesoporous materials (FSM4 and FSM7; pore diameters of 4 and 7 nm, respectively)	The enzyme was adsorbed/encapsulated by the silica-based FSM4 and FSM7 nanoparticles. The enzyme-FSM was immobilized on PDMS microchannel by polymerization of a PDMS prepolymer layer	[25]
Fused-silica capillary	Asparaginase	Nanoparticle	Gold nanoparticles (AuNPs), MPTMS	The enyme was immobilized on AuNPs through passive adsorption. The inner wall of a fused-silica capillary was modified with MPTMS by sol–gel technique, followed by immobilization of enzyme-attached AuNPs on the free thiol-functionalized inner surface of the capillary	[26]

Fused-silica capillary	Adenosine deaminase, xanthine oxidase	Nanoparticle	Gold nanoparticles (AuNPs), polyethylenimine (PEI)	Multiple enzymes were simultaneously immobilized on AuNPs through passive adsorption. A capillary surface was coated with positively charged PEI through electrostatic interaction, followed by immobilization of negatively charged enzyme-AuNPs on the positively charged capillary inner wall	[27]
Fused-silica capillary	α-Glucosidase	Monolith nanoparticle	MAPTS, ethylene dimethacrylate (EDMA), glycidyl methacrylate (GMA), 2,2-azobis(2-methylpropionitrile) (ABMP), cysteamine, gold nanoparticles	The poly(GMA-*co*-EDMA) monolith was formed by polymerization of EDMA, GMA, and ABMP in a capillary whose surface was functionalized with MAPTS. The resulting epoxide-modified monolith was functionalized with cysteamine to introduce a thiol group, followed by modification with gold nanoparticles (AuNPs) through coordinate bonding with the thiol–Au. The enzyme was immobilized on AuNPs through passive adsorption	[28]
PET microchip	Trypsin	Channel wall	Poly(diallyl dimethyl ammonium chloride) (PDDA), gold nanoparticle	The channel surface coating was assembled via a layer-by-layer electrostatic binding of PDDA and gold nanoparticles. The enzyme was adsorbed in the two-bilayer assembly	[29]

(*continued*)

Table 5.1 (*Continued*)

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
Microfluidic paper-based analytical device (μPAD)	Lactate dehydrogenase, diaphorase	Channel wall	Chromatography paper	A μPAD was prepared by the following steps: printing of a microchannel pattern on a chromatography paper using hydrophobic solid ink on a wax printer to form the paper-based microchannel surrounded by the hydrophobic ink walls, followed by adsorption of two enzymes by spotting their solutions as a circle on a channel line, which are located apart from one another	[30]

PDMS, poly(dimethyl siloxane); PMMA, poly(methyl methacrylate); PS, polystyrene; PET, poly(ethylene terephthalate); EDC, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; APTES, 3-(aminopropyl)triethoxysilane; MTES, methyltriethoxysilan; TEOS, tetraethoxysilane; TMOS, tetramethoxylsilane; GPTES, γ-glycidoxypropyltrimethoxysilane; PEI, polyethyleneimine; PTFE, *polytetrafluoroethylene*; MAPTS, *g*-methacryloxypropyl trimethoxysilane; MPTMS, 3-mercaptopropyl-trimethoxysilane.

5.2.1.1 Monoliths and Particles

He *et al.* reported the polyethyleneimine (PEI)-coated silica-monolithic grass microchip [18]. Glucose oxidase and choline oxidase were immobilized on the PEI layer through electrostatic adsorption between electronegative enzymes and electropositive PEI polymer.

Liang *et al.* developed a graphene oxide (GO)/magnetite nanocomposite-based on-chip enzymatic microreactor [24]. Nanoparticles (NPs) of magnetic iron oxide such as magnetite γ-Fe_2O_3 and magnetite Fe_3O_4 show not only a powerful magnetic force but also low toxicity and biocompatibility in physiological environments. They inferred that the introduction of Fe_3O_4 NPs into graphene could combine the high adsorption capacity of graphene and the manipulation convenience of the magnetic materials, which is favorable for further immobilization of the enzyme and easy retrieval and separation of graphene from the dispersion.

The use of folded-sheet mesoporous materials (FSM) as an enzyme-immobilized support was described by Matsuura *et al.* [25]. FSM is a silica-based particle with controlled pore size and a uniform pore structure. The developed microchip with enzyme-adsorbed FSM enabled the evaluation of the effect of pore size on the reaction efficiency of the enzyme microreactor.

Gold nanoparticles (AuNPs) have often been used as a support material for passive adsorption of the enzyme in reactor systems. Enzyme-adsorbed AuNPs have been immobilized in reactors using the following approaches: AuNPs were coordinatively bonded to thiol groups functionalized on a capillary surface [26]; negatively charged enzyme-adsorbed AuNPs were immobilized on a positively charged PEI-coated capillary surface through electrostatic interactions [27]; AuNPs were attached to a copolymer monolith possessing thiol groups through coordinative Au–thiol bonding [26]; and AuNPs were adsorbed on the layer of poly(diallyl dimethyl ammonium chloride) assembled on a microchannel surface [29].

5.2.1.2 Synthetic Polymer Membranes and Papers

Nitrocellulose (NC) and poly(vinylidene fluoride) (PVDF) can strongly adsorb proteins. NC and PVDF membranes have been heavily used in biotechnological processes for protein capture such as western blot. Several groups have developed enzyme microreactors in which NC or PVDF membranes have been grafted for enzyme immobilization [20–22, 31].

Ferrer *et al.* developed a microfluidic paper-based analytical device (μPAD) wherein two enzymes are arranged on the "paper-based" microchannel. The two enzymes were adsorbed in the microchannel by spotting the enzyme solutions at the disk-shaped area on a paper-channel line. This device is inexpensive and easy to fabricate and effects the reaction based solely on buffer capillary action [30] (Figure 5.2). There have been considerable efforts to speed up the development of a μPAD system for diagnostics [32].

5.2.1.3 Adsorption to Channel Walls

Kataoka *et al.* described direct adsorption of lipase on a mesoporous silica film that was coated on the microchannel inner wall [12]. A similar approach was also

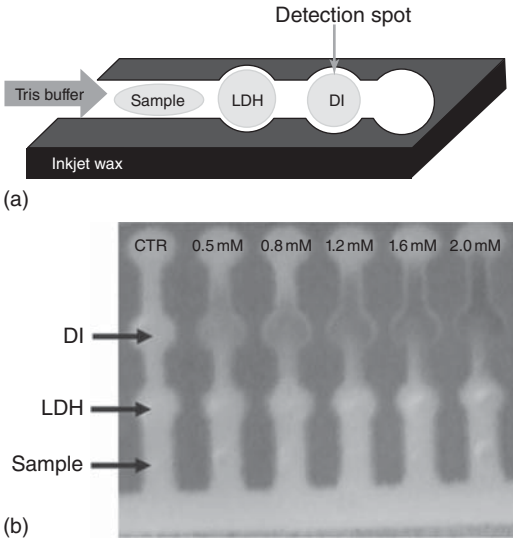


Figure 5.2 (a) Schematic representation of the double microreactor μPAD. The figure shows an eight-channel double microreactor μPAD. Diaphorase (DI), lactate dehydrogenase (LDH), and the sample are spotted on the μPAD prior to analysis. (b) Photograph of the double microreactor μPAD using a range (0.0, 0.5, 0.8, 1.2, 1.6, and 2.0 mM) of concentrations of resazurin. Running buffer is 0.2 mM Tris at pH 7.4. (From [30] with permission ©2008 American Chemical Society.)

reported in which the enzyme was directly adsorbed on the copper-functionalized surface of a capillary [13].

Enzyme immobilization based on layer-by-layer (LBL) assembly technology has been developed by several groups. The LBL structure on a microchannel surface was prepared from various charged polymers such as hexadimethrine [16], poly(diallyldimethylammonium chloride) (PDDA)/silica sol–gel matrix [14], PDDA/β-zeolite nanocrystals [15], and chitosan/hyaluronic acid [17]. Trypsin and tyrosinase were strongly and efficiently immobilized on the LBLs assembled on the microchannel surfaces through passive adsorption (Figure 5.3).

5.2.2 Enzyme Immobilization by Entrapment

Another approach for the immobilization of enzymes is entrapment, which consists in physically trapping enzymes into a film, polymer coating, gel, or monolith, or by microencapsulation [33]. The method involves a physical step, namely confining enzymes within the lattices of polymerized gels. This allows the free diffusion of low-molecular-weight substrates and reaction products. During the process, the enzymes are incorporated into a matrix network. The usual method is to polymerize the hydrophilic polymer and/or silica sol–gel matrix in an aqueous solution of the enzyme. This method basically contains no

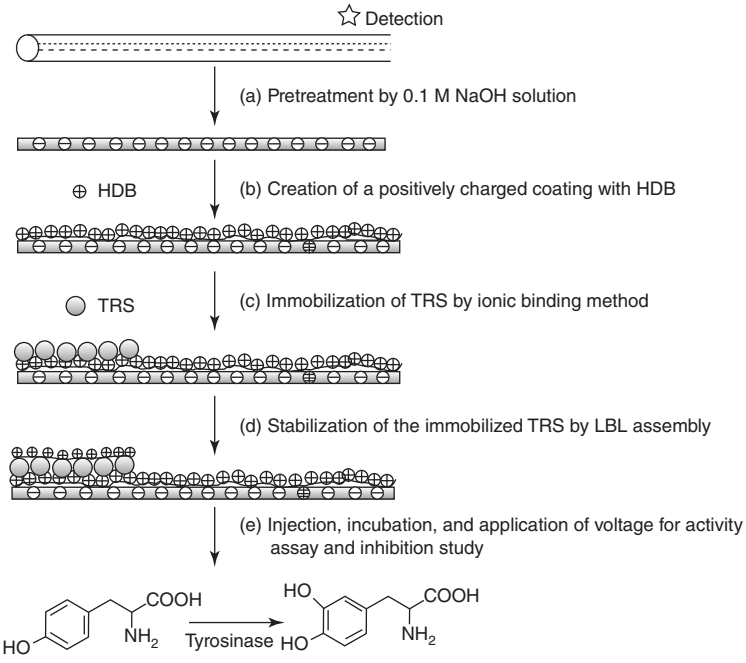


Figure 5.3 Schematic representation of the immobilized capillary enzyme reactor prepared by the layer-by-layer assembly. (From [16] with permission ©2013 Elsevier.)

covalent linkage between the enzyme and the polymer matrix, avoiding enzyme inactivation often caused by chemical modifications. Other advantages of this immobilization technique include the extremely large surface area between the substrate and the enzyme, within a relatively small volume, and the real possibility of simultaneous immobilization [34]. Table 5.2 presents a summary of enzyme immobilization methods using the entrapment technique.

5.2.2.1 **Silica-Based Matrices**

Simple entrapment in silica sol–gel monolithic supports, which were prepared by polymerization of tetramethoxysilane or by photopolymerization of methacryloxypropyl trimethoxysilane, was reported for the preparation of protease-immobilized microchips or capillaries [35, 36]. Enzymes are immobilized in the silica sol–gel matrix with a stable gel network through a Si–O–Si bridge by tethering to the silanol groups. Qu *et al.* developed a PMMA microreactor with trypsin-entrapped tetraethoxysilane (TEOS) polymer, wherein the TEOS sol–gel polymer was tethered to the microchannel surface that was coated with silanol-grafted polyacrylate [41] (Figure 5.4). TEOS polymerization was performed in the presence of the enzyme and polyethylene glycol (PEG). In a similar fashion, the developed enzyme-entrapped silica matrices were often assisted by hydrophilic and biocompatible polymers such as PEG [39, 40], polyethylene oxide (corresponding to PEG with long chains) [38], and dextrin [37]. Such

Table 5.2 Entrapment techniques for enzyme-immobilized microreactor preparation.

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
PMMA microchip	Trypsin	Monolith	TMOS	The enzyme was entrapped in silica-sol–gel monolithic support by *in situ* polymerization of TMOS in the presence of enzyme in a sample reservoir of the PMMA chip	[35]
Fused-silica capillary	Pepsin A	Monolith	MAPTS, TMOS	A foundational sol–gel monolith was prepared by photopolymerization of MAPTS in a limited section of a capillary. The enzyme was immobilized by coating of enzyme-entrapped TMOS sol–gel polymer on the foundational monolith surface	[36]
Poly(ether ether ketone) (PEEK) capillary	Protease P	Monolith	MTES, TMOS, dextrin	The enzyme was entrapped in silica monolith by *in situ* sol–gel polymerization of TMOS and MTES in PEEK capillaries. The sol–gel matrix was also doped with dextrin to prevent shrinkage of the gel	[37]
Glass microchip	Glucose 6-phosphate dehydroge-nase, cellulase	Monolith	Ehtyleneglycol modified silane (EGMS), TEOS, polyethylene oxide (PEO)	The silica monolith in microchannels was prepared by the following two-steps. A homogenous silane-precursor sol solution was prepared by hydrolysis of TEOS or EGMS, followed by addition of enzyme and PEO into the sol. The mixture was sufficiently polymerized into the microchannels, resulting in formation of enzyme-entrapped monolith in the microchannels	[38]

PET microchip	Trypsin	Channel wall	Sodium hydroxide, TEOS, PEG	The microchannel surface was hydrolyzed with aqueous NaOH in order to convert ester groups of PET to free hydroxyl or carboxyl groups. Silica sol–gel polymer, which was prepared from the reaction mixture of TEOS and PEG, was linked to the converted functional groups on the microchannel surface, and then an additional polymerization of TEOS containing enzyme was performed to entrap the enzyme in the silicamonolith in the PET microchannels	[39]
PMMA microchip	Trypsin	Channel wall	Zeolite nanoparticle, TMOS, PEG	The microchannel surface was modified with zeolite nanoparticles, called silicalite-1, to introduce the silanol groups on the surface by a dynamic coating method. Silica sol–gel polymer, which was prepared from the reaction mixture of TEOS and PEG, was linked to the silanol groups on the microchannel surface. The enzyme was mixed in the sol–gel solution, resulting in immobilization of trypsin in the silica sol–gel matrix with a stable gel network through a Si–O–Si bridge via tethering to the silanol groups	[40]
PMMA microchip	Trypsin	Channel wall	Butyl methacrylate (BMA), γ-methyl-acryloxypropyltri-methoxysilane (MAPTS), TMOS, PEG	The microchannel surface was coated with a copolymer of BMA/MAPTS. A silica sol–gel polymer, which was prepared from the reaction mixture of TEOS with PEG, was linked to the silanol groups of the co-polymer on the channel surface, followed by additional TEOS-polymerization containing enzyme to entrap the enzyme in the sol–gel matrix on the channel surfaces	[41]

(*continued*)

Table 5.2 (*Continued*)

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
PDMS microchip	Hydroxylamino-benzene mutase, soybean peroxidase	Beads	Silaffin-derived R5-peptide, TMOS	Enzymes were mixed with silaffin-derived R5 peptide, that facilitates the formation of siliceous nanostructures. Biosilicification and enzyme entrapment simultaneously occur by addition of TMOS into the mixture of R5-peptide and enzyme. The enzyme-immobilized biosilica particles were packed in the microchannels of a PDMS-based microchip	[42]
Silicon microchip	β-Galactosidase	Channel wall	1,1,3,3-Tetramethyl-disiloxane (TMDSO)	Organosilicon films of plasma-polymerized TMDSO (ppTMDSO) were deposited on the surface of microreactor substrate as carrier matrix by "cold remote plasma enhanced chemical vapor deposition" technique. The enzyme was entrapped in the growing organosilicon polymer network by polymerization of TMDSO in the presence of enzyme, or by overcoating of additional ppTMDSO layer on the enzyme-preadsorbed ppTMDSO layer	[43]
PDMS microchip	Trypsin	Channel wall	Alumina, titania	Alumina or titania sol–gel polymerization in the presence of enzyme was performed on the PDMS microchip with a plasma-oxidized surface, resulting in entrapment of enzyme in the sol–gel matrix	[44]

Fused-silica capillary	Acetylcholine esterase	Channel wall	PEI, alginate, chitosan	The procedure entrapping enzyme on a capillary inner wall includes the following steps: coating the surface with PEI, immobilization of enzyme/alginate complex to the PEI layer through electrostatic interactions, fixation of the enzyme in the calcium alginate gel network formed by addition of $CaCl_2$ solution, formation of a thin layer of chitosan on the calcium alginate layer, and coating with PEI to make the thin layer stable	[45]
Fused-silica capillary, Glass microchip	Glucose oxidase	Gel	MAPTS, acrylamide, bis-acrylamide, poly(acrylic acid), PEG	The channel surface was functionalized with MAPTS to enable covalent binding of the gel to the surface. The enzyme was entrapped in a polyacrylamide gel-based monolith by *in situ* polymerization of the reagent mixture containing acrylamide, bis-acrylamide, poly(acrylic acid), PEG, and enzyme	[46]
PDMS microchip	Alkaline phosphatase, urease	Beads	Ethylene glycol diacrylate (PEGDA)	PEG-based hydrogel microstructures were fabricated inside PDMS microchannels using UV initiated free-radical crosslinking of the PEGDA. The hydrogel patterns were dependent on the shapes of photomask. Enzymes were incorporated into the hydrogel by photopolymerization of the reaction mixture supplemented with enzyme	[47]

(*continued*)

Table 5.2 (*Continued*)

Medium	Enzyme	Support	Adsorption materials and reagents	Immobilization procedure	References
PDMS microchip	Glucose oxidase, horseradish peroxidase	Beads	4-Hydroxybuthyl acrylate (HBA), acrylic acid, ethylene glycol dimethacrylate (PEGDA), 2,2-dimethoxy-2-phenyl-acetonphenone (DMPA)	Enzyme-encapsulated polymeric microparticles were prepared by *in situ* photo-polymerization in a continuous flow of a water-immiscible fluid. The polymerizable hydrogel solution, including HBA, acrylic acid, PEGDA, and DMPA, uniformly mixed with enzyme was shaped as microdroplets surrounded with immiscible non-polymerizable sheath fluid (mineral oil) and polymerized by continuous UV exposure in the PDMS-microchannels	[48]
PTFE microchip	Protease	Monolith	Poly(vinyl alcohol) (PVA)	The procedure for encapsulation of protease in aqueous PVA matrix includes the following steps: loading the PVA solution mixed with enzyme into the channel of PTFE microchip reactor, cooling to −40 °C, and freeze-drying under vacuum	[49]

PDMS, polydimethylsiloxane; PMMA, polymethyl methacrylate; PS, polystyrene; PET, polyethylene terephthalate; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; APTES, 3-(aminopropyl)triethoxysilane; MTES, methyltriethoxysilan; TEOS, tetraethoxysilane; TMOS, tetramethoxylsilane; GPTES, γ-glycidoxypropyltrimethoxysilane; PEI, polyethyleneimine; PTFE, polytetrafluoroethylene; MAPTS, g-methacryloxypropyl trimethoxysilane; MPTMS, 3-mercaptopropyl-trimethoxysilane.

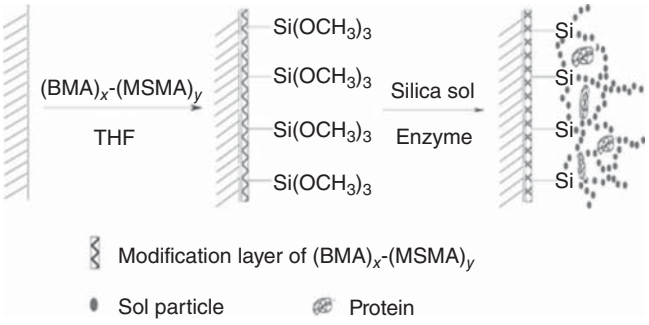


Figure 5.4 Process of functional PMMA surface modification followed by enzyme immobilization using silica sol-gel entrapment. (From [41] with permission ©2004 American Chemical Society.)

polymers are known to have a favorable effect on the stability of the enzymes. Naik *et al.* developed an interesting method for enzyme entrapment in a silica-based matrix using biosilicification [42]. The authors demonstrated that the biosilicification reaction could serve as an alternative method for the entrapment of enzymes using more biologically compatible reaction conditions and applied it to the entrapment of a variety of enzymes.

A novel and facile method for enzyme immobilization using an organosilicon thin film was developed by Elagli *et al*. Organosilicon thin films deposited by remote plasma-enhanced chemical vapor deposition (RPECVD) system have been used in various applications including protein immobilization [43, 50]. Cold plasma polymerization by RPECVD enabled the deposition of 1,1,3,3-tetramethyldisiloxane (TMDSO) monomer containing an enzyme on the substrate, resulting in the stable formation of enzyme-entrapped organosilicon film. β-Galactosidase was entrapped in the growing polymer network of the plasma-polymerized TMDSO (ppTMDSO) as carrier matrix or was coated by the ppTMDSO layer [51].

5.2.2.2 **Non-Silica-based Matrices**

Alumina and titania are metallic-oxide-based biocompatible materials and are able to form sol–gel polymers in a manner similar to silica sol–gel. Microchips with trypsin-entrapping metallic oxide sol–gel matrices have been developed to provide stable and efficient proteolysis in a biocompatible microenvironment [44, 52] (Figure 5.5).

Min *et al.* described an enzyme immobilization method combining electrostatic interaction and film overlay [45]. A complex of acetylcholinesterase (AChE) and the negatively charged alginate polymer was electrostatically adsorbed on the positively charged PEI-coated capillary surface, followed by entrapment of AChE through the formation of a chitosan thin film over the adsorbed enzyme.

Several groups have applied acrylic polymer-based hydrogels as support materials for enzyme entrapment. Mersal *et al.* developed a one-step procedure for enzyme entrapment in a polyacrylamide gel-based monolithic capillary [46].

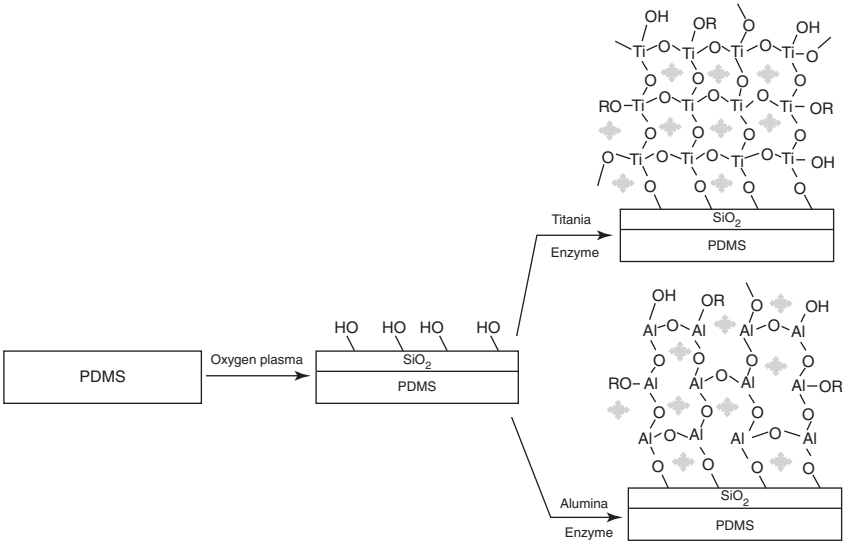


Figure 5.5 Process of forming enzyme-encapsulated sol–gel inside microchannel of PDMS functionalized by oxidation in oxygen plasma. (From [44] with permission ©2004 American Chemical Society.)

Enzyme entrapment was achieved by *in situ* polymerization in the presence of the enzyme, poly(ethylene glycol) (PEG), and negatively charged poly(acrylic acid). The authors optimized the composition of the polymers to maintain the enzymatic activity and to prevent the formation of bubbles and allow liquid transportation by electroosmotic flow.

Particle dispersion- and shape-controlled hydrogel microstructures, in which enzymes were entrapped for enzymatic microreactions, were prepared by UV-initiated cross-linking of PEG diacrylate in continuous microfluids [47, 48].

Aqueous polyvinyl alcohol (PVA) solutions are well known to form a "cryogel", which is a hydrogel formed upon freezing. Proteins are automatically entrapped in the PVA matrix by freeze-drying a PVA solution mixed with the protein. Therefore, PVA cryogels were successfully applied to the immobilization and/or encapsulation of biomaterials [53]. This simple method was applied to protease- and α-amylase-immobilized microreactors [49, 54] (Figure 5.6).

Immobilized lipase is a commonly used biocatalyst in organic synthesis in microreaction systems [55]. Using immobilized lipase on a polymer support allowed the enzymatic resolution of a key intermediate for the synthesis of odanacatib, which is a potent and selective cathepsin K inhibitor currently being evaluated in clinical trials [56]. The immobilized lipase was more active and 15 times more stable than the free enzyme. This permitted a continuous dynamic kinetic resolution process that was significantly less expensive than the original batch process, and with a threefold reduction in the E-factor of the process [56].

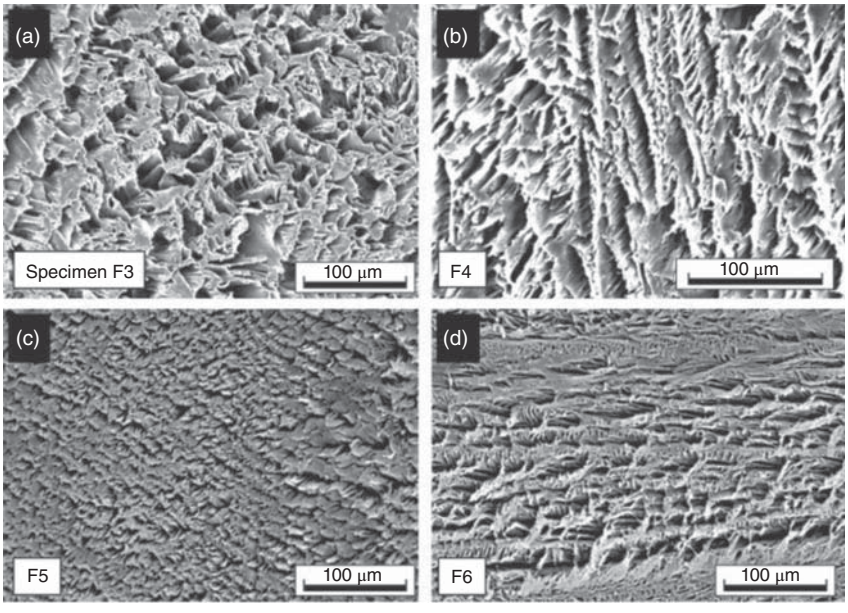


Figure 5.6 SEM images of bulk freeze-dried foams. (a) Specimen prepared in copper sample holder, slow cooling. (b) Copper sample holder, rapid cooling. (c) PTFE sample holder, slow cooling. (d) PTFE sample holder, rapid cooling. (From [54] with permission ©2014 Elsevier B.V.)

5.2.3
Enzyme Immobilization by Affinity Labeling

Noncovalent biological interactions such as His-tag/nickel ion-coordinated nitrilotriacetic acid (Ni-NTA), biotin/avidin, glutathione *S*-transferase (GST)/glutathione, and hybridization of complementary DNA strands show high binding affinity and specificity. These interaction systems have been essential in techniques for various biotechnological processes because they enable highly specific and orientational enzyme immobilization, reversible/repeatable immobilizations, and avoidance of enzyme inactivation. Therefore, affinity labeling systems have been used for the preparation of enzymatic reactors. Table 5.3 presents a summary of enzyme immobilization methods using affinity labeling.

5.2.3.1 **His-Tag/Ni-NTA System**

The simple preparation of an enzyme reactor utilizing a His-tag/Ni-NTA system was reported by Miyazaki *et al*. and Matosevic *et al*., wherein the His-tagged enzymes were immobilized to Ni-NTA introduced on the capillary inner walls modified with silane reagents [57, 70]. The commercially available Ni-NTA-conjugated agarose beads have been utilized by several groups by simply packing them in microreactors [58, 59, 71]. Muñoz *et al.* developed a glass microchip possessing Ni-NTA agarose beads trapped on the PEI/dextran sulfate LBL deposit

Table 5.3 Affinity labeling techniques for enzyme immobilized microreactor preparation.

Medium	Enzyme	Support materials	Linking reagents	Immobilization procedure	References
Fused-silica capillary	Lactic dehydrogenase	Channel wall	APTES, MTES, NTA, carbodiimide/NHS	The inner wall surface of fused-silica capillary was functionalized with amino groups by sol–gel technique utilizing APTES and MTES, followed by coupling NTA as a chelator of Ni ion using the carbodiimide/NHS technique. Histidine-tagged enzyme was immobilized to Ni-chelating NTA group on the capillary surface	[57]
PDMS microchip	Kromycin hydroxylase	Ni-NTA-modified magnetic agarose beads	Commercially available beads	Ni-NTA magnetic agarose beads were packed into the microchannel of PDMS microchip. Histidine-tagged enzyme was immobilized onto the beads using His-tag/Ni-NTA binding	[58]
Fluorinated ethylene propylene (FEP) microtube	Transketolase, transaminase	Ni-NTA-modified agarose beads	Commercially available beads	Ni-NTA agarose beads were packed into a FEP-microtube. Histidine-tagged enzymes were immobilized onto the beads using His-tag/Ni-NTA binding	[59]
PASS *flow* (polymer-assisted solution phase synthesis under flow conditions) microreactor	Benzaldehyde liase, *p*-nitrobenzyl esterase	Polymer beads	Poly(vinyl benzyl chloride), divinyl benzene, *N*-vinylpyrrolidinone, NTA	Polymer beads with high polarity were prepared by cross-linking of poly(vinyl benzyl chloride), divinyl benzene, and *N*-vinylpyrrolidinone. The beads introduced tyrosine- or lysine-based NTA group attached histidine-tagged enzymes using His-tag/Ni-NTA binding. The enzyme-immobilized beads were packed inside a PASS *flow* microreactor	[60]

Glass-microchip	Diacylglycerol acyl transferase	Ni-NTA-modified agarose beads	APTES, PEI, crotonaldehyde, dextran sulfate	The enzyme-immmobilization process includes the following steps: functionalization with APTES on the channel surfaces of glass-microchip, coating with PEI, activation of the channel surface using crotonaldehyde (propionaldehyde), re-coating with PEI and cross-linking between amino and aldehyde groups, attachment of polyanionic dextran sulfate on the PEI-layered channel surface through electrostatic interaction, additional coating of PEI, trapping of Ni-NTA-functionalized agarose beads on the layer-by-layer deposition, and immobilization of His-tagged enzyme to the beads through His-tag/Ni-NTA coordinate bonding	[61]
PDMS microchip	Glucose oxidase, horseradish peroxidase	Streptavidin-modified beads	Commercially available beads	Biotin-labeled enzymes were conjugated onto microbeads coated with streptavidin. These microbeads were subsequently packed into each of the two discreet reaction zones of a PDMS microchip with weirs for retaining the beads	[62]
PDMS-microchip	β-Galactosidase, glucose oxidase, horseradish peroxidase	Channel wall	Polymeric amine, glutaraldehyde, heparin, avidin, biotin-polyethylene glycol spacer-succinimide	The channel surface of a PDMS microchip was coated with avidin-attached multilayers prepared by the following steps: coating with polymeric amine, crosslinking by glutaraldehyde, coating with heparin, crosslinking by glutaraldehyde, coating with heparin, and attachment of avidin. Enzymes conjugated with biotin by biotinyl reagent (biotin–biotin-polyethylene glycol spacer–succinimide) were immobilized to the avidin-modified surface of the microchannel	[63]

(*continued*)

Table 5.3 (*Continued*)

Medium	Enzyme	Support materials	Linking reagents	Immobilization procedure	References
PDMS-glass microchip	Alkaline phosphatase, glucose oxidase, horseradish peroxidase	Channel wall	Fibrinogen, biotin-4-fluorescein	Plasma-oxidized channel surfaces of a PDMS glass microchip were coated with fibrinogen as passivating protein. Biotin-4-fluorescein (B4F) was adsorbed on the fibrinogen thin layer, followed by photo-attachment of B4F to fibrinogen by Ar/Kr laser beam. Streptavidin-linked enzymes were immobilized on the channel surface through avidin/biotin binding	[64]
PDMS microchip	Alkaline phosphatase, glucose oxidase, horseradish peroxidase	Channel wall	1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-Cap-PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-biotin (*N*-biotinyl-Cap-PE)	Phospholipid small unilamellar vesicles (SUVs) were prepared from DLPC, NBD-Cap-PE, and *N*-biotinyl-Cap-PE. SUVs were flowed through the channels of PDMS microchip oxidized by oxygen plasma treatment, or borosilicate-based capillary, resulting in fusion of SUVs onto the channel surfaces and formation of single planner supported membranes containing biotinylated lipid. Avidin-conjugated enzymes were immobilized on the membrane-coated channel surface through avidin/biotin binding	[65]

Fused-silica capillary	Peptide-*N*-glycosidase F	Monolith	Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 2,2′-azobisisobutyronitrile (AIBN), ammonium hydroxide, succinimidyl-6-[(iodoacetyl)amino] hexanoate (SIAH), glutathione	Glutathione-modified monolithic capillary was prepared by the following steps: coating of GMATES sol–gel polymer on the inner surface of a capillary, formation of a co-polymer monolith using a polymerization of GMA, EDMA, and AIBN, amination of the poly(GMA-*co*-EDMA) monolith by ammonium hydroxide, introduction of an iodoacetyl group using amino-reactive SIAH to the monolith, and covalent attachment of glutathione to the monolith using a thiol/iodoacetate reaction. GST-fusion enzyme was immobilized to the monolith through the glutathione/GST binding	[66]
PDMS/glass-microchip	Horseradish peroxidase (HRP)	Channel wall	APTES, disuccinimidyl-glutarate, amino-modified oligonucleotide, thiol-modified oligonucleotide, sulfo-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexan-1-carboxylate (sSMCC)	A microchip was prepared from a PDMS substrate containing microfluidic channels and a glass slide bearing the DNA microarray. The process for immobilizing oligonucleotides for capturing a target DNA on the glass surface includes the following steps: amination of the glass surface with APTES, generating the amino-reactive surface by disuccinimidylglutarate, and covalent attachment of 5′ amino-modified capture oligonucleotide on the active surface. HRP was conjugated with a "target" oligonucleotide thiolated at the 5′-terminus by covalent cross-linking using sSMCC as a hetero-bifunctional cross-linker. Alternatively, a conjugated HRP-oligonucleotide was prepared by reconstitution of apo-HRP (HRP without heme) with a heme group-modified oligonucleotide.	[67]

(*continued*)

Table 5.3 (*Continued*)

Medium	Enzyme	Support materials	Linking reagents	Immobilization procedure	References
				The HRP-oligonucleotide was immobilized on the channel surface by hybridization of the enzyme-conjugated "target" oligonucleotide and its complementary "capture" oligonucleotide on the channel surface	
Fused-silica capillary	Lipase B, horseradish peroxidase, glucose oxidase	Channel wall	2,2,2-Trifluoroethyl undec-10-enoate (TFEE), amino-modified oligonucleotide, azide-modified enzyme, alkyne-modified oligonucleotide	The fused silica capillaries were covalently coated with TFEE by UV irradiation. "The capture" oligonucleotide modified amino group at the 5′-terminus was attached to the capillary wall through amine-reactive trifluoroethanol group on the capillary inner surface. Azide group-functionalized enzymes were covalent linked to 5′-acetylene-modified "target" oligonucleotides by copper-catalyzed alkyne-azide cycloadditions (Click reaction). Enzymes were immobilized on the capillary surface by the complementary hybridization of the enzyme-conjugated target-oligonucleotide and the capture oligonucleotide	[68]
Poly(ether ether ketone) (PEEK) microcolumn	Chymotrypsin	Channel wall	APTES, glutaraldehyde, amino-modified aptamer specific to enzyme	Amino-modified silica beads were treated with glutaraldehyde, resulting in introduction of aldehyde groups on the surface of the beads. ADNA aptamer specific to chymotrypsin, which had a modified amino group at the 5′-terminus, was immobilized on the silica beads through Schiff's base reaction, followed by packing of aptamer-modified silica beads into a PEEK microcolumn	[69]

NTA, *N*-(5-amino-1-carboxypentyl)iminodiacetic acid; GST, glutathione-*S*-transferase.

on a microchannel surface [61]. Drager *et al.* prepared an enzyme-immobilizing support from a Merrifield resin having NTA groups that were synthesized based on tyrosine or lysine [60].

5.2.3.2 GST-Tag/Glutathione System

Proteins fused with GST (i.e., GST-tagged proteins) often have higher specificity and solubility than His-tagged proteins. A glutathione-modified monolithic capillary was developed by Krenkova *et al.* [66]. Glutathione was covalently attached to the polymer monolith with the iodoacetyl group using a thiol/iodoacetate reaction. The GST-fused enzyme was immobilized on the polymer monolith through glutathione/GST binding.

5.2.3.3 Avidin/Biotin System

Rusmini *et al.* inferred that the His-tag/Ni-NTA interaction is relatively weak ($K_d = \sim 10^{-6}$ M) and not too specific, thus a gradual loss of the enzyme is a considerable risk [72]. The avidin/biotin system is the strongest noncovalent biological interaction known ($K_d = \sim 10^{-14}$ M), and is one of the most widely used affinity pairs owing to the high affinity and specificity of the interaction. A simple immobilization method using streptavidin-coated microbeads was developed for a sequential enzymatic reaction by two enzymes [62, 73]. Seong *et al.* conjugated biotin-labeled glucose oxidase and horseradish peroxidase onto streptavidin-coated microbeads. These microbeads were subsequently packed into each of the two discreet reaction zones. Boehm *et al.* attached biotin-labeled enzymes to the avidin-modified microchannel surface of a PDMS microchip [63].

An interesting biotin attachment method for patterning avidin-linked enzymes inside microchannels was developed by Holden *et al.* [64]. Biotin-4-fluorescein was photo-attached to the fibrinogen thin layer coated on the microchannel using a laser beam. Streptavidin-linked enzymes such as glucose oxidase, horseradish peroxidase, and alkaline phosphatase were immobilized on the microchannel surface at the laser-illuminated site through avidin/biotin binding. Furthermore, the authors demonstrated that fibrinogen was more suitable for a passivating the thin-film coating than lysozyme, bovine serum albumin (BSA), and immunoglobulin G. The fibrinogen coating generated the greatest density of specifically bound streptavidin molecules and the lowest nonspecific adsorption (Figure 5.7).

A unique technique using phospholipid small unilamellar vesicles (SUVs) was developed to functionalize a microchannel surface with biotin [65]. The contact of the phospholipid vesicles with the surfaces of the plasma-oxidized PDMS and borosilicate substrates triggers the vesicle fusion, resulting in the formation of a single planer supported membrane on the substrate surfaces [65]. Biotinylation of the microchannel surface for immobilization of avidin-conjugated enzymes was achieved by using SUVs doped with biotinylated phosphatidylethanolamine.

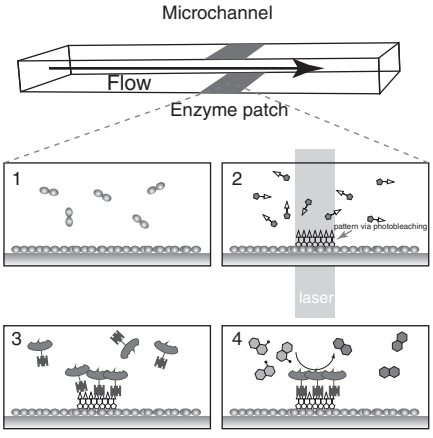


Figure 5.7 Schematic diagram of the photoimmobilization process. Enzyme patches are formed on the top and bottom of a microchannel using the following procedure. (1) Passivation of the surface with a fibrinogen monolayer is followed by (2) biotin-4-fluorescein surface attachment. This is accomplished by photobleaching with 488-nm laser light. (3) Next, the binding of streptavidin-linked enzymes can be exploited to immobilize catalysts and (4) to monitor reaction processes on-chip. (From [64] with permission ©2004, American Chemical Society.)

5.2.3.4 **DNA Hybridization System**

DNA/RNA oligonucleotides hybridize to its complementary strands with high specificity and affinity ($K_d = 10^{-7} - 10^{-9}$ M in a 21-base oligonucleotide with a random sequence) [74]. The unique molecular recognition property has led to several researchers attempting to convert DNA microarrays into protein arrays using a DNA-directed immobilization strategy [75]. Schröder *et al.* also applied the DNA recognition property to the addressable enzyme immobilization for multiple enzymatic cascade reaction in a continuous flow reactor [67]. A "target" oligonucleotide and its complementary "capture" oligonucleotide were covalently attached to an enzyme and a microchannel surface, respectively. The captured DNA microarray surface inside the microfluidic channels was configured through conventional spotting, and the resulting DNA patches could be conveniently addressed with enzymes containing complementary target DNA tags. Vong *et al.* also described a DNA-based method for dynamic positional enzyme immobilization inside silica microchannels [68].

5.2.3.5 **Other Techniques Using Nucleotides for Enzyme Immobilization**

An alternative oligonucleotide-based method was reported by Xiao *et al*. They applied a DNA aptamer, which is a 40-mer oligonucleotide that shows highly specific recognition and binding to the target molecule [69]. Enzyme immobilization was achieved by packing microbeads that are modified with a DNA aptamer specific to chymotrypsin in the microreactor.

5.2.4
Enzyme Immobilization by Covalent Linking

This technique of immobilization involves the formation of a covalent bond between the enzyme and the support material. Covalent bonds usually provide the strongest linkages between the enzyme and the carrier. Leakage of enzyme is often minimized with covalently bound immobilized enzymes. Moreover, immobilization of proteolytic enzymes on solid supports eliminates unwanted enzyme autodigestion and interfering fragments, and an extremely high local concentration of proteolytic enzymes provides rapid catalytic turnover. Table 5.4 presents a summary of enzyme immobilization methods using covalent linking.

5.2.4.1 Immobilization to Solid Supports

Various enzyme reactors have been prepared by packing enzyme-immobilized solid supports such as monoliths, beads, fibers, and nanotubes into microchannels or capillaries. An optimal support should be structurally stable, be chemically inert, and present a very large, solvent-accessible surface area. It should also offer low resistance to fluid flow and the ability to be patterned within microdevices.

Immobilization to Embedded Monolith Almost all polymer monoliths used as enzyme-immobilizing supports in reactors have been prepared by *in situ* polymerization in the microchannels and capillaries. The functionalization of monolith supports for enzyme attachment is mainly done by two methods: polymerization of monomer molecules possessing functional groups that enable enzyme immobilization, or generation of such functional groups by additional chemical treatment after monolith formation in the microreactors.

Several groups have reported enzyme microreactors with inorganic and organic hybrid silica monoliths such as silica sol–gel polymers containing fatty amine, PEG, and poly(glycidyl methacrylate-*co*-acrylamide-*co*-ethylene glycol dimethacrylate). As examples of the former, PEG-based hydrophilic monoliths containing the amino-reactive succinimide groups were prepared using *N*-acryloxysuccinimide as the active monomer to form trypsin-immobilized support in a microchip and a capillary [98, 99]. Also, Peterson *et al.* utilized a polymer possessing azlactone groups for the preparation of trypsin-immobilized monoliths in a capillary [100]. The azlactone group is reactive toward amino and thiol groups of enzymes.

Silica-containing monoliths are tightly packed in glass- and/or PDMS-based reactors through covalent linkage of the monolith to the microchannel surfaces. Anuar *et al.* employed a hybrid monolithic capillary prepared by a simple mixing of PEG and tetraethoxysilane [101]. The embedded monolith was functionalized with 3-aminopropyl triethoxysilane (APTES), and then lipase was covalently linked to the monolith through Schiff's base reaction using glutaraldehyde. Similarly, in order to immobilize enzymes, the hybrid monoliths formed in the microreactors were functionalized by additional modifications by glutaraldehyde [102–104] and trimethoxysilyl butyraldehyde [105]. Lin *et al.* developed

Table 5.4 Covalent linking techniques for enzyme immobilized microreactor preparation.

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
PMMA microchip	Trypsin	Channel wall	EDC, NHS	Carboxylic acid termini were yielded on the surface of PMMA microchannels by exposure to UV light. Trypsin was covalently bound to the carboxyl groups using the activation reagents: EDC and NHS	[76]
UV-activated PS microchip	Trypsin	Channel wall	Glutaraldehyde	PS substrate sheets were irradiated with UV through a microchannel-designed photomask, resulting in the generation of carboxylic acid termini on the PS sheet surface. The carboxyl groups were activated by EDC, and then aminated with ethylenediamine. An aldehyde-functionalized PS sheet was obtained by reacting glutaraldehyde with amino groups. The enzyme was covalently bound to the PS sheet surface using a Schiff's base reaction	[77]
Hydrolyzed Nylon microtube	Carbonic anhydrase II	Channel wall	Glutaraldehyde	Hydrolysis of the inner surface of a nylon microtube with HCl to generate amino groups on the surface, aldehyde-functionalization of the surface by glutaraldehyde, covalent linkage of chitosan to the aldehyde groups, reactivation of the surface by attachment of glutaraldehyde to the coated chitosan, and coupling of the enzyme on the Nylon surface using a Schiff's base reaction	[78]
Fused-silica capillary	Trypsin	Channel wall	APTES, glutaraldehyde	The inner wall surface of a fused-silica capillary was functionalized with APTES, followed by introduction of aldehyde groups with glutaraldehyde. The enzyme was covalently linked to the aldehyde group of the capillary surface	[79]

Silica-based photonic crystal fibers	Lipase	Channel wall	APTES, glutaraldehyde	The inner wall surfaces of silica-based photonic crystal fibers were functionalized with APTES, followed by introduction of aldehyde groups using glutaraldehyde. The enzyme was covalently linked to the aldehyde group of the channel surface	[80]
Fused-silica capillary	Lipase	Channel wall	APTES, MTES, EDC, NHS	The inner wall surface of a fused-silica capillary was functionalized with amino groups by sol–gel technique utilizing APTES and MTES, followed by coupling the enzyme using the EDC/NHS technique	[81]
Silicic acid-supplemented PDMS microchip	Thermophilic β-glycosidase	Channel wall	APTES, glutaraldehyde	A PDMS microchip was prepared by curing dimethylsiloxane with pyrogenic silicic acid as a supplement, which enables the introduction of hydroxyl groups on the microchannel wall. The channel surface was functionalized with APTES, followed by linking glutaraldehyde to the amino groups of the APTES-sol–gel polymer. Enzymes were covalently immobilized on the channel surface through the aldehyde groups	[82]
Fused-silica capillary	Pepsin A	Channel wall	GPTES	The enzyme was covalently linked to the aldehyde group of the wall surface of fused-silica capillary functionalized with GPTES. The aldehyde group was generated by hydrolysis of the epoxide group and oxidation of the hydroxide groups	[83]

(*continued*)

Table 5.4 (*Continued*)

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
Plasma-oxidized PDMS microchip	Urease	Channel wall	APTES, carbodiimide, NHS, cysteamine, 3-nitro-2-pyridinesulfenyl chloride (Npys-Cl)	The plasma-oxidized channel surface of a PDMS microchip was coated with APTES by sol–gel technique, followed by carboxylation of the aminated surface by treatment with succinic anhydride. Urease was covalently linked to carboxyl groups of the channel surface using the carbodiimide/NHS technique. For enzyme immobilization through the disulfide bond, cysteamine was reacted with the activated carboxyl group of the channel surface by carbodiimide/NHS, followed by activation of the incorporated thiol groups on the channel surface by treating with Npys-Cl. The thiol groups of the enzyme were immobilized on the channel surface through disulfide bond between the enzyme-thiol and the active thiol of the channel surface	[84]
Fused-silica capillary	Pepsin	Channel wall	APTES, MTES, dextran, EDC/NHS	The inner wall surface of a fused-silica capillary was functionalized with APTES, followed by coupling carboxyl-modified dextran and additional amino-modified dextran using the EDC/NHS technique. The enzyme was covalently linked to EDC-activated carboxyl group of dextran polymerized on the capillary surface	[85]
Glass microchip	Soybean peroxidase	Channel wall	APTES, poly(maleic anhydride-alt-α-olefin) (PMA)	The microchannel surface of glass microchip was functionalized with amino groups by sol–gel polymerization of APTES. The amino-functionalized surfaces were coated with PMA, followed by immobilization of enzyme. Maleic anhydride groups enable the PMA matrix to covalently link to amino groups of the channel surface and enzyme	[86]

Fused-silica capillary	Trypsin	Channel wall	APTES, *N*-(4-azidobenzoyloxy)-succinimide (ABS)	The inner wall surface of a fused-silica capillary was functionalized with APTES, followed by photo-initiated polymerization of ABS. The enzyme was immobilized through the succinimide group of ABS	[87]
Fused-silica capillary	Lipase	Channel wall	APTES, glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 4,4′-azobis(4-cyanovaleric acid) (ACA)	The inner surface of a fused-silica capillary was coated with APTES sol–gel polymer. The mixture of GMA, EDMA, and ACA was polymerized inside the capillary, resulting in formation of co-polymer monolith of poly(GMA-co-EDMA) that was attached to methacrylate group of the capillary surface. The enzyme was immobilized to the glycidyl groups on the monolith by coupling of the epoxide groups and amine groups of enzymes	[88]
Silicic acid-supplemented PDMS microchip	Thermophilic β-glycosidase	Channel wall	APTES, glutaraldehyde, polyamidoamine-dendrimers (PAMAM generation 5 dendrimers)	Covalent attachment of the enzyme onto the microchannel walls via a dendrimeric linker. Immobilization of PAMAM-dendrimers was performed after the APTES-functionalized microchannel surface had been activated with glutaraldehyde	[89]
Alumina-coated stainless steel microchip	β-Glucosidase	Channel wall	APTES, glutaraldehyde	A stainless steel microchip, which has γ-aluminum oxide-coated microchannels, was functionalized with APTES, followed by introduction of aldehyde groups with glutaraldehyde. The enzyme was covalently linked to the aldehyde group of the channel surface.	[90]

(*continued*)

Table 5.4 (*Continued*)

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
Hydrated ceramics microchip	Urease	Channel wall	APTES, glutaraldehyde	Hydroxyl groups were yielded on the surface of LTCC (low temperature co-fired ceramics) microchannels by hydration with a powerful etching solution (HNO_3/H_2O_2), followed by silanization of the hydrated surface with APTES. The aminated surface was functionalized with aldehyde groups using glutaraldehyde. The enzyme was covalently attached to the aldehyde groups on the channel surface through the formation of a Schiff's base	[91]
PMMA-microchip	Trypsin	Channel wall	TEOS, graphene oxide GO), EDC, NHS	The channels of PMMA-microchip were coated with a layer of hydrophilic silica using an *in situ* hydrolysis of TEOS. GO mixed with TEOS-sol was loaded into the silica-coated channels and polymerized, resulting in a coating of GO–silica composite on the channel surface. The enzyme was covalently linked to the carboxy group of GO using the EDC/NHS technique	[92]
PDMS-microchip	Trypsin	Channel wall	Poly(acrylic acid) (PAA), EDC, NHS	PAA polymer was graft-polymerized onto the surface of PDMS microchannel by UV exposure. The enzyme was covalently bound to carboxyl groups of the PAA using the activation reagents; EDC and NHS.	[93]
Hydrolyzed PMMA microchip	Glucose oxidase	Channel wall	Sodium hydroxide, PEI, glutaraldehyde	The channel walls of a PMMA microchip were heated and treated with sodium hydroxide solution to hydrolyze the ester groups from the surface of the PMMA, followed by adhesion of PEI on the carboxyl-group-generating channel surface through electrostatic interaction. The enzyme and glutaraldehyde were loaded into the PEI-coated channels, resulting in a covalent linkage of oxidase to PEI through a Schiff's base reaction with glutaraldehyde	[94]

PC microchip	Alkaline phosphatase	Channel wall	PEI, poly(ethylene glycol) diglycidyl ether (PEGDE), carbodiimide	The channel walls of a PC microchip were adsorbed with PEI. The enzyme was immobilized to the surface by covalent crosslinking between the enzyme and the PEI using PEGDE, homo-bifunctional crosslinker, or by direct binding between PEI and the carbodiimide-activated enzyme	[95]
Au-electrode-integrated glass microchip	Cholesterol oxidase	Au-electrode in channel	Thioglycolic acid (TGA), EDC	TGA was coated as a self-assembled monolayer (SAM) on the gold (Au) electrode in a microsensor chip through coordinate bonding of thiol-Au, followed by activation of carboxylic group of TGA-SAMs by EDC. The enzyme was immobilized onto the amino-reactive SAM through the amide bond formation between TGA-SAM and the enzyme	[96]
Silicon/PTFE/glass microchip	β-Galactosidase	Channel wall	Silica-based nanospring, APTES, *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), dithiothreitol	Silica-based nanospring mats (60-μm thick) were functionalized with amino groups by sol–gel polymerization of APTES, followed by modification with SPDP in order to introduce thiol-reactive pyridyl disulfide (PDS) groups. The active nanosprings were treated with dithiothreitol, resulting in generation of free thiol groups on the nanospring surface. The PDS-modified enzyme was immobilized by covalent disulfide linkages with the surface of nanosprings. The enzyme-coated nanospring mat was placed into a microchannel, with the mat partially occluding the channel	[97]

(*continued*)

Table 5.4 (*Continued*)

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
Glass microchip	Trypsin	Monolith	Poly(ethylene glycol) diacrylate (PEGdA), *N*-acryloxysuccinimide (AS)	A hydrophilic monolithic support was prepared in glass-microchannels by photopolymerization using PEGdA as hydrophilic crosslinker and AS as active monomer. The enzyme was covalently linked to the monolithic support through the succinimide groups	[98]
Fused-silica capillary	Trypsin	Monolith	Acrylamide, *N*-acryloxysuccinimide (AS), ethylene dimethacrylate (EDMA)	Succinimide-activated monolith in a fused-silica capillary was prepared by in situ polymerization of acrylamide, *AS* and EDMA in the presence of a dodecanol/cyclohexanol mixture, which enables the formation of porogenic structures in the monolith. Enzyme was immobilized through the succinimide group of the monolith	[99]
Fused-silica capillary	Trypsin	Monolith	2-Vinyl-4,4-dimethyl azlactone, ethylene dimethacrylate, acrylamide	Trypsin was immobilized by covalent bonding of the amine and thiol groups of the enzyme with the azlactone groups on porous monoliths, which formed by polymerization of 2-vinyl-4,4-dimethyl azlactone, ethylene dimethacrylate and acrylamide in a capillary	[100]
Fused-silica capillary	Lipase	Monolith	TEOS, PEG, APTES, glutaraldehyde	A monolith prepared by simple mixing of PEG and TEOS in a fused-silica capillary was functionalized with APTES, and then lipase was covalently linked to the monolith using a Schiff's base reaction with glutaraldehyde	[101]

Fused-silica capillary	Trypsin	Monolith	glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), MAPTS, ammonium hydroxide, glutaraldehyde,	Poly(glycidyl methacrylate-co-ethylene dimethacrylate) (poly-GMA-EDA) monolith was prepared by in situ polymerization of GMA and EDMA in a capillary that was functionalized with MAPTS on the inner surface. The process for immobilizing the enzyme to the monolith includes the following steps: amination of the monolith with ammonium hydroxide, activation by glutaraldehyde, and linking trypsin to the aldehyde group of the monolith	[102]
Fused-silica capillary	Trypsin	Monolith	TEOS, APTES, cetyltrimethyl ammonium, glutaraldehyde	An inorganic and organic hybrid silica monolith was prepared with TEOS, APTES, and cetyltrimethyl ammonium bromide in a fused-silica capillary. The enzyme was covalently linked to the amino groups of the monolith through the intermediary of glutaraldehyde	[103]
Fused-silica capillary	Trypsin	Monolith	Ethyleneglycol dimethacrylate (EGDMA), acrylamide, glycidyl methacrylate (GMA), MAPTS, ammonium hydroxide, glutaraldehyde	Poly(glycidyl methacrylate-co-acrylamide-co-ethylene glycol dimethacrylate) monolith was formed in a fused-silica capillary, functionalized with MAPTS on the surface by in situ polymerization of EGDMA, acrylamide and GMA. The enzyme immobilization process includes the following steps: amination of the grafted glycidyl groups on the monolith with ammonium hydroxide, covalent linkage of glutaraldehyde to the amino groups and binding of enzyme through the aldehyde groups	[104]

(*continued*)

Table 5.4 (*Continued*)

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
Fused-silica capillary	Trypsin	Monolith	PSG-PEG, MAPTS, trimethoxysilyl-butyraldehyde (TMSBA)	MAPTS-sol–gel monolith incorporated with PEG was prepared by photo-polymerization in a capillary, followed by functionalization with TMSBA. The enzyme was attached to the aldehyde-activated monolith	[105]
Fused-silica capillary	V8 protease	Monolith	Butyl acrylate, 1,3-butanediol diacrylate (BDA), MAPTS, 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPSA), benzoin methyl ether (BME), carbonyldiimidazole	Tandem enzyme-immobilized capillary was prepared by the following steps: preparation of monolithic support in a fused-silica capillary by *in situ* photo-polymerization of butyl acrylate, BDA, MAPTS, AMPSA, and BME (photo-initiator), additional photo-graft polymerization with glycidyl methacrylate at one limited area of the monolith by masking the other area during exposure, and immobilization of an enzyme to the glycidyl group with carbonyldiimidazole. Enzymes were sequentially immobilized by repeating the photografting and immobilization steps on separate sections of the monolith	[106]
PDMS microchip	Trypsin	Magnetic beads	EDC, sulfo-NHS (*N*-hydroxy-sulfosuccinimide), carbodiimide, 2,4,6-trichloro-1,3,5-triazine	Magnetic particles differentially coated with various polymers, which were covalently attached with enzyme, were packed in a PDMS-based microchip.	[107]

Glass microchip	Trypsin	Nanoparticles	Glutaraldehyde	Aldehyde-functionalized magnetic nanoparticles were prepared by treating the amine-functionalized magnetic nanoparticles with glutaraldehyde. The nanoparticles were covalently attached with trypsin through the aldehyde groups. The enzyme particles were packed onto the glass microchip by a strong magnetic field	[108]
PDMS microchip	Proteinase-K	Carboxyl-modified polystyrene-magnetic beads	EDC, NHS	The enzyme was covalently attached with carboxyl group-functionalized polystyrene-coated magnetic beads by the EDC/NHS technique. The enzyme-grafted beads were packed inside a PDMS microchannel using a magnetic field	[109]
Adhesive PMMA microchip	Pepsin	Agarose beads	Commercially available pepsin beads	Pepsin-immobilized agarose beads were adhered onto the adhesive PMMA surface, which was functionalized using dichloromethane in a microchannel	[110]
Glass tube	L-allo-threonine aldolase	Eupergit beads	Ethylenediamine, glutaraldehyde	Direct method: the enzyme was mixed with Eupergit, resulting in covalent linking of enzyme-amino group to Eupergit-epoxy group. Indirect method: amination of Eupergit with ethylenediamine, activation by glutaraldehyde, and linking enzyme to aldehyde group of the activated Eupergit	[111]
PMMA microchip	Trypsin	Glass-fibers	Chitosan, glutaraldehyde	Fiberglass bundles were sandwiched between a PMMA cover plate and a PMMA base plate, resulting in packing of the bundles into a microchannel of the PMMA chip. The glass fibers were coated with positively charged chitosan through physical adsorption. The chitosan was functionalized with aldehyde groups by treating with glutaraldehyde, followed by attachment of the enzyme to the aldehyde groups	[112]

(*continued*)

Table 5.4 (*Continued*)

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
PDMS microchip	β-Galactosidase	Carboxylated multi-walled carbon nanotubes (MWNTs), single-walled carbon nanotubes (SWNTs)	EDC, glutaraldehyde, oligonucleotide	MWNTs microreactor: MWNTs were oxidized by H_2SO_4/HNO_3, resulting in the formation of high-density carboxylic acid groups on the surface of the MWNTs. The carboxylated MWNTs, β-galactosidase, and the aminated surface of PDMS microchannels were covalently linked by an EDC coupling reaction. SWNTs microreactor: aldehyde groups were introduced on the PDMS microchannel walls by glutaraldehyde. SWNTs were wrapped with 15-mer oligonucleotide, in order to functionalize with amine groups. The aminated SWNTs and enzyme were covalently linked by EDC coupling. The enzyme-bound SWNTs were immobilized on the surface of the PDMS microchannels through aldehyde groups	[113]
PTFE/indium tin oxide-coated glass microchip	Trypsin, pepsin	Cylindrical agarose discs	Glycidol (2,3-epoxypropanol), sodium periodate	Preformed agarose discs were functionalized with glyoxyl group using glycidol (2,3-epoxypropanol), followed by oxidation with sodium periodate to form aldehyde groups. The enzyme was covalently linked to on the aldehyde group of agarose via Schiff's base reaction	[114]

PDMS, polydimethylsiloxane; PMMA, polymethyl methacrylate; PS, polystyrene; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; APTES, 3-(aminopropyl)triethoxysilane; MTES, methyltriethoxysilan; TEOS, tetraethoxysilane; TMOS, tetramethoxylsilane; GPTES, γ-glycidoxypropyltrimethoxysilane; PEI, polyethyleneimine; PTFE, polytetrafluoroethylene; MAPTS, g-methacryloxypropyl trimethoxysilane; MPTMS, 3-mercaptopropyl-trimethoxysilane.

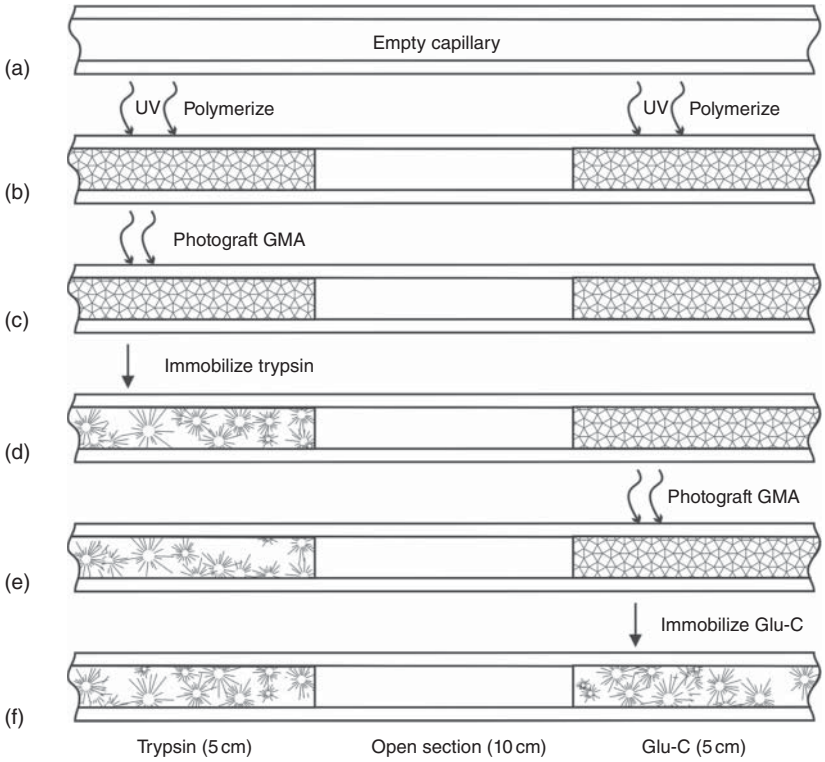


Figure 5.8 Scheme for the preparation of enzyme reactors with two proteases. (a) Empty Teflon-coated capillary (100 μm id/365 μm od). (b) Fabrication of the monolith column. (c) One section of monolith photografted with glycidyl methacrylate (GMA) by masking the other section during exposure. (d) Trypsin immobilized onto the GMA grafted monolith. (e) The second section of monolith photografted with GMA. (f) V-8 protease (Glu-C) immobilized onto the second GMA grafted monolith. (From [106] with permission ©2009, John Wiley and Sons.)

a tandem-type dual-enzyme-immobilized capillary using a glycidyl group-containing hybrid monolith. The amino groups of the enzyme were activated by carbonyldiimidazole and immobilized through the reaction with glycidyl groups of the monoliths [106] (Figure 5.8).

Immobilization to Beads (Micro/Nanoparticles and Nanotubes) Magnetic beads have been often used as solid support for covalently linked enzymes, which could be easily packed at the desired positions in the microchannel using a magnetic field. Aldehyde-modified magnetic NPs that were prepared by treating the amine-functionalized NPs with glutaraldehyde were developed as a glass microchip for trypsin digestion [108]. Direct attachment of the enzyme on the surface of the magnetic beads was achieved by activation of the carboxyl-functionalized beads using the carbodiimide technique [109, 115]. This immobilization method was

adapted for the preparation of a replaceable dual-enzyme capillary microreactor [116].

Tibhe *et al.* developed an enzymatic microreactor using Eupergit® oxirane acrylic beads, which provide a rapid and simple support for protein immobilization [111]. Eupergit has a high density of epoxy groups that can covalently link to the nucleophilic groups of the protein, such as amino, hydroxyl, and thiol groups, without any additional reagents. Therefore, Eupergit enables efficient enzyme immobilization because of the multipoint attachment of the enzyme.

A simple method for the fixation of nonmagnetic beads on a microchannel was reported by Liuni *et al.* They showed the adhesion of pepsin-immobilized agarose beads onto the PMMA surface, which was adhesive owing to treatment with dichloromethane in a microchannel [110]. Carbon nanotubes (CNTs) are of interest to many researchers because of their high tensile strength, high resilience, flexibility, and other unique structural, mechanical, electrical, and physicochemical properties [117]. The use of two types of CNTs, namely single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs), in a PDMS microchip for enzyme immobilization was described by Song *et al.* [113]. To introduce the functional group on the CNTs and increase the water solubility, the SWNTs were wrapped with DNA and the MWNTs were oxidized by strong oxidants. Such modifications enabled the generation of a high density of carboxylic groups on the MWNTs and amino groups on SWNTs (Figure 5.9).

Immobilization to Other Supports A stainless steel microchip with alumina-coated microchannels was developed as an enzyme-immobilized microreactor [90]. Alumina (γ-aluminum oxide) is attachable with silane coupling reagents. Therefore, β-glucosidase was immobilized on a layer of alumina (γ-aluminum oxide) modified with APTES using glutaraldehyde.

Malecha *et al.* developed ceramic-based enzyme-immobilized reactors [91]. Modern low-temperature cofired ceramic (LTCC) technology is much cheaper

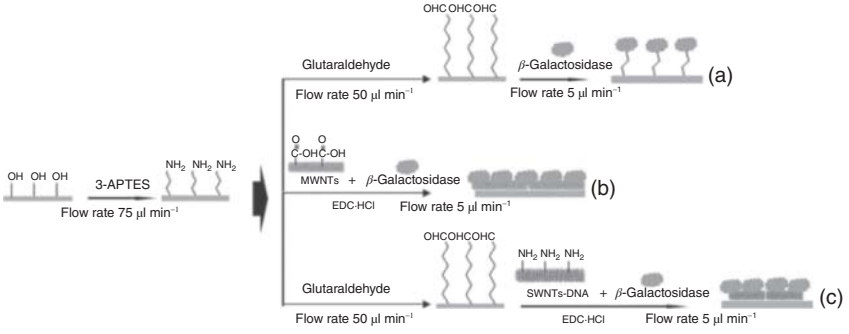


Figure 5.9 Scheme of β-galactosidase immobilization on a microchannel surface. (a) Glutaraldehyde (GA)-microreactor. (b) MWNTs-microreactor. (c) SWNTs-DNA-microreactor. (From [113] with permission ©2012 Elsevier B.V.)

than silicon/glass technology, and the process of developing a new device is much easier and faster. The LTCC material is more chemical- and temperature-resistant than silicon and polymers. Hydration of the ceramics enables the generation of hydroxyl groups linkable to APTES [91].

Non-silica methods have been developed by several groups, wherein the wall surfaces of microchannels and capillaries were covalently coated with the functionalized synthetic polymers. Wu *et al.* utilized poly(acrylic acid) graft-polymerized onto the surface of a PDMS microchannel [93]. Cerdeira *et al.* reported PEI-mediated enzyme immobilization in a PMMA microchip. Glucose oxidase was immobilized by cross-linking the carboxylated PMMA surface, the surface-coated PEI, and the enzymes using a glutaraldehyde cross-linker [94].

As a surface-modification method using non-polymer molecules, Rahman *et al.* proposed a method for enzyme immobilization mediated by self-assembled monolayers (SAMs) with a functional group [96]. Cholesterol oxidase was covalently attached to thioglycolic acid SAM coated on the gold (Au)-electrode in a microchip using the 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC)/NHS (*N*-hydroxysuccinimide) technique, which is discussed in Section 5.2.4.2.

A study by Schilke *et al.* demonstrated the application of nanosprings as novel and highly efficient carriers for immobilized enzymes in microreactors [97] (Figure 5.10). Nanosprings are formed from helical silicon dioxide, which can be grown by a chemical vapor deposition process on a wide variety of surfaces.

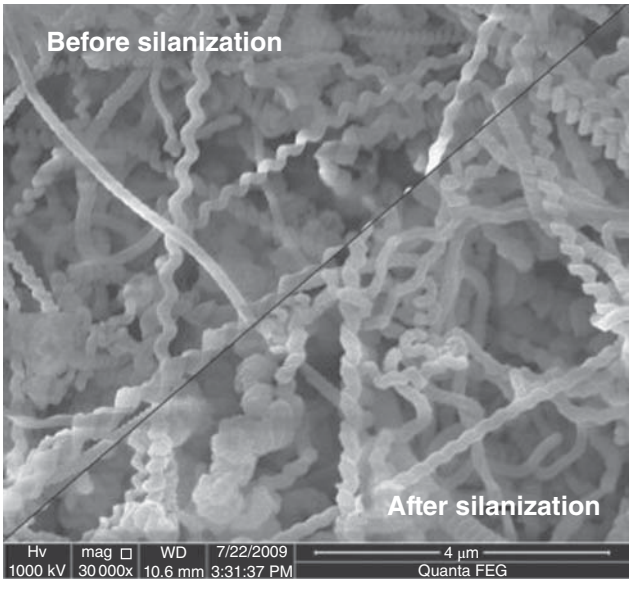


Figure 5.10 Morphology of silicon dioxide nanosprings before (top) and after (bottom) vapor-phase silanization with APTES. (From [97] with permission ©2010 John Wiley and Sons.)

The unique properties of nanosprings are their mechanical flexibility and ability to store potential energy when stretched or compressed, which enables low brittleness and resistance to fluid flow [118].

Silica-based nanospring mats grown on the gold-coated area of a silicon wafer were functionalized with APTES, followed by the introduction of free thiol groups through the amino groups on the nanospring surface. β-Galactosidase modified with a thiol-reactive pyridyl disulfide group was immobilized through covalent disulfide linkages with the surface of nanosprings. The enzyme-coated nanospring mat was placed into a microchannel, with the mat partially occluding the channel. A similar method was adapted for the preparation of a threonine aldolase-immobilized microreactor by Fu *et al.*, in which 3-mercaptopropyltrimethoxysilane was used instead of APTES [119].

5.2.4.2 Direct Immobilization to a Channel Wall

The simplest approach for immobilization is direct attachment of the enzyme to the "wall" itself of the microchannel or the capillary. The direct wall modification with enzymes was achieved by generating functional groups on the channel surface of the polymer-based microreactors using UV irradiation or chemical hydrolysis methods [76–78].

A variety of surface coating methods have been developed for enzyme immobilization on the inner wall of microchannels and capillaries. Silanization of the channel surface by sol–gel polymerization is the most commonly used technique for the preparation of microreactors made of silanol-containing materials such as glass and PDMS (Figure 5.11). In particular, the use of APTES is convenient

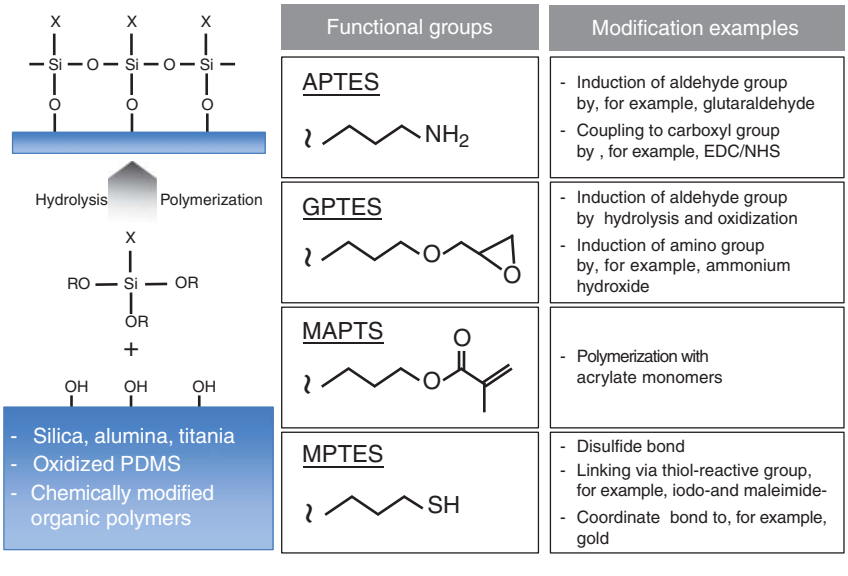


Figure 5.11 Functional silanization techniques.

for introducing a functional group (amino group) on the channel surface as a base for surface modification. Enzymes have been immobilized to the amino-functionalized surface of microchannels and capillaries using activation reagents. The combination of *N*-hydroxysuccinimide (NHS) and a carbodiimide reagent, such as EDC, has been used for the formation of an amide bond between the carboxyl group of the enzyme and the amino group of the microchannel surface [81]. Glutaraldehyde is frequently used for enzyme immobilization as an amine-reactive homo-bifunctional cross-linker. Cross-linking of the enzyme's amino group and the surface amino group of the microchannel wall by glutaraldehyde using a Schiff's base reaction has been reported as a simple enzyme-immobilizing technique [79, 82]. This method has been applied to the preparation of an enzyme microreactor utilizing photonic crystal fibers [80, 120]. This fiber has a micro structured arrangement of air channels within a flexible acrylate polymer-coated silica tube (Figure 5.12).

Tang *et al.* reported the carboxylation of PDMS surface aminated using APTES sol–gel by treatment with succinic anhydride. Immobilization was achieved through bonding between the amino group of urease and carboxyl group on the microchannel surface by using the EDC/NHS technique [84]. An alternative simple method using γ-glycidoxypropyltrimethoxysilane (GPTES) was proposed by Krenkova *et al.* GPTES could generate aldehyde groups for enzyme immobilization by hydrolysis/oxidation of glycidoxy groups [83].

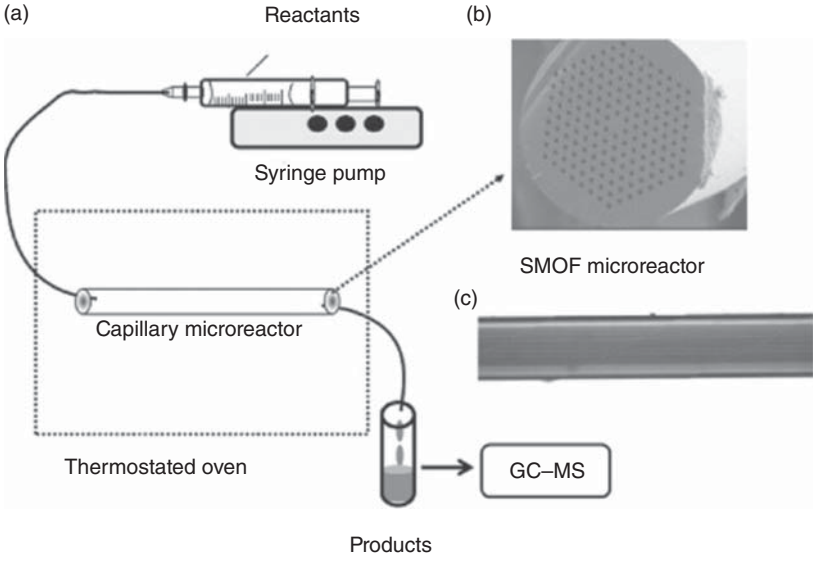


Figure 5.12 (a) Schematic setup of the flow-through silica microstructured optical fiber (SMOF) microreactor. (b) SEM image of a cross-section of the SMOF microreactor. (c) Micrograph of the SMOF microreactor. (From [80] with permission ©2010 Elsevier B.V.)

Table 5.5 Enzyme polymerization techniques for enzyme-immobilized microreactor preparation.

Medium	Enzyme	Support	Linking reagents	Immobilization procedure	References
PTFE tube	α-Chymotrypsin	Channel wall	Glutaraldehyde, paraformaldehyde	The enzyme was immobilized onto the internal surface of the PTFE tube by forming an enzyme-polymerized membrane through crosslinking enzymes between each other with glutaraldehyde and paraformaldehyde	[121]
PTFE tube	Acylase	Channel wall	Poly-L-lysine, glutaraldehyde, paraformaldehyde	The enzyme was immobilized onto the internal surface of the PTFE tube by forming an enzyme-polymerized membrane through crosslinking enzymes and poly-L-lysine as a support polymer with glutaraldehyde and paraformaldehyde	[122]
Fused-silica capillary	Aminoacylase	Controlled pore glass particles	Formaldehyde	L-Aminoacylases precipitated with ammonium sulfate were crosslinked using formaldehyde. The crosslinking enzyme aggregates were combined with controlled pore glass particles and then packed in capillary reactors and trapped by a silica frit	[123]
Fused-silica capillary	γ-Lactamase	Controlled pore glass particles	Formaldehyde	Dried crosslinked enzyme polymeric aggregates were mixed with controlled pore glass, followed by packing the mixture into capillary tubes with a frit at one end	[124]
Fused-silica capillary	Chymotrypsin	Channel wall	APTES, glutaraldehyde	A capillary inner surface was functionalized with APTES, followed by introduction of aldehyde groups with glutaraldehyde. The enzyme was covalently linked to the aldehyde group on the channel surface. The sequence of the aldehyde functionalization and then enzyme linkage was repeated three times, resulting in the formation of a heterogeneous solid material made of crosslinked enzyme polymer on the channel surface	[125]

PDMS microchip	Choline oxidase (ChOx), acetylcholine esterase (AChE)	Gold/silicon electrode in microchannel, magnetic microparticles	Cystamine, poly(L-lysine), glutaraldehyde, aldehyde-modified dendrimers	The procedure of enzyme immobilization includes the following steps: (i) ChOx immobilization: fabrication of a self-assembled monolayer (SAM) of cystamine on the micropatterned gold/silicon electrode in a channel of a PDMS microchip, mixing of ChOx, poly(L-lysine) (PLL), and glutaraldehyde, injection of the mixture into the microchannel to crosslink ChOx/PLL/SAM surface using a Schiff's base reaction between the aldehyde groups and amine groups from SAM, PLL, and ChOx. (ii) AChE immobilization: AChE was conjugated to magnetic microparticles (MPs) coated with aldehyde-group-terminated dendrimers through a Schiff's base reaction. Subsequently, AChE-modified MPs were deposited on the ChOx-immobilized electrode using a magnet	[126]
PDMS microchip	Horseradish peroxidase, alkaline phosphatase	Agarose gel	Ure2 prion domain fused to enzyme	Enzyme modified with amyloid fibril-forming module were prepared by self-assembly of chimeric enzyme fused with Ure2 prion domain. The enzyme-polymeric fibrils were immobilized in the agarose gel-packed channels of a PDMS microchip by stacking the fibrils on the agarose interface	[127]

5.2.4.3 Enzyme Polymerization

An enzyme polymerization method that covalently connects enzymes to each other by using a cross-linker, partly categorized as covalent linking, enables the formation of miniature structures made of the polymerized enzymes in a reactor.

Enzyme immobilization methods depend on the formation of enzymatic microstructures by the polymerization of enzyme molecules. The enzymatic microstructures basically do not require any solid supports and are composed of highly condensed enzyme molecules. Table 5.5 presents a summary of enzyme immobilization methods using enzyme polymerization.

Our group has developed a facile and inexpensive preparation method for an enzyme-immobilized reactor using the enzyme cross-linking reaction [121]. The immobilization of enzymes can be achieved by the formation of an enzyme-polymeric membrane on the inner wall of a PTFE tube through cross-linking polymerization in laminar flow [121] (Figure 5.13). The formation of a protein-based cylindrical microstructure is unique to microfluidic systems. The enzyme-polymerized membrane was based on a cross-linked enzyme aggregate (CLEA) prepared by using a cross-linker with aldehyde groups, which react with the amino groups of the enzyme. Preparation of a CLEA-based enzyme microreactor (CEM) using electronegative enzymes is not possible because of the relative scarcity of amino groups, which results in inefficient formation of CLEA. To expand the generality of CEM preparation, we developed an improved method using poly-L-lysine (poly-Lys) as an aggregation booster/adjunct for the effective polymerization of electronegative enzymes [122] (Figure 5.14). The CEM preparation could be expanded to microreactors with a broad range of functional proteins. Utilizing this method, several enzymatic microfluidic systems have been developed [128–131].

Ghafourifar *et al.* developed a microreactor with chymotrypsin-CLEA that covalently attaches on the surface of amine-functionalized fused-silica capillaries [125]. Modified CLEA-based microreactors have been reported in which packed aminoacylase- or γ-lactamase-CLEA have been combined with pore-glass particles [123, 124]. Han *et al.* have described a CLEA-based bienzyme microchip

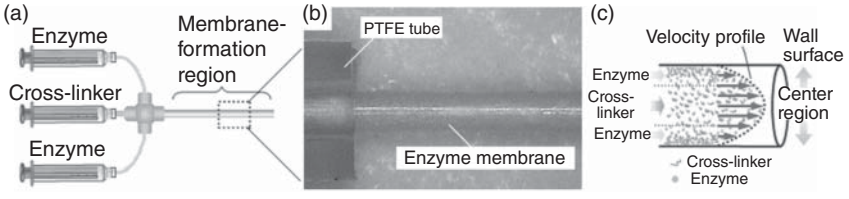


Figure 5.13 Preparation of enzyme-membrane on the inner wall of a PTFE tube. (a) Enzyme and aldehyde solutions were each charged into a 1-ml syringe, and the solutions were supplied to a PTFE tube using a syringe pump. (b) Cylindrical enzyme-membrane (dry state) exposed from PTFE tube, which forms on the inner wall of the tube. (c) Possible mechanism of polymerization process of enzyme and cross-linker reagent in a microchannel. (From [121] with permission ©2005 Royal Society of Chemistry.)

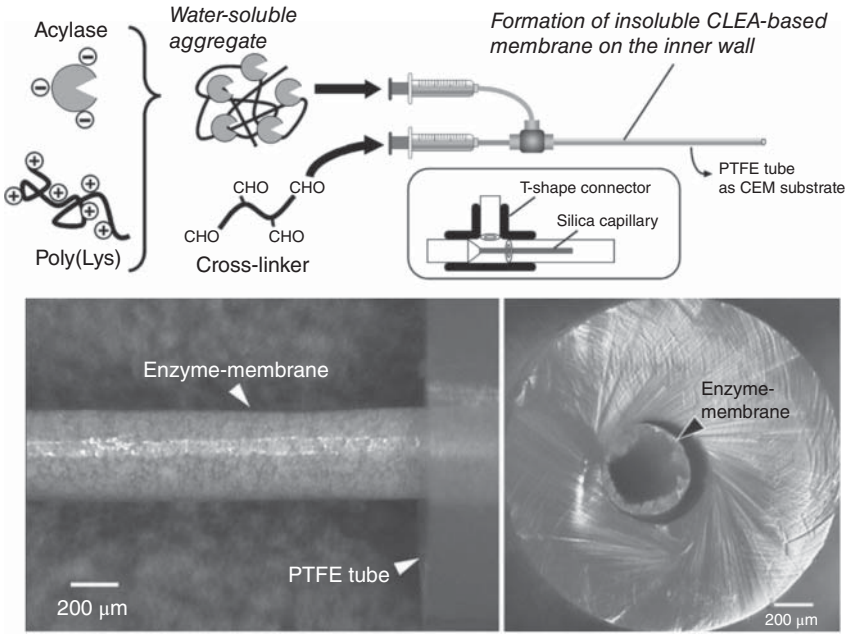


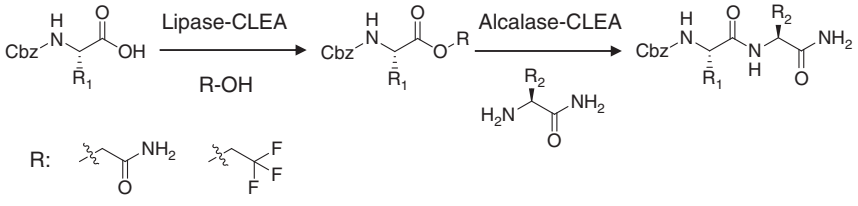
Figure 5.14 Schematic illustration of the procedure used to prepare an acylase-CEM (top). The cross-linking polymerization was performed in a concentric laminar flow. A silica capillary was fitted to the outer diameter of the T-shaped connector by attaching to a PTFE tube using heat-shrink tubing. The capillary was set in the connector located at the concentric position of the CEM tube. The cross-linker solution was supplied to the substrate PTFE tube through the silica capillary, corresponding to a central stream in the concentric laminar flow. A solution of acylase-poly-Lys mixture was poured from the other inlet of the T-shaped connector, and formed an outer stream of the laminar flow. Charge-coupled device (CCD) images (bottom) of cylindrical enzyme-membrane (dry state) exposed from the PTFE tube, which forms on the inner wall of the tube and a sectional view of the obtained CEM. (From [122] with permission ©2006, John Wiley and Sons.)

[126]. The bio-electrocatalytic microreactor has the two distinct interlaced enzyme systems that were immobilized by cross-linking and beads modification. Choline oxidase (ChOx), the signaling redox enzyme, was polymerized with poly-Lys through cross-linking and then attached on the electrode surface. AChE conjugated to magnetic microparticles was deposited in proximity to the ChOx electrode surface to obtain a strong electrochemical signal from the AChE/ChOx reaction.

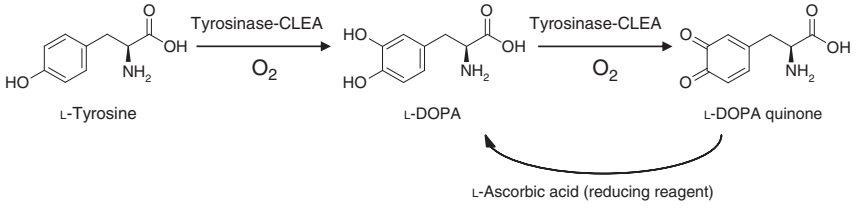
Peptides composed of <10 amino acid residues are of interest because they can be utilized either as a therapeutic or as a prodrug [132]. Therefore, chemo-enzymatic peptide synthesis is a potentially cost-efficient technology for the synthesis of short and medium-sized peptides. However, there are still some limitations in synthesizing challenging peptides (e.g., peptides containing sterically demanding acyl donors, non-proteinogenic amino acids, or proline

residues). Recently, immobilized proteases such as lipase-CLEA- and alcalase®-CLEA-mediated peptide synthesis have been reported [133]. In that case, special ester moieties were used that are specifically recognized by the enzyme (e.g., guanidinophenyl, carboxamidomethyl, or trifluoroethyl (Tfe) esters). The carboxamidomethyl and Tfe esters are very useful for alcalase®-CLEA mediated peptide synthesis using sterically demanding and non-proteinogenic acyl donors as well as poor nucleophiles, and combinations thereof. In addition, these esters, which are difficult to synthesize chemically, can be efficiently synthesized by using lipase-CLEA or alcalase®-CLEA. In turn, ester synthesis by lipase-CLEA and subsequent peptide synthesis by alcalase®-CLEA can be performed simultaneously using a two-enzyme, one-pot approach (Scheme 5.1).

L-3,4-Dihydroxyphenylalanine (L-DOPA) is the drug of choice for the treatment of Parkinson's disease. Tyrosinase-CLEA was used as the catalyst for the production of L-DOPA from L-tyrosine (Scheme 5.2) [134]. Using tyrosinase-CLEA, a conversion of 53% was obtained after 2 h with a productivity of 209 mg l^{-1} h^{-1}, which is much superior to other batch processes catalyzed by the enzyme immobilized with traditional carrier-bound immobilization methods. The effects of pH, temperature, and L-ascorbic acid as the reducing agent on the L-DOPA production were examined to improve the production yield. In the continuous synthetic processes carried out in a continuously stirred tank reactor and a packed-bed reactor, a productivity of 103 and 49 mg l^{-1} h^{-1} was obtained, respectively. The operational stability of the tyrosinase-CLEA could be improved by entrapment into calcium alginate gels. The CLEA/alginate beads in the continuously stirred tank reactor achieved a long life time of >104 h, producing L-DOPA with a productivity of 57 mg l^{-1} h^{-1}.



Scheme 5.1 Simultaneous esterification and peptide synthesis using a two-enzyme, one-pot approach.



Scheme 5.2 Synthesis of L-DOPA from L-tyrosine by tyrosinase-CLEA.

A unique enzyme polymerization method for enzyme immobilization was developed by Zhou *et al.* [127], which utilizes an enzyme modified with an amyloid fibril-forming module. This method is different from traditional technique such as adsorption, covalent attachment of enzymes to carriers, entrapment, and chemical cross-linking. Ure2 protein has the ability to form amyloid fibrils through its own N-terminal domain, the prion domain, and the fibrils propagate in a manner analogous to mammalian prions [135]. The extraordinary stability and tunable assembly of amyloid fibrils make them attractive targets as nanomaterials. Zhou *et al.* applied the prion domain-fused enzyme to an enzymatic microreactor and showed that the prion domain is an ideal scaffold for the immobilization of active enzymes in enzymatic reactor systems [127].

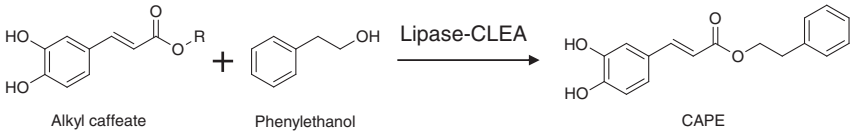
5.2.5
Enzyme Immobilization by Other Techniques Using Organisms

A cell is a protein production plant. Cells expressing enzymes of interest are able to act as biocatalysts. Therefore, various cell-integrated bioreactors, including microreactors, have been developed. Immobilization of the cells is one of the techniques used to improve the productivity of the bioreactors. Adherent cells that possess a functionality that adheres onto a suitable solid support enable facile immobilization of the cells in bioreactors. Akay *et al.* developed microreactors with immobilized bacteria using PolyHIPE polymer as a monolithic support for cell attachment [136]. Several groups have applied covalent coupling for immobilizing the cells on the surfaces of solid supports, including the inner walls of microreactors and microbeads in miniaturized reactors [137–140].

5.2.6
Application of Immobilized Enzymes in Microfluidics

The advantages of enzymes in industries are pertinent when combined with microfluidics. Microfluidic systems are employed to provide an integrated environment in which sample preparation and fluid control measurement take place in a confined space with volumes in the nanoliter to milliliter range [141]. In addition, they offer the possibility to be used with automation systems in industry. When combining enzymes with microfluidic systems, enzymes can be integrated into the system by immobilization techniques on supporting surfaces such as microfluidic channels or microparticles, as described previously. A variety of methods ranging from simple adsorption to covalent binding or bio-affinity binding are available. All of them have their advantages and drawbacks, depending on the specific application.

Caffeic acid phenethyl ester (CAPE) is a rare natural ingredient with several biological activities, but the industrial production of CAPE using lipase-catalyzed esterification of caffeic acid and 2-phenylethanol in organic solvents and ionic liquids is hindered by low substrate concentrations and a long reaction time [142]. A novel continuous flow enzymatic synthesis of CAPE in an ionic liquid using a



Scheme 5.3 Synthesis of CAPE using lipase-catalyzed esterification of caffeic acid and 2-phenylethanol.

packed-bed microreactor has been reported (Scheme 5.3) [143]. Novozyme 435®, which is a commercially available immobilized lipase, was used in the microreactor. Under the optimum conditions, a 93% CAPE yield was achieved in 2.5 h using an immobilized lipase microreactor, compared with 24 h using a batch reactor. The reuse of Novozyme 435® for 20 cycles and continuous reaction for 9 days did not result in any decrease in activity.

Continuous-flow, lipase-catalyzed, ring-opening polymerization of ε-caprolactone to polycaprolactone in a microreactor has been reported [144]. Although similar polymerization reactions by free protease have been studied well, this is the first report of a solid-supported, enzyme-catalyzed polymerization reaction in the continuous mode. Lipase was immobilized on macroporous poly(methyl methacrylate). A microreactor was designed to perform these heterogeneous reactions in the continuous mode in organic media at elevated temperatures. Faster polymerization and higher molecular mass were achieved using microreactors compared to using batch reactors. Although this study focused on polymerization reactions, it is evident that similar microreactor-based platforms can readily be extended to other enzyme-based systems in industries.

Biosynthesis of a variety of important compounds has been achieved by multi-enzymatic processes. Recently, a flow microreactor with a three-step enzyme pathway was developed as a reference platform for *in vitro* synthetic biology [63]. Biotin-modified enzymes, namely β-galactosidase, glucose oxidase, and horseradish peroxidase, were individually immobilized on streptavidin-coated microbeads. A packed-bed microreactor was shown to be optimal for enzyme compartmentalization. The specific substrate conversion efficiency could significantly be improved by an optimized parameter set. Thus, the designed microreactor provides a platform to explore new *in vitro* synthetic biology solutions for industrial biosynthesis.

5.3 Novel Techniques for Enzyme Immobilization

In addition to the fundamental techniques for enzyme immobilization that were described in Section 5.2, several new techniques have been reported recently. In this section, we introduce recent examples of enzyme mobilization using various methods, including noncovalent and covalent linking methods. These new techniques may provide a variety of bioreactor designs.

5.3.1
Polyketone Polymer: Enzyme Immobilization by Hydrogen Bonds

A polyketone polymer has been reported as a new support for direct enzyme immobilization [145]. The polyketone polymer, prepared by the copolymerization of ethene and carbon monoxide, was utilized for the immobilization of three different enzymes, namely one peroxidase and two amine oxidases. The immobilization procedure was carried out in diluted buffer, at pH 7.0 and 3 °C, by gently mixing the proteins with the polymer. A large number of hydrogen bonds between the carbonyl groups of the polymer and the –NH groups of the polypeptidic chain were formed. Therefore, this technique represents an easy immobilization procedure because bifunctional reagents were not required as a cross-linker. High immobilization yields were obtained for peroxidase and two different types of amine oxidases. The apparent concentration of the immobilized enzyme on the polyketone was >2.36 mg ml^{-1} [145]. Moreover, activity measurements demonstrated that immobilized amine oxidase from lentil seedlings totally retained the catalytic characteristics of the free enzyme. A slight increase in the K_m value was observed, suggesting that restricted mobility of the linked enzyme also occurred. The peroxidase-immobilized polymer was used as an active packed bed of an enzymatic reactor for continuous-flow conversion and flow-injection analysis. Furthermore, the immobilized enzymes retained their catalytic activity for several months.

5.3.2
Thermoresponsive Hydrogels

Environmentally responsive hydrogels, which have the ability to turn from solution to gel under specific conditions, have been applied for enzyme immobilization. Thermoresponsive hydrogels transition from solution to gel upon a change in temperature [146]. Typically, aqueous solutions of hydrogels used in enzyme immobilization are liquid at ambient temperature and a gel at physiological temperature. Poly(*N*-isopropylacrylamide) hydrogel has been widely studied in biochemical applications [146]. This polymer is biocompatible and exhibits a sharp phase transition. The lower critical solution temperature (LCST) of poly(*N*-isopropylacrylamide) is about 32 °C [147]. Below the LCST, the polymer assumes a flexible state and dissolves in aqueous solutions. In contrast, it becomes hydrophobic above the LCST and the polymer chains seem to collapse prior to aggregation (insoluble form) [148]. Because the transformation of the polymer between solution and gel is carried out under very mild conditions (temperature or pH), the catalytic activities of the entrapped enzymes are retained because there are no conformational changes when the hydrogels are formed [146].

Laccase was immobilized on a conductive support prepared by attaching the poly(*N*-isopropylacrylamide) hydrogel to an indium-tin oxide electrode [149]. The entrapped laccase in the gel matrix remained enzymatically active longer than in the solution. The laccase enzymatic activity in the gel matrix depended on the

reaction temperature. Reversible swelling/shrinking of the matrix was studied at 30 and 35 °C (around the LCST). Shrinking of the gel at higher temperature considerably decreased the efficiency of the catalytic reaction; however, it did not lead to irreversible changes in the enzyme conformation. At temperatures below LCST, the catalytic properties of the electrode were fully restored.

The thermal properties of a copolymer composed of poly(*N*-isopropylacrylamide) and different amounts of polyethylene oxide were studied in a buffer solution [150]. The number of polyethylene oxide grafts affected the LCST and viscosity of the solutions. Because enzymatic activity depends on an optimum temperature, the ability to control the LCST of the support material may be advantageous for the immobilization of different enzymes.

5.3.3 Immobilization Methods Using Azide Chemistry

Classical covalent immobilization methods utilize amino groups, carboxyl groups, or thiol groups that expose the enzyme surface. These methods are not site-specific chemical reactions. Recently, site-specific (selective) immobilization methods have been developed and carried out under mild physical conditions. Among them, the chemical reaction using azide compounds is of wide interest for the immobilization of biomolecules including enzymes [151].

5.3.3.1 Staudinger Ligation

The reaction between an azide with a phosphinothioester forms a stable amide bond [152]. This reaction is known as a traceless version of Staudinger ligation [153, 154]. Recently, Staudinger ligation was applied for enzyme immobilization [155]. An azido group was installed at the C-terminus of ribonuclease by using the method of expressed protein ligation and a synthetic bifunctional reagent. This azido protein was immobilized by Staudinger ligation to a phosphinothioester-displaying SAM on a gold surface. Immobilization proceeded rapidly and selectively via the azido group. The immobilized enzyme retained its catalytic activity and was able to bind to its natural ligand [155]. Although the method involves the introduction of an azide moiety on the enzyme of interest, this method results in site-specific immobilization in high yield at room temperature in an aqueous solution or an organic solution containing water.

5.3.3.2 Click Chemistry

The copper(I)-catalyzed 1,2,3-triazole-forming reaction between azides and terminal alkynes is called Click chemistry [156, 157]. Click chemistry has been used not only for enzyme immobilization but also for a variety of applications to enable the attachment of biomolecules bearing either the azide group or alkyne group with various fluorophores, polymers, or other biochemical compounds. A large number of its application can be found in nearly all areas of modern chemistry from drug discovery to materials science [157].

Another related cross-linking reactions is photoclick chemistry, whereby the formation of pyrazoline between tetrazoles and alkenes is carried out by UV irradiation. In the first literature report on photoclick chemistry [158], the selective functionalization of *O*-allyl-tyrosine was genetically encoded in a Z-domain protein in *Escherichia coli*. The obtained *E. coli* that expressed mutant Z-domains was suspended in a buffer containing a tetrazole compound. The bacterial cell suspensions were irradiated with light at 302 nm for 4 min, indicating that the cross-linking reaction was carried out in living bacteria cells. This reaction procedure was simple and nontoxic to *E. coli* cells, with only tetrazole and photons required as external reagents. The same research group also reported a *p*-(2-tetrazole)phenylalanine-containing protein for photoclick reactions [159]. Although these interesting methods have not been applied for enzyme immobilization yet, further development will allow the preparation of novel bioreactors.

5.3.4
Graphene-Based Nanomaterial as an Immobilization Support

More recent studies have reported that graphene-based nanomaterials are suitable for use in various applications, including enzyme immobilization [160]. The large surface area and exceptional physicochemical properties of graphene- or GO-based nanomaterials create an ideal immobilization support for enzymes. Graphene-based nanomaterials interact with enzymes mostly through physicochemical properties such as electrostatic interactions or hydrophobic interactions [160]. GO is one of the most widely studied materials because of its unique structural features and chemical, electrical, and mechanical properties [160, 161] and has been applied in many fields [160–162]. GO can be grafted with desirable functional groups such as epoxide and carboxylic and hydroxyl groups [160]. These functional groups provide a negative surface charge to the materials [162]. Enzymes can be immobilized on GO through covalent or noncovalent binding using the functional groups on the GO surface [160].

Early studies on the binding of enzymes to GO have reported that enzyme immobilization on the GO surface could take place readily without the use of any cross-linking reagents or additional surface modification. The binding of peroxidase or lysozyme as model enzymes to GO is mainly through electrostatic interaction [163]. However, electrostatic interactions as the driving force for enzyme binding to GO severely affected the catalytic activity owing to the conformational change of the enzyme induced by its binding to GO [163]. In similar results, significant structural changes often lead to lower catalytic activity [164, 165]. It is known that multiple interactions between the immobilization substrate and the enzyme molecule could change the enzyme conformation [166]. These immobilization studies suggest that the effect of graphene-based nanomaterials on the catalytic activity and conformation of the enzyme is difficult to predict and

depends on the nature of the enzyme (hydrophobicity, hydrophilicity, tertiary structure, or quaternary structure). Therefore, to retain the conformation and catalytic activity of the enzyme, the number and properties of the functional groups on the GO surface must be optimized.

To improve the catalytic activity of immobilized enzymes on a GO surface, a chemically reduced graphene oxide (CRGO) was utilized for immobilization of peroxidase and oxalate oxidase [167]. The enzymes can bind on the CRGO surface directly with 10 times the enzyme loading capacity of GO. In this case, hydrophobic interactions play a major role [168]. Interestingly, immobilized enzymes on CRGO exhibited higher catalytic activity and stability than those on GO. The results indicate that CRGO is a potential substrate for efficient enzyme immobilization. Similar results on improved catalytic activity of immobilized enzymes were also observed for the interaction of hydrolases with amine-functionalized GO [168]. Enzymes were covalently immobilized on GO using glutaraldehyde as a cross-linking reagent between the enzyme and amino groups on GO. The covalently immobilized enzymes exhibited comparable or even higher catalytic activity compared to the enzymes that were prepared by electrostatic interaction [168]. More recent reports suggest that covalently immobilized lipase shows high thermal and solvent stability [169, 170]. Enhanced thermal stability and solvent tolerance of the immobilized enzyme on graphene-based materials will be useful for industrial-scale use.

The application of immobilized enzyme systems for the degradation of pollutants and wastewater treatment has been reported. The removal of phenolic compounds from aqueous solution using GO-immobilized horseradish peroxidase was explored with seven phenolic compounds as model substrates [171]. The GO immobilized horseradish peroxidase exhibited high removal efficiency of several phenolic compounds in comparison to the free enzyme, especially for 2,4-dimetheoxyphenol and 2-chlorphenol, the latter being a major component of industrial wastewater.

5.3.5
Immobilization Methods Using Proteins Modified with Solid-Support-Binding Modules

Silica is one of the most frequently used support materials for enzyme immobilization in a wide range of biotechnological and biomedical analytical applications. However, the unmodified silica surface is unsuitable for attachment of proteins because it induces denaturation of the proteins. Bolivar *et al.* developed a novel and facile method for the direct immobilization of enzymes on an underivatized silica surface [172]. They prepared chimeras of target enzymes, which possess a small module that binds efficiently and tightly to solid silica at physiological pH conditions. The module, called Z_{basic2}, forms a three-α-helix bundle mini-protein of 7 kDa size that exposes clustered positive charges from multiple arginine residues on one side.

5.4 Conclusions and Future Perspectives

Immobilized enzymes are more robust and more resistant to environmental changes such as reaction temperature and organic solvents than free enzymes in solution. The storage stability depends on the immobilization method. In addition, immobilization results in heterogeneity of the catalyst. This heterogeneity enables ease of separation of both the enzyme and the product, as well as reuse of the enzymes. Thus, applications of immobilized enzymes are of wide interest across various industries.

Immobilization techniques have been successfully applied to a broad range of enzymes. Enzymes that catalyze hydrolysis reaction, such as lipases and proteases, have been widely applied in industrial processing. However, there are many useful enzymes that catalyze other important reaction in cells and/or organs that have yet to be applied in industry. In most cases, enzyme activity needs to improve the catalytic performance and enantio- and/or regioselectivity. For such a purpose, modifications of enzyme molecules are essential tool to obtain better catalytic activity. The current trend in protein engineering based on directed evolution, combined with computational algorithms such as ProSAR [173], may overcome these obstacles. Directed evolution with ProSAR was used in the development of ketoredactase and *R*-selective transamidase to enhance the catalytic activity and enantioselectivity [174, 175]. These studies show that the combination of different technologies is a promising approach and could improve the results of current enzyme immobilization techniques.

Oxidation and reduction are two of the most important chemical reactions in chemical and pharmaceutical industries; the development of immobilized enzymes with redox activity is still in its primitive stage. Most oxidoreductases need a coenzyme, such as NADH or NADPH, for their catalytic activities. This is a serious drawback because coenzymes increase the cost of processing. To reduce the cost caused by coenzymes, efforts to establish a co-immobilization technique of enzyme and coenzyme, or a regeneration technique of the coenzyme, are under way. Recently, the regeneration of NADH in a microreactor by alcohol dehydrogenase immobilized on magnetic NPs has been reported [176]. Like multiple enzymatic steps such as oxidoreductase and alcohol dehydrogenase, the combination of the different immobilized enzymes in a microreactor sytem could provide good results for industrial use.

There are still obstacles that need to be overcome for the development of useful immobilized enzymes as biocatalysts. As noted in this chapter, miniaturized reactors are useful for the development of novel enzymatic reaction technique. With the developed new technologies, immobilized enzymes will see more industrial applications.

Abbreviations

AChE	acetylcholinesterase
APTES	3-aminopropyl triethoxysilane
AuNPs	gold nanoparticles
CAPE	caffeic acid phenethyl ester
CEM	CLEA-based enzyme microreactor
ChOx	choline oxidase
CLEA	cross-linked enzyme aggregate
CNTs	carbon nanotubes
CRGO	chemically reduced graphene oxide
L-DOPA	L-3,4-dihydroxyphenylalanine
EDC	1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide
FSM	folded-sheet mesoporous materials
GO	graphene oxide
GPTES	γ-glycidoxypropyltrimethoxysilane
GST	glutathione *S*-transferase
LBL	layer-by-layer
LCST	lower critical solution temperature
LTCC	low-temperature co-fired ceramics
MWNT	multi-walled carbon nanotube
NC	nitrocellulose
NHS	*N*-hydroxysuccinimide
Ni-NTA	nickel ion-coordinated nitrilotriacetic acid
NPs	nanoparticles
μPAD	microfluidic paper-based analytical device
PDDA	polycarbonate polydiallyldimethylammonium chloride
PDMS	polydimethylsiloxane
PEI	polyethyleneimine
PEG	polyethylene glycol
PMMA	polymethyl methacrylate
ppTMDSO	plasma-polymerized tetramethyldisiloxane
PVA	polyvinyl alcohol
PVDF	polyvinylidene fluoride
RPECVD	remote plasma enhanced chemical vapor deposition
SAM	self-assembled monolayer
SUVs	small unilamellar vesicles
SWNT	single-walled carbon nanotube
TEOS	tetraethoxysilane
Tfe	trifluoroethyl
TMDSO	1,1,3,3-tetramethyldisiloxane

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Part II
Microbial Process Engineering

Applied Bioengineering: Innovations and Future Directions, First Edition. Edited by Toshiomi Yoshida.

6
Bioreactor Development and Process Analytical Technology

Toshiomi Yoshida

6.1
Introduction

The contribution of microorganisms to modern human society is immense. It includes the industrial manufacturing of materials that are important for humanity. The processes of industrial manufacturing that use microorganisms must be optimized to survive economically in a highly competitive society. Appropriate control of a microbial process for industrial application can be accomplished by accurate, near-real-time monitoring of the state of the process. The bioreactor was developed as a key component of bioprocessing for industrial manufacturing, which must provide appropriate conditions for the organisms to grow and produce the target products under optimal conditions.

A remarkable development was made during the last two decades: the scaling-down of bioreactors. Small-scale bioreactors comprise a group of micro-bioreactors with sub-milliliter volumes and a group of mini-bioreactors with a volume above 1 ml. These reactors have been utilized in high-throughput bioprocessing for the design of large-scale bioreactors in industrial-scale manufacturing and the optimization of operational conditions in production plants.

Conventional stainless steel fermentors can be used repeatedly with clean-up after cultivation and sterilization before inoculations for other batches. Those procedures are complicated, requiring special care to prevent contamination with foreign organisms, and the capital cost of devices is high. To overcome such difficulties, there is a demand for simpler and more flexible devices for microbial cultivation. Single-use disposable bioreactors, in particular, have received much attention as one of the most promising alternatives, and their popularity has rapidly risen.

Bioprocess engineers have worked on developing technologies to obtain information on microbial cultivation by analyzing microbial physiology in relation to its environment and manipulating operational conditions to control the microbial cultivation. The demand for real-time and near-real-time monitoring has led to significant innovation of analytical technologies and automation systems in the

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field of the process monitoring. In 2004, the U.S. Food and Drug Administration (FDA) issued [1] "Guidance for Industry PAT (Process Analytical Technology)" as a framework to assist innovative development of pharmaceutical manufacturing and quality assurance processes. In the PAT initiative, FDA identified the potential for continuous improvement in the fermentation and downstream processing technologies. FDA has promoted the PAT initiative as implementing process controls so that quality by design (QbD) approaches can "build quality into" manufacturing processes. In the last decade, the understanding and adoption of the PAT initiative has increased remarkably, and its implementation has become widespread, especially in the pharmaceutical industry.

This chapter will review the development of technologies for (i) parallel bioreactor systems for high-throughput processing, (ii) single-use disposable bioreactor systems, (iii) sensor and monitoring technologies, and (iv) process analytical technology. Validation and practical implementation of these technologies and future perspectives are discussed.

6.2 Bioreactor Development

The bioreactor is a main constituent of the bioprocess in industrial manufacturing. Various biological catalysts, including enzymes, bacteria, fungi, plant cells, animal cells, and other means, have been used to produce various chemicals, biochemicals, foods, proteins, biologics, and other biological materials. The bioreactors, which is the heart of the bioprocess, should have various functions based on their purpose, including containment of the materials to ensure sterility, introduction of oxygen into the medium, removal of carbon dioxide and other gaseous byproducts, introduction of nutrients for the organisms to utilize, control of the physiological environment such as pH, temperature, shear rate, and suspension of cells and dispersion of solid materials or other nonaqueous liquids [2].

Oxygen supply to the medium is a crucially important issue in the cultivation of aerobically growing organisms. The amount of oxygen dissolved in the medium is at any time quite limited because of its low solubility, so oxygen must be continuously supplied. Thus, the oxygen supply rate to the medium is one of the important parameters in the configuration design of a bioreactor and the optimization of its operational conditions. Consequently, oxygen transfer performance is considered the first priority in selecting a bioreactor and its scale-up. There have been many investigations on the design of bioreactors, such as continuously stirred tank reactors (STRs), and bubble column reactors or air-lift fermenters to increase the oxygen transfer rate (OTR). In transferring oxygen from air bubbles to cells, the rate-limiting step is the bubble-to-bulk transfer and diffusion at the surface of the air bubble, and the OTR absolutely depends on the surface area and oxygen transfer coefficient. These characteristics are strongly affected by the hydrodynamic conditions of the gas and liquid dispersion in the medium. It is widely accepted that the speed of the impeller tip as a parameter representing the shear

rate in the bioreactor is a good and representative engineering parameter that governs the mass transport phenomenon between immiscible phases in the bioreactor. During the cultivation, operational conditions including the agitation and aeration rates should be manipulated for an oxygen supply rate that will meet the oxygen uptake rate (OUR) by the respiration of the organisms involved. Therefore, the volumetric mass transfer coefficient, $k_L a$, of oxygen is a key parameter in the design, scale-up, and operation of bioreactors [3, 4].

Regions of low oxygen concentrations may occur in a large bioreactor. This heterogeneity results from the flow conditions and reaction kinetics of cellular metabolism. From an engineering perspective, it is necessary to understand what biochemical engineers have learned about the design of bioreactors, such as mixing, rheology, and process optimization, that ties engineering to microbial kinetics [5–10]. A computational fluid dynamics (CFD) model was applied to analyze the hydrodynamics within a bubble column [11], and application of an airlift bioreactor in the large-scale production of a biofuel using oleaginous *Rhodotorula mucilaginosa* growing on glycerol and yeast extract in a sea water medium was investigated as an economically competitive process [12].

6.2.1
Parallel Bioreactor Systems for High-Throughput Processing

There have been remarkable developments in the scale-down of various types of bioreactors. As an example of small-scale bioreactors, so called micro-/mini-bioreactors (MBRs) can be divided in two groups: one is a micro-bioreactor with a volume below 1 ml, and the other is a miniature bioreactor with a volume of 1–100 ml, as suggested by Lattermann and Büchs [13]. Büchs' group has extensively investigated the effective utilization of microtiter plates (MTPs) in the development of high-throughput technology (HTPT), as described in the following. MTPs have been utilized for microbial cultivation in laboratories for many years; it came into practical use in the 1990s. MTPs have 6, 24, 48, 98, or even 9600 wells per plate, and they have been utilized for screening microbial strains. In the biotechnology industry, it is especially useful at an early stage of the process development in order to screen promising clones with the potential to produce certain physiologically active chemicals or proteins. For this purpose, researchers have to examine a large number of samples, for example, several thousands or more. To increase the speed of screening, many larger MTPs that can handle many samples at a time must be utilized. Nevertheless, several technical developments are required to establish the basis of a production plant, starting from strain screening and moving to characterizing strains for efficient cultivation, determining operational conditions for cultivation, designing a fermentor, and scaling up to industrial manufacturing. HTPTs have been developed for quick scale-up. A parallel bioreactor system could be helpful and should be considered in HTPTs.

As illustrated in Figure 6.1, several types of MBRs have been utilized in the process of scaling up. A shake flask is one of the most common devices used for

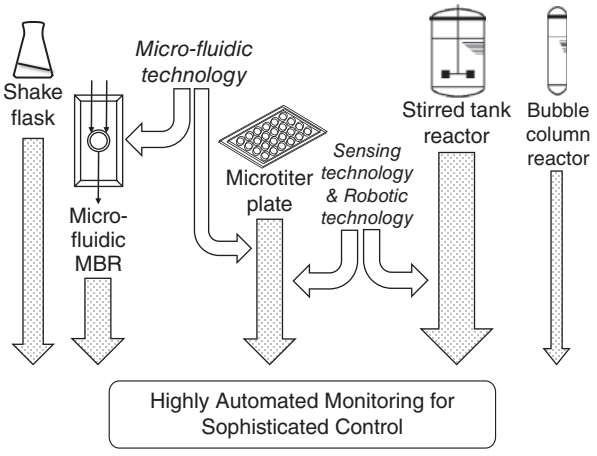


Figure 6.1 Development of micro-/miniature bioreactors in parallel use for high-throughput processing.

the cultivation of various microorganisms and other cells in the laboratory. MTPs have been utilized to screen strains and to incorporate various inputs from other disciplines, such as microfluidics, monitoring, and robotic technologies, which has facilitated miniaturization and automation of more sophisticated cultures. Parallel use of many STRs can lead to powerful systems for quick scale-up of cultivation. Furthermore, the automation of manufacturing plants will be accelerated by introducing modern sensing and robotic technologies, as shown in Figure 6.1.

Two prototype 24-unit MBRs, a standard 24-well plate and 24 discrete units of MBRs, were compared in the cultivation of *Escherichia coli*. Continuously monitoring and controlling the dissolved oxygen (DO) concentration was successfully achieved in the system of milliliter-scale MBRs, and it was claimed that HTPT could provide important insights into the dynamics of operational parameters at such a small scale [14]. Some drawbacks of conventional MTPs or shake flaks, such as low density of data due to the simple end-point measurements, could be circumvented in new MBRs using continuous monitoring technologies. The parallel use of MBRs offers the flexibility and controllability of bench-scale reactors and provides results that are directly comparable to those of large-scale fermentations. Practical experience in the manufacturing section utilizing MBR technology with automated sampling has indicated a significant improvement in developing timelines and close integration with the purification section [15].

6.2.1.1 **Microtiter Plate Systems**

Miniaturization allows a reduction in use of reagents, and quick optimization is possible by use of a large number of wells on MTPs in parallel and use of liquid handling robotics, thereby greatly increasing the throughput capabilities in a laboratory. Table 6.1 shows progress on MTP-type MBRs that has been made in the last 15 years.

Table 6.1 Development of microtiter-plate (MTP) bioreactors for high-throughput processing.

Reactor/ device [developer]	Volume (ml)	Multi-plexity	Rotation (rpm) <shaking scale, mm>	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
Early development									
MTP [ETH]	0.5	96	300 <50>	188 [16]	*Pseudomonas*, *Rhodococcus*, *Alcaligenes*, *Streptomyces*	9 g l^{-1} [16] 9.5 g l^{-1} [17]	Optimization of OTR and microbial growth in microbioreactors [16] Effect of shaking parameters on OTR in deep, square wells [17]	[16] [17]	(2000)
MTP deep-well [NIBR]	10	24	150, 250, 300	130	Insect cell clone *Sf*21	~5.2 × 10^9 cells l^{-1}	Optimization of operational conditions for cell growth	[18]	(2005)
Development of monitoring in microbioreactor									
MTP	0.2	96	1 020 <1>	130 [19] 10–40 [20]	*Corynebacterium glutamicum* and *Escherichia coli*	5 g l^{-1}	Monitoring of DO and pH by fluorophores immobilized on the bottom of each well for online monitoring [19, 20]	[19] [20]	(2003) (2005)
Optical sensing [Saar. U] [Aachen U]	1.2	24	250 <25>						
BioLector MTP [Aachen U]	0.2	96	995 <3>	100–270 [21] OTR > 0.03 M h^{-1} [22]	*E. coli*, *Vibrio natriegens* [21], *Hansenula polymorpha* [22], and *Kluyveromyces lactis* [23]	14 g l^{-1} [22] μ: 0.6 l h^{-1} [23]	A new device for quasi-continuous parallel monitoring of light scattering, and fluorescences in each well [21] Scale-up 7000-fold: MTP (200 nl) to STR (1.4 l, $k_L a$: 370–600 h^{-1}) [22] A new MTP system for high-throughput temperature optimization; BioLector for monitoring DO, pH in MTP wells [23]	[21] [22] [23]	(2005) (2009) (2014)

(*continued*)

Table 6.1 (*Continued*).

Reactor/ device [developer]	Volume (ml)	Multi-plexity	Rotation (rpm) <shaking scale, mm>	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
BioLector MTP Robo-Lecter [Aachen U]	1	48	1000 <3>		Mixed acetic acid bacteria	<0.5 g l^{-1}	Scaling down of vinegar fermentation from 9 l to MTP cum BioLector in Robo-Lector	[24]	(2014)
BioLector MTP Robotics [FZJ]	1	48	200–1200		*C. glutamicum*	μ_{max} = 0.28 $l\,h^{-1}$	Embedding BioLector in robotic workstation; growth monitoring at 1-h intervals	[25]	(2015)
24-Well MTP [UR]	3	24	670		*Pseudomonas putida*		An integrated dual sensor for DO and pH; oxygen-sensitive organosilica microparticles; pH-sensitive polymethacrylate derivative	[26]	(2008)
Engineering analyses and development of minibioreactors									
MTP CFD [UCL]	1.5/4.5	96/24	500–1500 <3>	200–360 18–100	*E. coli*	OD_{625}: ca. 5–6	Chemical engineering characterization by CFD simulation	[27]	(2008)
MTP Math-model [CWRU]	1	96			GS-CHO	<6×10^9 viable cells l^{-1}	Monitoring pH by SNARF and controlling with the aid of a mathematical model	[28]	(2009)

MTP deep, square-well [Merck]	1	96	300 <2>		CHO	$\sim 5.0 \times 10^9$ viable cells l^{-1}	Oxygen transfer study using an oxygen carrier, fluorinert; DO monitoring with ruthenium-based O-responsive dyes	[29]	(2012)
MTP air-sparge [UEP]	0.9	96	–	NA	*E. coli*	OD_{600}: 5.3–5.9	An air-well sparging minifermenter system enabled a considerable improvement and threefold higher yield of recombinant protein	[30]	(2014)
Expansion of applications									
Mini bioreactor [MicroReactor, Inc.]	10	24	500 <2>		*Shewanella oneidensis*	OD_{600}: 1–1.5	Single-use casette [MicroReactor Technologies] enabled control of temperature, pH, and DO in each bioreactor individually. Effects of culture conditions on Cr(VI) reduction	[31]	(2006)
M24 [MicroReactor, Inc.]	10	24	800 <5>		CHO	$\sim 2.0 \times 10^9$ viable cells l^{-1}	The same monitoring and control system as same as in [31] Scale-down (2 l to 5 ml) study on cell culture	[32]	(2009)
SimCell [Seahorse BP]	0.8	>1000	Vertical rotation 5–20 rpm	~7	CHO	$\sim 1.2 \times 10^{10}$ cells l^{-1}	Factorial design of experiment (DoE) varying four parameters: pH, DO, feed rate, and glutathione in 180 runs	[33]	(2009)

Abbreviations: Aachen U: RWTH Aachen University; CFD: computational fluid dynamics; CWRU: Case Western Reserve University; ETH: Swiss Federal Institute of Technology in Zurich; FZJ: Forschungszentrum Jülich; NA: not available; NIBR: National Institute for Biomedical Research, UK; OD: optical density; OTR: oxygen transfer rate; RAMOS: respiration activity monitoring system; Saa U: Saaland University; UEP: University of Eastern Piedmont; UMBC: University of Maryland, Baltimore County; UR: University of Regensburg.

Nevertheless, oxygen supply to the medium is a key issue in the cultivation of aerobic microorganisms on MTPs. It is important to ensure OTRs similar to those achieved in regular cultivation systems such as shake flasks and stirred bioreactors. Duetz *et al*. [16] reported that OTRs in a 1-ml-deep well on 96-well MTPs were 38 mmol l^{-1} h^{-1} or 188 h^{-1} of $k_L a$ at 300 rpm of orbital shaking with a shaking diameter of 50 mm, and that various strains of the genera *Pseudomonas*, *Rhodococcus*, and *Alcaligenes* were successfully cultivated without cross-contamination by using a cover of spongy silicone and cotton wool on top of the MTP. Mycelium-forming *Streptomyces* strains were grown in 1-ml liquid micro-cultures in square, deep-well MTPs, and the spore suspensions were prepared in the 96 wells of an MTP [17]. Rapid optimization of insect cell cultivation was possible because 24 deep-well blocks on MTPs were utilized [18].

John *et al*. [19] reported optical sensing of DO in each well on a 96-well polystyrene MTP by immobilization of two fluorophores at the bottom of each well; the $k_L a$ values obtained by the dynamic method using a commercial MTP reader were ~130 h^{-1}, which is in the lower range of values from typical stirred fermentors. In addition to DO, an optical sensing technique was applied for online measurement of pH in the wells of a continuously shaken 24-well MTP. The MTP was placed on a sensor dish reader, which could be fixed on an orbital shaker [20].

A novel quasi-continuous online measuring technique for shaken MTPs was examined in the development of HTPTs. A fiber-optical measurement system to measure turbidity by the scattered light and monitor fluorescent protein (e.g., NADH, yellow fluorescent protein) and a fluorimeter to monitor DO were established. An $x-y$ positioning device (BMG Lab Technologies; Offenburg, Germany) was utilized for the two-dimensional displacement of the fiber-optic bundle below the MTP [21] (Figure 6.2).

By utilizing the above-mentioned device, a successful 7000-fold scale-up from a 0.2-ml culture on MTPs with an online monitoring technique, BioLector™, to 1.4-l STR fermentation was performed by Kensy *et al*. [22]. A novel 96-well MTP with a fluorescence thermometry arrangement was designed to investigate the temperature profiles of growing organisms as well as enzyme reactions [23]. Schlepütz and Büchs [24] presented work on scaling down vinegar fermentation to optimize operational conditions, maintaining the DO level and suppressing ethanol and acetic acid losses from evaporation by using a 48-well MTP that utilized a custom-made lid for appropriate oxygen supply and ventilation. Unthan *et al*. [25] integrated the BioLector into a robotic platform to conduct liquid handling tasks, including control of the operation parameters of pH, temperature, DO, and optical density (OD). The pipetting robot allowed timed sampling or dosing events for sophisticated control of cultivation and reliable selection of the best producers, media optimization, and induction profiling. All biological and bioprocess parameters were optimized almost perfectly for the MTP scale.

An integrated dual sensor to detect DO and pH was developed using oxygen-sensitive organosilica microparticles and pH-sensitive microbeads of a polymethacrylate derivative, which were embedded into a polyurethane hydrogel.

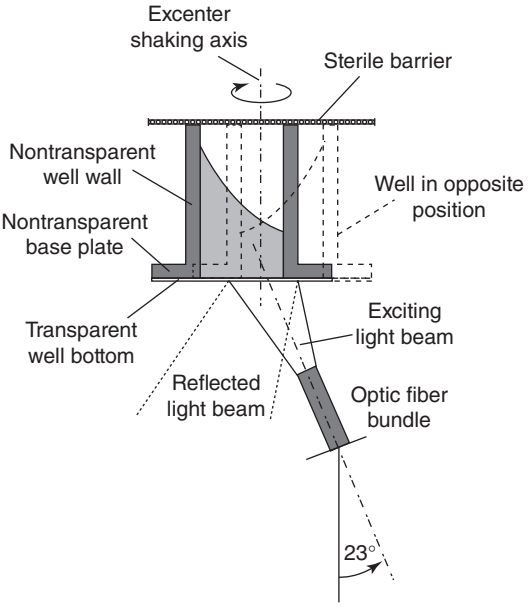


Figure 6.2 Measurement principle of the microtiter plate fermentation via back scattering of light from cells and fluorescence emission of molecules in a microtiter plate platform. (From [21] with permission ©2005 Wiley Periodicals Inc.)

The readout was based on a phase-domain fluorescence lifetime-based method referred to as "modified frequency-domain dual-lifetime referencing" with the use of a commercially available detector system for 24-well microplates [26].

The characterization of shaken MTP bioreactors was made using CFD techniques to evaluate fluid mixing, energy dissipation, and mass transfer in bioreactors with deep, square 24-well and 96-well MTPs [27]. An economical, rapid, and robust pH measurement method was developed by employing SNARF-4F 5-(-and 6)-carboxylic acid, and a mathematical model was established to regulate pH in multiwell plates and shake flasks [28]. The 96-well plate format has been widely used for HTP screening. However, it is often claimed that constraints in OTR prevents successful scale-up. Fluorinert, a biologically inert perfluorocarbon, has been employed to improve oxygen transfer in 96-well plates and to enable the growth of a Chinese hamster ovary (CHO) cell line [29]. An air-well-sparging mini-fermentor system was made from a homogeneous air-sparging device, and a stainless steel 96-needle plate integrated into a deep 96-well plate (0.9 ml culture volume) system was evaluated, resulting in considerable improvement, that is, a threefold higher yield in recombinant protein [30].

Tang *et al*. [31] reported a MBR system of 24 units from 10-ml reactors in a cassette that could monitor and control pH, DO, and temperature individually for each reactor. Independent monitoring and control of each reactor allowed

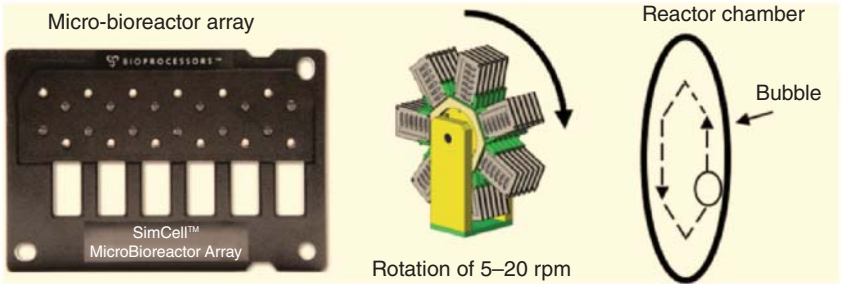


Figure 6.3 Micro-bioreactor array and agitation scheme. The bubbles comprising the headspace traverse the perimeter of each reactor as the array is rotated at 20 rpm. CFD calculations predict that the average shear stress on cells ranges from 0.11 to 0.34 dyne cm^{-2}. (From [34] with permission ©2009 Wiley Periodicals, Inc.)

the exploration of a matrix of environmental conditions known to influence microorganism metabolisms. A 24-well MTP system was developed as a scale-down model with noninvasive online monitoring and the capability of controlling several process parameters such as pH, DO, and temperature at the individual well level [32].

Optimization of glycoprotein production using CHO cells requires a large number of runs of cultivation experiments. The SimCell™ system was utilized to run 180 cultivations in accordance with a factorial design of experiments (DoE) that varied four process parameters: pH, DO, feed rate of a supplement, and reduction in glutathione level [33]. The results showed that the SimCell system could be an excellent model for handling a large number of MBRs (Figure 6.3).

6.2.1.2 Stirred-Tank Reactor Systems

Several types of MBRs, such as MTPs or microfluidic MBRs, have many advantages over STR-type MBRs, including low investment in MBR manufacturing test kits, lower costs in conducting experiments, and technical ease of handling higher order multiplex kits during scale-down. One group of stirred tank-type MBRs has a peculiar characteristic: maintaining the same configuration as conventional industrial fermentors, allowing greater certainty in comparing the results of scaled-down MBRs and other MRBs. The miniaturization of conventional fermentors seems to widely accepted, and there have been a large number of reports on STR-type MBRs. Table 6.2 summarizes the development of stirred tank-type MBRs in HTPTs.

Highly parallel bioprocessing can be accomplished with adequate design of bioreactors of small volume and a compact monitoring system to measure important parameters involved in bioprocesses. Kostov *et al.* [35] presented approaches for scaling down the working volume of the bioreactors to 2 ml, with a system to monitor pH, DO, and OD by means of optical sensors. A cuvette-based microbioreactor with a magnet stirrer, a sparger, and a couple of optical

Table 6.2 Development of stirred-tank bioreactors for high-throughput processing (HTP).

Bioreactor/ device [developer]	Volume (ml) multiplexity	Impeller (rpm)	Aeration	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
Early development of miniature bioreactor (MBR)									
Cuvette-bioreactor [UMBC]	2 Single	Magnetic stir bar	Pipet tip with 3 tubes	9.8 (1 vvm) 27.5 (2 vvm) 44.4 (3 vvm)	*Escherichia coli*	OD_{600}: ~8	Fluorescent dyes immobilized on the walls of the minibioreactor to monitor DO; semiconductor light sources and detectors for a very compact and low-cost detection system	[35]	(2000)
Mini-bioreactor (MBR) [UCL]	6 24 units	3 stage, 6-blade turbine	Single tube	100–400	*E. coli*	1.4 g l^{-1}	DO, OD, and pH monitoring by fiber optic probes; analyses of $k_L a$, P_g, energy dissipation by CFD system	[36]	(2010)
Development of HTBD (high-throughput bioprocess design) MBR									
HTBD MBR (miniature bioreactor) [TUM]	8–15 48 units	Gas-inducing impeller 1500–4000	Surface aeration	>1440 [37–39]	*E. coli* [37, 38, 40–43], *Bacillus subtilis* [44], and *Saccharomyces cerevisiae* [45]	30 g-dcw l^{-1} [44] 8.4 g l (batch) 18.2 g l^{-1} (fed-batch) [45] 12 g l^{-1} [39]	Automated liquid handling of HTDB MBR [37, 38] Comparison of ml-scale STRs [40] Engineering HTDB MBR by CFD [41] Microfluidic 48 parallel reactors with 144 micropumps for fed-batch cultures including riboflavin production [44, 45] Biphasic ionic liquid/water system [42] Multichannel peristaltic pumps for parallel continuous cultivations [39]	[37] [38] [40] [41] [44] [45] [42] [39]	(2005) (2005) (2005) (2010) (2007) (2011) (2012) (2015)

(*continued*)

Table 6.2 (*Continued*).

Bioreactor/ device [developer]	Volume (ml) multiplexity	Impeller (rpm)	Aeration	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
High-throughput bioreactor (HTBR) [UMBC]	12.5–30 12 units	Two-stage paddle 150	Sparger	2–4	SP2/0 myeloma/mouse hybridoma [43, 46]; CHO, NS0, and HEK 293 [47]	1.0×10^9 cells l^{-1} [46] 1.18×10^9 cells l^{-1} [47] 2.5×10^9 cells l^{-1} [43]	Validation of HTBR with optical sensors [46] Analysis of mixing in HTBR by optics [48] CultiBag (2 l/3.5 l; $k_L a = 2–3$) could be scaled down to HTBR [47] Clone selection by HTBRs [43]	[46] [48] [47] [43]	(2006) (2006) (2009) (2010)
Various systems in broad applications									
MicroReactor μ-24 [MicroReactor Technologies]	10 24 units	500–800 (shaking)	Surface aeration	32.6–56.1	*S. cerevisiae*, *E. coli*, and *Pichia pastoris*	278 g-wcw l^{-1}	MicroReactor was validated in aerobic cultivation of bacteria and yeasts	[49]	(2007)
SMBR (Stirred minibioreactor) [UCL]	100 4–16 units	6-blade turbine 100–2000	Sintered sparger 0–2 vvm	<400	*E. coli* and *B. subtilis*	7.6 g l^{-1} (*E. coli*) [50] 3.8 g l^{-1} (*E. coli*), 9 g l^{-1} (*B. subtilis*) [51]	Predictive scale-up; analysis of power consumption and OTR [50] SMBR (60 mm ø, 60 mm high, magnetic stirrer, and sintered sparger [51]	[50] [51]	(2008) (2008)

AMBR® 15 system [TAP]	15 24–48 units	Rushton or pitched marine <1500	Sparger, single nozzle	2.18	CHO	μ_{max}: 0.34–0.39 l/d [52] ~1.4×10^{10} viable cells l^{-1} [53] VCD: ~4.5×10^{9} cells l^{-1} [54]	Disposable MBRs in a workstation [55] Optical monitoring and control DO and pH; fed-batch cultures [52] To obtain a high $k_L a$, 2.18 h^{-1} in ambr®15, much higher power input (~400 Wm^{-3}) is required than (~20 $W m^{-3}$) that in large-scale culture Equations for estimation of $k_L a$ [53] Scale-down to manufacturing scale (15 000 l) and bench scale (3, 5, 15 l) [54, 56]	[55] [52] [53] [54] [56]	(2010) (2012) (2013) (2014) (2015)
Ambr® 250 workstation [TAP]	250 12 or 24 units	2-stage turbine 150–3500	Sparger, single nozzle	200–1000 (*Pichia* and *E. coli)* 1–1.15 (cell culture)	CHO, *P. pastoris,* and *E. coli*	>400 g-wcw l^{-1} [57] OD_{max}: ~160 [58]	A highly automated workshop with parallel use of disposable miniature bioreactors; Rushton-type impellers, two-stage, for cell culture (20 mm ø, 30 mm spacing) and two pitched blade impeller for microbial culture (26 mm ø, 30 mm spacing) [57] Application on DoE and DSD [58]	[57] [58]	(2013) (2015)

Abbreviations: BioLector: a system for optical monitoring and control in each individual bioreactor; CultiBag: disposable wave-type bioreactor; dcw: dry cell weight; DO: dissolved oxygen; DoE: design of experiments; DSD: definitive screening design; NR: not reported; UCL: University College London; OD: optical density; RAMOS: respiration activity monitoring system; Seahorse BP: Seahorse BioProcessors; STR: stirred-tank reactor; TAP: The Automation Partners Biosystem; TUM: Technische Universität München; UCL: University College London; UMBC: University of Maryland, Baltimore County; VCD: viable cell density; wcw: wet cell weight.

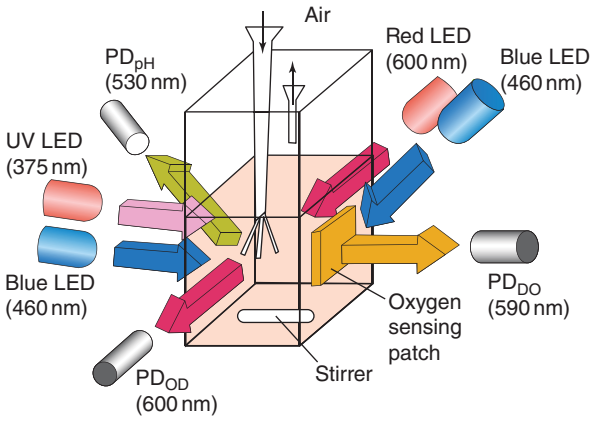


Figure 6.4 Cuvette-based micro-bioreactor. At the left wall, blue and UV LEDs with 530 nm photodetector are used to measure pH; at the right wall, blue LED, oxygen sensing patch, and a 590-nm photodetector are used to measure DO; red LED and 600-nm photodetector are used to measure OD through the front and back wall. (From [35] with permission ©2000 John Wiley & Sons, Inc.)

measurement components is shown in Figure 6.4. The use of semiconductor light sources and detectors has enabled the design of a very compact and low-cost detection system. DO and pH were monitored using fluorescent dyes and the absorbance of the cell suspension. pH measurements were performed using a ratiometric pH-sensitive dye, 1-hydroxypyrene-3,5,7-sulfonic acid, in the culture medium and DO Ru(diphenylphenanthroline)$_3^{2+}$ immobilized in silicone rubber attached to the wall of the reactor. The $k_L a$ of the cuvette was evaluated under different conditions such as sparging position and air flow rates, as shown in Table 6.2. The performance of the cuvette microbioreactor was finally tested by parallel fermentations of *E. coli* in the microbioreactor and a 1-l fermentor. Profiles of the pH, DO, and OD were very similar in the two processes.

An MBR with a culture volume of 6 ml and vessel diameter of 16 mm, which is equal to that of one well of a 24-well MTP, was investigated for its engineering performance as a fermentor. A set of three impellers was installed in the MBR for mixing. The DO and cell biomass concentration were monitored continuously by means of fiber-optic probes. The volumetric oxygen transfer coefficient, $k_L a$, was estimated experimentally in air–water and in *E. coli* culture. The local and average power input, energy dissipation rate, and bubble size were derived from an analysis of the multiphase flow in the MBR using CFD, and $k_L a$ was estimated using Higbie's penetration model with the contact time obtained by CFD simulation. Predicted and measured values of $k_L a$ were in the range 100–400 h^{-1}, which was typical of values reported for large-scale fermentation [36].

Weuster-Botz's group has extensively investigated STR-type MBRs with gas-inducing impellers as high-throughput bioreactors (HTBRs) for the development of HTPT to address various issues and problems such as automation [37, 38], comparability of MBRs [40], engineering by CFD simulation [41], microfluidic technologies [44], adaptation of the HTBR with 144 micro-pumps for highly parallel fed-batch cultures of industrial riboflavin fermentation by *Bacillus subtilis* [45],

and biphasic biocatalysis of the reduction of 2-octanone to (*R*)-2-octanol by a recombinant *E. coli* [42]. Monitoring and control, automation, and robotics are enhancements in the promotion of multifunctional screening required for sophisticated fermentations. Increasing the number of measured parameters as well as the quality of measurements is the intent, and pH, DO, and OD have been monitored optically by single-use patches from the outside of bioreactors. Installation of multichannel peristaltic pumps has enabled fine control of pH and also highly parallel continuous cultivations [39].

Liquid mixing in an MBR was investigated with a novel optical sensor system. The system was designed to monitor fluorescence with a tracer in a glass vessel. The MBR, a 12.5-ml stirred vessel, was operated in the transitional regime (Reynolds number (*Re*): 14–1350), which is an industrially relevant scenario. A linear correlation between the mean circulation time and the inverse of the impeller rotational speed was observed, similar to that seen in large-scale stirred vessels [48]. The performance of an HTP MBR was compared with that of a lab-scale single-use wave-type bioreactor, CultiBag, for cell growth and monoclonal antibody (mAb) production. Similar oxygen supply rates were achieved in both systems, leading to approximately equal culture performances (growth and mAb production) across scales and modes of mixing. Therefore, the MBR showed promise as an HTP scale-down model for wave-type bioreactors [47]. After comparing HTP MBRs with a conventional MTP used for clone screening, the stirred bioreactor environment was found to be a superior method for clone selection [43].

A 24-well plate MBR system, μ-24, which is composed of 24 cylindrical tube bioreactors, was assessed for cultivations of *Saccharomyces cerevisiae*, *E. coli*, and *Pichia pastoris*. The μ-24 system was capable of controlling DO, pH, and temperature under batch and fed-batch conditions with subsequent substrate shot feeds [49]. A detailed investigation of the mixing, power consumption, and oxygen mass transfer characteristics of a 100-ml MBR with a single stirrer was undertaken to create predictive engineering correlations useful for scale-up studies [50]. The influence of the established geometrical and dynamical parameters, including dimensionless terms such as *Re*, in MTPs was evaluated to establish a robust scaled-down approach to identifying and optimizing relevant operational conditions [59]. The Merck group developed a small-scale, 250-ml, automated, disposable stirred-tank bioreactor for HTP bioprocessing with a performance equivalent to that of industrial biologics processes used to develop therapeutic proteins and mAb production using CHO cells, *E. coli*, and *P. pastoris* [57].

The Automation Partnership (TAP) Limited [55] designed a series of HTP MBR systems as an advanced MBR workstation, ambr™. This workstation contains 24 or 48 disposable bioreactors with 10–15 ml of working volume and an internal impeller. Gases are supplied by sparging. The operations, including supply of gases and liquids and all monitoring, are performed by an automated workstation. Currently, Sartorius Stedim Biotech offers a new ambr station with a larger bioreactor, ambr® 250, which has been built on TAP's successful microbioreactor technology, allowing increased cultivation volumes as well as individual control of the bioreactor temperature and impeller speed. This system offers independent, parallel control of 12 or 24

single-use bioreactors (100–250 ml working volume) and is fully automated to allow feeding of media, inoculation, and sampling. Each bioreactor vessel is operated individually to control the temperature, impeller speed, pH, and DO. Practical applications in many pharmaceutical companies are documented in research reports based on the cultivation of CHO cells in an ambr station [52–54, 56]. These studies confirmed that ambr provided the same performance as large-scale STRs [52–54], while the cultivations in shake flasks did not show comparable performance to ambr and STRs [52, 53]. However, Nienow *et al*. [53] investigated the physical characteristics of the ambr system (15 ml) and found that these were quite different from those of large-scale stirred bioreactors, with much higher specific power input (~400 W m^{-3}) required in the microbioreactor to achieve sufficiently high $k_L a$ values to satisfy the oxygen demand of the cells, compared to a typical large-scale culture (~20 W m^{-3}). The empirical equation used for $k_L a$ correlations in ambr is the following:

$$k_L a = A(P/V)^{0.3}(Q_G)^{0.15}$$

where $k_L a$ is in h^{-1}, P/V is in W m^{-3}, Q_G is in ml min^{-1}, and A is 2.04 for water and 1.74 for the medium. For more detail, see [53].

A scale-down model was developed in an ambr system (15 ml) using statistical multivariate analysis techniques, which showed a comparable manufacturing scale and bench scale. The scale-down model was used for process characterization in the DoE study and the results of product qualification. Upon comparison with DoE data from bench-scale bioreactors, similar effects of process parameters on process yield and product quality were observed between the two systems [56].

Tai *et al*. [58] demonstrated the power of the 250-ml automated mini-bioreactor system, ambr250™. Using the ambr250 system to grow *E. coli* for recombinant protein production, many factors were screened for use as process controls with the data processing program, DoE, and definitive screening design (DSD). A systematic experimental workflow that efficiently characterized the *E. coli* fermentation process for recombinant protein production was developed using a 24-bioreactor ambr250 system and a 10-factor DSD program. The resulting strategy was validated by laboratory-scale experiments, and the use of an HTP automated bioreactor system with the efficiency of DSDs allowed systematic and rapid development of processes useful for QbD approaches to late-stage process characterization.

6.2.1.3 Microfluidic Microbioreactor Systems

Microfluidics emerged in the 1980s and was used for inkjet printers. This technology appeared in the field of bioprocess engineering in the early 2000s [34, 60–66]. The fabrication of microfluidic systems in polydimethylsiloxane (PDMS) was summarized by McDonald, Whitesides *et al*. [60]. From the early 2000, microfluidic devices have been applied as analytical systems, biomedical devices, tools for chemistry and biochemistry, and bioreactors for biotechnological research and development. Methods to fabricate microfluidic devices have advanced, and soft lithography has become one of the most common methods for faster, less expensive processes (Table 6.3).

Table 6.3 Development of microfluidic bioreactors (MFLBR) for high-throughput processing.

Bioreactor/ device [developer]	Volume (ml), multiplexity	Agitation (rpm)	Aeration	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
Early development of microfluidic bioreactors (MFLBR)									
Micro-fluidic bioreactor [Harv U]	NA						Review: Soft lithography provides faster, inexpensive fabrication of microfluidic devices in poly(dimethylsiloxane) (PDMS) for biological applications	[60]	(2000)
10 × 10-array microchambers [UCB]	~0.03 100 chambers		Diffusion through PDMS membrane	NA	HeLa cells	~2.5 × 10^5 cells cm^{-2}	The first microfluidic cell culture array for 100 runs in parallel. Repeated cell cycles, reagent introduction, and OD analysis. T_d: 1.4 days	[63]	(2005)
Membrane-aerated MBR									
PDMS-MBR [MIT]	0.005–0.05 Single well	Magnetic stirrer 200–1500 [61–63] 700 [66]	Diffusion through PDMS membrane	ca. 60 ~170	*Escherichia coli*	(1–4) × 10^{12} cfu l^{-1}	Mathematical analyses on OTR and calculation of $k_L a$	[61]	(2004)
				NA	*E. coli*	(2–4) × 10^{12} cells l^{-1}	Screening microbial strains by gene expression analysis	[62]	(2005)
PMMA-PDMS-MBR [MIT]	0.15 4 wells			NA	*E. coli*	OD_{600}: ~5.5	Multiplexing for high-throughput bioprocessing	[63]	(2005)
	0.1 8 wells			ca. 360	*E. coli*	13.8 g-dcw l^{-1}	Highly organized system with mixer and microfluid injectors for scalable application	[65]	(2006)

(*continued*)

Table 6.3 (*Continued*).

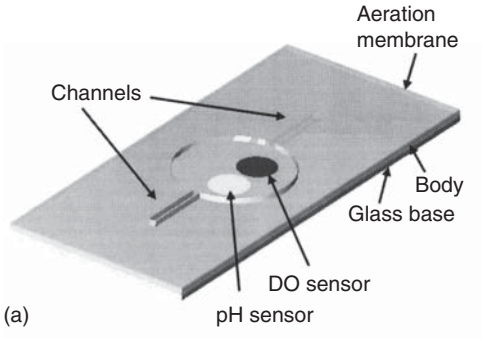
Bioreactor/ device [developer]	Volume (ml), multiplexity	Agitation (rpm)	Aeration	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
	0.15 3 wells			NA	*Saccharomyces cerevisiae*	OD_{600}: ~7.0	Application to profile eukaryote gene expression	[66]	(2006)
	0.15 8 wells			NA	*E. coli*	OD_{600}: ~6.2	Active mixing by magnetic stir bar. Plug in-and-flow microfluidic connectors for fast set-up of batch, continuous, and fed-batch operations	[33]	(2007)
Application of microfluidics in MTP									
Microfluidic MTP [Aachen U]	0.5 ml 48 wells	800	Surface aeration with shaking	NA	*E. coli*	NA	Microfluidics enforce MTP utilities and performances	[67]	(2009)
		1.000		460	*E. coli*	Equivalent to 11.7 g-dcw l^{-1}	User-friendly and disposable microfluidic system; fed-batch culture	[68]	(2010)
	1–25 48 wells	700–1200		<587	*E. coli*, *Gluconobacter oxydans*, and *Kluyveromyces lactis*	OD_{600}: ~14 (*E. coli*) OD_{600}: ~25 (*L. lactis*)	A fast way to gain comprehensive and quantitative data by parallel use of 25-ml shake flasks with RAMOS, and MTPs in the BioLector system	[69]	(2015)

Abbreviations: Aachen U: RWTH Aachen University; BioLector: a system for optical monitoring and control in each individual bioreactor; cfu: colony forming unit; dcw: dry cell weight; Harv U: Harvard University; Inst Pasteur: Institute of Pasteur; MIT: Massachusetts Institute of Technology; MTP: microtiter plate; NA: not available; OTR: oxygen transfer rate; RAMOS: respiration activity monitoring system; UCB: University of California, Berkeley.

Sinskey's group [61–63, 65, 66] has developed a series of MBRs that utilized PDMS and microfluidics. They first fabricated a micro-scale MBR with PDMS and glass with a working volume of 100–150 µl. Supply of oxygen to microbial cultures was made through a gas-permeable PDMS membrane. A bubble-free oxygen supply allowed direct measurements to observe subtle changes in the concentration of cells. Sensors were installed for online monitoring of the OD to estimate cell concentration, as well as DO and pH. All three measurements were based on optical methods [61, 62]. A miniature motor was installed for magnetically driven stirring of the culture in the MBR [63], and a microfluidic injector was installed to feed medium components [65]. Differential gene expression was analyzed in the eukaryotic model *S. cerevisiae* grown in galactose and glucose media in the 150-µl MBR system with online monitoring of OD, pH, and DO. The result from the highly instrumented MBR system allowed integration of HTP studies on physiology with global gene expression analyses of microorganisms [66]. A polymer-based microbioreactor with a magnetic stir bar was presented as an example of an MBR fabricated with a PDMS and poly(methylmethacrylate) multilayer by micromachining for online optical sensing of OD, pH, and DO. Plug in-and-flow microfluidic connectors and fabricated polymer micro-optical lenses/connectors were integrated into the microbioreactors for fast setup and easy operation. Online optical measurements of OD, pH, and DO were integrated, and active mixing was made possible with a miniature magnetic stir bar. Examples of applications demonstrate the feasibility of culturing microbial cells, specifically *E. coli*, in 0.150-ml bioreactors in batch, continuous, and fed-batch operations [34] (Figure 6.5).

A new multiplex microfluidic cell culture array composed of 10×10 cultivation units was developed for long-term cellular monitoring. This array can potentially allow 100 different cell-based experiments to be run in parallel. Major functions include repeated cell growth, reagent introduction, and real-time optical analyses. HeLa cells were cultivated in the device with continuous perfusion of medium at 37 °C. This cell culture array offers a platform for a wide range of drug screening assays [64].

Buchenauer [67] investigated the incorporation of microfluidic elements into an array of micro-bioreactors based on a 48-well MTP format. In this system, the process parameters of pH and biomass were monitored online using a combination of different sensors with BioLector technology and conductance measurements, and a microfluidic device dispensed two fluids individually into each well to maintain pH within a narrow range. Funke *et al*. [68] developed a user-friendly system for routine laboratory work. All of the disposable microfluidic MTPs were fabricated to be automatically actuated. This novel microfermentation system was tested in fed-batch fermentations of *E. coli* in a BioLector system. The scale-up to a 2-l laboratory-scale fermentor was examined by holding $k_L a$ at 460 h^{-1}. The same growth of *E. coli* was observed at both scales. Wewetzer *et al*. [69] investigated the parallel use of two small-scale culture systems to obtain more quantitative information for scale-up. One is the respiration activity monitoring system (RAMOS) device to monitor oxygen uptake and carbon dioxide evolution rates in



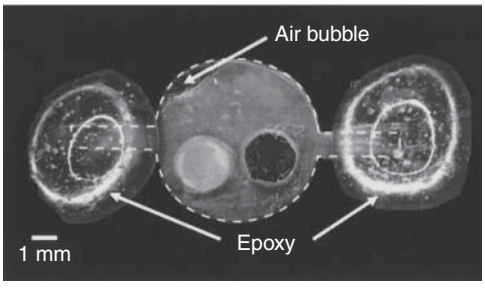


Figure 6.5 Microbioreactor built of three layers of PDMS on top of a layer of glass. (a) Solid model drawn to scale. (b) Photograph of microbioreactor at the end of a run. (From [61] with permission ©2000 John Wiley & Sons, Inc.)

flasks. Another is a 48-well MTP BioLector device. They both provide information of pH, cell growth, metabolites, and DO tension. Consequently, the number of experiments needed in a laboratory-scale STBR for process development could be substantially reduced.

6.2.1.4 Bubble Column Systems

Doig *et al*. [70] developed a miniature microplate bioreactor system that consisted of 48 static, deep wells in a bubble column configuration. The value of $k_L a$ could be up to 220 h^{-1}, which is in the same range as that of large-scale bioreactors. Another multiple microbioreactor battery was designed to produce HTP recombinant protein using *E. coli* [71]. The new system was comprised of aerated glass bioreactors with a working volume of 80 ml and optical sensors for measuring OD, pH, and DO. All the bioreactor process parameters, namely pH, DO, and turbidity, were controlled online. Cultivation of *E. coli* on a high-density medium that was enriched by aeration enabled the cells to grow to an OD_{600} of 100. The miniature bubble column bioreactor system appeared to be well suited for automated parallel cultivations and process optimization. A miniature bubble column system would also be suitable for the cultivation of phototrophic microorganisms. Havel *et al.* [72] reported such a system with 16 parallel bubble columns equipped with artificial light sources supplying a homogeneous light spectrum directly above the

bioreactors. Cylindrical light-reflecting tubes were positioned around every single bubble column to avoid light scattering and to redirect the light from the top onto the cylindrical outer glass surface of each bubble column.

6.2.1.5 Comparison of Various Parallel-Use Micro-/Mini-Bioreactor System

Parallel-use micro-bioreactor technologies have resulted in a significant expansion in use and increase in the sophistication of biotechnological engineering. Table 6.4 provides an overview of state-of-the-art technologies in the fields of MBRs for high-throughput processing. Areas for significant development include devising bioreactor configurations, instrumentation in monitoring and control systems, technologies for fluid handling for material supply and culture monitoring, machinery automation to integrate all of the parts into an organized system, and data processing, including optimization of the process. Developments in the function and quality of devices, instrumentation, and software back-up are comparable to those in industrial manufacturing sites. Well-developed monitoring systems are now able to quasi-continuously provide data on the growth, respiration, substrate consumption, and other information reflecting changes in the physiological state of cells. Further, these systems now enable the evaluation of cultures in various environmental conditions to effectively screen clones, develop and optimize culture procedures, and scale up to the manufacturing stage.

Along with the development of the high-throughput technologies shown in Table 6.4, standard equipment has been built to monitor MBR systems, including direct measurements of pH, DO, OD, and fluorescence almost on a real-time basis by using miniature probes or by fiber-optic methods. State-of-the-art MBRs have high specifications and useful features such as good mixing, high OTR and $k_L a$, and high quality monitoring, which all contribute to speeding up the screening of clones, optimization of media and cultivation procedures, data processing with the aid of software for DoE, DSD, and predictive scale-up. Now it is possible to run a large number, even more than 1000, of parallel cultivations using single-use disposable fermenters that facilitate automatic liquid handling with a number of micro-pumps and mini-valves. The results of mixing analyses and chemical engineering studies on fluid dynamics allow us to understand, quantitatively, the hydrodynamic phenomena in MBRs. Because of this, we are able to perform chemostat, fed-batch, and more sophisticated cultures.

Table 6.4 provides some information on the availability of well-developed and ready-to-use micro/mini-bioreactor systems in four categories, as summarized below.

(i) MTP-type: SimCell [Searhorse BP], BioLector [Archen U], μ-24 MTP [MicroReactor], (ii) STBR-type: HTBD MBR [TUM], HTBR [UMBC], SMBR [UCL], ambr15®/250 [TAP], (iii) MFLBR-type: 10×10 array micro-chamber [UCB], PDMS-MBR [MIT], Microfluidic MTP [Aachen U], and (iv) BCBR-type: MMF [Inst. Pasteur], where the names of bioreactors are abbreviated and the developers are presented in square brackets [ ].

Table 6.4 Overview of various mini/micro bioreactors for high-throughput processing – key technologies and performance specifications.

Bioreactor/ device [developer]	Volume (ml), multiplexity	Agitation (rpm)	Aeration	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
Group 1. Microtiter plate (MTP)									
M24 MTP [MicroReactor]	10 24	Horizontal rotation 800	Headspace aeration	56	CHO	~2.0×10^9 viable cells l^{-1}	It enabled to control temperature, pH and DO in each bioreactor individually Scale-down (2 l to 5 ml) study on cell culture	[32]	(2009)
SimCell [Seahorse BP]	0.8 >1000	Vertical rotation 5–20	Diffusion through membrane	~7	CHO	1.2×10^{10} cells l^{-1}	Factorial design of experiment (DoE) varying four parameters: pH, DO, feed rate, and glutathione in 180 runs	[33]	(2009)
MTP cum BioLector [Aachen U]	0.2 96	Horizontal rotation 995	Headspace aeration	100–350 [22] OTR > 0.03 M h^{-1} [23]	*Hansenula polymorpha* [22] and *Kluyveromyces lactis* [23]	14 g-dcw l^{-1} [22] μ: 0.6 1 h^{-1} [23]	Scale-up 7000-fold: MTP (200 nl) to STR (1.4 l) [22] A new MTP system for HTP temperature optimization [23]	[22] [23]	(2009) (2014)
Group 2. Stirred tank bioreactor (STBR)									
HTBD MBR (miniature bioreactor) [TUM]	8–15 48	Gas-inducing impeller 1500–4000	Surface (Gas-inducing impeller)	>1440 [37, 39]	*Escherichia coli* [37], *Bacillus subtilis* [44], and *Saccharomyces cerevisiae* [45]	30 g-dcw l^{-1} [44] 8.4 g l^{-1} (batch) 18.2 g l^{-1} (fed-batch) [45] 12 g-dcw l^{-1} [39]	Gas-inducing bioreactor [37] Fed-batch cultures for riboflavin production [44, 45] Many parallel continuous cultivations [39]	[37] [44] [45] [39]	(2005) (2007) (2011) (2015)

HTBR (high-throughput bioreactor) [UMBC]	12.5–35 12	2-Stage paddle 150	Sparging	2–4 (HTBR)	SP2/0 myeloma/mouse hybridoma [43, 46]; CHO, NS0, and HEK 293 [47]	1.0×10^9 cells l^{-1} [46] 1.18×10^9 cells l^{-1} [47] 2.5×10^9 cells l^{-1} [43]	Validation of HTBR with optical sensors [46] Analysis of mixing in HTBR by optics [48] CultiBag (2 l/3.5 l; $k_La=2-3$) scaled down to HTBR [47] Clone selection by HTBRs [43]	[46] [48] [47] [43]	(2006) (2006) (2009) (2010)
SMBR (Stirred minibioreactor) [UCL]	100 4–16	6-blade turbine 100–2000	Sintered sparger; 0–2 vvm	<400	*E. coli* and *B. subtilis*	7.6 g l^{-1} (*E. coli*) [50] 3.8 g l^{-1} (*E. coli*), 9 g l^{-1} (*B. subtilis*) [51]	Predictive scale-up, analyses of power consumption and OTR [50, 51]	[50] [51]	(2008) (2008)
Ambr® [TAP]	250 12/24	Rushton or pitched-blade 150–3500	Sparger	1–15 (cells), <770 (microbes)	CHO, *Pichia pastoris*, and *E. coli* [57]; Recombinant CHO [53]; *E. coli* [58]	>400 g-wcw l^{-1} [57] ~1.4×10^{10} cells l^{-1} [53] OD_{max}: ~160 [58]	Ambr 250, highly automated workstation with parallel use of disposable fermentors [57] Ambr 15 required a higher power input (~400 W m^{-3}) than large-scale (~20 W m^{-3}) to provided a same level of k_La [53] DoE/DSD using ambr 250 [58]	[57] [53] [58]	(2013) (2013) (2015)
Group 3. Microfluidic bioreactor (MFLBR)									
MFLBR [MIT]	0.1/ 0.15 8/3	Magnetic stirrer 700 [66]	Diffusion through membrane	~360	*E. coli* [34, 65] and *S. cerevisiae* [66]	13.8 g-dcw l^{-1} ($OD_{650}>40$) [65] OD_{600}: ~7.0 [66]	Highly organized MFLBR with bubble-free oxygen supply [65] Profiling eukaryote gene expression [66] Batch, continuous, and fed-batch operations [34]	[65] [66] [34]	(2006) (2006)

(*continued*)

Table 6.4 (*Continued*).

Bioreactor/ device [developer]	Volume (ml), multiplexity	Agitation (rpm)	Aeration	$k_L a$ (h^{-1})	Organism	Cell growth	Research on properties and performances	References	Year
Microfluidic MTP [Aachen U]	0.5/(1–25) 48	Horizontal rotation 800 [67], 1 000 [68], and 700–1 200 [69]	Surface aeration with shaking	460 [68] 183–603 [69]	*E. coli*, *Gluconobacter oxydans*, and *Kluyveromyces lactis*	OD_{600}: 10.8 [69]	Introducing microfluidics to MTP [67] Fed-batch culture [68] Parallel use flasks (250 ml) with RAMOS and microfluidic MTP in BioLector system [69]	[67] [68] [69]	(2009) (2010) (2015)
Group 4. Bubble column bioreactor (BCBR)									
MMF (Multiple Microfer-mentor) [Inst. Pasteur]	80 8		Sintered glass	750	*Mycobacterium tuberculosis* and *Mycobacterium leprae*	OD_{600}: 100	Miniature probes for DO, pH, and temperature; individually temperature-controlled in each bioreactor	[71]	(2006)

Abbreviations: Aachen U: RWTH Aachen University; BioLector: system for optical monitoring and control in each bioreactor; CultiBag: disposable wave-type bioreactor; dcw: dry cell weight; DO: dissolved oxygen; DoE: design of experiments; DSD: definitive screening design; Inst Pasteur: Institute of Pasteur; MIT: Massachusetts Institute of Technology; OD: optical density; RAMOS: respiration activity monitoring system; Seahorse BP: Seahorse BioProcessors; STR: stirred-tank reactor; TAP: The Automation Partners Biosystem; TUM: Technische Universität München; UCL: University College London; UMBC: University of Maryland, Baltimore County; wcw: wet cell weight.

It is worthwhile to cite several review papers highlighting the progress on key issues in the field of parallel-use bioreactors. Betts and Baganz [73] reviewed the research on MBRs, identifying best practices and future opportunities and discussing the use of different types of MBRs to cultivate various organisms in parallel, to speed up bioprocessing. MBRs are based on several existing bioreactor platforms such as shaking devices, STRs, and bubble columns. Bareither and Pollard [74] noted that significant technological improvement is still required to provide automated solutions that will speed up upstream process development. The authors envisage an automated solution that integrates liquid handling decks. Disposable reactors for fast set-up time with automated media dispensing, inoculation, and growth control, which enable variable conditions and feed regimes should be used. The integration of parallel, disposable, and well-controlled systems will have a significant impact on improving process efficiency (estimated at a 4–5-fold increase). Kirk and Szita [75] presented the oxygen transfer characteristics of miniaturized bioreactor systems. The applicability of $k_L a$ measurements over a wide range of reactor scales, from the microliter level to the kiloliter level, was confirmed experimentally and mathematically. The need to develop measurement methods for DO and OUR should be appreciated. The oxygen transfer performance was examined using sub-milliliter micro-bioreactors and 1–10 ml MBRs. MBRs have reached $k_L a$ values of 460 h^{-1} in microfluidic MTPs [68], and 770 h^{-1} in ambr® 250 [57]. The highest $k_L a$ value reported was 1440 h^{-1} in a 10-ml stirred-tank-type bioreactor with a gas-inducing impeller [37]. Kim *et al*. [76] reviewed the miniaturized bioreactors for their performance in animal cell cultivation, describing novel designs for active mixing and microfluidic cell culture devices that incorporated information on cell density and product titer, in batch or fed-batch modes. Mini-scale tools for process performance evaluations of animal cell cultures were also discussed.

6.2.2 Single-Use Disposable Bioreactor Systems

Conventional stainless steel fermentors were invented for various purposes and developed as durable devices. They are used repeatedly with cleaning after cultivation and sterilization and before inoculation. Those procedures for using a fermentor are technical and complicated, requiring special care to prevent contamination from foreign organisms, and manufacturing such permanent devices is costly. To overcome such difficulties, there is a need to develop alternative simpler and more flexible devices and procedures to win in severe competition among manufacturers who have to handle more diversified targets seeking for successful commercial products. The setup costs for plants, including costs for fermentors and ancillary equipment, are increasing with the expansion and sophistication of R&D requirements. Single-use disposable bioreactors have received much attention as one of the most promising alternatives to conventional fermentors, and their use has grown rapidly.

6.2.2.1 Features of Single-Use Bioreactors

Single-use disposable bioreactors are used to grow microorganisms [57, 77–79], plant and insect cells [80], animal cells [81–83], and human cells [57, 84]. Important features of a single-use fermentor are a reduction in the regulatory risk, low capital and energy costs, and simplicity, which includes flexibility in choice of the facility, quick start time, ease of use, less risk of cross-contamination, and fewer requirements for training, cleaning, sterilization, and validation [81, 83, 85–87].

One of the most important issues in the selection of a single-use bioreactor is an appropriate oxygen supply [79, 88]. As an example, the single-use bioreactor Biostat CultiBag® (Sartorius-Stedim Biotech) is capable of providing oxygen with sufficiently high $k_L a$ values to propagate bacterial and animal cells, including CHO cells, up to a high concentration, similar to the levels reached in a reusable stirred tank [79, 87–90]. The single-use bioreactor family has been thoroughly characterized, with measurements of the power input per volume, mixing time, and $k_L a$ value. These systems are suitable for the cultivation of mammalian cells, even for high cell density and high titer applications [88, 90].

Stirred disposable bioreactors have been the most commonly used bioreactors, although the first models came onto the market only in 2006. In a comparison of commercially available stirred disposable bioreactors, several types with different cultivation container designs, either flexible bags or rigid cylindrical vessels, and scales of operation were identified [81]. However, the contribution of conventional methods, which have been frequently used in industrial manufacturing of various biologics, should not be discounted. Novo Nordisk, for example, still uses conventional 75 m^3 steel tanks to produce commercial insulin (personal communication). The current movement does not imply the lack of value of single-use bioreactors, and their advantages should be incorporated into the development of new reactors.

6.2.2.2 Sensors and Monitoring

The development of disposable sensor technology still lags behind that of bioreactors [85]. Nevertheless, many bioreactors have pH and DO monitoring and control systems with the use of an optical probe [57, 68, 78, 88], in addition to monitoring and control of temperature. Monitoring of cell growth is possible online, based on the use of many single-use bioreactors [57, 68, 81]. Cell viability is an important issue in the propagation of fragile mammalian cells such as CHO cells and human mesenchymal stem cells [57, 84]. Viability should be correlated with cell growth, which can be monitored online using dielectric spectroscopy in a disposable reactor [91].

In many bioreactors, DO and pH can be monitored individually online by measuring the fluorescence decay time of two chemical sensors immobilized at the bottom of each single-use bioreactor [68, 78, 88]. In microfluidic MTPs of a single-use MBR in the BioLector system, pH-controlled batch as well as fed-batch fermentation was performed. A disposable microfluidic system was established that allowed scalable and fully controlled and monitored fermentation in working

volumes below 1 ml [68]. Sensors for all critical parameters on the same detection platform in single-use bioprocess components would be highly desirable. A new approach utilizing passive radio frequency identification (RFID)-based sensing was explored. The developed RFID sensors combined several parameters measured by the resonant sensor antenna with a multivariate data analysis for a multiparameter sensing technique [92]. Disposable device technology has rapidly advanced in terms of cell culture, liquid handling, and storage, but it has lagged in other areas. There is a need for more improvement, with the evolution of disposable sensors, control systems, supplier increases, platform diversity, and support package standardization [85, 87].

6.2.2.3 Single-Use Bioreactors in Practical Use

The commercialization of disposable equipment, including bioreactors, sensors, and monitoring and control systems, has increased significantly during the last several years [57, 77–79, 82, 83, 87, 89, 90]. A mass transfer characterization of a novel mechanically driven/stirred process scouting device (PSD; SuperSpinner D 1000®, SSD) was performed. This novel device can be viewed as a disposable bioreactor [88]. DO and pH were monitored in the SSD with patch-based optical sensors. Dreher *et al*. [89] developed a stirred single-use bioreactor family at 50–2000 l scale with geometrical ratios similar to conventional reusable systems. Using a unified single-use cell culture platform, PacificGMP, consisting of a presterilized, flexible, disposable plastic bag on a rocking platform, was filled with media and a gas mixture that supported the growth of *E. coil*, CHO cells, and hybridoma cells [83].

Eible *et al.* [81] provided an overview of commercially available disposable bioreactors. These bioreactors can be classified into various categories: wave-mixed, orbitally shaken, and stirred disposable bioreactors, with animal-cell-derived production at the cubic meter scale reported from these reactors. For laboratory use, various sizes of STRs have been developed. Kustere *et al*. [78] presented a system of STBRs coupled with a control system based on the assembly of a single-use bioreactor from 48 units at a 10-ml scale, with individual stirrer speed control, DO, and pH monitoring and control [78]. Another small-scale stirred tank with a disposable 250-ml reactor was developed by Bareither *et al*. [57], who also introduced a stirred single-use bioreactor family at a 50–2000 l scale [87].

Löffelholz described parametric, experimental, and computer-based numerical methods for biochemical engineering to characterize single-use bioreactors in terms of their practical application [90]. Wave-mixed bioreactors are characterized by low shear forces, with gas exchange realized by the large gas–liquid interface [79, 83, 87, 89, 90]. Junnes *et al*. [79] reviewed the applications of wave-mixed disposable bag bioreactors to the cultivation of microorganisms. In these applications, volumetric OTRs were obtained in the range of 50 and 300 h^{-1}. They successfully scaled up *E. coli* fed-batch cultivation from the 12 to 120 l scale using a disposable wave-mixed bioreactor, CELL-trainer®, resulting in a final cell concentration of 45 g l^{-1}.

The single-use bioreactors should be developed for higher scalability and configurability, which is needed. Especially important is the connection with downstream for overall performance of the manufacturing plant.

6.3 Monitoring and Process Analytical Technology

It is important for bioprocess engineers to develop the technologies for adequate control of microbial cultivation processes, first, by recognizing and analyzing the microbial physiology in relation to the surrounding environment accurately, and, second, by manipulating operational conditions in the most appropriate way. In the past two decades, the demand for real-time and near-real-time monitoring for the automatization of bioprocesses has resulted in significant innovation and automation in the field of process monitoring [93].

6.3.1 Monitoring and State Recognition

6.3.1.1 Sensors for Monitoring Bioprocesses

Modes of monitoring used by sensors and analyzers are classified in various ways such as "offline," "at-line," and "online." The monitoring systems of bioreactors have advanced toward automation for real-time acquisition of information (Figure 6.6). Previously, samples were obtained manually and then transferred to a separate chemical laboratory to be analyzed, as shown in (1) offline monitoring. The transfer of the sample to the laboratory causes a certain delay in the analysis. In (2) at-line monitoring, the collected samples are analyzed by an analytical system in the immediate vicinity of the bioreactor. In both cases, samples can be automatically collected using an autosampler. After development of an auto-analyzing system, the assays of some medium constituents can be made continuously by (3) inline monitoring with continuous sampling, or (4) inline monitoring of the circulating culture broth outside of the reactor. Lastly, direct measurements can be made by (5) online monitoring with an invasive probe or (6) online monitoring with a noninvasive probe.

***In Situ* Sensors** Real-time monitoring of bioreactors is now regarded as a key issue for effective bioprocess control, which can lead to increased efficiency, productivity, and reproducibility, and also improved quality control and reduced environmental pollution. Thus, overall costs are minimized by the newly developed PAT [93]. Previously, only a small number of variables were commonly measured online in bioreactors, namely temperature, pressure, DO, pH, stirring speed, and gas and liquid flow rates. These processes are generally referred to as process variables or engineering data. With PAT, additional probes are available

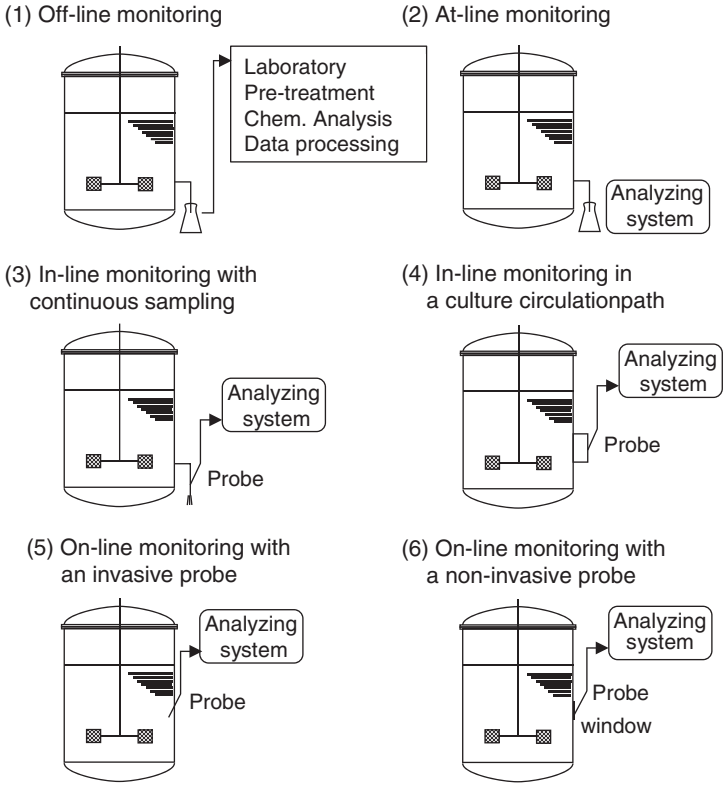


Figure 6.6 Bioreactor monitoring systems advancing toward the automation for real-time information acquisition. (1) Manual sampling and analysis in a separated chemical laboratory, (2) at-line monitoring with manual sampling, (3) iline monitoring with continuous sampling, (4) inline monitoring in a culture circulation path, and (5) online monitoring with an invasive probe, and (6) online monitoring with a noninvasive probe through a window or indirect detection.

to measure variables such as dissolved carbon dioxide, oxidation–reduction potential, OD, or turbidity. Also, various components of the culture medium and metabolites in the cells were monitored inline and online modes, and three were additional gaseous components in the inlet and outlet gas stream.

A typical comprehensive review on *in situ* sensor techniques for bioprocess monitoring was made by Beutel and Henkel [94] and Biechele *et al*. [95]. Wang and Wolfbeis [96] and Pospíšilová *et al*. [97] provided reviews on fiber-optic chemical sensors and biosensors. Wang and Wolfbeis also comprehensively reviewed optical methods for sensing oxygen materials, spectroscopies, and applications [98].

Biomass Sensor and Flow Cytometry Kiviharju *et al*. [99] reviewed online biomass measurement, summarizing various studies with *in situ* measurements and a software sensor; Suhr *et al*. [100] and Bittner *et al*. [101] reported the *in situ* determination the of cell population, and Bluma *et al*. [102]; and Höpfner *et al*. [103] reviewed *in situ* sensors for biomass monitoring and inline characterization of cell populations in agitated bioreactors using *in situ* microscopy (ISM) and other *in situ* sensors for imaging of cell morphology. Hewitt and Nebe-Von-Caron [104] reported industrial application of flow cytometry for the assessment of cell physiological state, enabling the assessment of population heterogeneity, and Silva *et al*. [105] reviewed the application of multiparameter flow cytometry technologies to monitor industrial bioprocesses for the production of biodiesel, bioethanol, biomethane, and biohydrogen, among others.

Sensor Systems of Electronic Tongues and Noses Requests for the development of online sensors in fermentation monitoring commonly stress on process control issues. One of the techniques suitable for this purpose is chemically based sensors, which has been recognized as an attractive tool for industrial process control, because of its low cost, relatively simple instrumentation, minimal sample preparation, and ease of automation. However, practical use of chemical sensors in complex media is often hindered by their insufficient selectivity. In practice, only pH and oxygen probes are routinely used in bioreactors. One of the emerging approaches that has allowed overcoming the selectivity problems is the use of systems instead of discrete sensors. Such systems for liquid and gas analyses are named "electronic tongues" and "electronic noses," correspondingly. They are both capable of performing quantitative analysis (component concentrations) and classifying or recognizing the multicomponent media. Rudnitskaya and Legin [106] reviewed recent achievements in the R&D, including the application of electronic tongues and noses to the monitoring of biotechnological processes.

EU Experts' Recommendations on Sensors in PAT An expert panel organized by the M^3C Working Group of the European Section of Biochemical Engineering Science (ESBES) in European Federation of Biotechnology (EFB) reviewed monitoring of MBRs and presented an expert opinion on future development needs [107]. The recommendations included online analytics such as chromatography or mass spectrometry that were added to bioreactors, preferably using noninvasive sensors and optical or electronic sensor methods that do not involve labeling or in other ways not affecting cellular functions. Sensors to be used online in the bioreactors should be selected based on three criteria: (i) detection limits in relation to analytes, (ii) stability in relation to the testing period, and (iii) the possibility of miniaturization to specific volume ranges and dimensions in the microfluidic system applied in the bioreactors. Nevertheless, mathematical models based on soft sensor principles should be exploited to reduce the number of sensors required.

Soft Sensor (EU Expert) The M^3C Working Group of the EFB gave a status report on soft sensors in bioprocessing and made recommendations [108].

Krause *et al*. [109] presented a method for the accurate detection of process parameters combined with expert knowledge of linguistic control mechanisms via adaptive sensor calibration.

6.3.1.2 Spectrometry

Spectrometric methods that have been extensively utilized for bioprocess monitoring include near-infrared (NIR) spectroscopy [110], Raman spectroscopy [111, 112], nuclear magnetic resonance (NMR) spectroscopy [113, 114], acoustic resonance spectroscopy [115], mass spectrometry [116], dielectric spectroscopy [117], fluorescence spectroscopy [118, 119], and photoacoustic spectroscopy [120, 121]. Calorimetry is utilized for studying microbial metabolism and growth [122, 123], and terahertz technology [94, 124] as well. Rathore *et al*. have extensively investigated the use of online HPLC [125, 126, 127].

Near-Infrared Spectroscopy for Process Monitoring The application of NIR spectroscopy (NIRS) has developed in parallel with the development of the personal computer and information technology, and its explosive growth has been visible in many industries. Recently, it has been particularly apparent in the area of monitoring and control of microbial and cell culture systems. Potentially, NIRS offers the prospect of real-time control of the physiology of cultured cells in fermentors, leading to marked improvements in authenticity, purity, and production efficiency. Despite this, NIRS is not yet as widely applied within the bioprocessing industry as its potential might suggest. A review by Scarff *et al*. [110] critically evaluated the development of this rapidly developing area as it pertains to the control system of microbial and cell culture and highlighted the critical stages in the development of the technology. More research must still be carried out if the full potential of NIRS is to be exploited in making proteins, hormones, and antibiotics via the fermentation route. The review has been particularly timely because NIRS stands on the threshold of widespread acceptance in bioprocessing.

Lourenço *et al*. [128] reviewed current conditions of sensor systems for bioprocess monitoring and claimed that various materials in the culture medium could be assayed by adequate spectroscopic methods. Several articles have claimed that NIRS is useful for monitoring glucose, lactate, antibodies, pH, fructose, acetic acid, and ethanol; UV–vis for bovine serum albumin (BSA) and toxic substances; proteins fluorescence spectroscopy for vitamins, pyruvate, ATP, NAD(P)H, cell mass, and ethanol; and Raman spectroscopy for glutamine and glutamate, phenylalanine, and TCD/VCD, where TCD for total cell density; and VCD for viable cell density.

Flow Injection Analysis Various problems associated with the use of biosensors in process control – for example, difficulties of sterilization, instabilities, short durability, calibration complications and sensor fouling – have often been pointed out. Ogbomo *et al*. [129] claimed the advantages of flow injection analysis (FIA), and reviewed examples for efficient sampling systems connected with this method. Special emphasis was given to problem-oriented sample

pretreatments, preventing fast inactivation of immobilized enzymes in the analysis system. Examples of sample pretreatment units were given. A proposal for a computer-controlled self-calibrating FIA system was also given.

Ion Mobility Spectrometry (IMS) Ion mobility spectrometry is now frequently applied in the field of process analytics. The method has the advantage of high sensitivity, with detection limits down to nanogram to picogram per liter and ppbv/pptv ranges, relatively low technical expenditure, and high-speed data acquisition. The time required to acquire a single spectrum is in the range of 20–50 ms. The working principles of IMS is based on the drift of ions at ambient pressure under the influence of an external electric field. The mean free path of the ions is smaller than in mass spectrometry. Therefore, a swarm of drifting ions experiences a separation process which is based on ions with different masses or structure having different drift velocities. Collecting these ions on a Faraday plate results in a time-dependent signal corresponding to the mobility of the arriving ions. This ion mobility spectrum contains information on the nature of the different trace compounds present in the sample gas. Different ionization techniques, sample introduction methods, and pre-separation methods were considered. The applications of IMS discussed relate to purity (gas insulated substations), ecology (contamination of water resources), plant and person safety (odorants in natural gas), food quality control (molds and bacteria), and human health (breath analysis) [130].

6.3.2 Process Analytical Technology (PAT)

As mentioned in the introduction of this chapter, the US FDA issued "Guidance for Industry PAT" as a framework to guide innovative pharmaceutical development, manufacturing, and quality assurance in 2004 [1].

Junker and Wang [131] reviewed the research on bioprocess monitoring and computer control as the key roots of the current PAT initiative, focusing on the impact of the work by D. Wang, who made a big contribution to the development of computer control and monitoring in the field of biochemical process engineering. It will extensively benefit PAT applications in the future as well. Examples of the past achievements are indirect estimation methods for cell density, online, inline, and at-line measurement techniques, and development of models and expert systems for control and optimization. Future applications are likely to include additional novel measurement technologies, measurements for multiscale disposable bioreactors, real-time batch release, and more efficient data utilization to achieve process validation and continuous improvement goals. Wang's substantial contributions in this arena have been recognized as one key factor in steering the PAT initiative toward realistic and attainable industrial applications.

In the PAT initiative, FDA identified the potential for continuous improvement in the fermentation and downstream processing technologies, and made concrete suggestions how this can be achieved. Jenzsch *et al*. [132] reviewed some of these

suggestions applied to recombinant protein production with *E. coli* and *P. pastoris* cultures. They discussed the development of process operational procedures that allow tighter supervision of the processes and the automatic control in cases where processes deviate from their set-point profiles.

As presented in two consecutive reviews [133, 134] by a group representing the FDA, PAT has been gaining momentum in the biotech community due to the potential for continuous real-time quality assurance, resulting in improved operational control and compliance. They addressed the evolution of the underlying concepts and applications in biopharmaceutical manufacturing, and also presented a literature review of applications in the area of upstream and downstream processing and drug product manufacturing, biological quality assurance and chemometrics, as well as evolution of PAT in biotech processing to illustrate how implementation of PAT can help in the realization of advanced approaches to ensuring product quality in real time. Implementing real-time product quality control meets one or both of the key goals outlined in FDA's PAT guidance: "variability is managed by the process" and "product quality attributes can be accurately and reliably predicted over the design space established for materials used, process parameters, manufacturing, environmental, and other conditions." The role of PAT as an enabling component of the QbD framework is emphasized, with discussions on the integration of PAT with the principles stated in the ICH Q8, Q9, and Q10 guidance documents.

An expert workshop was held at the 8th European Symposium on Biochemical Engineering Sciences (Bologna, 2010) and highlighted new opportunities for exploiting PAT in biopharmaceutical production [135].

6.3.2.1 PAT Tools

Rathore *et al*. [136] reviewed the various analytical methods that can enable the use of PAT. The selection of methods is a key issue for the success of PAT implementation. NIR spectroscopy is one of the most commonly used analyzers for PAT applications. Rathore *et al*. [137] presented a PAT application for one of the most commonly used unit operations in bioprocessing, namely liquid chromatography. The feasibility of using commercially available online high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) systems for real-time pooling of process chromatography column was examined. A model for a chromatography step of a different modality (hydrophobic interaction chromatography) was created. It was found that for the application under consideration, the online HPLC offers a feasible approach for analysis that can facilitate real-time decisions for column pooling based on product quality attributes. Implementing this analytical scheme resulted in meeting two of the key goals that have been outlined for PAT, as indicated by the FDA guidelines mentioned previously in the introduction of this chapter.

Observation of cell viability by applying inline radio frequency impedance measurement and online determination of intracellular recombinant target protein using the reporter protein T-sapphire green fluorescence protein (GFP) based on inline fluorescence measurement is indicative of the detection ability

of critical process states [138]. A high-resolution biocalorimeter was developed from a heat-flow calorimeter, and a simple estimator for biomass concentration and specific growth rate was formulated based on the heat flow values. It was applied to the fed-batch culture of three yeast strains: *Kluyveromyces marxianus*, *Candida utilis*, and *P. pastoris*, based on the inline measured metabolic heat flow signals [139, 140]. Performance of various online biomass sensors was compared during the early development of a filamentous growth of *Streptomyces coelicolor* in a fed-batch culture. The cultivation was monitored *in situ* using multiwavelength fluorescence spectroscopy, scanning dielectric spectroscopy, and turbidity measurements [141].

Streefland *et al*. [142] reviewed PAT tools for monitoring and controlling the biopharmaceutical cultivation step, focusing on the most common cell platforms (i.e., *E. coli*, yeast, and mammalian cells) used in biopharmaceutical manufacturing. The real challenge is to understand how intracellular mechanisms, from synthesis to excretion, influence the quality of biopharmaceuticals and how these mechanisms can be monitored and controlled to yield the desired end product quality. Modern "omics" tools and advanced process analyzers have opened up the way for PAT applications for the biopharmaceutical cultivation process step. Generally speaking, PAT must be an approach applicable to not only the pharmaceutical industry but also any production area, food industry, and other various biotechnological manufactures.

6.3.2.2 PAT Implementations

A multiauthor review article by Simon *et al*. [121] brought readers up to date with some of the current trends in the field of PAT by summarizing each aspect of sensor development and PAT-based process monitoring and control methods and presenting applications in industrial laboratories and in the manufacturing field. The benefits of applying PAT can be realized during process development as well as during commercial manufacturing. Typical advantages of using PAT include increased process understanding, faster process optimization, removal of the need for offline sampling, understanding and predicting the impact of scale-up, and the ability to control/correct processes before isolation of intermediates or product, leading to improved quality. When PAT applications are used in commercial manufacturing, the additional benefits may include the reduction in cost, time (offline analysis), and cycle time, allowing greater plant throughput, green metrics (energy, solvent usage, carbon footprint), safety (offline sampling of hazardous materials), further verification on the effect of scale-up on the process, and process variation monitoring as part of continuous verification during product lifecycle.

Chanda *et al*. [143] reported the current state of PAT for active pharmaceutical ingredient (API) development in pharmaceutical companies. They used an API process workflow and process steps from raw material identification through to the finished API, to provide a representative example, and explained why and how the pharmaceutical industry uses PAT tools in API development. The use of PAT can improve R&D efficiency and also minimize personnel hazards associated with sampling hazardous materials for in-process testing. PAT enables reliable,

rapid, real-time, or the like, analyses of processes that may involve highly hazardous materials. These measurements in PAT procedures can provide a significant amount of data for understanding process chemistry, which may include the detection of previously unknown reaction intermediates, mechanisms, or relationships between process variables.

The use of PAT tools aids in the complete understanding of the process, including determining the presence of transient species, which are difficult to sample and analyze offline, process components, mechanisms, and relationships between variables as well. Through process understanding, critical parameters are identified, and process parameter control limits might be established. Through the development of processes using QbD principles, the number of process steps requiring real-time control will reduce, and it will be possible to simplify the monitoring and control systems. This may result in controls being either offline or by simple measurements such as temperature and pressure being selected by PAT analyses. The industry does require more experienced users in PAT. It is essential that new university graduates, having the right skills and training, must continuously endeavor to ensure that PAT tools are effectively utilized. A successful PAT expert and practitioner should possess multiple skill sets, including understanding of and proficiency in engineering, sampling, process interfacing, chemistry, multiple spectroscopic tools, mathematics, and chemometrics.

6.4 Conclusion

Microbial and cell cultivation processes constitute one of the most influential platforms for industrial manufacturing of valuable and useful products. In particular, their contributions have been highly evaluated in the production of biologics and biochemical products, and consequently their economic and social impact has been well recognized. Recently, strong initiatives have yielded some notable changes in bioprocess engineering studies. They include the development of unique technologies to devise new or more advanced bioreactors for small-scale research on scaling-up toward large industrial scales, with the aim of optimizing the processes including high-throughput technology, single-use disposable bioreactors, monitoring technology, and also process analytical technology based on the concept of QbD control. This chapter provided an overview on state-of-the-art technologies, their widespread applications, and trends in technology advancement. For the individual validation of four types of bioreactors, MTPs are quite useful in the early stages of the process, including clone selection and medium optimization. Microfluidic devices are expected to contribute more in the investigation of cells in terms of basic science aspects, and STRs have demonstrated outstanding scalability, providing important information from the chemical engineering aspect. The use of bubble-type bioreactors could be more appreciated in the case of phototroph culture or where special protocols are required. The contribution of process analytical technology has been conspicuous

in bioprocess engineering, and an extensive multidisciplinary cooperation is expected to pave a new path for innovative sensor technology. We hope that this chapter will provide the reader with more insights into the existing technologies and possible future developments.

Abbreviations

CFD	computational fluid dynamics
CHO	Chinese hamster ovary
API	active pharmaceutical ingredient
DO	dissolved oxygen
DoE	design of experiments
DSD	definitive screening design
GMP	good manufacturing practice
HTP	high-throughput
HTPT	high-throughput technology
$k_L a$	the volumetric oxygen-mass transfer coefficient
MBR	micro-/mini-bioreactor
MTP	microtiter plate
OD	optical density
OTR	oxygen transfer rate
OUR	oxygen uptake rate
PAT	process analytical technology
PDMS	polydimethylsiloxane
QbD	quality by design
RFID	radio frequency identification
STR	stirred-tank reactor

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7
Omics-Integrated Approach for Metabolic State Analysis of Microbial Processes

Hiroshi Shimizu, Chikara Furusawa, Takashi Hirasawa, Katsunori Yoshikawa, Yoshihiro Toya, Tomokazu Shirai, and Fumio Matsuda

7.1
General Introduction

Optimization of metabolic pathways is important in the development of bioprocesses that facilitate the production of chemicals and fuels by microbial cells. Ideally, this process should incorporate two approaches: (i) analysis of metabolic states under given environmental conditions and optimization of cellular metabolic pathways for targeted products, and (ii) comparative and multi-omics analysis under stress conditions, including the development of stress-tolerant cells that remain active even under severe stress conditions.

Recent progress in omics science has facilitated systematic analysis of microbial status, strategic molecular breeding, and the development of bioprocesses. Integration of *in silico* and experimental approaches for the analysis of microbial cells has enabled the rational design of logical metabolic pathways with modifications. Predictions of metabolic pathways by genome-scale metabolic (GSM) reaction models and evaluations of the performance of developed cell factories by experimental ^{13}C metabolic flux analysis can be used to elucidate the state of the cell. By comparative transcriptome analysis, genes that confer useful phenotypes are identified. Integration of transcriptome analysis, comprehensive phenotype analysis, and evolution engineering approaches can provide important tools for the analysis of cell states under stress conditions. In Figure 7.1, we summarize the integration of *in silico* and experimental approaches for the analyses of microbial status and the creation of cell factories. These analyses provide in-depth understanding of cellular processes and enhance the design of cell factories. Moreover, they produce novel data from metabolic systems, which in turn can facilitate engineering of strains that are suitable for industrial applications.

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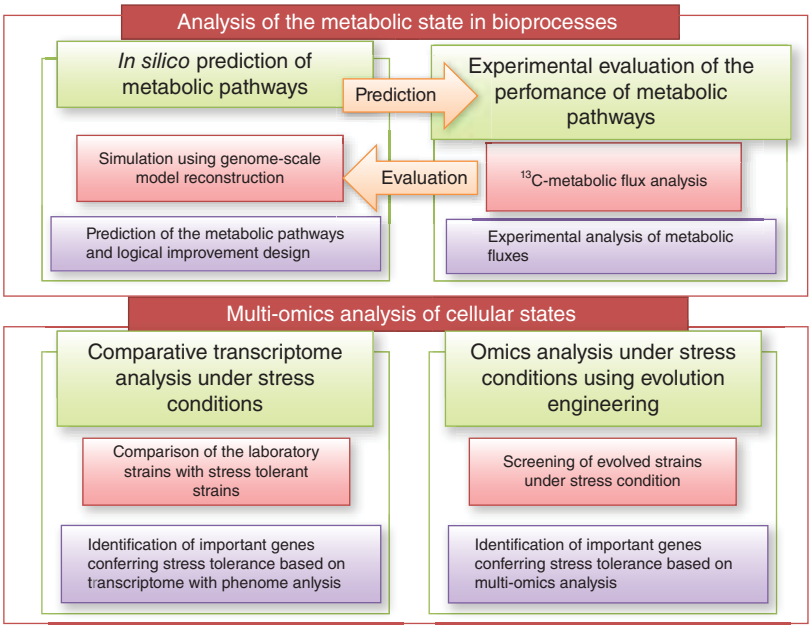

Figure 7.1 Schematic of the integration method, with *in silico* and experimental approaches, for the creation of cell factories.

7.2
Transcriptome Analysis of Microbial Status in Bioprocesses

7.2.1
Introduction

There are many reasons to perform transcriptome analysis of microbial processes: for example, to understand the cellular state, to improve the productivity of target products, and to identify target genes that could manifest useful phenotypes in host cells. DNA microarray is a powerful tool used to analyze the transcriptome. In microarrays, fluorescence intensities of labeled cDNAs, which are hybridized with corresponding probes, are measured and gene expression is thereby quantified. Recently, next-generation sequencing techniques, such as whole transcriptome shotgun sequencing (RNA-seq), have been developed and used to sequence and quantify all expressed RNA.

Inverse metabolic engineering involves the determination of factors that confer desired phenotypes, based on comparative analyses between wild-type strains and strains with superior properties [1]. Omics analysis has greatly influenced inverse metabolic engineering [2]. For example, in genome breeding, the mutations that facilitate high production of lysine were identified by comparing genome information from a wild-type strain of *Corynebacterium glutamicum* with that

of a high-yielding strain [3]. Furthermore, DNA microarrays have been used to identify genes responsible for improvement of galactose uptake in *Saccharomyces cerevisiae* [4] and promotion of antibiotic resistance in *Escherichia coli* [5], and they have facilitated molecular breeding of *E. coli* for lysine production [6] and the creation of osmotic-stress-tolerant strains of *S. cerevisiae* [7]. In the next subsection, we discuss the DNA microarray methodologies employed to analyze the state of the cells, identify targets for gene manipulation, and propagate useful phenotypes such as stress tolerance.

7.2.2
Microbial Response to Stress Environments and Identification of Genes Conferring Stress Tolerance in Bioprocesses

In this section, we describe the identification of genes conferring ethanol stress tolerance, which is an example of transcriptome analysis of microbial status in yeast processes [8]. Gene expression levels in two strains of *S. cerevisiae* were compared: a laboratory strain and a brewing strain (used in Japanese sake brewing). The brewing strain showed higher tolerance to ethanol stress than the laboratory strain.

The expression pattern of individual genes in these two strains was compared using cluster analysis. The self-organizing map (SOM) [9, 10] is a commonly used clustering method in bioinformatics. SOM clusters are further analyzed using the hierarchical clustering (HC) method [11], which is used to assess the similarity in weights of the vectors in SOM-clusters. The boundaries of HC-obtained clusters are determined from the experimental error of DNA microarray analysis.

Clustering analysis was applied to time course gene expression data from the laboratory and brewing strains before and after the addition of ethanol. More than 700 clusters were obtained using SOM, which were further classified into around 30 clusters using the HC method. Three typical gene expression patterns were found, and these are shown in Figure 7.2.

Clusters with specific expression patterns were screened to identify genes important to the status of the microorganism under conditions of high ethanol concentration. Three clusters were selected (Figure 7.2): (i) genes significantly expressed only in the brewing strain (Cluster 10); (ii) genes expressed in both laboratory and brewing strains following the addition of ethanol, but where expression ratios were higher in the brewing strain (Cluster 27); and (iii) genes significantly expressed more in the laboratory strain than in the brewing strain (Cluster 7). In clusters (i) and (ii), genes expressed in higher quantities in the presence of ethanol were screened to facilitate ethanol stress tolerance in the brewing strain. Despite high concentrations of ethanol, the genes in cluster (iii) were not required to facilitate ethanol stress tolerance in the brewing strain, because the genes responsible for ethanol stress tolerance are expected to be expressed at much higher levels in the brewing strain than in the laboratory strain in the absence of ethanol.

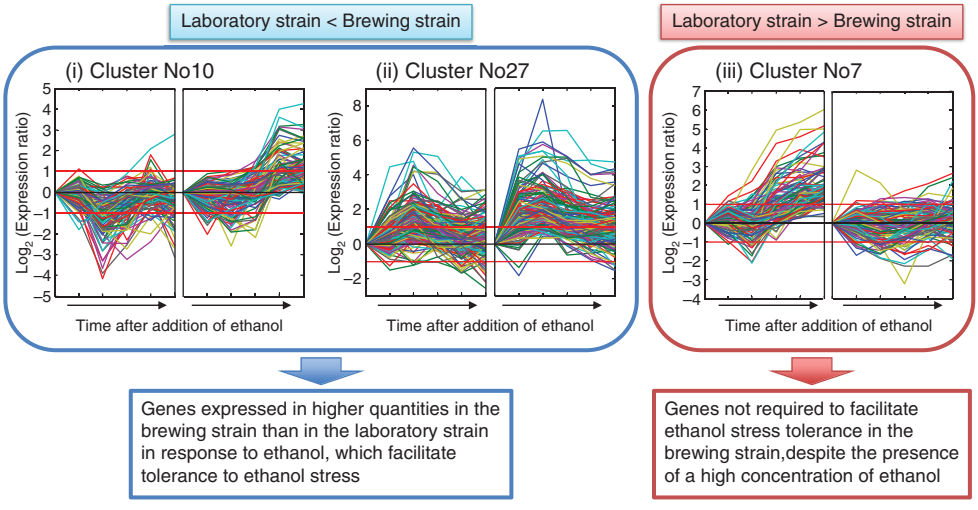

Figure 7.2 Typical patterns of gene expression among 29 clusters. Each chart (i–iii) indicates the expression pattern of genes in individual clusters. The horizontal axes indicate time points of the data, and vertical axes indicate $\log_2$ expression ratios. The left panel represents the gene expression pattern of the laboratory strain, and the right panel represents that of the brewing strain. In each chart, the distance between the two red lines represents a twofold expression change. (i) The genes significantly expressed only in the brewing strain (Cluster 10). (ii) The genes expressed in both laboratory and brewing strains following the addition of ethanol; the expression ratios of these genes were higher in the brewing strain than in the laboratory strain (Cluster 27). (iii) Genes were significantly expressed more in the laboratory strain than in the brewing strain (Cluster 7).

Sensitivity to ethanol was also examined in selected genes in terms of the growth of single-gene deletion mutants. The gene deletion mutants of the tryptophan biosynthesis genes, namely *TRP2*, *TRP3*, and *TRP4*, which were clustered in Cluster 7 (see (iii) in Figure 7.2), showed ethanol-sensitive growth. Additionally, the deletion mutants of other tryptophan biosynthesis genes, namely *TRP1* and *TRP5*, also showed ethanol sensitivity. Based on these results, candidate genes for construction of ethanol-tolerant strains were identified, and subsequently laboratory strains in which these genes were overexpressed were constructed. The overexpressed gene related to tryptophan biosynthesis (in Cluster 7) was found to confer ethanol stress tolerance to yeast cells. Overexpression of genes related to tryptophan uptake and the addition of tryptophan to culture were also effective in conferring ethanol stress tolerance. These important results validate the methodology employed for analysis. In general, the role of tryptophan biosynthesis is still somewhat unclear, although it is significantly related to

ethanol stress tolerance. One possible explanation is that the content of the membrane is affected by acceleration of tryptophan biosynthesis [8].

7.2.3 Transcriptome Analysis of the Ethanol-Stress-Tolerant Strain Obtained by Evolution Engineering

Experimental evolution is a method used to study the evolution of emergent properties in biological processes and uncover the changes in phenotype and genotype that are responsible for adaptive evolution [12, 13]. Parallel-evolution experiments are performed under identical stress conditions to clarify the phenotypic and genotypic changes necessary for adaptive evolution. Such evolution experiments have the potential to provide valuable information for the rational creation strategy of stress-tolerant strains. Moreover, it is expected that identification of the factors essential for higher tolerance will be easier using the experimental evolution approach.

A series of six independent repetitive inoculation (evolution) experiments were performed in *E. coli* cells under ethanol stress conditions [14]. Over 1000 generations were performed in a culture experiment to obtain ethanol-tolerant strains that exhibited nearly twofold higher specific growth rate than the parental strain. To understand the phenotypic changes in tolerant strains, their transcriptome was analyzed in a DNA microarray experiment.

Six parallel-evolution experiments were carried out in a 5% (v/v) ethanol stress condition, beginning with strain P (parental strain), using methods similar to those of the experiments in which ethanol was absent. Figure 7.3a shows the changes in specific growth rates during these evolution experiments. The specific growth rates gradually increased, resulting in an approximately twofold increase compared to strain P. The evolved strains (named strains A–F in descending order of the final growth rate) were stored at −80 °C.

To investigate the phenotypic changes that occur during adaptive evolution to ethanol stress, transcriptome analysis was performed on strains P and A–F in M9 medium with or without 5% ethanol. Principal component analysis (PCA) was employed to identify the factors important in the gene expression induced by ethanol and the adaptive evolution to ethanol stress. Figure 7.3b shows the PCA of 14 expression profiles, in which the principal components (PCs) 1 and 2 explain 42% and 14% of the variance in the expression profile, respectively. Data points corresponding to the addition of ethanol (depicted in Figure 7.3 as P5, A5–F5) cluster to the right of the PCA, and those corresponding to absence of ethanol cluster to the left. This indicates that PC1 represents change in the expression level due to ethanol stress, regardless of phenotypic changes produced by adaptive evolution. Furthermore, with or without the addition of ethanol, data for strain P is located at the top of the PCA figure, while data for the evolved strains are located at the bottom. We also note that the expression

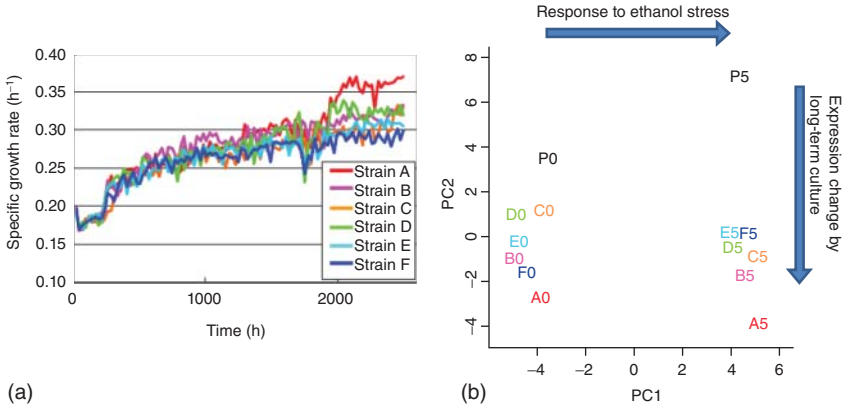

Figure 7.3 Experimental evolution of *E. coli* under 5% ethanol stress condition. (a) The time course of specific growth rates in six parallel evolution experiments. The cells obtained after 2500 h of cultivation under ethanol stress were named "strain A–strain F," in descending order corresponding to the final growth rate. (b) PCA score plot of the first and second principle components (PC1 and PC2). P0 and A0–F0 represent the expression profiles of strain P (parental strain) and tolerant strains A–F, respectively, obtained without addition of ethanol. P5 and A5–F5 indicate data obtained in the 5% ethanol condition.

profiles obtained under ethanol stress (P5, A5–F5) are ordered along the PC2 axis in a pattern that roughly corresponds to growth rate under ethanol stress. This result indicates that PC2 represents the change in expression levels that occur during adaptive evolution to ethanol stress in order to achieve the highest possible growth rates. In summary, PCA of gene expression data discriminated between changes that occur in response to ethanol stress and adaptive evolution to the stress.

Changes in gene expression were analyzed in further detail by screening for functional categories in which genes contributing to PC1 or PC2 were found to be statistically over-represented. A functional category groups together genes based on their functions. The functions of gene products were classified using Gene Ontology Annotation [15]. Information on gene regulation was obtained from RegulonDB [16]. To screen functional categories in which orthologous gene sets with different expression levels were significantly overrepresented, we used a hypergeometric distribution method [14].

We found that genes involved in the iron-ion transport and biosynthesis pathways of some amino acids, including tryptophan, histidine, valine, leucine, and isoleucine, were commonly upregulated in tolerant strains, which suggests that the functions of these genes influence ethanol tolerance. One possible explanation for the activation of iron-ion metabolism genes is that the enhancement of iron-ion uptake is involved in ethanol stress tolerance. However, it may also be caused by the change in the intracellular redox respiratory system, which results in an increased intracellular O_2 or hydrogen peroxide level. Further study is necessary to understand the mechanism of iron-ion transport with regard to ethanol tolerance.

7.3
Analysis of Metabolic State Based on Simulation in a Genome-Scale Model

7.3.1
Introduction

Based on whole-genome data, reconstruction of genome-scale cellular metabolic networks and application of the model of metabolic flux balance analysis (FBA) were performed [17, 18]. FBA is a simple approach to analyze metabolic flux distributions using linear programming (LP) and GSM reaction models. Although GSMs do not include any kinetic information and cannot compute the detailed dynamic behavior of metabolic reactions in a cell, these models enable us to describe the range of possible metabolic states based on constraints defined by stoichiometry of metabolic reactions and transport steps at a steady state. Furthermore, it is possible to develop a solution containing a set of all metabolic fluxes that maximizes an objective function using LP.

Biomass production rate is widely adopted as an objective function. The metabolic profiles calculated by maximization of biomass production can explain the metabolic states obtained experimentally in a number of organisms, which indicates that organisms are capable of maximizing their growth rate by adaptation. Using the appropriate GSM and objective function, FBA can predict the relationships among genotype, environmental condition, and production yields at steady states, and these can be used to improve microbial production [19–21]. In this section, we discuss using GSM and experimental evaluations for *in silico* metabolic simulations of the state of metabolism.

7.3.2
Reconstruction of GSMs and Simulation by FBA

All known reactions in the metabolic network of microorganisms can be collected from public databases and scientific publications. To simulate metabolic fluxes, it is also necessary to obtain information on biomass composition, which is thought to account for consumption of precursors and building blocks of cellular growth. Biomass synthesis is represented by a linear combination of components including amino acids, DNA, RNA, lipids, and cell envelope components. The energy requirement for cellular growth is also considered by taking into account adenosine triphosphate (ATP) consumption.

Simulation by FBA of metabolic fluxes in *C. glutamicum* is described in the following. For a metabolic network consisting of N metabolites and M metabolic reactions, assuming a pseudo-steady state of metabolite concentrations, the stoichiometric balance of metabolic fluxes is represented by the following equation:

$$\sum_{j=1}^{M} S_{ji} v_i = b_j, \quad i = 1, 2, \ldots, N \tag{7.1}$$

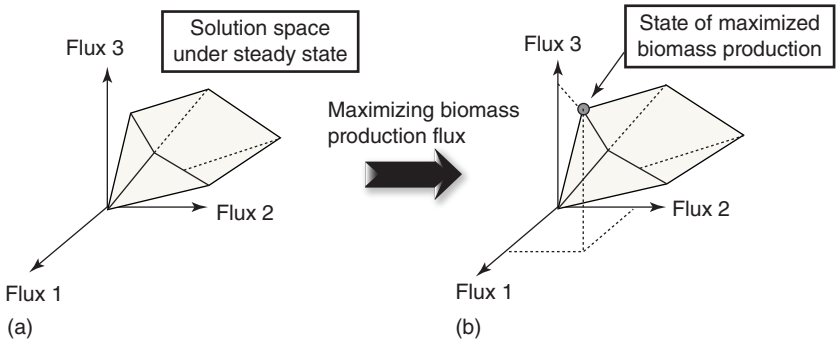

Figure 7.4 Schematic representation of the constraint-based flux balance analysis. The axes represent metabolic fluxes. (a) By applying the steady-state assumption, we obtained the feasible solution space. (b) When the biomass production flux was used for the objective function, optimal solutions that maximize the objective function could be calculated by linear programming (LP).

where S_{ji} represents the stoichiometric coefficient of metabolite j in reaction i, and v_i denotes the flux of reaction i. The constraints b_j contain substrate uptake and product rates. The upper and lower bounds, α_i and β_i, represent the ith flux, which defines constraints for the maximum enzymatic rate, irreversibility of reaction, or constant uptake from the environment.

Figure 7.4 represents the concept of estimation of metabolic states by LP. Based on the steady-state assumption, the possible flux profile is bound within a closed, finite space. However, it is not possible to determine a unique solution for the flux profile by employing only the steady-state assumption. Therefore, to determine the flux profile, the second assumption, namely the maximization or minimization of an objective function, is generally used to obtain a unique solution. The most widely used objective function in FBA is the biomass synthesis flux, which is represented as a linear combination of metabolic fluxes of building blocks and coenzymes required for biomass synthesis. Mathematically, maximization of the biomass synthesis flux is solved by LP, which corresponds to obtaining the optimal solution at one corner in the feasible flux space (Figure 7.4b). For a detailed examination of the processes of reconstruction and experimental verification of GSM reaction models, we studied the FBA of *C. glutamicum* as an example [22].

C. glutamicum is a facultative, aerobic, gram-positive bacterium that can grow on various sugars or organic acids. This organism shows a high-yield production of various amino acids such as glutamate and lysine; therefore, it is widely used for the large-scale production of amino acids. The production of ethanol and organic acids, such as lactate and succinate, by *C. glutamicum* under oxygen deprivation conditions has recently been studied. *C. glutamicum* is an industrially important microbial host used in metabolic engineering. Therefore, it is highly desirable to construct and explore appropriate *in silico* metabolic models that can predict both the cellular state and target compound production of this organism.

A GSM reaction model of *C. glutamicum* consisting of 277 genes, 502 metabolic reactions, and 423 metabolites was constructed. The reconstructed metabolic models, and *in silico* simulations by these models, were verified experimentally. To construct a GSM of *C. glutamicum*, a series of experiments were performed under different environmental conditions in which the existence of oxygen varied. Since metabolic states can differ dramatically because of differences in oxygen uptake rate (OUR), this was used as a parameter for changing the metabolic state of *C. glutamicum*. Culture experiments with five different OURs were performed, and the glucose uptake rate (GUR), OUR, and production rates of CO_2 and organic acids were quantified. The experimental results indicated that under anaerobic and micro-aerobic conditions, that is, under conditions of low OUR/GUR ratios, cells convert most of the glucose to organic acids, such as lactate and succinate, in order to oxidize NADH generated in the glycolytic pathway. As the OUR/GUR ratio increased, the cells changed their metabolism to produce acetate, and a further increase in the OUR/GUR ratio resulted in a metabolic shift to a CO_2 production phase, in which the tricarboxylic acid (TCA) cycle was activated.

The results of the simulation were evaluated using the experimental data summarized in Figure 7.5a. Maximization of the biomass synthesis flux was the objective function used to determine metabolic flux. The production yields of organic acids, CO_2, and biomass were calculated by the FBA scheme using the experimentally obtained OUR and GUR values as parameters. The predicted yields are shown in Figure 7.5b. Additionally, a scatter plot of carbon yields taken from five sets of experimental and simulation results is shown in Figure 7.5c. The predictions of the GSM showed good agreement with the experimental data (Figure 7.5c).

Constraint-based metabolic simulations are expected to provide quantitative predictions of flux changes caused by genetic modifications. This technique could be applied as an *in silico* screening strategy to identify possible gene manipulations that would improve the productivity and production yield of target products. It could also reduce experimental costs of strain improvements. Indeed, the process has already been applied to the production of several targets used in production of biochemicals and biofuels [23].

7.3.3
Using Prediction of Metabolic State for Design of Metabolic Modification

Using constraint-based metabolic simulations, *in silico* screening of effective gene knockouts for the overproduction of three useful metabolites in the host microorganism was performed. Heterologous metabolic pathways were added in order to produce the target metabolites in the GSM of the host cell. The changes in metabolic fluxes caused by all possible triple reaction knockouts were predicted by assuming the maximization of biomass production for increased production yields of the metabolites [23].

OptKnock, which is a bi-level programming framework for screening knockout targets, is widely used to search for gene knockout candidates [24]. This method utilizes a nested optimization framework to identify the combination of knockout

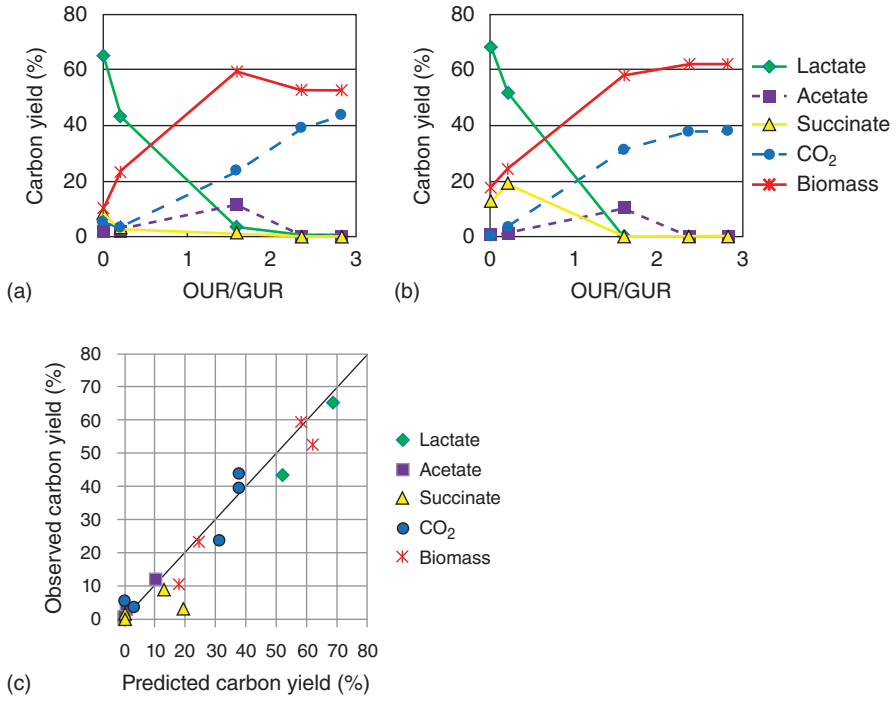

Figure 7.5 Changes in yield of organic acids, biomass, and carbon dioxide when the OUR/GUR ratio was altered. (a) Experimental results obtained from different OUR/GUR ratios. GUR, OUR, and the production rates of CO_2, lactate, acetate, succinate, and biomass are represented in mmol gDW^{-1} h^{-1}. (b) Predictions by FBA simulations. The simulation results were obtained using the GUR and OUR values from the experimental data. (c) A scatter plot of carbon yield. The *x*-axis corresponds to the result of FBA simulation, while the *y*-axis shows the experimentally observed carbon yield. The carbon yield in five sets of experimental and simulation results are presented. The diagonal line corresponds to $y = x$.

targets that optimize the production yield of target metabolites. OptKnock is publically available, and can be used to predict metabolic strategies such as gene amplification or reaction additions. However, one disadvantage of the algorithm is that it outputs only one set of knockouts for optimal productivity and an increase of computational cost, whereas other knockout targets, with suboptimal productivities, could be valuable for strain improvement.

To reduce computational time, reduced metabolic models were constructed that provided flux estimations completely identical to those of the original GSM. However, in a multiple knockout simulation, the increase in possible combinations dramatically affects computation time, limiting the number of knockouts that can be simultaneously analyzed. The Fast algorithm of knockout screening can be employed for target production based on shadow price analysis (FastPros) [25]. Using this method, the potential for a given reaction knockout to produce a specific metabolite is evaluated by shadow pricing of the constraint in the FBA.

This process generates a screening score to obtain candidate knockout sets. In addition, *in silico* design of non-native metabolite production was developed by recruiting heterologous genes and pathways [26].

7.4 ^{13}C-Based Metabolic Flux Analysis of Microbial Processes

7.4.1 Introduction

Metabolism is directly related to cellular activity and target compound productivity. Therefore, accurate evaluation of the metabolic state and regulatory mechanisms is important for metabolic modification and bioprocess development. One effective way to evaluate the metabolic state is to measure the concentration of intracellular metabolites. Recent advances in metabolomics techniques have enabled the measurement and analysis of various metabolites. Additionally, ^{13}C-based metabolic flux analysis (^{13}C-MFA) can be applied to determine metabolic reaction rates at steady state, and it provides quantitative information regarding metabolic reaction activity and metabolic flow. In this section, we discuss recent techniques and application of bioproductions related to the evaluation of the metabolic state by metabolomics and ^{13}C-MFA.

7.4.2 Principles of ^{13}C-MFA

^{13}C-MFA is a method for determining flux distribution based on experimental data [27, 28]. A schematic of the process is shown in Figure 7.6. For ^{13}C-MFA, cells are cultured with labeled substrates. These carbons are taken up and converted through various metabolic pathways before they become the amino acids that constitute cellular proteins. Therefore, flux distribution is reflected in the ^{13}C-enrichment of proteinogenic amino acids, which can be measured using mass spectrometry (MS). Generally, a ^{13}C-based experiment is conducted at metabolic steady-state conditions to keep cellular metabolism constant. A model representing ^{13}C-enrichment of metabolites from an arbitrary flux distribution can be constructed to determine the flux distribution using the measured ^{13}C-enrichments. The isotopomer method can be used to model isotopic mass balances. In recent years, elementary metabolite units (EMUs) based on the decomposition of the isotopomer network have been proposed [29], and the sophisticated framework drastically alleviates the computational cost of ^{13}C-MFA. Using this method, the flux distribution is estimated so that the measured values for ^{13}C-enrichments of amino acids can be sufficiently explained. The ^{1313}C-MFA software, OpenMebius, was developed to include functions such as automatic generation of metabolic models, simulation of mass distribution vectors, and a method of metabolic flux analysis [30].

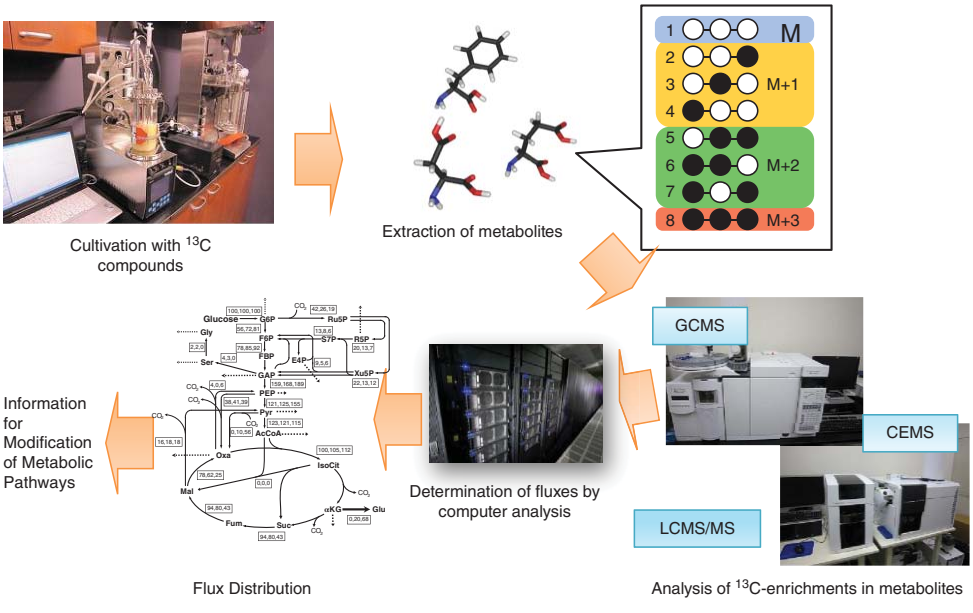

Figure 7.6 Overview of flux estimation using ^{13}C-metabolic flux analysis.

7.4.3 Examples of ^{13}C-MFA in Microbial Processes

The coryneform bacterium *C. glutamicum*, which was isolated in the 1950s, is capable of producing a large amount of amino acids, including glutamate and lysine. In glutamate fermentation, there are certain triggers for glutamate overproduction in *C. glutamicum*: the depletion of biotin, which is required for cell growth; the addition of detergent; the addition of lactam antibiotics, such as penicillin; and the temperature upshift of fermentation. If these are triggered, they cause a dramatic change at the branch point of 2-oxoglutarate in the TCA cycle because of a decrease in the activity of the 2-oxoglutarate dehydrogenase complex (ODHC) [31].

Here, we describe the use of ^{13}C-based MFA to examine the glutamate production phase of *C. glutamicum* compared with the growth phase. *C. glutamicum* is cultivated on natural glucose in the growth phase, and then glutamate production is induced by the addition of a detergent (Tween 40) at the mid-log growth phase. The amount of Tween 40 added is precisely adjusted to achieve both stable growth and glutamate production, and a ^{13}C label is incorporated into proteinogenic amino acids in the production phase. Subsequently, ^{13}C-labeled glucose is added in the glutamate production phase, and the time course of ^{13}C labeling patterns of amino acids is measured by gas chromatography–mass spectrometry (GC–MS) [32].

The results of ^{13}C-MFA in the growth phase and two glutamate production phases are shown in Figure 7.7. The anaplerotic pathways of *C. glutamicum* have the greatest correlation with the glutamate production based on ^{13}C-based MFA. As shown in Figure 7.7, reactions from phosphoenolpyruvate (PEP) to oxaloacetate catalyzed by phosphoenol-pyruvate carboxylase (PEPc), and from malate to pyruvate catalyzed by malic enzymes, are mainly active in the growth phase. The flux catalyzed by PEPc, which shows a certain extent value in the growth phase, is kept almost constant, despite the fermentation phase changing from cell growth to glutamate production by addition of the detergent.

The flux of the reaction from pyruvate to oxaloacetate catalyzed by pyruvate carboxylase (Pc) is almost zero in the growth phase. Production of glutamate increases with the addition of detergent, and the flux of this reaction, catalyzed by Pc, increases accordingly. The fluxes of the other anaplerotic pathways, from oxaloacetate to PEP catalyzed by phosphoenol-pyruvate carboxykinase (PEPck) and glyoxylate shunt, are almost zero. The flux of Pc proportionally increases with glutamate production; in contrast, the flux of PEPc remains constant throughout fermentation. In order to enhance glutamate production, increased flux of anaplerotic pathways is necessary because the TCA cycle produces two molecules of CO_2 per unit cycle from one molecule acetyl Co-A. Despite this enhancement from the TCA cycle, it does not contribute to increased glutamate production, and an increase of anaplerotic pathway activity is important. This result indicates that, in anaplerotic pathways associated with glutamate overproduction induced

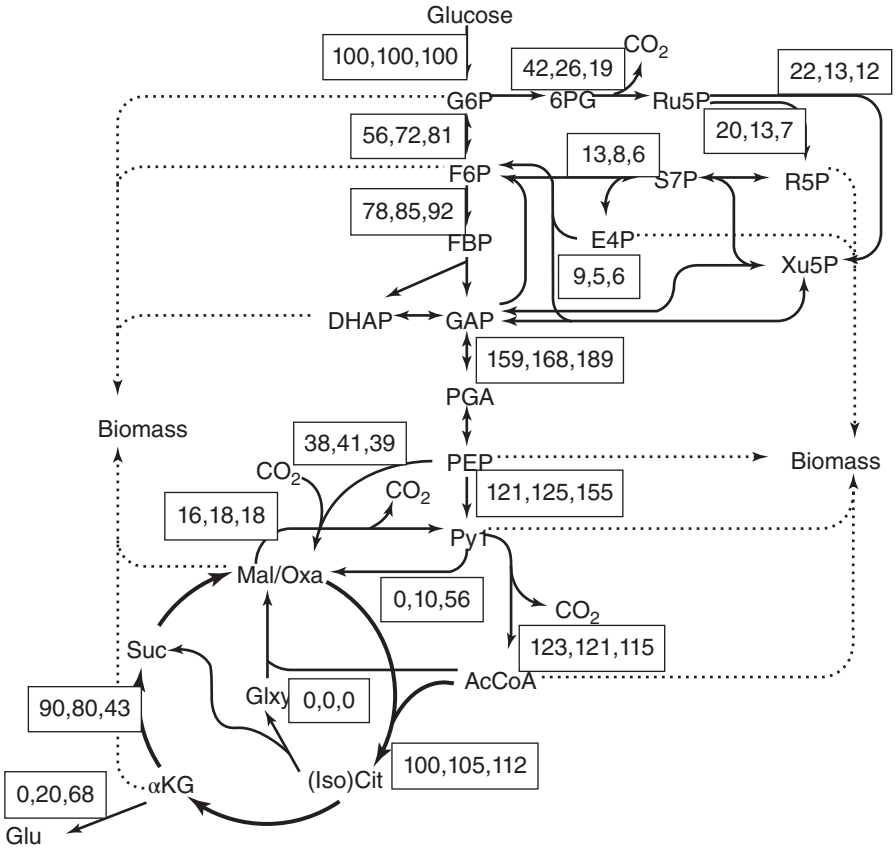

Figure 7.7 In *C. glutamicum*, metabolic fluxes in growth and production phases of two different glutamate production activities. Dotted arrows indicate fluxes for biomass. Left, middle, and right values in boxes indicate fluxes in the growth phase, low production phase, and high production phase, respectively, of two different activities caused by two levels of Tween 40 addition, where glutamate fluxes were 0, 20, and 68, respectively. In this study, the fluxes with backward (exchange) reactions, that is, those in glycolysis, the pentose phosphate pathway, the latter steps of the TCA cycle (succinate to oxaloacetate), and C1 metabolisms, are shown as net values. Abbreviations: Gly, glycine; Ser, serine; Glu, glutamate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; DHAP, dihydroxyacetone phosphate; PGA, phosphoglycerate; AcCoA, acetyl-CoA; IsoCit, isocitrate; aKG, 2-oxoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; Oxa, oxaloacetate.

by detergent addition, the flux of Pc is most important. We suggest that the flux of Pc is more active as glutamate production increases.

Many studies have been conducted to investigate mechanisms of cellular metabolism using ^{13}C-based MFA. For example, the flux distribution between the wild type and the gene-knockout mutants has been compared [33, 34]. Since changes in metabolic fluxes reflect changes in gene expression, protein expression, and metabolite concentration, these varying levels of information must be integrated to understand metabolic regulation. A wild-type *E. coli* and *pykFA*-knockout mutant, produced in batch culture using glucose as a carbon source, were analyzed for intermediate metabolite concentrations and the fluxes in central carbon metabolism [35]. Glycolytic intermediate metabolites accumulated because of the decrease and feedback inhibition caused by an accumulation of PEP in the *pykFA* mutant, but the overall flux distribution, including glycolysis and the pentose phosphate pathway, remained unchanged because of rerouting via anaplerotic reactions such as PEPc and malic enzymes. Usui *et al*. [36] constructed both genetic deletion and conditional mutant strains of *pgi* and *eno*, in which gene expression could be artificially controlled by the addition of varying amounts of isopropyl β-D-1-thiogalactopyranoside (IPTG), and they examined how perturbations in gene expression affects fluxes in central metabolic pathways. ^{13}C-based metabolic flux analysis of *S. cerevisiae* [37] and *Bacillus subtilis* [38] has also been reported.

7.5
Comprehensive Phenotypic Analysis of Genes Associated with Stress Tolerance

7.5.1
Introduction

Microorganisms are widely used in the production of various organic compounds. To improve the productivity of these compounds, genetic perturbations such as gene deletion and overexpression are generally used. However, obtaining the desired strains by genetic perturbations is not easy, since the intracellular interaction networks among genes, proteins, and metabolism are complex, and the response of cells to genetic perturbations is difficult to predict. Thus, it is important to investigate the nature of cellular response to genetic perturbations, including the robustness of cells, in order to develop a systematic method for constructing the desired strains of microorganisms.

In this section, we describe the comprehensive quantitative analysis of growth in *S. cerevisiae* following genetic perturbations, including gene deletion. The effects of genetic perturbations on a phenotype can be comprehensively investigated using the library of yeast strains with a single gene deletion [39]. Cell growth is a representative phenotype, and the effect of genetic perturbation on cellular growth is investigated because it is an essential function. Data on changes in the growth behavior following genetic perturbations is useful for designing a

desired strain with both high productivity and sufficient growth rate. The library of deletion strains is cultured individually in the same liquid medium to evaluate the effect of genetic perturbation on the cell growth rate.

7.5.2 Development of a High-Throughput Culture System

Specific growth rates of individual deletion strains are quantified independently in a liquid medium, which allows us to evaluate the effect of genetic perturbation on growth activity. Using this high-throughput cultivation method, the genes whose deletion results in significantly high or low growth rates, and the functional categories in which these genes are significantly overrepresented, can be identified. This screening provides information on the robustness and fragility of cellular functions, which is fundamental for state analysis and systematic gene manipulation.

7.5.3 Calculation of Specific Growth Rate

The optical density (OD) of the samples measured using the microplate reader is produced as follows. The OD of blank wells, that is, wells containing only the medium, is subtracted from the OD of the experimental samples. Since there is a small systematic error in the growth rate measurements in different plates, normalization must be carried out. In order to compare the specific growth rates of the deletion and overexpression strains cultured in different plates, specific growth rates are normalized with the respective control deletion strains cultured in each plate using the following formula:

$$\mu'_{i,j} = \mu_{i,j} \cdot \left(\frac{\mu_{\mathrm{con}}}{\overline{\mu}_{\mathrm{con},j}} \right) \tag{7.2}$$

where $\mu_{\mathrm{i,j}}$ is the specific growth rate of strain i in plate j, $\overline{\mu}_{\mathrm{con},j}$ is the mean specific growth rate of the standard strain from four wells in plate j, μ_{con} is the median of all specific growth rates of the standard strain in all inspected plates, and $\mu'_{i,j}$ is the normalized specific growth rate.

7.5.4 Results of Comprehensive Analysis of Yeast Cells Under Conditions of High Osmotic Pressure and High Ethanol Concentration

Here, we describe the findings of the comprehensive analysis of yeast cells under conditions of high osmotic pressure and high ethanol [40]. Specific growth rates of deletion strains under ethanol and osmotic stress conditions (high NaCl concentration) are shown in Figure 7.8, which shows specific sensitivity under ethanol stress or osmotic stress conditions (high NaCl concentration) following gene deletion.

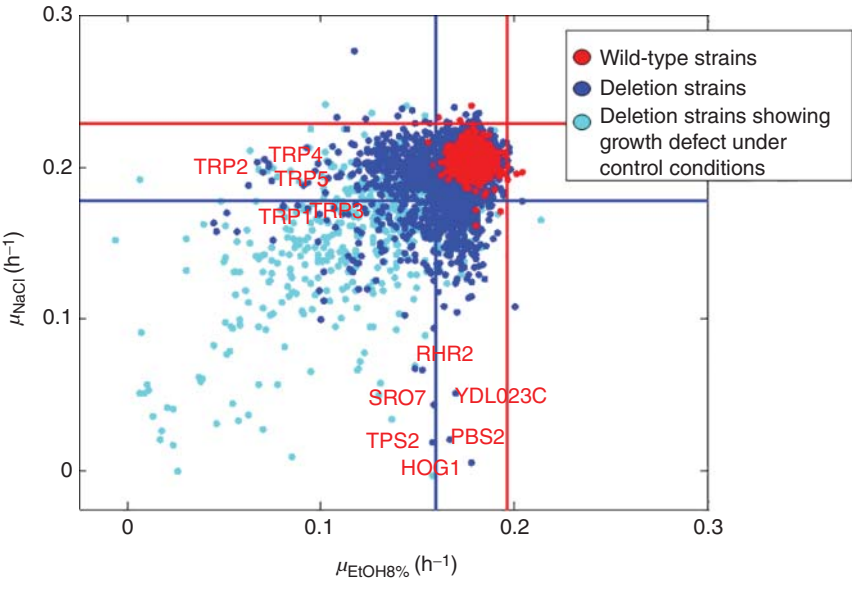

Figure 7.8 Sensitivity analysis of ethanol and osmotic stress conditions. Red plots indicate specific growth rate of standard strain; dark blue plots indicate specific growth rate of gene deletion strain, which does not show sensitivity under non-stress condition; light blue plots indicate specific growth rate of gene deletion strain, which shows sensitivity even under non-stress condition. Blue line denotes the threshold value of growth sensitivity under stress conditions, and the red line the threshold value of growth tolerance under stress conditions.

In the osmo-specific sensitive region, genes related to the high osmolality glycerol (HOG) and glycerol synthetic pathways were found. Gene deletion strains are known to be osmo-sensitive. In the ethanol-specific sensitive region, genes that were related to the tryptophan synthetic pathway (*TRP*) were found. Additionally, in strains in which the *TRP* gene was overexpressed, growth in osmotic stress conditions was found to be similar to that of the wild-type strain. In contrast, strains overexpressing the *TRP* gene showed growth under ethanol stress conditions that was superior to that of the wild-type strain. These results suggest that the *TRP* gene is specifically important to the ethanol stress condition.

There are many common strains that show both osmotic and ethanol sensitivity. However, many of them show sensitivity under normal culture conditions without any additional stress. Thus, they are known as the control sensitivity strain. When these control sensitive strains were excluded, the number of strains sensitive to both stress conditions decreased, and this number was lower than that of the ethanol- or osmo-specific sensitive strains. High-throughput culture systems, as well as the evaluation of the gene deletion library collections sensitivity under stress conditions, enables us to perform comprehensive analyses of the phenotype changes caused by gene deletion or overexpression (phenome) and evaluation of

the efficiency in the selection of candidate genes from transcriptome data by comparison with phenome data.

7.5.5
Identification of Genes Conferring Desirable Phenotypes Based on Integration with the Microarray Analysis Method

As described in Section 7.1, the integrated method of transcriptome and compressive phenotypic analysis facilitates the screening of genes that are important under stress conditions. In strains with different phenotypes, genes related to a target phenotype are identified from those that exhibit different levels of expression or dynamics [4, 7, 8, 41].

First, identification of candidate genes by transcriptome analysis is performed by comparative transcriptome analysis between strains of different phenotypes, and further screening is performed by sensitivity analysis based on the library of gene deletion mutants. For example, Hirasawa *et al*. [42] used microarrays to quantify gene expression profiles in two lactate-producing *S. cerevisiae* strains and a non-lactate-producing strain, and compared these profile differences with the lactate productivity of single-gene deletion strains. About 400 genes were upregulated or downregulated in the two lactate-producing strains in comparison with the non-lactate-producing strain, suggesting that these particular genes were involved in lactate production. In order to verify this hypothesis, the human lactate dehydrogenase (LDH) gene was introduced into the collection of yeast with a single gene deletion, and lactate production was evaluated. Genes for which deletion increases or decreases lactate productivity are significantly enriched among the collection of genes whose expression levels are commonly upregulated or downregulated in the lactate-producing strains. Extraction of genes whose expression levels differ between strains of different phenotypes is an effective method for identifying candidate genes for manipulation toward desired phenotypes.

7.6
Multi-Omics Analysis and Data Integration

Recent developments in analytical technologies have enabled the comprehensive analysis of various cellular components, such as the genome, proteome, metabolome, and fluxome. Several studies employ integrative analyses using multi-omics data.

Lee *et al*. [43] performed *in silico* simulations with a GSM reaction model. In many cases, after identification of the candidate genes, deletion and overexpression analyses are performed to evaluate the effect of these genes on the target phenotype. In order to increase threonine production in *E. coli*, the importance of optimizing the expression of the candidate genes using *in silico* simulation based on transcriptome data was reported.

Other studies have reported successful breeding using integration of transcriptome data with genomic information, such as metabolome and metabolic flux analysis [44], transcription factor binding sites [45], or metabolite profiles. Integrative analyses inform microbial state analysis and molecular breeding, providing data that cannot be extracted from transcriptome data alone. Recent rapid development of analytical technology will allow us to analyze multi-omics data with ease, and integrative analysis of these data with transcriptome data will clarify the molecular mechanisms of superior strains and accelerate molecular breeding.

Yoshikawa *et al*. studied the integration of transcriptome and metabolome in cyanobacteria [46]. Cyanobacteria have been analyzed as a model organism for the study of photosynthesis. As a sustainable energy resource – because of their organic material production capacity using light energy and CO_2 as a carbon source – cyanobacteria offer a next-generation microbial cell for bioproduction. Therefore, it is important for metabolic engineering, as well as for other scientific applications, to understand the cellular metabolism of cyanobacteria. To shed light on the central metabolism of cyanobacteria, transcriptomic and metabolomic analyses of a glucose-tolerant strain of the cyanobacterium, *Synechocystis* sp. PCC 6803 (cultured under autotrophic and mixotrophic conditions), were performed. Photoheterotrophic conditions were created by the addition of glucose and the photosynthesis inhibitor atrazine, and the effect of atrazine on central metabolism was investigated. Comparable transcriptomic and metabolomic changes were measured under mixotrophic and photoheterotrophic conditions and under autotrophic conditions. Metabolomic and transcriptomic data indicated the activation of the oxidative pentose phosphate pathway and glycolysis under mixotrophic and photoheterotrophic conditions. Oxidative pentose phosphate pathways activate gene expression and metabolism under photoheterotrophic conditions. Under the mixotrophic condition, a similar tendency was observed and pools of metabolites associated with glycolysis were increased. Additionally, GSM of cyanobacteria can provide a good understanding of metabolism change under different trophic conditions [47].

Recently, multi-omics integration analysis was performed with ^{13}C flux analysis. Compared with changes in transcriptome and metabolome data in the entire central carbon metabolism, fluxes under heterotrophic and mixotrophic conditions were significantly different. This suggests that a few key genes may control metabolic fluxes and have an important role in control of metabolic flows [48]. In the future, by comparative analysis of omics data, we expect to find new controls for metabolic pathways as a system.

7.7
Future Aspects for Developing the Field

In this section, we describe the integration of omics data for the development of bioproduction processes. An overview of the interaction of omics technologies is shown in Figure 7.9. GSMs of microorganisms can be constructed, and

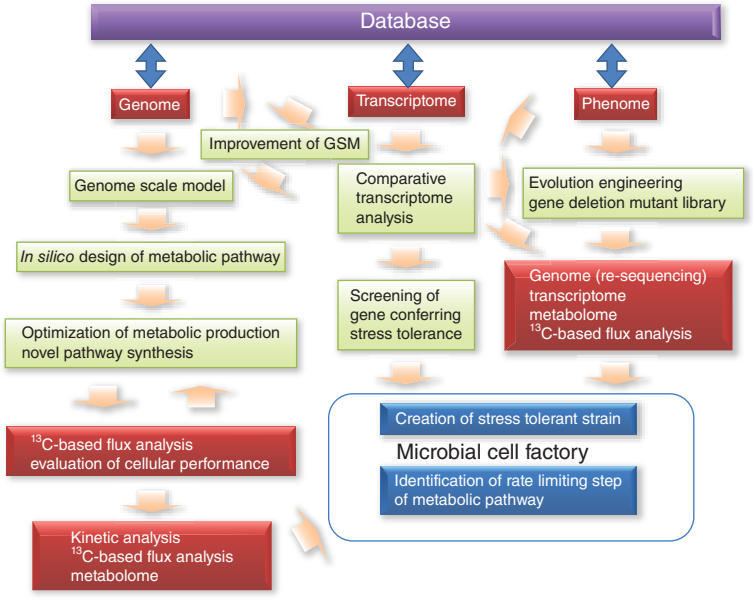

Figure 7.9 Interactions between omics technologies and future aspects for developing the field.

the metabolic flux profiles well predicted, under given environmental conditions. Prediction by GSMs enables us to design an *in silico* metabolic pathway modification strategy for increasing productivity and production yield of target products in bioprocesses. Recently, production yield of 3-hydroxypropionate from glycerol by *E. coli* was realized, and the concept was proved experimentally [49]. Transcriptome and ^{13}C-MFA data are particularly important for evaluating and upgrading the prediction accuracy of GSMs.

^{13}C-MFA is important for monitoring cellular performance of genetically modified cells. By comparison with fluxes of parental and recombinant microorganisms, the impact of the obtained genetic modification is effectively evaluated. In the future, in addition to stoichiometric analysis, rate-limiting step identification technology will be established. Metabolic control analysis is a systematic way to identify the rate-limiting step or the largest responsibility analysis in the pathway [50]. Metabolomics and proteomics data become important for analyzing the rate-limiting step in a pathway, based on the kinetic model and thermodynamics information of metabolism. Recently, a new method of ensemble modeling [51] was presented for this specific purpose.

Identification of factors conferring stress tolerance is a complicated process, as previously described. Where a rational approach is not effective, omics science with a random approach can be a powerful tool for inverse metabolic engineering. A comparative approach, combining transcriptomics with phenotypic analysis of the library of gene deletion mutants, produces results that can identify

important genes. Recent progress in next-generation sequencing technologies has led to substantial progress in evolution engineering. Data on important mutations in the genome that confer phenotypic changes is likely to be found as a result. In repetitive inoculation experiments conducted under stress conditions, changes in the genome, as well as transcriptome, proteome, metabolome, and ^{13}C-based fluxome, will be mapped to changes in phenotype, such as an increase in growth rate under conditions of stress. Therefore, inverse engineering technology will enable us to identify important factors that confer superior phenotypes to cells and improve our understanding of industrially useful microorganisms.

Acknowledgments

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8
Control of Microbial Processes

Kazuyuki Shimizu, Hiroshi Shimizu, and Toshiomi Yoshida

8.1
Introduction

Valuable microbial production processes have been developed [1] with key issues in process operation involving medium optimization and control of the environmental conditions in the culture. In particular, the prime concern is the maintenance of the substrate concentration at an appropriate level to avoid by-product formation caused by overflow metabolism resulting from carbon catabolite repression. The conventional approach is to employ a fed-batch cultivation strategy. Another approach is continuous culture, which allows the carbon source concentration to be held constant. Continuous culture is also attractive for post-genomic studies, which require reliable and biologically homogeneous datasets to study global transcriptomics, proteomics, and metabolomics [2].

The ability to maintain fermentation processes at their optimal states precisely and automatically is of considerable importance to many fermentation industries, because it allows them to reduce their production costs and increase yield and productivity, while maintaining the quality of the metabolic products. However, the control system design of bioprocesses is not straightforward because of the following:

1) Significant model uncertainty
2) The time-varying and nonlinear nature of these processes
3) Lack of reliable sensors that can detect the physiological state of cells
4) Slow response with slow dynamics of these systems.

In order to cope with the first problem, the control system design must be robust in the face of model uncertainties with disturbance rejection. For the second problem, adaptive control strategies with online tracking of the system's state should be considered. As for the third problem, observers and/or online filtering/estimation need to be incorporated into online measurements. For the

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fourth problem, a predictive control strategy and/or combined use of offline data should be considered [3, 4].

Although advanced and sophisticated monitoring and control strategies have been investigated, and their power and usefulness has been demonstrated by computer simulation and by small-scale laboratory experiments in academia, their practical applications are quite limited. Instead, primitive, simple control systems with standard measurements of temperature, pH, dissolved oxygen (DO) tension, and possibly carbon dioxide (CO_2) and O_2 concentrations in the off-gas in the vent line are commonly employed in industrial manufacturing processes [5].

Tight and effective control systems contribute significantly to improving performance in industrial fermentation; therefore, it is important to fill the gap between theoretical approaches and their implementation in practice. In other words, there is still enormous potential for significant improvement in the performance of manufacturing processes. Tight control depends on the extent to which we understand specific fermentation processes, which, unlike physical and chemical processes, depend on living organisms with sophisticated dynamics. Moreover, maloperations, contamination, or other failures must be prevented in industrial fermentation, with stringent safety requirements and operational constraints imposed in food and pharmaceutical production processes.

In the present chapter, therefore, comprehensive control system design is explained with examples of applications. The chapter is subdivided into topics such as online monitoring, estimation, control system design, and experimental implementation.

8.2 Monitoring

Accurate monitoring of the cell's physiological state using available online sensors for tight control of fermentation processes is a basic requirement [5–10]. The problem with bioprocess monitoring is a shortage of online sensors that can directly measure important state variables for efficient bioprocess control [5]. Therefore, several software sensors have also been developed to overcome the above issues [5, 11, 12].

8.2.1 Online Measurements

Typical online measurements in industrial fermentation are temperature, pO_2, fermentor head pressure, agitation rate, and possibly CO_2 and O_2 concentrations in the off-gas in the vent line [13, 14]. The volume or mole fraction of CO_2 and O_2 can be determined by infrared sensors, paramagnetic sensors, or mass spectrometers [15–18]. A mass spectrometer can also detect ethanol and methanol concentrations in the off-gas, and thus can be used for monitoring and control of yeast cultivation.

In many cases, information on biomass concentration (or even cell viability) is important to understand the physiological state and to control fermentation processes. Online measurements of the biomass concentration may be made by determining the optical density (OD) of a culture by means of a laser light transmission and backscattering technique (using a turbidimeter) [19]. Other than OD, dielectric spectroscopy, near-infrared (NIR) spectroscopy, Raman spectroscopy, *in situ* microscopy, and acoustic resonance densitometry may be used to monitor viable cell density [20–28].

The online information obtained by the above sensors is usually noise-contaminated, and therefore some sort of filtering is required, with the quality of the online data evaluated by the signal-to-noise (S/N) ratio. This value is significantly affected by operating conditions such as the rotating speed of the stirrer.

8.2.2 Filtering, Online Estimation, and Software Sensors

The ability to measure the primary process variables such as biomass, substrate, and metabolite concentrations is critical for efficient operation and tight control of bioprocesses at their optimal state. However, direct online measurements of such state variables are limited, or in many cases are not available in practice, because of limitations of the cheap and reliable sensors. Therefore, it is important to develop the so-called software sensors that can estimate primary state variables from available online data, where model uncertainty and the nonlinear, time-varying nature of the system are taken into consideration [11, 12, 29–33].

In order to monitor the physiological state of cells, specific rates such as the specific growth rate μ, the specific substrate consumption rate ν, and the specific metabolite production rate ρ should be estimated based on the online measured variables. However, the estimated values of these specific rates are highly sensitive to noise, in general. When the specific growth rate is estimated from biomass concentrations measured by a laser turbidimeter, the online measured values are usually corrupted by noise, which prevents accurate estimation. This problem may be overcome to some extent by model-based observers [34] or by filtering techniques such as the Kalman filter. The biomass concentration can be also estimated from the CO_2 evolution rate (CER) and oxygen uptake rate (OUR) computed from data from a CO_2/O_2 gas analyzer, and the base consumption rate, where cumulative data may be used to reduce the effect of noise [35]. A simple feed-forward-type artificial neural network (ANN) may also be applied to estimate the biomass concentration for the repeated batch operations [35].

8.2.3 Algorithm of Extended Kalman Filter and Its Application to Online Estimation of Specific Rates

In the case where not enough online information is available, an appropriate model may be considered based on mass balances with stoichiometric constraints

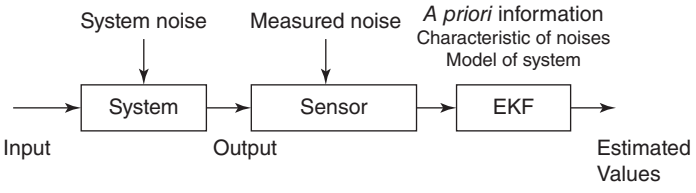

Figure 8.1 Estimation of specific rates using the extended Kalman filter (EKF).

for the development of extended Kalman filters (EKFs). EKFs are one-step predictors with extrapolation from a model and correction based on the observed data, where at each time point when new data becomes available the EKF determines a new estimate from the preceding step, model prediction, and the value obtained by the newly measured data. The new estimate is a weighted average of such data, with the EKF determining the weights based on the reliabilities that are attributed to the measured value, measurement uncertainties, model equations employed, and least estimate based on measurement uncertainty. A schematic diagram of the Kalman filter algorithm is shown in Figure 8.1.

Dynamics of nonlinear systems are generally represented as follows:

$$\mathbf{x}(t+1) = \mathbf{f}(\mathbf{x}(t), \mathbf{u}(t)) + \mathbf{G}(\mathbf{x}(t), \mathbf{u}(t))\mathbf{v}(t) \tag{8.1}$$

$$\mathbf{y}(t) = \mathbf{h}(\mathbf{x}(t)) + \mathbf{w}(t) \tag{8.2}$$

where $\mathbf{x}(t)$, $\mathbf{u}(t)$, and $\mathbf{y}(t)$ are the state, input, and measured vectors, respectively, at time t. The variables $\mathbf{v}(t)$ and $\mathbf{w}(t)$ are white noise vectors. The covariance matrices of $\mathbf{v}(t)$ and $\mathbf{w}(t)$ are represented as follows:

$$\begin{aligned} E[\mathbf{v}(t)\mathbf{v}(\tau)^T] &= \mathbf{Q}(t), \quad t = \tau \\ &= 0, \quad t \neq \tau \end{aligned} \tag{8.3}$$

$$\begin{aligned} E[\mathbf{w}(t)\mathbf{w}(\tau)^T] &= \mathbf{R}(t), \quad t = \tau \\ &= 0, \quad t \neq \tau \end{aligned} \tag{8.4}$$

and $\mathbf{v}(t)$ and $\mathbf{w}(t)$ are not correlated with each other at any time t as

$$E[\mathbf{v}(t)\mathbf{w}(t)^T] = \mathbf{0} \tag{8.5}$$

$\mathbf{f}(\mathbf{x}(t),\mathbf{u}(t))$, $\mathbf{G}(\mathbf{x}(t),\mathbf{u}(t))$, and $\mathbf{h}(\mathbf{x}(t))$ are nonlinear vector functions, and $E[\cdot]$ indicates the expectation values.

The algorithm of the EKF is presented as in Equations (8.6–8.15).

1) Initial condition of one-step prediction

$$\hat{\mathbf{x}}(0/-1) = \mathbf{x}_0 \quad \text{initial state estimation} \tag{8.6}$$

$$\hat{\mathbf{P}}(0/-1) = \mathbf{P}_0 \quad \text{initial state covariance matrix of estimation errors} \tag{8.7}$$

2) Estimation by filtering at time (t) with data collected at time $(t-1)$

$$\hat{\mathbf{x}}(t/t) = \hat{\mathbf{x}}(t/t-1) + \mathbf{L}(t)[\mathbf{y}(t) - \mathbf{h}(\hat{\mathbf{x}}(t/t-1))] \tag{8.8}$$

3) Kalman gain

$$\mathbf{L}(t) = \widehat{\mathbf{P}}(t/t-1)\mathbf{C}(t)^T[\mathbf{C}(t)\widehat{\mathbf{P}}(t/t-1)\mathbf{C}(t)^T + \mathbf{R}(t)]^{-1} \tag{8.9}$$

4) Predicted covariance matrix to filter estimation errors

$$\widehat{\mathbf{P}}(t/t) = \widehat{\mathbf{P}}(t/t-1) - \widehat{\mathbf{P}}(t/t-1)\mathbf{C}(t)^T [\mathbf{C}(t)\widehat{\mathbf{P}}(t/t-1)\mathbf{C}(t)^T + \mathbf{R}(t)]^{-1}\mathbf{C}(t)\widehat{\mathbf{P}}(t/t-1) \tag{8.10}$$

5) One-step prediction of state vector at time $(t+1)$ with data collected at time t

$$\hat{\mathbf{x}}(t+1/t) = \mathbf{f}(\hat{\mathbf{x}}(t/t), \mathbf{u}(t)) \tag{8.11}$$

6) Covariance matrix of one-step prediction errors

$$\widehat{\mathbf{P}}(t+1/t) = \mathbf{A}(t)\widehat{\mathbf{P}}(t/t)\mathbf{A}(t)^T + \mathbf{B}(t)\mathbf{Q}(t)\mathbf{B}(t)^T \tag{8.12}$$

where

$$\mathbf{A}(t) = \frac{\partial \mathbf{f}(\hat{\mathbf{x}}(t/t), \mathbf{u}(t))}{\partial \mathbf{x}} \tag{8.13}$$

$$\mathbf{B}(t) = \mathbf{G}(\hat{\mathbf{x}}(t/t), \mathbf{u}(t)) \tag{8.14}$$

$$\mathbf{C}(t) = \frac{\partial \mathbf{h}(\hat{\mathbf{x}}(t/t-1))}{\partial \mathbf{x}} \tag{8.15}$$

Figure 8.2a,b shows the estimates of the specific growth rate, μ, cell concentration X, the specific glucose consumption rate ν_g, and glucose concentration S

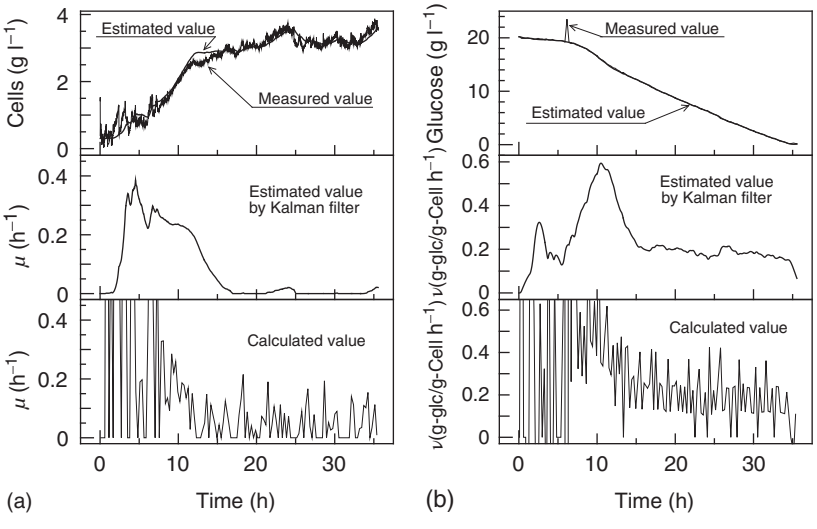

Figure 8.2 Estimates using a modification of the extended Kalman filter. (a) Cell concentration and specific growth rate. (b) Glucose concentration and specific glucose consumption rate. The bottom sections show the directly calculated values (the differences between the current and the last data).

determined by EKF. The cell concentration and glucose concentration are monitored by an online turbidimeter and an enzyme sensor, respectively. Random noise included in the measured cell and glucose concentrations was removed successfully. As a result, the specific growth and specific glucose uptake rates (μ and ν_g) were accurately estimated. For comparison, the calculated values of μ and ν_g by simple subtraction of the current from the last data are also shown. The estimated values of μ and ν_g, obtained by applying the EKF are clearly much more accurate.

EKF can be applied to many bioprocesses in practice. For example, in lysine production processes, the dynamic behaviors of cell growth and substrate consumption can be represented by a dynamic model in which the specific growth rate and specific glucose consumption rate can be estimated simultaneously by eliminating noise [36]. This information can also be used for state recognition in lysine production.

Note that specific rates such as μ, ν, and ρ are based on mass balances. When a large quantity of data is available, data-driven models with artificial intelligence (AI) such as ANNs and fuzzy inference, for example, may be considered.

8.3 Bioprocess Control

For an efficient control system design, the objective of fermentation must be clear, and then input and output variables must be carefully chosen, with online measurements critical to controlling quality. Moreover, appropriate control variables must also be carefully monitored. The primary control variable may be the substrate feeding rate in a fed-batch culture. In typical batch and fed-batch cultivations, the DO concentration decreases with time in accordance with an increase in biomass concentration, and the DO concentration can be controlled by either the aeration rate or the rotational speed of the stirrer, and therefore, some adaptive type of controller may be designed [37].

Open-loop control system should be considered only if accurate information about the critical state variables is not available. For example, a predetermined substrate feeding rate, such as an exponential feeding rate, may be applied with respect to time in fed-batch cultures. However, it must be noted that even minor deviations from the nominal set value during the initial period of cultivation may lead to large deviations at the end of the exponential growth phase. Closed-loop or feedback control systems can typically be applied to temperature and pH control, and the base feeding rate can also be measured by monitoring the balance (weight) of the feed tank or the pump speed.

8.3.1 Control of Fed-Batch Culture

The most important task for the control system of a bioreactor is the appropriate control of substrate feeding rate. That is, if substrate uptake exceeds

some threshold value, overflow metabolism occurs, with acetate produced in *Escherichia coli* cultures and ethanol in yeast cultures. As these by-products accumulate to higher levels, cell growth is inhibited and the yield is decreased. Therefore, the substrate feeding rate must be carefully controlled to maintain the highest substrate uptake rate without overflow fermentation. Since metabolism changes with overflow metabolism [38, 39], the threshold value may be detected by available online sensors. For example, OUR can be used to detect the substrate feeding rate [40–43] because the specific OUR reaches an apparent maximum just prior to the onset of acetate formation by *E. coli* [44]. Moreover, the ethanol concentration can be directly measured by an infrared sensor in the vent line to correct the predetermined feeding rate with an adaptive controller [45].

Conventional approaches to fed-batch cultures are feedback control with a DO-stat and a pH-stat, making use of signal changes in response to nutrient starvation. The main purpose of a fed-batch culture is to control the substrate concentration at a low level, by manipulating the substrate feeding rate, so that by-product formation is avoided or minimized. Konstantinov *et al*. [46] devised a model-independent two-loop control structure with a balanced DO-stat for phenylalanine production by recombinant *E. coli*. Ferreira *et al.* [47] reported a fed-batch culture of *Pichia pastoris* expressing a single-chain antibody fragment with adaptive DO-stat feeding of glycerol under the constraint of available oxygen transfer capacity. The pH-stat control strategy was also applied to vitamin B_{12} fermentation by *Pseudomonas denitrificans* in a 120 m^3 industrial fermentor [48].

A drawback of the DO-stat and pH-stat control systems is that the cells are often nutrient-starved, which may affect the fermentation performance. An alternative control strategy may be to stop the feeding periodically for a short time (e.g., 2 min) to see whether the pO_2 signal rises instantly. If the substrate in the fermentor is beyond the threshold value for overflow metabolism and some by-product is formed, there might be a time delay in increasing the pO_2 signal, indicating the need to modify the substrate feeding rate accordingly [49].

In a repeated batch or fed-batch fermentation, optimizing the trajectory to maximize productivity and yield is desired. For example, temperature profiles for a temperature induction system, based on optimal control theory [50] or on past industrial experience, should be monitored, and then a model predictive control (MPC) system should be designed [51, 52]. The basic idea of MPC is that deviations between the predicted and a prespecified set point are minimized over a time range (horizon) by controlling the input variables. The interval over which this mean deviation is computed may be of fixed length or move stepwise as each new measurement became available, with a receding time horizon. A generic model control strategy can be also considered to control the specific growth rate at prespecified values [53].

Knowledge-based supervision may be a powerful tool for high-performance control of microbial processes. Konstantinov *et al.* [54] developed a computer system for knowledge-based supervision of bioprocesses, and it was applied to the control of fed-batch cultures of a recombinant *E. coli* for phenylalanine production. Such a strategy constitutes a new approach to monitoring and handling

various important phenomena that usually remain outside of the scope of a conventional control approach.

8.3.2 Online Optimization of Continuous Cultures

In the case of continuous fermentation, an online strategy to optimize control may be effective. The objective of online optimizing control is to track the optimal state for fermentation based on changes in cell state (or enzyme activities) and the culture environment. It is particularly useful and convenient to consider this in a hierarchical structure consisting of online optimization (at a higher layer) and dynamic model identification (at a lower layer), as illustrated in the block diagram in Figure 8.3 [3].

Consider a continuous culture system, where the performance index may be expressed as

$$J = J(y, u, p) \tag{8.16}$$

where y, u, and p are the output, input, and parameter vectors of an appropriate dimension. As a performance index, the yield of the product per substrate used, product concentration (titer), and product formation rate (productivity) may be considered. Let the nonlinear process be expressed as

$$y = g(u, p) \tag{8.17}$$

Then the problem is to determine input u that maximizes J based on the identified model as expressed in Equation (8.17). In direct applications of a steady-state optimization, reaching the optimum can be very slow.

Let the observed input–output relationship be expressed as

$$A(z^{-1})y(k) = B(z^{-1})u(k-1) + \varepsilon \tag{8.18}$$

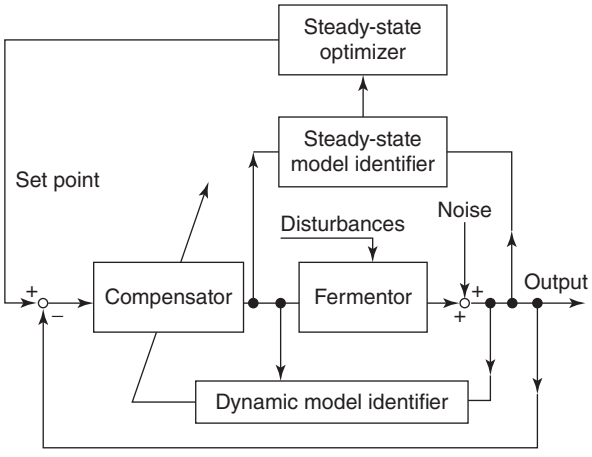

Figure 8.3 Block diagram for the online optimizing control.

where z^{-1} is the backward-shift operator, and ε is the process bias. The sampling instant is denoted as k (0, 1, 2,…). A is a diagonal polynomial matrix with elements

$$A_{ii}(z^{-1}) = 1 + a_{ii}{}^{(1)}z^{-1} + \cdots + a_{ii}{}^{(n)}z^{-n}, \quad (i = 1, 2, \ldots, m) \tag{8.19}$$

and B is a polynomial matrix with elements

$$B_{ij}(z^{-1}) = b_{ij}{}^{(0)}z^{-1} + b_{ij}{}^{(1)}z^{-1} + \cdots + b_{ij}{}^{(r)}z^{-r}, \quad (i = 1, 2, \ldots, n;\ j = 1, 2, \ldots, m) \tag{8.20}$$

Let the data vector be denoted as φ_{i} and defined as

$$\varphi_i{}^T(k) = [-y_i(k-1), -y_i(k-2), \ldots, -y_i(k-n), u_1(k-1), \ldots, u_1(k-r-1), \ldots, u_m(k-1), \ldots, u_m(k-r-1), 1] \tag{8.21}$$

and let θ_{i} be the parameter vector defined as

$$\theta_i^T = [a_{ii}^{(1)}, a_{ii}^{(2)}, \ldots, a_{ii}^{(n)}, b_{i1}^{(0)}, \ldots b_{i1}^{(1)}, b_{i2}^{(0)}, \ldots, b_{i2}^{(r)}, \ldots, b_{im}^{(0)}, \ldots, b_{im}^{(r)}, \varepsilon], \quad (i = 1, 2, \ldots, m) \tag{8.22}$$

Then recursive least square parameter estimation can be made by

$$\hat{\theta}_i(k) = \hat{\theta}_i(k-1) + \frac{P_i(k)\varphi_i(k)}{1 + \varphi_i{}^T(k)P_i(k)\varphi_i(k)}[y(k) - \theta_i{}^T(k-1)\varphi_i(k)] \tag{8.23}$$

where

$$P_i(k+1) = \frac{1}{\lambda_1(k)}\left[P_i(k) - \frac{P_i(k)\varphi_i(k)\varphi_i{}^T(k)P_i(k)}{\lambda_1(k)/\lambda_2(k) + \varphi_i{}^T(k)P_i(k)\varphi_i(k)}\right] \tag{8.24}$$

where $\hat{\theta}_i$ is the identified parameter vector, $P_i(k)$ is the error covariance matrix, and $\lambda_{\mathrm{i}}(k)$ are adjustable parameters.

Let $g_{ij}(z^{-1})$ be the (ij)th element of the input–output transfer function matrix such as

$$g_{ij}(z^{-1}) = B_{ij}(z^{-1})/A_{ii}(z^{-1}) \tag{8.25}$$

If a gradient search algorithm was employed for online optimization, the input $u(k)$ may be updated by

$$u(k+1) = u(k) + \delta S(k)\nabla_u J|_k \tag{8.26}$$

where δ is the step size and $S(k)$ is a positive definite matrix. $\nabla_u J|_k$ is a gradient of the objective function at $u(k)$, and can be obtained from

$$\nabla u^T J(y,u)|_k = \partial J(y,u)/\partial u^T|_k + \partial J(y,u)/\partial y^T|_k (dy/du^T)_k \tag{8.27}$$

where $(dy/du^T)_k$ can be evaluated from Equation (8.25).

Consider the practical application of the above algorithm to lactic acid fermentation. Lactic acid fermentation is one of the very important processes in the food industry. An online optimization strategy may be applied to control the cell recycling system with cross-flow filtration, as shown in Figure 8.4 [55, 56].

The input variables are the draw-off rate through the filter and the bleed stream flow rate. The output variables are the cell and lactate concentrations. The cell

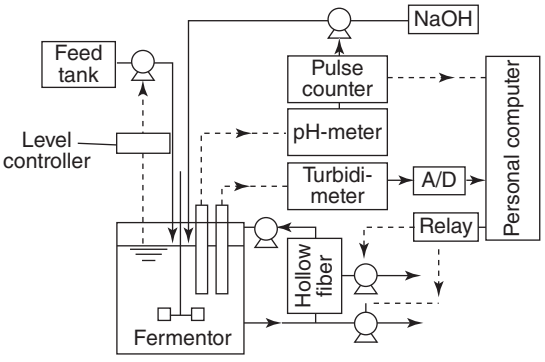

Figure 8.4 Process scheme for the cell recycle system with cross-flow filtration for lactic acid fermentation.

concentration can be measured online by a laser turbidimeter. Since lactate is the only organic acid to be produced by the homo-fermentative lactic acid bacteria (e.g., *Sporolactobacillus inulinus* ATCC15538), the lactate concentration can be estimated online by the amount of alkaline solution supplied to neutralize the culture broth. Experiments were conducted to maximize the productivity of lactate while keeping the lactate concentration at 30 g l^{-1} Figure 8.5 shows the experimental result, where productivity of ~22 g l^{-1} h^{-1} was attained by online optimization, as explained above, where Q_p denotes the productivity of lactate, ν_p indicates the specific lactate production rate, and μ gives the specific growth rate estimated online during fermentation [56].

This online optimization strategy can be applied to a wide variety of fermentation processes; for example, some attempt has also been made to optimize the glutamic acid fermentation by *Corynebacterium glutamicum* by adjusting the respiratory quotient (RQ) online in response to measurements of DO concentration [57].

8.3.3
Cascade Control for Mixed Cultures

Mixed cultures, or co-cultures, where multiple strains are cultured together in a fermentor, have received recent attention because of their potential in biofuel and biochemical production [58]. The design of the control systems is a new challenge for such systems. In order to control the conditions in a mixed culture, a cascade control strategy may be useful. This strategy takes into account the interactions among different strains in a co-culture system. For example, pH can be maintained at an optimal value using information on the interaction between *Lactococcus lactis,* which produces lactate, and *Kluyveromyces marxianus*, which assimilate lactate, in the production of the antimicrobial peptide nisin by *L. lactis* subspecies *lactis.* The bacterium *L. lactis* is a nisin producer that assimilates maltose and produces nisin and lactate. *K. marxianus*, which was originally isolated from kefir

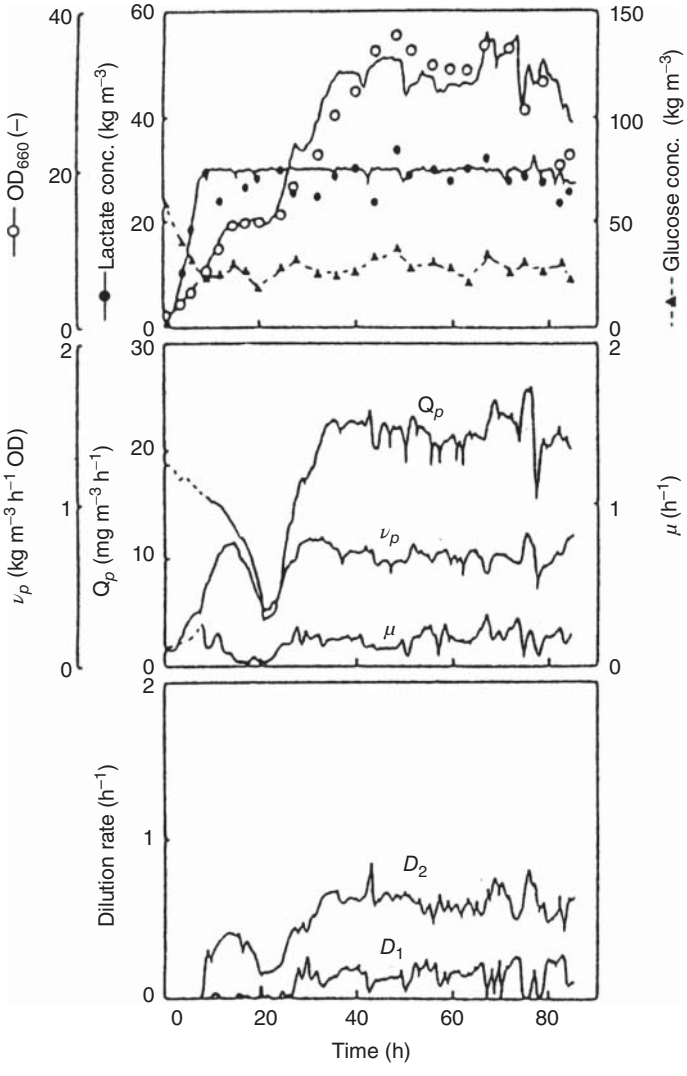

Figure 8.5 Application of the optimizing control to lactic acid fermentation.

grains, cannot assimilate maltose but can assimilate lactate. Because its lactate consumption rate is affected by the DO concentration and lactate concentration, and therefore by pH, nisin production can be controlled by manipulating the DO concentration. The concept of control systems for interacting microbes is illustrated in Figure 8.6.

A system has been developed with a cascade pH controller and DO control, which allows control of the specific lactate consumption rate of *K. marxianus* by changing the DO concentration.

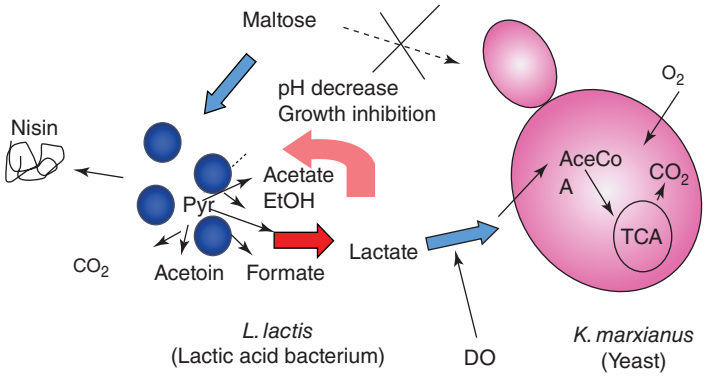

Figure 8.6 Concept of microbial interaction control.

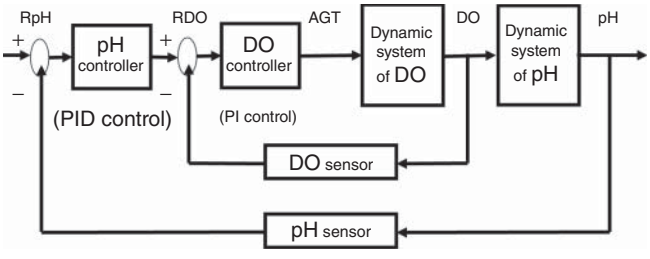

Figure 8.7 Schematic diagram of the cascade control in the co-culture system.

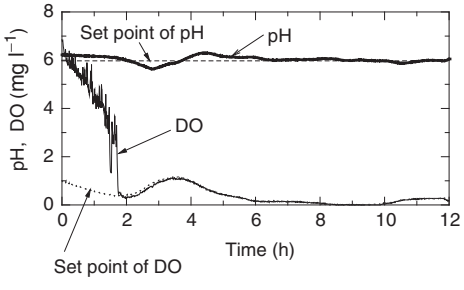

Figure 8.8 Experimental result for the cascade control of pH and DO in the co-culture system.

Figure 8.7 shows a schematic diagram of a cascade pH controller that incorporates DO control with pH control in the mixed culture. Proportional–integral (PI) and proportional–integral–differential (PID) control strategies can be employed for automatic control of the DO and pH. The pH and DO levels can be controlled as shown in Figure 8.8.

A time course for high production of nisin is shown in Figure 8.9. The pH was controlled near a set point value of 6.0. The final nisin concentration reached 200 mg l^{-1}, which is 3 times higher than that without pH control. When the

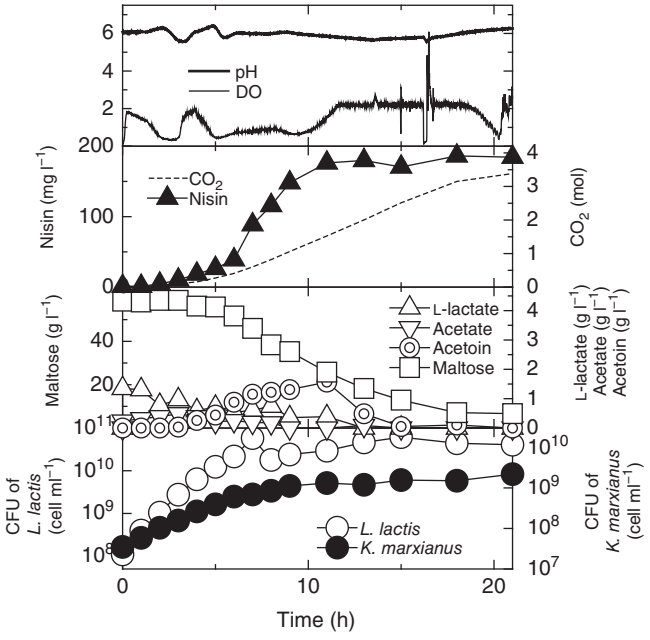

Figure 8.9 Cascade control results of pH and DO in the mixed culture of *L. lactis* and *K. marxianus*. The pH was stabilized at 6.0 by the cascade controller [59].

inoculum size of *L. lactis* is greater than the expected value, or the inoculum size of *K. marxianus* was less than the expected value, lactate was not completely assimilated by *K. marxianus,* and the pH decreased. In this case, growth of *L. lactis* was inhibited. If the *K. marxianus* population increased, the lactate concentration decreased, and both microorganisms would once again be able to grow. However, if *L. lactis* growth stopped during a period of low pH, growth of both microorganisms would cease.

Figure 8.10 shows the result of a stability test for uncertainty in inoculum sizes of both microorganisms. This experiment compared the inoculation with the control amount of *K. marxianus* cells with inoculation of 0.10 of the control amount. The imbalance in microbial concentrations resulted in the accumulation of lactate and a decrease in pH to 4.9. Growth of *L. lactis* ceased at 4.5 h. However, lactate was gradually assimilated by *K. marxianus,* and both microorganisms grew well after 8 h. The nisin concentration reached 190 mg l^{-1}. These results show that this control system is robust to changes in inoculum size of the microorganism [60].

8.3.4
Supervision and Fault Detection

The process data available must be checked for consistency based on mass balances and the absence of failures. With industrial-scale fermentation, fault

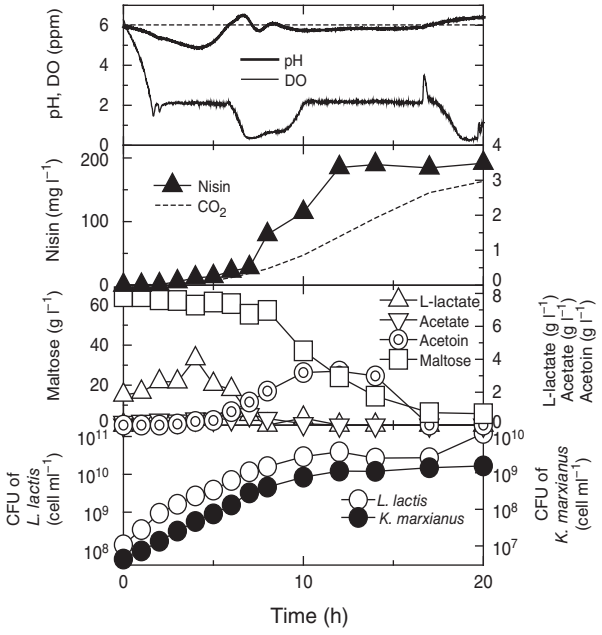

Figure 8.10 Stability test for uncertainty in inoculum size for cascade control results of pH and DO in the mixed culture [59].

detection and identification is mandatory. In the hierarchical control scheme, process supervision must be implemented at the highest level to detect and identify failures and inconsistencies in the data that may be due to sampling and instrumental errors [5, 49]. The supervision system must allow analysis of the process data to determine whether they are valid or not and force the control task to change to a safe open loop control mode, for example [5]. AI techniques such as ANN may be used to detect failures [61].

8.4 Recent Trends in Monitoring and Control Technologies

The developments of technologies in monitoring and control of bioprocesses in the previous half decade are discussed in the following to identify future directions in the fields. The first section deals with sensors that have been utilized in monitoring fermentation processes to provide information about the number of state variables involved in cultures and the physiological state of culture, and also analytical methods for supporting the sensing devices. The second section deals with control systems to optimize the process operation, including real-time assurance of quality of the product(s). Discussion will be expanded to involve matching of control with functions of monitoring devices and an emerging technology called "continuous bioprocessing."

8.4.1 Sensor Technologies and Analytical Methods

Many fermenters for the cultivation of microbial cells are installed with sensors or equivalent devices for online monitoring and control of temperature, pH, and DO. Additional probes are now available to measure variables such as dissolved carbon dioxide, oxidation–reduction potential, OD, and turbidity. In addition, various medium components and cell metabolites are monitored inline or online, and there are systems for sensing additional gaseous components in the inlet and outlet gas stream. Comprehensive reviews of *in situ* sensor techniques for bioprocess monitoring have been made by Beutel and Henkel [62] and Wang and Wolfbeis [63]. Moreover, Pospíšilová *et al.* [64] have provided a review on fiber-optic chemical sensors and biosensors. Wang and Wolfbeis also comprehensively reviewed optical methods for sensing oxygen materials, as well as spectroscopic methods and their applications [65].

Online biomass measurement was described by Bluma *et al*. [66] and Höpfner *et al*. [67]. Silva *et al.* [68] reviewed the application of multiparameter flow cytometry technologies to monitor industrial bioprocesses for the production of biodiesel, bioethanol, biomethane, and biohydrogen.

An expert panel organized by the M^3C Working Group of the European Section of Biochemical Engineering Science (ESBES) in the European Federation of Biotechnology (EFB) presented an expert opinion on future development needs for sensors in microbioreactors [69]. The recommendations suggested that online analytics such as chromatography or mass spectrometry be added to bioreactors, preferably using noninvasive sensors such as optical or electronic sensors that do not involve labeling or otherwise affecting cellular functions. The M^3C Working Group of the EFB also provided a status report on soft sensors in bioprocessing and made recommendations for their extended use [70]. Krause *et al.* [71] presented a method for the accurate detection of process parameters combined with expert knowledge of linguistic control mechanisms via adaptive sensor calibration.

Spectrometric methods that have been extensively utilized for bioprocess monitoring include NIR spectroscopy [72], Raman spectroscopy [73], nuclear magnetic resonance (NMR) spectroscopy [74], dielectric spectroscopy [75], fluorescence spectroscopy [76], and photoacoustic spectroscopy [77, 78]. Calorimetry as well as terahertz technology is used for studies on microbial metabolism and growth [62, 79]. Rathore *et al.* utilized online high-pressure liquid chromatography extensively [80].

Near-infrared spectroscopy (NIRS) has been developed in parallel with personal computers and information technology, and explosive growth in this technology can be seen in many industries. A review by Scarff *et al.* [72] critically evaluated this development pertaining to the control of microbial and cell culture, and highlighted critical stages in the development of this technology. More research is needed if the full potential of NIRS is to be exploited in making proteins, hormones, and antibiotics via fermentation routes.

Lourenço *et al.* [81] reviewed current conditions of sensor systems for bioprocess monitoring and claimed that various materials in the culture medium could be assayed by spectroscopic methods. These methods included IR spectroscopy for the monitoring of glucose, lactate, antibodies, pH, fructose, acetic acid, and ethanol; NIRS for glucose and lactate, glutamine, ammonium pH, and biomass; UV/vis spectroscopy for proteins such as bovine serum albumin and toxic substances; fluorescence spectroscopy for vitamins, pyruvate, ATP, NAD(P)H, cell mass, and ethanol; and Raman spectroscopy for glutamine, glutamate, and phenylalanine.

8.4.2 Control Technologies

In comparison with the remarkable achievements in molecular biology, improvements in industrial fermentation technologies have been less spectacular. Hence, an imbalance between new cellular systems and production technologies has occurred, resulting in a decrease in the annual rate of approved production processes. In its process analytical technology (PAT) initiative, the U.S. Food and Drug Administration (FDA) identifies the potential for continuous improvement and makes concrete suggestions on how this can be achieved. Some of these suggestions were applied to recombinant protein production by *E. coli* and *P. pastoris* cultures. According to two consecutive reviews [82, 83] by a group representing the FDA, PAT has been gaining momentum in the biotech community because of its potential to be applied to continuous real-time quality assurance for improved operational control and compliance. An expert workshop was held at the 8th European Symposium on Biochemical Engineering Sciences (Bologna, 2010), which highlighted new opportunities for exploiting PAT in biopharmaceutical production [84].

NIR offers the prospect of real-time control of the physiology of cultured cells in fermentors, leading to marked improvements in authenticity, purity, and production efficiency. A review by Scarff *et al.* [72] critically evaluated the development of this rapidly advancing technology as pertains to microbial and cell culture system control, and highlighted the critical stages in the development of this technology. Wang *et al.* [85] reviewed new advances in industrial bioprocess control and synergism between systems biotechnology and bioprocess engineering. Holistic viewpoints and ideas applied to industrial bioprocesses and future prospects were discussed by highlighting some successful cases.

Ferrero *et al.* [86] described available solutions in the area of automatic control systems for complex structured bioprocesses. They studied process control of a membrane bioreactor composed of two subprocesses, and compared several control strategies for integrated control systems. Existing knowledge is classified according to the variables manipulated, the operational mode (open-loop or closed-loop), and the control variables used. Simutis and Lübbert [87] discussed control of bioprocesses and presented important key issues for improving the performance of the processes. Productivity and process quality are important

contributors to variance in process variables. Identifying a straightforward way to reduce this variability should be one of the main tasks for quality assurance. Consequently, feedback techniques are the best to ensure reproducibility of industrial cultivation processes and quality of products.

Cooney and Konstantinov [88] introduced an emerging technology called "continuous bioprocessing" in pharmaceutical manufacturing. Innovative researches to realize continuous processing systems that integrate upstream and downstream processes for biologics manufacturing are appearing from several industrial and academic contributors who have been presenting their investigations in recent FDA conferences. The advantages of continuous bioprocessing include steady-state continuous operation, consistent product quality, reduced equipment size, high volumetric productivity, streamlined process flow, low cycle times, and reduced capital cost. These researchers reviewed the opportunities, technical needs, and strategic directions for continuous bioprocessing, and presented several examples from the recent literature that illustrate various forms of continuous bioprocessing systems.

8.5 Concluding Remarks

The technologies available for monitoring and control of microbial cultivation processes have been a key issue in the long history of fermentation and bioprocess engineering, and they have advanced significantly in the last decade. This chapter first described the development of technologies in process monitoring, including the measurement of process parameters and biological variables, data processing, and real-time estimation of the physiological state of a culture. Sensing technologies in bioprocess engineering were summarized to highlight the ingenious design and application of various sensors, and analytical methods using spectrometry, chromatography, and other detecting techniques. Thus, there have been significant advancements with respect to our ability to analyze and monitor key process and quality attributes in the biotechnology industry. In cases where not enough online information is available, an appropriate model may be considered based on mass balances with stoichiometric constraints for the development of EKFs. However, we need to do more to use the data collected for process control to achieve optimal production and product quality.

In examining the development of methodologies for the control of bioprocesses, the design and application of several methods were presented. Online optimization for continuous culture and cascade control for mixed culture were described. There have been various process control methods developed for biotechnological manufacturing, such as feedback control of a single process parameter, feed-forward control of physiological activities, specific growth rate control by exponentially increasing the substrate feed rate, cascade control of process parameters, control of multilateral variables, integrated control for total optimization in a manufacturing plant, and online optimizing control for continuous culture.

Finally, updated technologies for monitoring and control of bioprocesses were highlighted to identify future directions in the fields. They included *in situ* sensor technologies, recommendations by experts on future development needs for sensors, the contribution of NIR as a versatile method for monitoring biotechnological processes, computational intelligence methods, synergetic effects of systems biotechnology that increase the sophistication of bioprocesses, integrated control of multiplexing subprocesses for total process control, and feedback technologies ensuring high reproducibility of product quality. An emerging technology called "continuous bioprocessing" now presents a new scenario for the maturation of biomanufacturing as a true modern industry.

Abbreviations

ANN	artificial neural network
DO	dissolved oxygen
EKF	extended Kalman filters
FDA	U.S. Food and Drug Administration
NIR	near-infrared
NMR	nuclear magnetic resonance
OD	optical density
OUR	oxygen uptake rate
PAT	process analytical technology

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Part III
Plant Cell Culture and Engineering

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9
Contained Molecular Farming Using Plant Cell and Tissue Cultures

Stefan Schillberg, Nicole Raven, Rainer Fischer, Richard M. Twyman, and Andreas Schiermeyer

9.1
Molecular Farming – Whole Plants and Cell/Tissue Cultures

Molecular farming is the use of plants, plant tissues or plant cells to produce valuable recombinant proteins [1, 2]. Following many years of research to demonstrate that plants can express a multitude of diverse pharmaceutical proteins, attention is now turning to the commercial potential of the technology [3], and especially whether plants can compete with mainstream production systems such as microbes and mammalian cells [4].

Whole plants are advantageous because upstream production is less expensive and more scalable than fermenter-based microbial and mammalian cells, and they do not support the growth of human pathogens [5]. Plant-based systems are also versatile because different species, gene transfer strategies, expression strategies, protein targeting, and recovery strategies have been developed, which means a particular system can be chosen to suit the product, rather than the product being forced to work with the small number of platforms that have been adopted by industry (e.g., the bacterium *Escherichia coli*, yeast such as *Saccharomyces cerevisiae* and *Komagataella* (formerly *Pichia*) *pastoris*, and a small number of mammalian cells lines such as Chinese hamster ovary (CHO) and NS0). The diversity of suitable plant host species also means that molecular farming strategies can be adapted so that they are compatible with local environments and infrastructures, that food crops with generally recognized as safe (GRAS) status can be used to deliver recombinant oral vaccines and topical microbicides with limited need for processing, and that unique characteristics can be exploited to facilitate product purification, such as the targeting of proteins to oil bodies in oilseed crops. On the other hand, the same diversity is thought to have held molecular farming back, because it is difficult to make progress in a commercial environment without focusing on a small number of platforms. These and other attributes have been discussed in detail elsewhere [3, 6–8].

Although the advantages of plants have been widely discussed, it has been difficult to persuade the pharmaceutical industry to embrace molecular farming,

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and the few recombinant proteins derived from whole plants that have reached the market are approved for research use, as diagnostics or as cosmetic ingredients, and in one case as a medical device [6]. The slow uptake of the technology reflects many underlying factors, including perceived technical limitations that have largely been overcome [9], the negative public perception in Europe of any technology linked to genetically modified (GM) crops even if supported by patient advocacy groups [10], and the unwillingness of industry to switch from established platforms with a good track record and existing infrastructure [11]. Perhaps the most important advantage of the established platforms from an industry perspective is the robust intellectual property and regulatory landscape, which means the path to the market is defined and well tested. In contrast, plant-derived pharmaceuticals fall between the jurisdictions and competencies of agencies responsible for GM agriculture and biotechnology-derived drugs, and until recently there was no specific regulatory framework for such products. This meant they fell by default under the existing regulations for pharmaceutical proteins, which had been developed around the concept of cells grown in fermenters (e.g., master/working cell banks, virus inactivation steps) and were not easily translated to plants [12]. All pharmaceuticals intended for human use (even in phase I clinical trials) must be produced in compliance with good manufacturing practice (GMP), and this demands precise, documented growth conditions to ensure the batch-to-batch consistency of the product, which is challenging to implement with plants growing in greenhouses and next to impossible to implement in the open field. The use of controlled-environment greenhouses reduces much of the variation caused by weather, climate, soil heterogeneity, and interactions with other organisms, and once the plant material is harvested and the initial extraction steps are complete, it can be transferred to a GMP-compliant cleanroom for final purification and formulation. Even so, GMP processes for whole transgenic plants and transient expression systems have been realized only in the last few years, and the first products are only now undergoing clinical trials [11].

Whereas the use of whole plants required a new regulatory pathway to be laid down, plant tissues and cell suspension cultures can be grown in containment in exactly the same way as mammalian cells and microbes, making regulatory compliance much more straightforward. Molecular farming has therefore branched into three main approaches: transgenic plants suitable for the bulk production of pharmaceuticals over a long term; transient expression in whole plants suitable for rapid production over a shorter term; and, finally, the use of plant cell and tissue culture platforms to produce pharmaceuticals in a manner similar to established fermenter platforms, but with the advantages of inexpensive cultivation (like bacteria), higher eukaryotic protein processing (like mammalian cells) and the absence of bacterial endotoxins and mammalian pathogens. Based on this unique combination of advantages, the first plant-derived pharmaceutical protein approved by the U.S. Food and Drug Administration (FDA) for use in humans was produced using plant suspension cell cultures [13].

9.2
Plant Cell and Tissue Culture Platforms

The first examples of molecular farming involved the expression of an antibody in transgenic tobacco leaves [14] and the production of human serum albumin in transgenic tobacco and potato leaves, as well as tobacco cell suspension cultures [15]. The latter report is therefore the first example of molecular farming in plant cells, which thereafter was more widely used for the production of small-molecule compounds [16]. Many pharmaceutical proteins have been produced in cultured plant cells and tissues such as hairy roots since those initial reports (reviewed by Hellwig *et al*. [17] and Schillberg *et al*. [18]). The different platforms require diverse media and cultivation conditions to achieve maximum productivity, but a major distinction can be drawn between the cultivation of cells and tissues derived from roots (e.g., tobacco hairy roots, and the current commercial tobacco and carrot cell suspension culture platforms) or embryonic tissues (rice cell suspension cultures), and those based on green tissues (e.g., moss, algae, and duckweed). The former do not need light and grow to high densities in or on media containing sugar, whereas the latter can grow in water without carbohydrates but require constant illumination [19–23].

9.2.1
Cell Suspension Cultures

Plant cell suspension cultures grow in a liquid medium as individual cells or small aggregates, and are usually derived from callus tissue by the disaggregation of friable callus pieces in shake flasks. Recombinant proteins can be produced either by transforming the cells directly, usually by cocultivation with *Agrobacterium tumefaciens* to achieve transient or stable expression, or by the derivation of cell cultures from transgenic plants [24, 25]. Cell suspension cultures can be theoretically derived from any plant species, but the most widely used for molecular farming are tobacco cells derived from the embryonic root meristem of cultivars BY-2 and NT-1 [26], and cells derived from carrot [23] and rice [27], all of which have been used commercially, although only carrot cells have been used to produce approved human pharmaceuticals [18, 28]. Other species that have been used for proof-of-principle studies include Arabidopsis [29, 30], alfalfa [30], soybean [31], and tomato [32]. Recombinant proteins expressed in plant cell suspension cultures are usually secreted into the supernatant to facilitate recovery by endowing the expression construct with a signal peptide for secretion, although some of the protein may be retained within the cells depending on its size relative to the pores in the cell wall. The recombinant protein may also be targeted to alternative intracellular compartments such as the cytosol or vacuole. Cell suspension cultures are amenable to scale-up, and production capacities exceeding 1000 l have been reported [18].

9.2.2 Tissue Cultures

Recombinant protein expression has been achieved in various types of *in vitro* plant tissue culture, including callus cultures, shooty teratomas, and hairy roots. Shooty teratomas and callus cultures are less robust, scalable, and productive than hairy roots [33], although callus cultures can be used to screen for high expression levels before deriving suspension cell cultures [27, 34]. The hairy root phenotype is caused by infection with *Agrobacterium rhizogenes*, a soil bacterium carrying genes that promote the rapid and indefinite growth of highly branched plant roots even in the absence of exogenous hormones. Hairy roots are phenotypically and genetically stable, and selected hairy root lines can be exploited indefinitely. Like cell suspension cultures, transgenic hairy root lines can be generated either by converting the roots of existing transgenic plants into a hairy root phenotype using wild-type *A. rhizogenes*, or by creating the transgenic hairy roots directly by infecting wild-type plants with genetically modified *A. rhizogenes* carrying both the root-inducing genes and a transgene expressing a recombinant protein. Also, like cell suspension cultures, recombinant proteins are often secreted by hairy roots into the culture medium to facilitate recovery [35]. Stably transformed hairy root cultures have been established from many species, although tobacco has proven the most sustainable and scalable [36]. Recombinant proteins have also been produced using plant viruses as vectors in hairy roots [37] and in cell suspension cultures [38].

9.2.3 Light-Dependent Expression Platforms

Moss, algae, and aquatic plant cultures can also be used to produce recombinant proteins in containment, and all depend on photosynthesis to produce the energy and precursors required for protein synthesis. Like cell suspension cultures and hairy roots, the recombinant protein is generally secreted into the culture supernatant for ease of recovery. The advantage is compounded because these systems do not require a nutrient culture medium.

The moss *Physcomitrella patens* is a haploid bryophyte that can be grown in bioreactors under controlled conditions, using synthetic growth media and artificial light [20]. The advantage of this species as a production host for recombinant proteins is its unusual propensity to undergo homologous recombination, which means that transgenes can be introduced into the genome to allow the production of recombinant proteins under the control of strong endogenous promoters, and endogenous genes that have undesirable effects can be knocked out or replaced by gene targeting. The latter has been used to modify the protein glycosylation pathway, removing genes responsible for the synthesis of plant glycans and in some cases replacing them with genes promoting human-type glycosylation [39, 40]. The moss system has been commercially developed by the German biotechnology company greenovation Biotech GmbH, which offers a transient expression

platform for feasibility studies as well as stable production lines that can be scaled up to several hundred liters [41, 42].

Photosynthetic microalgae such as *Chlamydomonas* spp. can also be used as a platform for the production of recombinant proteins. They grow rapidly, thus producing large amounts of biomass in a short time, and whereas nuclear transformation is the typical approach in other cell and tissue culture platforms, the single plastid found in algal cells is much easier to transform, thus stable transplastomic lines can be produced within a few weeks [43]. This advantage is somewhat offset by the fact that proteins expressed in the plastids do not undergo post-translational modifications that are typical in the other platforms and are not secreted to the culture medium, which makes subsequent purification more challenging. The U.S. company PhycoBiologics Inc. is using *Chlamydomonas* spp. plastid transformation to produce oral aquaculture vaccines such as the *Staphylococcus aureus* fibronectin-binding domain D2 fused to the cholera toxin B (CTB) mucosal adjuvant to induce antigen-specific immune response in mice [44].

Finally, we consider the *in vitro* cultivation of whole aquatic plants, particularly duckweeds such as *Wolffia* spp. [45], *Spirodela* spp. [46], and *Lemna* spp. [47]. These species can be transformed by *A. tumefaciens*, and recombinant proteins can be secreted into the culture medium or accumulated in the plant, which is advantageous in the case of *Lemna minor* because this species contains a pectic polysaccharide with adjuvant properties, and oral administration of plant extract containing the target antigen promotes a stronger immune response than the pure protein [48]. The American company Biolex Therapeutics Inc. developed *L. minor* and *Spirodela oligorrhiza* as platforms for the production of recombinant human pharmaceutical proteins such as IFN-α2b (Locteron), which is ready for Phase III clinical trials. The company has also humanized glycosylation in *L. minor* by RNA interference, targeting the endogenous α-1,3-fucosyltransferase and β-1,2-xylosyltransferase genes. A human CD30-specific antibody produced in these engineered cells did not contain any plant-specific *N*-glycans and showed an improved cell-mediated cytotoxicity and effector cell reporter binding than the corresponding antibody produced in CHO cells [49]. The company sold its technology and product portfolio to the Dutch company Synthon BV in 2012 and later filed for bankruptcy [50].

9.3 Comparison of Whole Plants and *In Vitro* Culture Platforms

As stated previously, the main advantage of *in vitro* culture platforms is the ability to grow the cells, tissues, or floating plants in containment, under sterile conditions that are fully defined and controlled. Whole plants can also be grown in degrees of containment, ranging from pots of soil in greenhouses (thereby removing some but not all biological variables) to fully controlled glasshouses and growth rooms where the temperature, light intensity, and atmosphere can be regulated, and where soil is replaced with sterile support substrates and

a defined nutrient solution. The recent development of automated cultivation systems, incorporating light-emitting diode (LED) illumination and nutrient film technology, aims to eliminate almost all environmental fluctuation [51, 52]. Even so, plant cells and tissues remain advantageous in terms of growth, product recovery, product quality, and process consistency. We discuss each of these aspects in turn.

Plant cells and tissues cultivated *in vitro* grow more rapidly than whole plants, with cell doubling times as short as 1 day for higher plants and 8–10 h for algae [53]. This means that a single batch fermentation using tobacco BY-2 cells takes 1–2 weeks, compared to months for a single batch of transgenic tobacco plants assuming that a working seed bank is already available [18]. Although whole plants are more scalable, in that production can be boosted by sowing more seeds without extending the cultivation period, plant cell and tissue cultures can be scaled up by preparing additional precultures as required for the projected final cultivation volume, and even when a final volume of 10 000 l is considered, the overall campaign lifecycle is still shorter than that required for a single batch of transgenic plants. Alternatively, parallel cultivation or continuous fermentation can be used to shorten the production periods for larger fermentation volumes. Shorter campaigns are possible with whole-plant transient expression systems [54–56], but transient expression platforms introduce an extra regulatory burden, such as the endotoxins released by infiltrated bacteria [57] and the large-scale use of genetically modified bacteria or viruses [11].

Recombinant proteins produced by sterile cultures of plant cells and tissues can be secreted into the medium if an N-terminal signal peptide is used to direct the protein into the secretory pathway. The signal peptide is cleaved off during transit and the mature protein accumulates in the well-defined and sterile medium, which contains few other proteins, facilitating recovery and purification. In contrast, proteins expressed in plants are usually retained in the cell and must be released by mechanical disruption, which liberates the entire cell contents. Recovery and purification therefore involve separating the target protein from particulates, soluble host cell proteins, and host cell metabolites, some of which may damage recombinant proteins by oxidation or the formation of adducts and conjugates [58].

The secretion of recombinant proteins into the culture medium has an important knock-on effect on product quality because all the recovered protein molecules are fully processed and therefore homogeneous. The signal peptide must be removed and glycan chains must be added before secretion, whereas the release of intracellular proteins from whole plants liberates proteins distributed throughout the secretory pathway as well as proteins retained in other compartments, often yielding a heterogeneous mix of processing variants [59]. The improved product homogeneity and integrity ensures superior product and process consistency, which is a critical aspect of GMP compliance. Plant cell and tissue cultures are grown in contained bioreactors, under precise and documented conditions that are fully controlled, so that the nutritional and physical process parameters can be monitored and adjusted. Fermentation processes are

therefore less prone to biotic and abiotic variations that reduce batch-to-batch consistency, which is an inherent risk when producing pharmaceutical proteins in greenhouse-grown plants.

9.4 Technical Advances on the Road to Commercialization

The first approved veterinary and pharmaceutical proteins produced by molecular farming were manufactured using cell suspension cultures (Box 9.1). This required several technical, regulatory, and commercial barriers to be addressed. The principal technical barriers were the low specific productivity (the amount of product produced per unit of biomass per unit of time), low recovery (the amount of final purified product compared to the amount present in the harvested biomass), and variable quality (lack of consistency between batches) associated with plant-derived pharmaceuticals. The overall productivity of plant cell cultures can be increased by improving the intrinsic yield (amount of product per unit biomass) and/or the amount of biomass produced per unit time, and the recovery can be improved by increasing the efficiency of downstream processing. This has required a combination of genetic, biological, and physical approaches applied during both upstream production and downstream processing, focusing on the *quality* and *quantity* of the final product.

9.4.1 Improving the Quantity of Recombinant Proteins Produced in Cell Suspension Cultures

Many genetic strategies have been used to improve the specific productivity of molecular farming platforms, for example, enhancing transcription using optimized promoters, ensuring more efficient mRNA processing and translation, and protein targeting tailored to improve product stability and facilitate recovery (reviewed by Twyman *et al*. [9]). These approaches apply to all molecular farming platforms, whereas other strategies are more relevant to cell suspension cultures and tissue bioreactors. A great deal of progress has been made by optimizing the cultivation process to increase cell-specific productivity, involving a combination of cell line development and process development to achieve synergy between biological and physical process parameters [18].

Transformation methods for plant cells usually produce multiple primary transformants with different transgene copy numbers and insertion sites. The independent transgenic events differ significantly in terms of productivity, so one or more rounds of screening and selection are necessary to identify the most productive cells that are suitable for the generation of production lines [60]. Single-cell DNA microinjection can be used to produce a single transgenic progenitor cell that multiplies to yield a monoclonal cell culture from the outset [61], but this approach is cumbersome and labor-intensive, and the transgene integration site is still random so a panel of monoclonal cultures must be generated and screened

to identify those that are most productive [62]. Genome editing, using engineered nucleases and more recently the CRISPR/Cas9 system [63], can be used to preselect a specific and productive integration site, although screening is still required to remove the background of nonspecific integration events. Zinc finger nucleases have recently been used to engineer BY-2 cells in this manner [64].

In most cases, it is therefore necessary for polyclonal transformants to be screened to isolate clones with optimal productivity. Screening can be carried out at the callus stage or following the cultivation of multiple events in a microtiter dish or in small bioreactors, the latter offering the advantage that cells are exposed to the same conditions as they will later experience during scale-up. Although screening and selection help eliminate this initial heterogeneity, further variation can appear *de novo* during prolonged cultivation with periodic subcultures. This phenomenon, known as somaclonal variation, involves a combination of genetic and epigenetic mechanisms that can reduce the productivity of initial jackpot clones and even cause complete transgene silencing [65]. Highly productive cells also tend to dedicate a significant proportion of their resources to protein synthesis, which means they grow more slowly than their less productive peers and eventually become diluted from the population [66]. It can therefore be useful to isolate elite individual cells and use them to seed highly productive monoclonal cell suspension cultures. Recently, flow sorting has been used to separate the most productive cells from a heterogeneous tobacco BY-2 cell culture producing a full-length human antibody, by selecting the coexpressed fluorescent marker protein DsRed located on the same T-DNA [67]. Using a feeder strategy, single cells selected by flow cytometry were regenerated into stable monoclonal cell lines with homogeneous DsRed fluorescence and antibody yields up to 13-fold higher than the parental culture.

Cell line development can improve productivity only within the boundaries of efficiency permitted by the cultivation process, so for the best results it is necessary to combine an optimized cell line with an optimized process. The first consideration is the culture medium, which is responsible for nurturing the cells and maintaining them under conditions that favor recombinant protein production. As discussed previously, aquatic plant cultures such as moss and duckweed are maintained in water with minimal mineral nutrition, with biomass derived by photosynthesis. In contrast, most plant cell suspension cultures require a more complex medium that provides one or often two nitrogen sources (nitrate and ammonium) in a defined ratio, as well as mineral nutrients, a carbon/energy source, other complex organics as necessary, and plant growth regulators to maintain the cells in an undifferentiated state. Examples include the widely used Murashige and Skoog (MS) medium [68] and Gamborg's B5 medium [69], but these are often varied to take account of species-dependent nutrient requirements and the characteristics of particular cell lines. Indeed, it has recently been appreciated that specific media are required for the production of different recombinant proteins, which has resulted in a shift away from trial-and-error testing toward more systematic approaches based on statistical experimental designs [18]. The addition of more nitrogen to the MS medium

can improve the productivity of BY-2 cells by up to 150-fold in the stationary phase, ultimately improving yields by up to 20-fold [70, 71]. The online analysis of oxygen consumption using a respiration activity monitoring system (RAMOS) is a useful tool for process and medium optimization. This allowed the detection of metabolic changes in transgenic BY-2 cells cultivated in shake flasks, and further analysis of the nutrients in the spent MS medium revealed that the metabolic shift was caused by ammonia depletion. Supplementing the medium with additional ammonium doubled the amount of recombinant green fluorescent protein (GFP) produced by these cells, and the same medium also doubled the productivity of transgenic NT-1 cells producing the influenza hemagglutinin protein [71]. In later studies, multiple parameters were tested in statistical experimental designs to optimize the culture medium and thereby improve the productivity of BY-2 cells [72] and tobacco hairy roots [73].

9.4.2
Improving the Quality and Consistency of Recombinant Proteins Produced in Cell Suspension Cultures

The quality and consistency of recombinant proteins produced in plant cells depends on two types of processing, namely the processing associated with protein synthesis and activity (e.g., folding, glycosylation, phosphorylation), and the processing associated with protein damage and degradation (e.g., oxidation, proteolysis). Ideally, the production process handles these forms of processing using distinct strategies – the processing associated with protein synthesis is encouraged and tailored for specific applications, whereas the processing associated with protein degradation is inhibited or prevented.

Although plants carry out most of the post-translational modifications that occur in animal cells, by far the most important in terms of its impact on pharmaceutical quality is glycosylation. This is because ~30% of all approved biopharmaceuticals carry N-linked glycans, and these can have a profound impact on protein structure, activity, stability/longevity, and immunogenicity. The glycosylation machinery in plants and mammals is similar but not identical. In both cases, nascent polypeptides with the consensus motif NXS/T (where X is any amino acid except proline) are augmented with an oligosaccharide precursor, which is attached to the asparagine residue during co-translational import into the endoplasmic reticulum (ER). As the protein matures, the oligosaccharide precursor is trimmed to a high-mannose-type glycan that is identical in plants and mammals, and then further modified in the Golgi apparatus by enzymes that are species-dependent, thus resulting in different complex-type glycans in different species. Plant glycoproteins tend to contain core β-1,2-xylose and α-1,3-fucose residues that are not present in mammalian glycoproteins, whereas mammalian glycoproteins contain β-1,4-galactose and terminal sialic acid residues that are not present in plants [74].

Plant glycans were initially considered a risk because of their potential immunogenicity and potential impact on pharmacokinetics and pharmacodynamics,

but there is little evidence that they pose any danger. For example, the only plant-derived pharmaceutical protein currently approved for injection into humans (Elelyso, see Box 9.1) contains the aforementioned core β-1,2-xylose and α-1,3-fucose residues but demonstrated no immunogenicity or other adverse effects, even after repeated doses. Even so, there has been a significant effort to remove or replace plant glycans, which, although unnecessary in hindsight, has nevertheless allowed the development of a plethora of technologies to achieve the control of glycosylation in plants. This has achieved two major benefits – the creation of "biobetters" (i.e., products with tailored glycans that improve their properties as drugs) and the ability to produce glycoproteins with homogeneous glycan profiles, which is advantageous from a regulatory perspective.

The simplest way to avoid plant-like glycan residues is to add the tetrapeptide H/KDEL to the C-terminus of pharmaceutical proteins, causing them to be retrieved to the ER before the plant-type complex glycans are added. This strategy has been used to produce many high-mannose recombinant glycoproteins in whole plants and plant cell suspension cultures [75], but it has two significant drawbacks. First, the presence of the additional peptide extension alters the sequence of the protein and therefore may affect its activity and stability, also attracting a higher regulatory burden. Second, ER retention means the protein cannot be secreted, thus removing one of the principal benefits of plant cell suspension cultures, which is the ability to recover homogeneous proteins from the culture supernatant. Alternative strategies have therefore focused on preventing the enzymatic addition of plant complex glycan structures by mutating or inhibiting the corresponding enzymes, for example, by gene targeting [39, 42] or RNA interference [49]. In the latter case, the human anti-CD30 antibody MDX-060 was produced in *L. minor* without plant glycans, and this variant demonstrated a >10-fold higher affinity for the human FcγRIII receptor and >20-fold higher antibody-dependent cell-mediated cytotoxicity (ADCC) against a tumor cell line *in vitro* than its counterpart produced in CHO cells. This provides an excellent example of glycomodification being used to develop "biobetter" pharmaceutical products. Although glycan structures are typically controlled using genetic strategies, the medium composition can also have an important impact. For example, the addition of *N*-acetylglucosamine to tobacco BY-2 cells resulted in a dose-dependent shift from paucimannosidic or hybrid type to complex type *N*-glycans [76], whereas sugar depletion in rice suspension cell cultures changes the glycan profiles of secreted glycoproteins [77].

As discussed previously, recombinant proteins are modified not only during synthesis but also during damage and degradation. Degradation caused by intracellular or extracellular proteases reduces both the yield and quality of recombinant proteins, and the resulting protein fragments must be removed during downstream processing. Degradation may occur *in vivo*, during extraction and/or during subsequent downstream processing, but the extracellular compartments of the plant cell (apoplast and cell wall) and the external environment (the culture medium) appear to be particularly rich in proteolytic enzymes [17, 18, 78]. Extracellular degradation can be avoided by ER retention, because the ER

contains abundant molecular chaperones that facilitate protein folding/assembly and enhance their stability [9]. Many recombinant proteins accumulate to higher levels (one or two orders of magnitude) in the ER compared to the apoplast [79], but as stated previously, this affects the glycan structures of the resulting proteins and also means they have to be liberated from the cell, releasing all the endogenous proteins and other contaminants. It is also unclear which proteases contribute most to intracellular and extracellular proteolytic activity, because hundreds of different proteases are produced by plant cells and their abundance varies throughout the production cycle [80]. Certain cysteine and serine proteases have been implicated in the degradation of human granulocyte-macrophage colony-stimulating factor [81] and antibodies [82–84], whereas specific metalloproteinases have been invoked in the degradation of recombinant DSPAα1 produced by tobacco BY-2 cells [85], but generally it is difficult to identify specific culprits on a case-by-case basis [18]. Once the specific proteases responsible for recombinant protein degradation are known, it should in theory be possible to target the corresponding genes or inhibit the enzymes by RNA interference, but this may have unintended effects on the plant cell if the proteases fulfill a critical housekeeping role [86]. An alternative strategy is to inhibit protease activity specifically in the vicinity of the recombinant protein by coexpressing a protease inhibitor [87, 88]. Further strategies include the coexpression or external addition of a "decoy" protein such as human serum albumin or gelatin, to mop up excess protease activity [89], or the more straightforward approach of capturing the recombinant protein immediately after secretion using an appropriate affinity reagent [90].

9.5
Regulatory and Industry Barriers on the Road to Commercialization

Technical barriers affecting the quality and quantity of recombinant proteins produced using *in vitro* plant-based expression platforms have been largely addressed, but the commercialization of plant cell technology also requires plant cells to match their mammalian counterparts in terms of regulatory compliance. The cryopreservation of mammalian cells is an industry standard that allows the provision of master and working cell banks whose quality and productivity are consistent over time. Similarly, the performance of plant cell lines can change over time (in terms of growth rate and recombinant protein productivity), reflecting the impact of somaclonal variation as discussed above. Therefore, the cryopreservation of plant cells/tissues in liquid nitrogen is necessary for long-term genetic conservation, as well as the establishment of master cell banks under GMP guidelines. Whereas the gold standard mammalian cell lines behave in a similar manner, allowing generic cryopreservation strategies to be employed, the properties of plant cells are diverse and species-dependent, requiring unique cryopreservation protocols for different cell lines [91, 92]. These protocols are currently based on three main approaches, namely controlled-rate cooling

(two-step/equilibrium freezing), which requires a slow cooling gradient (1 °C min^{-1}) to an intermediate low temperature (−30/−40 °C) before rapid cooling in liquid nitrogen, or vitrification with 5–8 M cryoprotectants at nonfreezing temperatures to promote dehydration before ultrarapid cooling in liquid nitrogen, or desiccation with or without encapsulation in alginate beads. Successful cryopreservation has been achieved in many species, but the protocols are usually optimized using nontransgenic cell lines and evaluated using criteria such as cell growth and morphology. There are limited data for transgenic lines and, as is the routine approach with mammalian cells, it is important to quantify the success of plant cell cryopreservation in terms of productivity after recovery [93].

Once regulatory barriers have been overcome, the final hurdle is the conversion of laboratory-based demonstrations into industrial processes. Plant cell cultures must therefore be scaled up during process development to achieve commercial productivity. Cell line development and optimization are usually carried out using small-scale vessels such as microtiter plates, shake flasks, or disposable 50-ml bioreactors. However, there are many different bioreactor designs for larger scale cultivation. Floating plants and hairy root cultures require specialized bioreactors, but most plant cell cultures are grown as homogeneous suspensions similar to microbial and mammalian cell fermentations, and these can be maintained in stirred-tank bioreactors, bubble column bioreactors, air-lift bioreactors, single-use stirred-tank bioreactors, wave-mixed bioreactors, and membrane bioreactors. The choice depends on the species and culture type, based on characteristics such as cell or tissue growth, morphology and aggregation, shear sensitivity, oxygen demand, and culture rheological properties. For example, moss cultures need light and cannot be grown in stainless steel fermenters, whereas tobacco cells are heterotrophic and are well suited to such vessels. The status and characteristics of different types of bioreactors for recombinant protein production in plant cell/tissue suspension cultures have recently been discussed [94]. The manufacture of antibodies in tobacco BY-2 cells can be scaled up from shake flasks to 200-l disposable bioreactors [95].

Plant cell/tissue suspension cultures are often maintained in reusable bioreactor systems made of glass or stainless steel. These tend to be stirred-tank bioreactors with large, slow-moving axial and radial flow impellers, delivering maximum biomass values of 60–70% packed cell volume [96]. The typical design incorporates marine or pitched blade impellers and a ring sparger for aeration, which ensures cell growth without cell damage even though many cultures become non-Newtonian fluids with increasing culture broth viscosity [97] and can form surface foams if appropriate antifoaming agents are not used [98].

Reusable bioreactors require regular cleaning to avoid contamination, which involves the use of harsh chemicals for cleaning-in-place (CIP) and steam venting for sterilization-in-place (SIP). These expensive and time-consuming operations can be avoided by using disposable bioreactors, where the fermentation takes place within a disposable plastic bag [99]. The disposable systems are expensive in terms of consumables, but the additional costs are more than offset by the savings accrued by omitting CIP/SIP procedures and the reduced risk of

contamination. Elelyso, the only plant-derived pharmaceutical protein currently approved for human use by the FDA, is produced in carrot cells cultivated in bubble column-type bioreactors fitted with disposable polyethylene bags [100].

Most biopharmaceuticals are formulated as a purified product, so the majority of pharmaceutical proteins produced in plants must be extracted from plant tissue or recovered from culture medium and then purified and formulated in the same way as conventional biopharmaceutical products. Regardless of the upstream production platform, downstream processing can account for up to 80% of the total manufacturing costs for a pharmaceutical protein [58]. The early processing steps are largely platform-dependent, so intracellular proteins must be released by mechanical disruption in the presence of an appropriate extraction buffer, and the extract must be clarified by filtration and/or centrifugation to remove debris, fibers, and other particulates, which increase the overall downstream processing costs. Therefore, the secretion of recombinant proteins into the cell culture medium is advantageous, because the purification process can begin immediately after the cells are removed (e.g., by filtration or centrifugation) or even during production, if appropriate affinity reagents are available. Many natural and synthetic affinity ligands can be used for the capture of antibodies, for example, the natural ligand protein A and the synthetic ligand mercaptoethylpyridine (MEP HyperCel™) [101]. The captured recombinant protein is "polished" by the application of two or more orthogonal separation methods, which remove process-related impurities such as host cell proteins and nucleic acids, and product-related impurities such as degradation fragments, unfolded proteins, and aggregates. In the well-characterized process of antibody purification, up to 85% of the product can be recovered from the harvested culture broth [18].

9.6 Outlook

Plant cell suspension cultures have now been accepted as a commercially viable production platform for niche pharmaceutical proteins, namely those whose quality, activity, or delivery can be improved by virtue of using plant cells. It is unlikely that plant cells will ever replace mammalian cells as the mainstream industry platform for pharmaceutical proteins given that the latter have an enormous head start in terms of strain and process development and regulatory compliance, but the first wave of products derived from plant cells have ensured that the platform can never again be dismissed solely for its emergent status, unproven commercial viability, or lack of regulatory framework. In this sense, plant cells qualify as a disruptive technology, that is, a technology that does not achieve incremental improvements on an incumbent platform but one that offers the potential for game-changing market reorganization. Disruptive technologies tend to become established in niche, high-end markets and then gradually spread to displace their rivals once their credentials are established and they are no longer regarded

as a risk. Although the productivity of BY-2 cells is more than an order of magnitude lower than CHO cells, the advantage of plant cells is not their yield but the simple and inexpensive cultivation medium, their inability to support the replication of human pathogens, the lack of endogenous viruses and oncogenic DNA sequences, the ability to tailor the post-translational modification of proteins to generate "biobetter" drugs, and the ability to encapsulate protein antigens in edible plant-cell pellets to prolong their contact with the mucosal immune system [5]. Process development in the context of plant cell suspension cultures is an emerging field, comprising strain and medium optimization and the statistical testing of different upstream and downstream process designs to promote recombinant protein accumulation. The FDA approval of recombinant glucocerebrosidase produced in carrot cells has demonstrated the competitiveness of plant-cell-based production platforms in niche markets, but further candidates must now be produced using GMP-compliant, large-scale processes and the products entered into clinical trials to demonstrate the overall sustainability of contained and controlled plant cell and tissue culture systems. The benefits of plant-type glycans and the use of the plant matrix to enhance the immunogenicity of animal vaccines and avoid cost-intensive purification processes are unique selling points that can be exploited in the next generation of pharmaceutical products from plant cells.

Box 9.1 The Pioneers

Hemagglutinin-neuraminidase glycoprotein

The Newcastle disease vaccine for poultry was developed by Dow AgroSciences LLC and was the first plant cell-derived pharmaceutical product to be approved. The vaccine is the recombinant hemagglutinin-neuraminidase (HN) glycoprotein of the Newcastle disease virus (NDV). HN is one of two viral surface glycoproteins and the major surface antigen that induces neutralizing antibodies. It is used as a vaccine to induce a protective immune response against NDV in domestic poultry and other avian species, thereby helping to prevent the spread of this acute and contagious disease. The vaccine was produced in transgenic tobacco NT-1 suspension cells and accumulated within the cells. Processing comprised a basic extraction followed by injection of the crude extract containing recombinant HN into chickens, thus reducing the production costs sufficiently to make a plant-derived veterinary vaccine economically viable. The activity of the unpurified vaccine was demonstrated *in vitro* using hemagglutination assays, and the HN protein conferred full protection in chickens that were challenged with the virus. Although the vaccine was approved by the USDA in February 2006 for use in poultry, a strategic decision was made by Dow AgroSciences not to introduce the product into the market [102].

Elelyso

Recombinant glucocerebrosidase (prGCD, taliglucerase alfa, Elelyso), manufactured in carrot cells by Protalix Biotherapeutics and distributed by Pfizer Inc.,

was approved by the FDA in May 2012 for human use (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm302549.htm). Recombinant glucocerebrosidase is needed to replace the nonfunctional enzyme in patients with the monogenic disorder Gaucher's disease, who otherwise cannot degrade glucosylceramides, which therefore accumulate in the lysosomes of phagocytes. Clinical symptoms of the disease include hepatosplenomegalia, anemia, and thrombocytopenia. Patients have been treated with a recombinant version of the enzyme (imiglucerase, Cerezyme) produced in CHO cells [103], which is one of the most expensive biopharmaceuticals on the market, with an annual treatment cost of USD 200 000 per patient [104]. The recombinant imiglucerase is purified from the CHO cell culture medium and must then be processed enzymatically to expose terminal mannose residues that are required for the efficient uptake of the enzyme into macrophages. Taliglucerase alfa from carrot cells does not require these additional processing steps because it is targeted to the cell vacuole where the complex type *N*-glycans are trimmed to the paucimannose form, exposing terminal mannose residues [105]. The marketing of glucocerebrosidase has taken advantage of an abbreviated approval process that allows direct progression from phase I to phase III trials because the drug falls within the scope of the Orphan Drug Act.

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10
Bioprocess Engineering of Plant Cell Suspension Cultures

Gregory R. Andrews and Susan C. Roberts

10.1
Introduction

In 1902 the Austro-German botanist Gottlieb Haberlandt reported the first attempts to cultivate plant cells *in vitro* [1]. Although Haberlandt failed to induce cell division, his bold hypotheses were eventually proven correct and inspired the work of numerous future scientists. For this reason, Haberlandt is credited with founding the science and technology of plant cell culture [2]. Routien and Nickell, in conjunction with Pfizer (New York, NY, USA), were granted the first patent for *in vitro* cultivation of plant cells in 1956, and describe the growth of a variety of plant cells in simple 20-l carboy systems [3]. It was not until 1983 that the first plant cell culture synthesized product, shikonin, a reddish-purple pigment used in the cosmetics industry, reached the market [4]. Since then, plant cell culture technology has experienced much success in the production of marketed, biologically active molecules (see Table 10.1) most notably the chemotherapeutic Taxol® (paclitaxel) and the therapeutic recombinant enzyme ELELYSO® (taliglucerase alfa). During the past 5 years, 288 patents have been issued pertaining to plant cell culture, more than twice the number issued in the previous 5-year interval, and a simple Web of Science search for "plant cell culture" returns 26 882 publications.

Specialized (formerly secondary) metabolites serve to protect plants from threats posed by their native environment; however, in many instances these molecules possess physical properties or biological activities of human interest. Plant cell culture offers a sustainable and economically favorable production platform for these valuable small molecules. Specialized metabolites typically represent <1% of the dry weight of the plant, rendering natural harvest low-yielding, environmentally destructive, and overall impractical [5]. For instance, 340 000 kg of *Taxus* bark or 38 000 trees are required to supply 25 kg of paclitaxel [5]. Harvesting from field-grown plants is further subject to seasonal availability, species abundance, and slow and variable growth rates [8]. Chemical synthesis of plant-derived natural

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Table 10.1 Products produced commercially via plant cell suspension culture [5–7].

Product(s)	Species	Manufacturer	Applications
Specialized metabolites			
Anthocyanins	*Euphorbia milii* *Aralia cordata*	Nippon Paint Co., Ltd (Japan)	• Textile dyes • Food colorants
Arbutin	*Catharanthus roseus*	Mitsui Chemicals, Inc. (Japan)	• Cosmetics (skin lightening agent) • Traditional medicine (antiseptic)
Arctic cloudberry-derived compounds	*Rubus chamaemorus*	Lumene Cosmetics (Finland)	• Cosmetic ingredients
Berberines	*Coptis japonica* *Thalictrum minus*	Mitsui Chemicals, Inc.	• Anticancer • Antibiotic • Anti-inflammatory
Betacyanins	*Beta vulgaris*	Nippon Shinyaku (Japan)	• Red-violet pigment • Food colorant
Bioactive compounds in Resistem™	Not disclosed	Sederma (France)	• Cosmetics
Carthamin	*Carthamus tinctorius*	Kibun Foods, Inc. (Japan)	• Red pigment • Food colorant and dye
Date palm-derived compounds	*Phoenix dactylifera* L.	XTEMcell (France)	• Cosmetic ingredients
Echinacea polysaccharides	*Echinacea* spp.	Diversa (now Phyton Biotech®, Germany)	• Immunostimulant • Anti-inflammatory
Geraniol	*Geraminea* spp.	Mitsui Chemicals, Inc.	• Essential oil • Primary component of rose, palmarosa, and citronella oils
Ginseng saponins	*Panax ginseng*	Nitto Denko Corp. (Japan) Unhwa Biotech Corp. (Korea)	• Dietary supplement • Dietary supplement • Cosmetics
Leontopodic acids A and B	*Leontopodium alpinum*	Istituto di Ricerche Biotecnologiche (IRB, Italy)	• Cosmetics

Paclitaxel	*Taxus chinensis*	Phyton Biotech®, and Samyang Genex (South Korea)	• Chemotherapeutic
Podophyllotoxin	*Podophyllum* spp.	Nippon Oil (Japan)	• Precursor to anticancer agents etoposide and teniposide
Rosmarinic acid	*Coleus blumei*	Sanofi (Germany)	• Anti-inflammatory
Scopolamine	*Duboisia* spp.	Sumitomo Chemical Co., Ltd (Japan)	• Anticholinergic • Antimuscarinic • Treatment of motion sickness, nausea, and intestinal cramps
Shikonin	*Lithospermum erythrorhizon*	Mitsui Chemicals, Inc.	• Dyes (deep red pigment) • Antibiotic
Recombinant proteins			
ELELYSO™ (Glucocerebrosidase)	*Daucus carota*	Protalix Biotherapeutics (Carmiel, Israel)	• Gaucher disease
Hemagglutinin-neuraminidase (HN) of Newcastle disease virus	*Nicotiana tabacum*	Dow AgroSciences LLC (Indianapolis, IN, USA)	• Poultry vaccine (currently not on market)
PRX-102 (α-Galactosidase-A)	*N. tabacum*	Protalix Biotherapeutics	• Fabry disease (currently in Phase I/II clinical trials)
PRX-106 (antitumor necrosis factor)	*N. tabacum*	Protalix Biotherapeutics	• Potential arthritis treatment (completed Phase I clinical trials)
PRX-110 (deoxyribonuclease I (DNase I, AIR DNase™))	*N. tabacum*	Protalix Biotherapeutics	• Cystic fibrosis (completed pre-clinical studies)
PRX-112 (oral glucocerebrosidase)	*N. tabacum*	Protalix Biotherapeutics	• Gaucher disease (completed Phase I clinical trials) • Therapeutic recombinant enzyme encapsulated in plant cells

products is of major scientific interest but often difficult to apply on a commercial scale. The presence of multiple rings and/or chiral centers complicates synthesis of specialized metabolites from commercially available precursors. Low product yields and harsh reagent requirements further limit the chemical synthesis of complex natural products on the often desired multi-kilogram scale. Many specialized metabolites are the product of complex and/or unknown biochemical pathways, eliminating the possibility of heterologous production in microbes.

Mammalian cell culture is currently the industrial workhorse of biopharmaceutical production, with 9 of the 11 biopharmaceuticals, or biologics, approved in 2014 produced with mammalian-cell-based platforms, 8 of which utilize Chinese hamster ovary (CHO) cells [9]. However, plant cell culture technology presents numerous advantages over traditional microbial and mammalian-cell-based recombinant protein production platforms. Plant cells, unlike their mammalian counterparts, are inexpensive to grow and maintain and, in contrast to microbes, are capable of producing complex, multimeric proteins possessing human-like post-translational modifications [10]. Plant cell culture processes are intrinsically safer, lacking the risk of human pathogen infestation or endotoxin production associated with mammalian culture and microbial fermentation, respectively. Compared to recombinant protein production in greenhouse or field-grown plants, plant cell culture processes occur on timescales of days or weeks compared to months and can be carefully monitored throughout the entire process, which facilitates regulatory compliance. Compared to differentiated plant cell cultures such as shoot and root cultures, suspensions of dedifferentiated plant cells are more convenient for large-scale production processes and can be cultivated in existing microbial and mammalian cell culture vessels with only minor modifications.

Here we describe general bioprocess strategies, considerations, and infrastructure applied to the cultivation of plant cell suspension cultures beyond the laboratory scale, and in particular how they are influenced by the unique properties intrinsic to plant cells. The chapter focuses on bioprocessing of plant cell suspensions for production and purification of specialized metabolites and recombinant proteins, and culminates in detailed summaries of two plant-cell-based culture processes used in the synthesis of United States Food and Drug Administration (FDA)-approved therapeutics.

10.2
Culture Development and Maintenance

Initiation of plant cell suspension cultures is preceded by callus formation. In short, an excised piece of plant tissue, or explant, is surface-sterilized and placed on a solid medium containing the appropriate nutrients and hormones, exact concentrations of which are largely determined by empirical methods (Figure 10.1).

Cells on the surface of the explant begin to grow, divide, and dedifferentiate to form an unorganized mass of cells, or callus. After generally 2–6 weeks, the primary callus will have grown sufficiently large to subculture onto fresh medium with selective removal of necrotic parts [12].

Suspension cultures are prepared by inoculating friable callus, typically 50–100 g l^{-1} or ~10% packed cell volume (PCV), in liquid medium [13]. Agitation of culture medium dislodges clumps of cells, or aggregates, which move freely in suspension. The heterogeneity exhibited by newly formed cell suspensions leads to unstable growth and inconsistent product formation, two characteristics

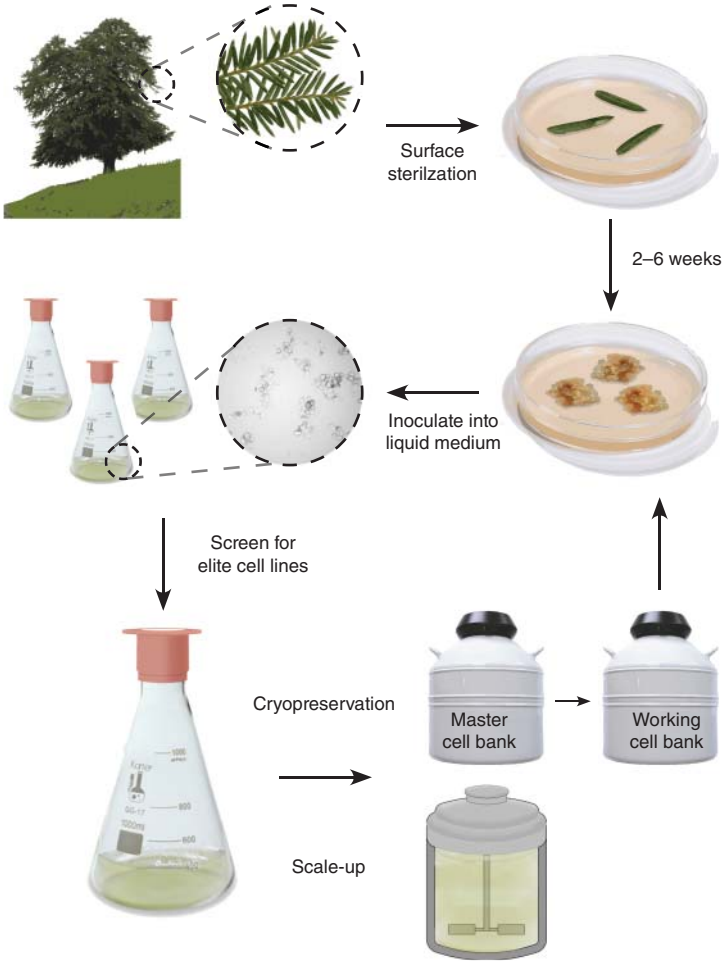


Figure 10.1 Initiation and propagation of plant cell suspension cultures. Cell image is from [11].

detrimental to bioprocesses at any scale. Cultures are typically homogenized during the first several subpassages by selecting aggregates of similar size through filtration or settling. Smaller aggregates are usually preferred for ease of handling, although the degree of aggregation has been shown to significantly impact specialized metabolite production (see Section 10.4.3). Generation times between 7 and 21 days are preferred; shorter times result in the rapid occurrence of genetic instability, often attributed to somaclonal variation (e.g., chromosome rearrangement, changes in ploidy, DNA methylation), while longer generation times increase the duration of the production processes. The entire process, from plant to stable suspension culture, may take 6–9 months; however, a stable cell line is critical for consistent product formation and compliance with current good manufacturing practices (cGMP) [12].

Although plant cells grow and divide indefinitely in culture, risk of contamination, high costs of culture maintenance, and decrease in culture production associated with long-term culturing predicate the need for a long-term storage solution. For example, production levels of hGM-CSF in tobacco NT-1 suspension cultures decreased >80% after 250 generations [14]. Cryopreservation of plant cultures, although a more complicated process than for microbial and mammalian cells, is a strict cGMP requirement for all cell-culture-based processes [15]. Regulatory agencies, such as the FDA and the European Medicines Agency (EMA), require a two-tiered cell banking system whereby a single, well-characterized clone is used to create a master cell bank. A working cell bank derived from the master cell bank is used for scale-up and production.

Cryopreservation techniques for plant cell suspension include controlled slow freeze, vitrification, and desiccation with or without encapsulation in alginate beads, although the exact procedure is cell-line-specific [12]. Phyton Biotech® has developed a robust, vitrification-based cryopreservation procedure that is used in the long-term storage of a plant cell culture collection comprised of ~500 species [6, 16]. Because of the plant cell's high water content, a critical component of all cryopreservation protocols is a controlled dehydration to prevent ice crystal formation upon freezing. A less sophisticated and more cost-effective solution to cryopreservation is the storage of *in vitro* cultures at 0–15 °C [17]. The reduced temperature greatly reduces metabolic activity and therefore culture growth. Although these conditions permit culture storage only for 4–6 months, the process is less damaging than cryopreservation and allows cells to be recovered more quickly and easily for use in scale-up and subsequent bioprocesses.

10.3 Choice of Culture System

In vitro specialized metabolite production is limited to cultures derived from species that synthesize the specific compound or possess a majority of the necessary biochemical pathway. Since *in vitro* production rates can be quite low, plant species known to produce the compound of interest are typically screened

to enable high yields. Preliminary studies in the development of the plant-cell-suspension-based production process of the chemotherapeutic paclitaxel determined that cultures derived from *Taxus chinensis* were the best source of the high-value molecule [18]. Additionally, cultures derived from different parts of the plant may produce varying amounts of the desired compound. For example, although paclitaxel accumulates in the bark of *Taxus* species, it was found that cultures generated from needles were the most productive *in vitro* [18].

Screening for elite cell lines can be performed while the culture of interest is in callus or suspension form, regardless of the nature of the desired product (specialized metabolite or recombinant protein). If the molecule of interest is a pigment, high-producing cell aggregates may be selected visually, as was performed in the development of commercial-scale processes for berberine and shikonin by Mitsui Chemicals, Inc. [19]. Brute-force screening techniques have given way to more sophisticated methods including flow cytometry and the feeding of precursor analogs [20, 21]. A large number of suspension cultures may be grown and screened in 50-ml Cultiflasks [22]; these modified polypropylene centrifuge tubes allow relatively high throughput screening under conditions most similar to those observed in large-scale cultivations.

Heterologous protein production on an industrial scale is limited to a handful of well-characterized systems, most notably those utilizing tobacco and carrot cells [23]. The time to initiate stable suspension cultures and develop effective gene transformation procedures makes using well-studied cell lines advantageous. Standardization of *in vitro* hosts for therapeutic protein synthesis also facilitates regulatory approval of plant-cell-based production platforms.

Tobacco plants served as host for the first plant-derived recombinant protein to be produced in the laboratory [24]. Fast-forward 25 years to today, where tobacco suspension cells, especially those from the closely related BY-2 and NT-1 cultivars, are used for large-scale recombinant protein production [25, 26]. Production of numerous therapeutic proteins including antibodies, enzymes, antigens, hormones, and cytokines has been achieved in tobacco suspension cultures [27–32]. Favorable growth characteristics, straightforward transformation procedures, and inexpensive nutritional requirements make tobacco cells ideal hosts for recombinant protein synthesis [10]. Under optimal conditions, tobacco BY-2 cells can multiply 80- to 100-fold in 1 week, and their extensive use and study has led them to be coined the "HeLa cells" of plant biotechnology [33]. Fraunhofer and Dow AgroSciences' BY-2 based production platform CONCERT® yielded the first plant-cell-derived recombinant protein to achieve market approval [27]. Although the vaccine antigen was intended for use in poultry and is no longer on the market, CONCERT® served as a powerful proof-of-concept of the capabilities of plant-cell-based production platforms.

Carrot cells have recently emerged as suitable hosts, especially with the FDA approval of ELELYSO® (taliglucerase alfa), a therapeutic recombinant enzyme produced on a commercial scale in carrot suspension culture. Indicated for long-term enzyme replacement therapy for patients with Gaucher disease, ELELYSO® is the first and only FDA-approved, plant-derived biotherapeutic

[34]. Carrot cells are a model *in vitro* system and their study has allowed for a greater understanding of the basic principles of plant cell culture including callus formation, totipotency, somatic embryogenesis, and somaclonal variation [35]. Like tobacco suspensions, carrot cultures are characterized by rapid growth and numerous published gene transformation procedures. Carrots are also a component of the human diet worldwide, making them ideal hosts for the oral delivery of recombinant therapeutics, a grand challenge in the biopharmaceutical industry.

Although large-scale recombinant protein production is limited to a handful of well-studied cell lines, the feat has been achieved on a laboratory scale in cultures derived from numerous plants including rice and soybean [36, 37]. The field is also not restricted to plant cell suspensions, as heterologous protein synthesis in differentiated organ cultures and whole plants has proven quite successful [38, 39]. Recombinant protein production in plant cell culture is of great interest, as plant cells are easily grown on simple media (similar to bacteria) but are capable of producing complex, properly folded, multimeric proteins complete with post-translational modifications (different from bacteria, see Table 10.2). Although both plant- and mammalian-cell-based platforms are capable of producing mature and properly folded proteins, the costs associated with the latter are far greater because of the complex medium requirements and the potential for human pathogen contamination which leads to an increase in downstream processing costs.

Table 10.2 Properties of recombinant proteins from various expression systems.

Feature	Bacterial	Mammalian	Plant
Protein expression level ($g\,l^{-1}$)	0.5–5	1–3	0.01–0.2
Molecular weight	Low	High	High
Post-translational modification	None/incorrect	Yes	Yes
Glycosylation	None/incorrect	Yes (human-like)	Yes
Protein folding accuracy	Low	High	High
Protein homogeneity	Low	High	High
Multimeric protein assembly	No	Yes	Yes
Disulfide bond formation	Limited	Yes	Yes
Cell-line stability	High	Low	Medium
Potential contaminants	Endotoxins	Virus, prions, oncogenic DNA	None
Production timescale	Short	Long	High, long
Downstream purification cost	High	High	Low
Production cost	Low	High	Low

Source: Adapted from [40].

Table 10.3 Comparison of characteristics of plant, mammalian, and bacterial cells [41, 42].

Characteristic	Bacterial	Mammalian	Plant
Shape	Spherical	Spherical	Spherical/cylindrical
Size (μm)	<1	10–50	20–200
Cell wall	Yes	No	Yes
Water content (%)	75	50–70	>90
Doubling time (h)	<2	20–48	20–200
Cultivation time	Days	Days/weeks	Weeks
Product accumulation	Typically extracellular	Typically extracellular	Typically intracellular
Variability in accumulation	Low	Low	High
Product yields	High	High	Low
Cellular aggregation	No	No	Yes
Contamination risk	Low	High	Low
Shear sensitivity	Not sensitive	Very high	High
Compartmentalization	None	Compartmentalized	Compartmentalized
Cryopreservation techniques	Well developed	Well developed	Immature
Oxygen uptake rate (mmol l^{-1} h^{-1})	10–90	0.05–5	5–10
$k_L a$ required (h^{-1})	10–1000	0.25–10	10–50

10.4 Engineering Considerations

Many of the large-scale cultivation techniques applied to plant cell suspensions are derived from microbial and mammalian cell fermentation and culture strategies, respectively. Plant cells, however, possess many unique characteristics that warrant special consideration during bioprocess design (Table 10.3).

10.4.1 Cell Growth and Morphology

Typical plant cells range from 10 to 100 μm in length and are semispherical or rod-like in shape. Extended subculture intervals promote cellular elongation, resulting in overall changes in morphology [13]. Cell shape has been shown to contribute significantly to the rheological properties of the medium, with an elongated cellular morphology associated with an increase in culture viscosity [43]. Plant cell suspension cultures typically exhibit doubling times of several days, far greater than those of microbes but comparable to cultured mammalian cells. Plant cells are also capable of achieving relatively high densities (>60% PCV) in suspension culture [44]. Biomass levels in large-scale cultivations are measured offline using traditional methods including gravimetric determination of fresh or

dry weight and measurements of packed or settled cell volume, or online by using the linear relationship between medium conductivity and culture growth [45, 46].

10.4.2 Gas Requirements

Most plant cell suspensions are heterotrophic, that is, are unable to fix carbon dioxide, because of the lack of chloroplasts (a result of culturing in the dark) and consequently consume oxygen for cellular respiration and metabolism while producing carbon dioxide. Oxygen uptake rates (OURs) of 5–10 mmol l^{-1} h^{-1} are typical, and lower than those observed in microbial cultivations due to the reduced growth rate. High oxygen concentrations may be even toxic to plant cells and displace other necessary gaseous components from the medium [47]. It is typical for culture OURs to increase with biomass levels during exponential growth. Dissolved oxygen is generally maintained above the critical oxygen concentration, found to be 15–20% of air saturation [45]. Low OURs allow conventional fermentation equipment to be easily modified to accommodate plant cell suspensions. Carbon dioxide, often considered an essential nutrient, can have a positive effect on cell growth but has also been shown to inhibit specialized metabolite production at high concentrations [48]. Ethylene, a plant hormone most often associated with fruit ripening *in vivo*, can accumulate in culture vessels at concentrations on the order of tens of ppm [49]. Ethylene has been shown to both inhibit and promote culture growth depending on the species, making it important to determine its specific effects on the culture of interest [49]. The optimized gas mixture composition for paclitaxel production in suspension cultures of *Taxus cuspidata* was found to be 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide, and 5 ppm ethylene [48].

10.4.3 Aggregation

Plant cells in suspension tend to grow as aggregates composed of two to a few hundred cells and ranging in size from 100 μm to >2 mm [50]. Aggregation is a result of a failure to completely separate following cell division, and is dependent on plant species, medium composition, growth stage, and culture conditions [27, 50]. Cellular aggregation is often necessary for proper cell growth and metabolism; however, large aggregates can settle to the bottom of a bioreactor resulting in decreased cell viability, undesired oxygen and nutrient gradients, and complicated downstream processing. The extent of cellular aggregation has been shown to significantly impact specialized metabolite production in an unpredictable manner. For example, aggregate size was shown to correlate positively with phenylpropanoid production in strawberry suspension cultures [51], while the opposite

phenomenon was observed for paclitaxel production in *Taxus* suspension cultures [52]. Large aggregates, prone to both sedimentation and damage due to hydrodynamic shear, pose a technical challenge in the development of agitation and aeration strategies. One must consider both the oxygen/nutrient gradients that arise within the aggregate itself, as well as the culture vessel configuration.

10.4.4 Medium Rheology

At low cell densities, plant culture medium behaves as a Newtonian fluid. However, as culture density increases, so does the viscosity. Plant cell cultures can grow to be incredibly dense (over 50% PCV), and are therefore often highly viscous [53]. Polysaccharides secreted into the growth medium during the later stages of culture growth also contribute to the increase in viscosity [54, 55]. As viscosity increases, culture broth begins to behave as a non-Newtonian fluid, restricting heat and mass transfer. The resulting nonuniform temperature and nutrient distributions lead to "dead zones" in a bioreactor [47]. Dense, viscous suspension cultures exhibit shear thinning or pseudoplastic properties [19]. Regions of high shear, for example, near the impeller, are characterized by a lower apparent viscosity and more efficient mixing. As you move further from the impeller, apparent viscosity increases, and mixing efficiency decreases. Medium rheology, especially at high cell densities, largely dictates the choice of the impeller system in a stirred-tank reactor (STR) and the air-flow rate of a pneumatically driven reactor.

10.4.5 Shear Sensitivity

Plant cells are relatively large in size (20–200 μm), contain water-filled vacuoles that may occupy 90–95% of their volume, and are surrounded by a thin, rigid, cellulose-based cell wall, making them susceptible to hydrodynamic shear, especially during late exponential and early stationary phases when the cellular water content is the highest [45, 47, 56]. Increasing shear has been associated with decreased cell viability, release of intracellular metabolites, changes in aggregation, and shifts in cellular metabolism. Shear susceptibility can vary among cultures of the same cell line, depending on culture age and cultivation regime [45]. Impellers are the largest source of shear stress; however, gas sparging is also known to induce shear damage, especially in the bubble generation zone, the rising zone through the bulk fluid, and the surface of the suspension culture. Early studies showed a negative correlation between specialized metabolite production and cultivation in stirred-tank fermenters as opposed to airlift fermenters, demonstrating the potential negative effects of shear stress on cultured plant cells [57]. However, studies published a decade later showed that shear forces were not

as detrimental as was once thought, and today most of the industrial plant cell suspension-based production processes use STRs [56, 58].

10.5 Bioprocess Parameters

10.5.1 Medium Composition and Optimization

Plant cell suspensions are generally cultivated in a basal medium composed of a carbon source and micro- and macronutrients, which is further supplemented with phytohormones and/or growth regulators. Sucrose (2–5%) is the most common carbon source and is hydrolyzed to monosaccharides such as glucose and fructose during autoclaving or by invertases secreted into the medium [13]. Numerous basal medium formulations exist, such as those developed by Murashige and Skoog (MS) [59], Linsmaier and Skoog (LS) [60], Schenk and Hildebrandt (SH) [61], and Gamborg (B5) [62]. All differ widely in composition, and the optimal medium is best determined empirically. For example, LS medium differs from the other formulations in its high levels of nitrate, potassium, and ammonia. Nitrogen source and concentration significantly impact plant cell growth and production. Increasing the amount of nitrate in the medium of transgenic tobacco BY-2 cultures increased accumulation of recombinant antibody 20-fold [31]. Phytohormones, cytokinins, and auxins may be supplemented alone or in tandem, and affect both the extent of differentiation or dedifferentiation and product formation in plant cell cultures [63]. The overall process of medium optimization may be performed "the old-fashioned way" by varying one factor at a time or by utilizing statistical experimental techniques such as factorial design and response surface methodology [64].

10.5.2 Temperature and pH

The temperature is generally maintained between 23 and 29 °C for plant cell cultivation. Attempts to optimize temperature are often futile because of gradients and fluctuations that arise in large vessels primarily due to insufficient mixing. The fresh culture medium pH is typically adjusted to be between 5.0 and 6.0 before sterilization and is generally not controlled throughout the process [6]. Plant cells can tolerate a wide pH range, and media components often serve as buffers, making the addition of acid and base during cultivation unnecessary. Conversely, the stability of secreted proteins and metabolites is highly dependent on pH, and optimum pH values must be determined to maximize the recovery of the desired product. For example, maintaining the pH between 6.4 and 6.8 during the production phase in transgenic tobacco cultures simultaneously increased stability of the human protein product and reduced protease activity [65]. The authors attributed the observed increase in stability to the medium pH closely resembling that of the

protein's native physiological environment. The production of shikonin in *Arnebia* spp. cell cultures was greatest at a pH of 9.75, consistent with the optimum pH range of a critical metabolic enzyme [66].

10.5.3 Agitation

Mixing promotes culture growth by dispersing nutrients to cells while preventing accumulation of both heat and toxic metabolites. Inadequate mixing in large-scale bioreactors causes a decrease in mass transfer efficiency and unwanted concentration gradients. Mechanical agitation is most frequently applied, often with impellers that have been specifically designed to cater to the physiochemical properties of plant cells (see Section 10.7). High agitation rates can be harmful to shear-sensitive cultures. For example, agitation rates >160 rpm resulted in a sharp decrease in viability and hCTLA4Ig production in transgenic rice suspensions cultivated in a 3-l STR [67]. Agitation can be decreased in shear-sensitive cultures by reducing the impeller speed; however, this strategy can result in inadequate mixing and reduced oxygen and heat transfer rates in high-viscosity cultures. Low agitation also promotes the formation of larger aggregates that can alter medium rheology and metabolic capabilities of the culture [50, 68].

10.5.4 Aeration

Aeration is responsible for maintaining the concentration of dissolved gases, desorption of volatile compounds, and dissipation of heat generated by metabolic processes. Agitation intensity, bubble dispersion, and the culture medium's capacity for dissolved gases (e.g., oxygen) influence aeration [69]. Optimum air flow rates range between 0.1 and 0.5 vvm (**v**olume of air (l) passing through a given **v**olume of medium (l) per **m**inute) for plant cell suspensions but may be adjusted to accommodate the physiological state of the cells [45, 70]. An aeration rate of 0.4 vvm was found to be ideal for the culture of ginseng suspension cells in 3-l balloon-type bubble bioreactors (BTBBs) but detrimental to the growth and recombinant protein production of transgenic rice suspensions in a 3-l STR, a result attributed to shear sensitivity [67, 71]. The volumetric mass transfer coefficient ($k_L a$) offers a direct measure of oxygenation of the culture medium and is modulated through agitation and aeration. As the viscosity of the culture medium increases, $k_L a$ decreases, requiring more intense agitation and increased oxygen transfer. High levels of aeration can lead to excessive foam production due to the presence of extracellular polysaccharides, proteins, and fatty acids. Cells become entrapped in the foam and begin to form a thick layer that adheres to the vessel and probes. As these cells change metabolically and become membrane-compromised, they secrete inhibitory substances such as proteases or broken down organelles. Bubble-free aeration with thin-walled silicone membrane tubing is capable of eliminating wall growth, but it is not suitable for scale-up, especially of viscous cultures, due to reduced oxygen transfer. Other strategies

to counteract foam formation include the addition of antifoam and mechanical foam disruption [44].

10.6 Operational Modes

The standard operational strategies applied to mammalian and microbial systems, such as batch, fed-batch, repeated-batch, two-stage culture, perfusion culture, and semicontinuous and continuous culture, can be applied to plant cell suspension systems [40]. The choice of the process mode is dependent on culture dynamics (e.g., growth rate, aggregation, foaming, and specialized metabolite production), desired product (i.e., heterologous protein or specialized metabolite), accumulation site (i.e., intracellular or cell-associated implying either extracellular or associated with the cell wall), product stability, and the relationship between culture growth and product accumulation. The production of growth-associated products is improved by increasing the growth rate and biomass density and prolonging the exponential growth phase [40]. When product accumulation is not coupled with culture growth, production is generally induced or elicited once a high biomass level is achieved during late or post exponential growth [72].

Batch processes are the simplest of all the cultivation strategies but are limited by the significant amount of time required between cycles to clean and sterilize the system as well as accumulation of toxic or inhibitory by-products that may significantly limit culture production [73]. Batch processes are the most straightforward to scale-up, but product accumulation does not often scale predictably because of nutrient gradients that arise when changing vessel size and geometry [74]. The simplicity of batch cultivations makes them convenient for use in small-scale experiments in the development of fed-batch or continuous operations. Culture growth typically slows at a PCV of ~60–70% in batch cultivations resulting from an overall reduction in cellular metabolism [40]. The cultivation of plant cell suspensions in fed-batch mode typically involves the intermittent or continuous addition of carbon sources (e.g., sucrose, glucose, fructose, and maltose), amino acids, or chemical elicitors [45, 75–77]. Fed-batch cultivation can be performed with or without feedback control, and an optimized feeding strategy can simultaneously increase biomass concentration and reduce the accumulation of inhibitory products [45]. An optimized sucrose feeding strategy improved paclitaxel production in *T. chinensis* cultures by nearly a factor of 5 compared to batch cultivation in a 5-l STR [78].

Continuous culture can be used for the production of growth-associated products. Product inhibition is eliminated by the addition of fresh medium and removal of culture medium and cells. Continuous cultivation requires that steady state has been reached, which for plant cell suspensions takes approximately 2–4 residence times [45]. Perfusion cultivation achieves higher biomass concentration than continuous culture and effectively separates active cells from the toxic medium. Dry cell weights exceeding $20\,g\,l^{-1}$ and twice that achieved during batch cultivation have been reported [79, 80]. Perfusion culture

is particularly advantageous when the desired product is secreted into the medium and/or prone to degradation [81]. High cell retention is key to successful perfusion cultivation and becomes more difficult to obtain with increasing scale. Specific growth rate can be modulated in both continuous and perfusion cultivation by adjusting the dilution rate and bleed stream, respectively [68].

Two-stage cultivation is most effective when growth and production phases are independent, as in elicited specialized metabolite production or metabolically induced recombinant protein production [82–85]. This mode of operation is particularly advantageous because it allows conditions for biomass growth and product accumulation to be independently optimized. Chemical inducers and elicitors require optimization of timing and concentration to maximize protein and specialized metabolite yields, respectively. Two-stage cultivation of American ginseng (*Panax quinquefolius*) suspension cultures in a 5-l STR resulted in a four-fold increase in total saponin yield compared to fed-batch cultivation. The second stage of the cultivation regime involved a combination of elicitors (100 mg l^{-1} lactalbumin hydrolysate and 2 mg l^{-1} methyl jasmonate) [86]. Recombinant protein production driven by the sugar-starvation rice α-amylase 3D (RAmy3D) promoter system requires medium exchange to a sugar-free medium to induce production; however, this shift can significantly reduce culture viability [85]. Two-stage cultivation can significantly increase non-growth-associated product yields compared to simpler process strategies, but can be technically challenging and labor intensive to apply at large scales.

10.7 Bioreactors for Plant Cell Suspensions

The purpose of any bioreactor is to provide optimum conditions for biomass cultivation and/or product formation by precisely controlling the chemical and physical properties of the culture environment. Plant cell suspensions pose unique challenges for bioreactor design by requiring a generous mixing of viscous, high-density culture medium while maintaining a low shear environment. Traditional bioreactor configurations, including STRs and pneumatically driven airlift and bubble column reactors, have been adopted from microbial fermentation with only minor modifications for plant cell suspension cultivation. Novel bioreactors have been designed that cater to the unique shear sensitivity of plant cells, many of which are disposable, allowing plant cell biotechnology to keep pace with recent trends in biopharmaceutical production. The most commonly used and promising configurations are described in the following and summarized in Table 10.4.

10.7.1 Conventional Bioreactors

10.7.1.1 Stirred-Tank Reactors

STRs are the most commonly used bioreactors for cultivation of plant cell suspensions, as they are characterized by simple scale-up, high volumetric mass

Table 10.4 Comparison of bioreactors for plant cell suspension culture.

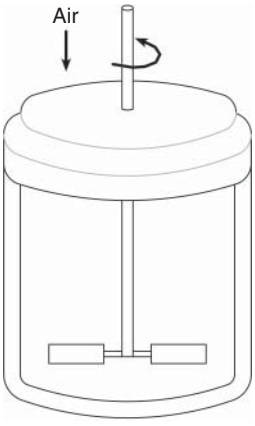
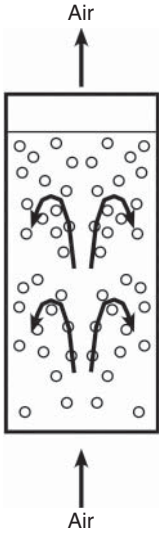
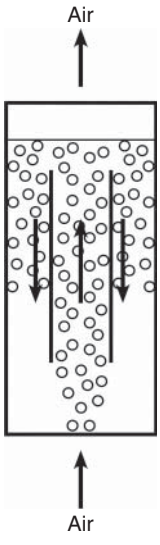
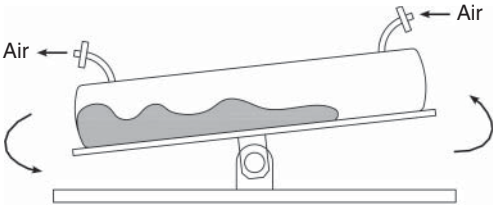
Bioreactor type	Advantages	Disadvantages
Stirred-tank reactor (STR) 	• Widely studied/implemented • Easily scaled-up • Enhanced transport • Can accommodate highly viscous cultures • Multiple impeller designs are available • Ease of process monitoring and control • Established compliance with cGMP regulations	• High-shear environment (particularly near impeller) • Significant capital investment and operational costs • High energy use • Risk of contamination due to mechanical seal
Bubble column reactor 	• Low-shear environment • Absence of mechanical mixing reduces heat generation • Simple construction and scale-up • Low contamination risk • Low operational costs	• Poor oxygen transfer • Inadequate mixing for high-density cell cultures • Excessive foam generation at high aeration rates

Table 10.4 (*continued*)

Bioreactor type	Advantages	Disadvantages
Airlift reactor 	• Low shear environment • Absence of mechanical mixing reduces heat generation • Simple construction and scale-up • Low contamination risk • Low operational costs • Multiple internal draft tube configurations • Enhanced oxygen transfer compared to bubble column • Circulating flow patterns aid in nutrient and gas transfer and prevent bubble coalescence	• Poor oxygen transfer • Inadequate mixing for high-density cell cultures • Excessive foam generation at high aeration rates
Wave reactor 	• Disposable • High oxygen and mass transfer • Low-shear environment • Bubble-free aeration • Ease of construction and operation	• Difficult scale-up • Difficult to apply advanced cultivation strategies • Low heat transfer rate

transfer, and an established history of compliance with cGMP [73]. However, STRs represent a significant upfront investment in capital, are difficult to seal, demand large amounts of power, and produce a high-shear environment. The impeller system is the defining component of an STR and largely determines the vessel's internal environment (Figure 10.2). Rushton turbines, most frequently used in microbial fermentation, have been used in the cultivation of plant cell suspensions but are not suitable for shear-sensitive plant cell cultures [87]. Although capable of providing complete culture mixing, Rushton turbines exhibit a high energy dissipation rate and may cause shear damage at modest agitation speeds [87]. To reduce the amount of damage due to hydrodynamic shear, numerous impeller designs have been evaluated for plant cell suspensions (e.g., helical ribbon, marine impeller, pitched-blade impeller, Intermig impeller,

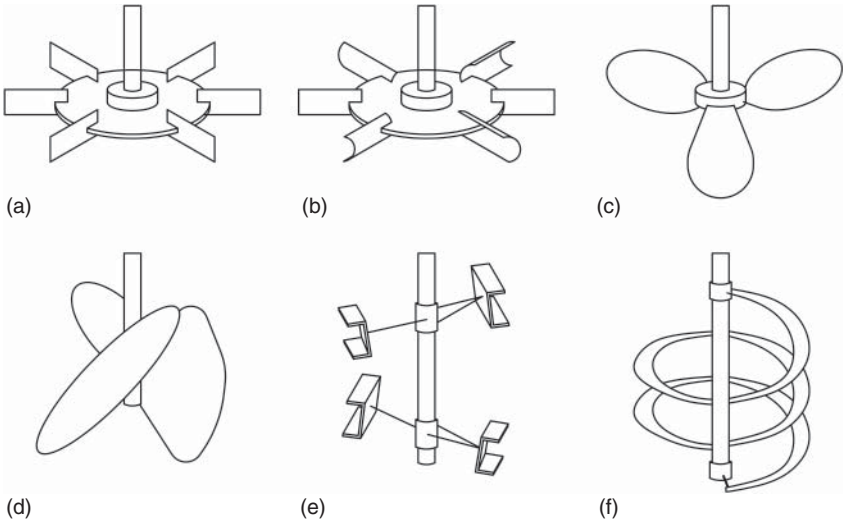


Figure 10.2 Impellers typically used for plant cell suspensions. (a) Rushton turbine, (b) rushton turbine (curved-blade), (c) marine impeller, (d) pitched-blade impeller, (e) Intermig® impeller, (f) helical-ribbon impeller.

see Figure 10.2), some developed specifically for plant cells and others adopted from mammalian cell cultivation. The centrifugal impeller bioreactor has been successful in the cultivation of high-density shear-sensitive plant cell suspensions [88–91]. The rotation of the propeller generates an area of negative pressure at the bottom of the vessel, drawing cells and the medium upward and forcing them into bulk flow. Generally, impeller systems exhibiting axial flow patterns and low tip speeds (up to 2.5 m s^{-1}) are considered suitable for plant cell cultivation [92].

Novel aeration systems (such as bubble-free aeration, gas basket, and cage aeration) have been developed for cultivating the most shear sensitive cells. Bubble-free aeration has been shown to provide sufficient aeration and completely eliminate foam formation [93, 94].The internal geometry of the STR contributes significantly to the reactor's internal environment and critical aspects such as the shape, location, and quantity of baffles, spargers, and impellers, and the ratio of height to the vessel diameter must be optimized to achieve the desired conditions. Most of the existing industrial-scale plant cell culture processes use conventional STRs. Phyton Biotech® operates the largest cGMP plant cell culture facility in the world, comprising a series of stainless steel STRs up to 75 000 l in volume and used in the production of paclitaxel from *Taxus* suspension cultures [58].

10.7.1.2 Pneumatic Bioreactors

Pneumatically driven bioreactors, including air-lift and bubble column bioreactors, are characterized by a cylindrical vessel in which aeration and agitation are

provided by the introduction of compressed air or a gas mixture through the bottom of the vessel. The lack of mechanical mixing presents a low-shear environment, which is ideal for the cultivation of even the most shear-sensitive cells. The absence of moving parts and seals ensures long-term sterility and reduces operation and investment costs compared to STRs. Air-lift bioreactors are simply bubble columns that contain a draft tube (internal loop) or an external loop. The resulting cyclic fluid motion prevents bubble coalescence, enhances oxygen transfer, evenly distributes shear forces, and provides overall shorter mixing times [40]. Pneumatic bioreactor operation at high cell densities is often plagued by insufficient fluid mixing and unwanted gradients in biomass, nutrient, and oxygen concentrations. Increasing aeration to support high biomass levels results in excessive foaming, which may trap cells and interfere with probes and filters, and creates regions of high shear, especially at the gas–liquid interface, the sparger, and the bulk flow area where bubbles rise, which can significantly impact cell growth and viability [95]. Pneumatic bioreactors are therefore typically used for the cultivation of the most shear-sensitive cells at a modest biomass concentration [40]. The ProCellEx™ platform developed by Protalix Biotherapeutics uses disposable pneumatic bioreactors to produce recombinant proteins in commercial quantities in transgenic tobacco and carrot cultures.

10.7.2 Disposable Bioreactors

Disposable bioreactors represent the current state of the art in cell cultivation and biopharmaceutical production [96–99]. Single-use vessels are discarded after harvesting, reducing the risk of cross-contamination and decreasing turnover time by eliminating the need for post-cycle sanitation and sterilization. Single-use cultivation vessels are composed of FDA-approved plastics (e.g., polyethylene, polystyrene, polytetrafluoroethylene, polypropylene, or ethylene vinyl acetate), facilitating regulatory approval of the overall production process [97]. Compared to traditional stainless steel vessels, disposable bioreactors require less up-front capital investment and simpler housing facilities.

Wave bioreactors consist of a sterile plastic bag containing the culture medium and cells placed on a platform capable of rocking in one or two dimensions [100]. Rocking generates a wave motion that provides agitation and bubble-free aeration with minimal shear stress and foam formation. The headspace is continuously aerated and ventilated through an exhaust. Mass and heat transfer are both functions of the rocking rate, rocking angle, bag type, and geometry and culture volume [100]. Shear stress is highest at minimum culture volumes and maximum rocking rate and angle. Wave bioreactors are characterized by simple operation, have a low risk of contamination, and are the most cited disposable vessel for the cultivation of plant cell suspensions [97]. In addition, wave bioreactors are capable of cultivating cells in batch, fed-batch, repeated fed-batch, continuous, and continuous perfusion modes [101]. Transgenic rice cultures grown in a wave bioreactor exhibited similar maximum biomass and recombinant protein levels as those grown in

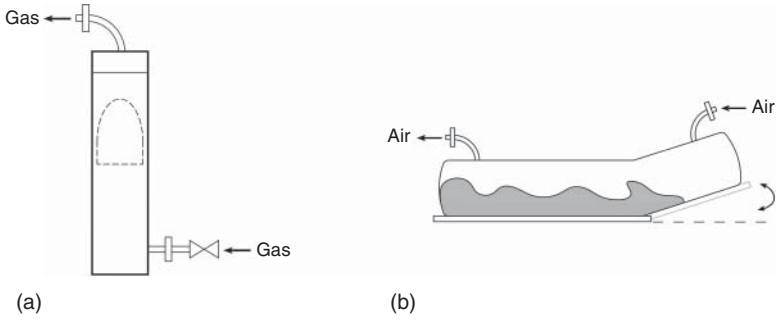


Figure 10.3 Disposable bioreactors developed specifically for plant cell suspensions. (a) Slug-Bubble bioreactor. (b) wave and undertow (WU) bioreactor.

an STR of the same volume (5 l) [102]. Although mixing times observed in wave bioreactors are comparable to those of STRs, they vary unpredictably with the bag size, thus complicating scale-up.

Two novel disposable bioreactors have been developed specifically for plant cell cultivation: the Slug-Bubble (SB) bioreactor and the Wave and Undertow (WU) bioreactor [103] (Figure 10.3). The SB bioreactor is comprised of a vertical cylinder in which agitation and aeration are provided by the intermittent generation of large cylindrical bubbles at the bottom of the vessel. Size and quantity of these "slug bubbles" are determined by the inlet pressure and valve opening time. These long, bullet-shaped bubbles occupy the entire cross section of the tube and are characterized at the tail end by a region of strong mixing and enhanced transport. SB bioreactors have been shown to exhibit $k_L a$ values comparable to those of STRs up to 70 l working volume. The WU bioreactor is similar to the wave-mixed bioreactor in that it consists of a plastic container placed on a moving platform. However, the intermittent raising/lowering of only one side of the platform provides culture agitation. Rising of the platform creates a wave that propagates through the vessel, generates an undertow at the opposite end, and returns back. The WU bioreactor provides low-shear agitation and bubble-free aeration. The WU bioreactor exhibits a $k_L a$ value less than that in traditional STRs but comparable to that in wave bioreactors of up to 100 l working volume. Nestle R&D Centre (Tours, France) has since reported increases in volumes of the SB and WU bioreactors to 175 and 750 l, respectively [104].

There are challenges that prevent the universal adoption of disposable bioreactor technology. Disposable vessels are limited in size because of the strength, or lack thereof, of the plastic materials of which they are made. This problem can be overcome by implementing a horizontal scale-up strategy, as opposed to the traditional vertical scale-up approach. Commercial availability of reliable, disposable components for process monitoring such as sensors and valves is also limited [105]. An important challenge is the leaching of components, such as polymer layers and adhesives, from the plastic vessel, which may interfere with product quality. Many of the materials that comprise single-use systems were "grandfathered

in" or approved before strict testing was a requirement, and therefore have not undergone rigorous evaluation regarding long-term exposure [106].

10.8 Downstream Processing

An economical and cGMP-compliant downstream process relies on the use of standardized infrastructure that blends well with upstream operations. Since the upstream processes associated with plant cell suspension cultures largely parallel those of animal and microbial cells, commercially available downstream processing equipment may be applied to large-scale plant cell culture with only minor modifications. Because of the complex biological matrix and variable product secretion associated with plant cells, the extraction process is largely tailored to the culture system while purification is customized to the desired product.

10.8.1 Specialized Metabolite Extraction and Purification

Extracellular metabolites may be extracted directly from the liquid phase after removal of biomass by filtration or centrifugation. Cell-associated metabolites require cell disruption via homogenization, sonication, steam explosion, or enzymatic digestions [45]. The use of cell-wall-digesting enzymes resulted in 90% of the total paclitaxel in *Taxus* cultures accumulating in the extracellular medium [107]. Liquid–liquid extraction is performed on the culture medium or the aqueous homogenate with the extraction solvent chosen by the adage "like dissolves like" based on the physiochemical properties of the product. The material can be selectively extracted with a series of organic solvents of increasing polarity or totally extracted with an alcoholic solvent that efficiently extracts a broad range of polar metabolites. Even if the metabolite is soluble in aqueous medium, a liquid–liquid extraction with an organic solvent is favorable because of the high boiling point of water and the presence of polar contaminants (e.g., pigments and tannins) in aqueous plant extracts [108]. Other factors contributing to solvent selection include selectivity, recoverability, viscosity, toxicity, stability, availability, and cost [108].

Organic solvents for use in the preparation of pharmaceuticals are regulated and classified by the FDA and EMA [108]. Solvents are divided into three classes. Class I solvents should never be used in the preparation of drug products because of their detrimental effects on human health and the environment. These solvents include benzene, a known carcinogen, and carbon tetrachloride, an ozone-depleting greenhouse gas. Class II solvents may be used; however, their residual concentration in any product is highly regulated because of their inherent toxicity. These solvents include acetonitrile, chloroform, methanol, and hexane.

Class III solvents are not known to be human health hazards at levels normally accepted in pharmaceutical preparation and include heptane, ethyl acetate, and acetone.

The physiochemical properties of the various classes of specialized metabolites dictate the exact extraction procedure. For example, alkaloids, containing one or more basic nitrogen atoms, are pH-sensitive. By adjusting the pH of the aqueous solution, the solubility of an alkaloid between the aqueous and organic phases can be altered. For example, by adjusting the pH of the aqueous extract-bearing solution to 10, indole alkaloids from *Catharanthus roseus* suspension cultures were successfully partitioned into ethyl acetate [109]. Phenolic compounds exhibit a broad structural diversity and must be handled on a case-by-case basis [110]. More polar phenolics such as benzoic acids and cinnamic acids require 70% aqueous methanol or acetone, while flavonoid glycones may be extracted with less polar solvents such as ethyl acetate or diethyl ether [110]. Less polar solvents are preferred, as they are more easily removed because of their lower boiling points. Liquid–liquid extractions remove not only the desired product but many closely related analogs; however, under optimized conditions, a high selectivity can be achieved, as was demonstrated with paclitaxel and cephalomannine (a structurally similar, unwanted by-product) [111].

The required purity of the final product depends on the application. Ultrapure products are not required for nutraceuticals, cosmetics, and fragrances, but the opposite is true for pharmaceuticals [108]. For example, the purification of the chemotherapeutic paclitaxel consists of liquid–liquid extraction with a mixture of three solvents, column chromatography, distillative exchange, and crystallization, whereas the preparation of a plant cell culture extract to be added to a cosmetic can simply involve lyophilization, extraction with 70% aqueous ethanol, and finally another round of lyophilization [112, 113]. Countercurrent chromatography (CCC) is the method of choice for the isolation of plant natural products. The lack of solid support eliminates the risk of substrate loss because of binding to a stationary phase, a major advantage in the isolation of high-value products present in low concentrations [114]. Structural analogs of similar polarity can have different partition coefficients in a specific two-phase system [115]. Modern CCC instrumentation is capable of purifying natural products on a kilogram scale [116].

10.8.2 Recombinant Protein Extraction and Purification

Downstream processing can account for up to 80% of the total manufacturing cost of a pharmaceutical protein [10]. Plant-cell-synthesized recombinant proteins may be retained within the cell or targeted for secretion; however, most plant-derived biotherapeutics in clinical development are targeted to specific organelles in the plant cell to provide the necessary post-translational processing, protect the recombinant product from post-translational degradation, or to eventually use the plant cell as a delivery vehicle for a biotherapeutic [117–120].

Both secreted and cell-associated proteins present technical challenges for extraction and purification. Secreted proteins are extracted without cellular disruption from a medium free of contaminating proteins, cellular debris, and small-molecule metabolites, but are present in extremely low concentrations and prone to degradation if not stable in the culture medium. The opposite is true for cell-associated proteins, whereby cellular disruption must be performed; however, the protein products are protected from degradation in the culture medium (see Section 10.9.3.2).

The first step in purifying secreted protein products is the removal of bulk cell mass with the use of a decanter, disk-stack separator, or semicontinuous or continuous centrifuge [10]. Cell-associated proteins must be first liberated from cells via wet milling, sonication, pressure homogenization, or enzymatic treatment [41]. Pressure homogenization is the method of choice for large-scale processes; wet-milling and sonication are difficult to scale-up, while enzymatic lysis is not cost effective at large scales [10, 41]. The addition of aqueous buffers is common to ensure proper protein folding and solubility. Generally, the pH is maintained between 7.0 and 8.0 during protein recovery from plant material [121]. For example, homogenization of transgenic carrot cells during the extraction of the recombinant biotherapeutic ELELYSO® is performed in an aqueous extraction buffer at a pH of 7.2 [122]. In addition, the buffer contains EDTA (ethylenediaminetetraacetic acid; chelating agent), ascorbic acid (antioxidant), polyvinylpyrrolidone (polyphenol scavenger), and Triton-X-100 (detergent) [122].

Cellular disruption generates unwanted debris, which must be removed to prevent fouling of chromatography resins and clogging of downstream filters [121]. This clarification process typically involves centrifugation and/or filtration. Centrifuges allow continuous operation but are prone to contamination, while filters are often disposable and easily scaled-up [121]. The clarification process can be further aided by the addition of flocculants, which are charged polymers that promote aggregation between dispersed particles, allowing for enhanced separation via filtration or centrifugation [123]. Cross-flow filtration is the method of choice for the clarification of plant cell suspension products; extracellular polysaccharides can form a layer over membranes and filters, which can trap the product of interest when performing dead-end filtration [10, 124].

Protein purification often involves a series of liquid chromatographic steps, including, but not limited to, size-exclusion chromatography (SEC), affinity chromatography (AFC), and ion-exchange chromatography (IEC), with AFC often first in the line because of its superior selectivity [121]. Protein A and Protein G AFC are particularly useful in the capture and purification of plant-derived antibodies [75, 125]. An AFC bioreactor was developed that circulated media through protein G and iminodiacetic acid metal affinity resins. Cultivation of transgenic tobacco cultures in this system afforded an eightfold increase in the production of the heavy chain of a mouse monoclonal antibody [126].

10.9
Yield Improvement Strategies

10.9.1
Specialized Metabolites and Recombinant Proteins

10.9.1.1 Cell Immobilization

Plant cells can be immobilized by encapsulation within a porous matrix such as alginate, agar, or polyurethane foam [127–130]. Retention of the biomass allows for high cell densities and continuous cultivation, which are considerable advantages considering the low growth and production rates associated with plant cells. Immobilization decreases exposure to shear stress, eliminates issues with foaming, and facilitates the recovery of excreted products. The efficiency of a production process using immobilized cells is largely determined by the rate of secretion of the desired product, as opposed to its biosynthesis. Limitations of plant cell immobilization strategies include cell leakage, bead disruption, and decreased mass transfer. Immobilization of transgenic tobacco NT-1 cells in alginate beads increased productivity of human secreted alkaline phosphatase (SEAP) by a factor of 33 compared to suspension culture [131]. Although the immobilized cells were protected from hydrodynamic shear, once cells began to leak in the medium, SEAP was readily denatured by endogenous proteases. *Taxus baccata* cells immobilized in alginate beads afforded the highest production rates of paclitaxel observed to date in a laboratory-scaled bioreactor [132]. Immobilization is best applied to the production of growth-associated products that are excreted into the medium.

10.9.1.2 *In Situ* Product Removal

Two-phase plant cell culture has been demonstrated to increase yields of both specialized metabolites and recombinant proteins [133, 134]. *In situ* removal protects the desired product from degradation and can reduce downstream processing costs. Two-phase cultivation is particularly advantageous in situations where the desired product is volatile or toxic, or inhibits its own production via negative feedback [135]. All two-phase systems are composed of an aqueous phase compatible with cell growth and a second phase, either liquid or solid, that extracts the desired material. Composition of the second phase depends on the physiochemical properties of the desired product and compatibility with the culture phase.

Aqueous–organic two-phase systems have been exclusively applied to the production/recovery of specialized metabolites because of their stability in organic solvents. The organic phase must be sufficiently lipophilic to be immiscible with the culture medium while effectively extracting the desired product. Typical organic solvents used include butyl acetate, cyclohexane, octane, and decanol [135]. Aqueous-organic two-phase systems have been successfully implemented to enhance the production of paclitaxel in *Taxus* cultures and elicit production of shikonin derivatives in *Echium italicum* suspensions [136–139]. Two-phase cultivation of *T. cuspidata* cells with 4%

(v/v) oleic acid increased paclitaxel accumulation 4.8-fold. Oleic acid not only effectively partitioned paclitaxel but also elicited a stress response in the cultured cells, resulting in overall higher productivity [140]. Limitations of aqueous-organic two-phase systems include difficulty in removing higher molecular weight solvents downstream and the inherent cytotoxicity of many organic solvents [135].

Aqueous two-phase systems (ATPSs) are formed by combining two water-soluble polymers (e.g., polyethylene glycol (PEG), dextran, starch, polyvinyl alcohol) or a polymer and a salt such as phosphate or sulfate [41, 135]. The large interfacial area between the two aqueous phases upon mixing allows efficient partitioning. ATPSs are ideal for bioprocesses because of the aqueous nature of both phases and the ability to partition nutrients, metabolites, proteins, cell particles, and whole cells effectively. Tobacco cells grown in 4% PEG and 7.5% dextran exhibited a growth rate and cell density comparable to that of cultures grown in standard medium, while the phosphodiesterase product was successfully partitioned to the dextran phase [141].

Solid–liquid two-phase systems are formed by the addition of solid adsorbents to the liquid medium. These adsorbents, typically polymer-based resins, remove the target compound(s) from the aqueous phase while maintaining equilibrium conditions [135]. This technique has been applied to increase productivity and facilitate recovery of specialized metabolites and recombinant protein production [134, 142–144]. The addition of paraffin oil to suspension cultures of *Lithospermum erythrorhizon* resulted in a 4.2-fold improvement in shikonin production [145]. Similarly, a solid–liquid two-phase system formed by the addition and frequent harvesting of hydroxyapatite resin to transgenic tobacco NT-1 cultures yielded an eightfold increase in recombinant antibody production [134].

10.9.2 Specialized Metabolite Specific Strategies

10.9.2.1 Elicitation

Specialized metabolites are produced by plants in response to stresses imposed by the environment including pathogen infestation, herbivore predation, and harsh UV radiation. The *in vivo* biological activity of specialized metabolites has led to their usefulness as components in pharmaceuticals, nutraceuticals, and cosmetics. Most importantly, plant stress and specialized metabolite production can be stimulated *in vitro* by altering the chemical and physical properties of the culture medium with the use of elicitors. Abiotic elicitors, including heavy metal ions, inorganic salts, temperature fluctuations, and even ozone, initiate a stress response by simulating a change in the plant's physical environment [41]. Heavy metal ions (Co^{2+}, Ag^{+}, and Cd^{2+}) increased resveratrol production by 60% in *Vitis vinifera* cell suspensions 4 h after administration. Biotic elicitors are most often of fungal, bacterial, or yeast origin and include whole cells or any part thereof (e.g., polysaccharides, glycoproteins, inactivated proteins, etc.) [146]. For example, chitosan, a component of fungal cell walls, has been effective in a variety

of systems [22, 147, 148]. The jasmonates, a family of lipid-derived compounds involved in plant growth and development, are the most often used elicitors of specialized metabolism [149, 150]. Elicited specialized metabolite production is typically performed using a two-stage cultivation regime [82]. Maximum biomass can be obtained before initiation of the production phase, where growth is often decreased, along with cell viability in some instances [22]. Elicitors require optimization of timing of addition and concentration, and the most productive strategy may involve the addition of multiple elicitors simultaneously [151, 152].

10.9.2.2 Metabolic Engineering

Efforts in the field of plant metabolic engineering have focused heavily on the heterologous expression of plant-specialized metabolite pathways in microbes [153–155]. However, the development of numerous transient transformation techniques such as particle bombardment, tissue electroporation, DNA injection, agroinfiltration, and viral expression systems opens the door for ongoing and future metabolic engineering studies in plant cell suspensions [5]. Although these techniques allow only short-term expression in some instances, they result in higher gene expression levels in nearly all plant species and are far less time consuming than stable transformation [5]. Transcription factors are an enticing target for metabolic engineering studies, as a complete knowledge of the biosynthetic pathway of interest is not required [156, 157]. Metabolic engineering efforts in plants have also been hindered by a poor understanding of the complex metabolic pathways that control specialized metabolite production. However, the declining costs of next-generation sequencing have made it affordable to obtain plant genomic and transcriptomic data. For example, 454 pyrosequencing was used to construct a library of expressed sequence tags from *T. cuspidata* needles [158]. This library can be studied to elucidate the underlying pathways and transcription factors that govern paclitaxel production. Genomic data can also be used to construct genome-scale metabolic (GSMs) models. GSMs have been used extensively in the study and manipulation of microbes and have recently been shown to predict observed complex phenotypes in plants. AraGEM, a GSM of Arabidopsis primary metabolism constructed largely from the publicly available Arabidopsis gene and reaction database from the Kyoto Encyclopedia of Genes and Genomes, consists of 1567 unique reactions spanning five cellular compartments [159]. The developed model accurately predicted the photorespiratory cycle as well as differences in redox metabolism in photosynthetic and nonphotosynthetic plant cells [159]. GSMs can be used in conjunction with metabolic flux analysis. By tracking the fate of an isotope-labeled precursor through metabolic pathways, those that regulate biosynthesis of desired compounds or are activated in response to a stressor can be determined. Labeling experiments with heterotrophic Arabidopsis cell cultures showed that flux through the tricarboxylic acid cycle remained unchanged when exposed to different oxygen concentrations, displaying the stability of central metabolism in plants [160].

10.9.3 Recombinant-Protein-Specific Strategies

10.9.3.1 Expression Systems

Recombinant protein production in any heterologous system is highly dependent on construct design and optimization. Constitutive promoters continuously drive protein production during culture growth, while inducible promoters require a chemical or environmental stimulus to initiate transcription.

The cauliflower mosaic virus (CaMV) 35S promoter is the most commonly used promoter in the production of recombinant proteins in plant cell culture and is capable of continuously driving expression until stationary growth phase [28, 31, 32]. However, constitutive production places a significant burden on the cellular machinery, potentially slowing growth, and the build-up of foreign protein may produce a toxic environment that impacts cell viability [41]. Inducible expression allows the separation of growth and production phases and, thus, independent optimization of both processes. An ideal inducer/promoter pair is characterized by high specificity, rapid response upon addition and withdrawal of inducer, low toxicity, and easy scalability [41]. The RAmy3D promoter, induced upon sucrose starvation, has been a successful inducible promoter system in plant cell culture systems [161–163]. Heterologous expression in rice suspension cultures under the influence of RAmy3D has resulted in the greatest recombinant protein yields of all plant cell expression systems (247 mg l^{-1}) [162]. However, use of the RAmy3D promoter is plagued by a decrease in cell viability upon transfer to sucrose-depleted medium.

10.9.3.2 Minimizing Post-Translational Loss of Recombinant Proteins

The low yields associated with heterologous protein production in plant cell suspensions are not just a result of slow production rates, but a consequence of post-translational loss and degradation, as suggested by the lack of correlation between mRNA levels and product accumulation in some systems [164]. Proteases, both intracellular and extracellular, can degrade a protein product before it can be effectively recovered. Human IgG1 accumulated in degraded form, while IgG4 was barely detectable in transgenic tobacco suspension cultures [165]. It is often difficult to discern low production rates from endogenous protease activity, especially if the recombinant protein product is degraded shortly after translation. Protease activity is dependent on species, culture conditions, and the recombinant protein's primary structure, specifically the number of protease-susceptible sites [165, 166]. Secreted proteins, although more easily purified, suffer from a lack of stability in plant cell culture medium and a tendency to irreversibly bind to the vessel walls [164]. Numerous strategies have been implemented at both molecular and process levels to reduce post-translational losses by reducing protease activity and increasing foreign protein stability.

Intracellular protease activity is compartment-specific [167]. The cytosol and apoplast are associated with relatively high levels of proteolytic activity, while the endoplasmic reticulum (ER), characterized by low protease activity and the presence of molecular chaperones and stabilizing agents, offers a favorable

environment for foreign proteins [27, 167]. Targeting recombinant proteins to the ER via the KDEL (Lys-Asp-Glu-Leu) or HDEL (His-Asp-Glu-Leu) C-terminal signal peptides has been shown to significantly increase heterologous protein production in whole plant systems, with a 10^4-fold improvement observed in tobacco plants [167, 168]. A similar strategy applied to tobacco BY-2 cells resulted in a fourfold increase in the production of human granulocyte colony stimulating factor (hG-CSF) when compared to apoplastic expression [169]. Protein targeting location is also dependent on the necessary post-translational modifications, which vary from organelle to organelle [170]. N-Glycosylation is a universal post-translational modification in eukaryotes; in plant cells the process begins in the ER, while mature glycoproteins are produced in the Golgi apparatus [170]. Other potential strategies to overcome protease activity include the addition of a protease inhibitor to the culture medium (assuming it can enter the cell), co-expression of recombinant protease inhibitors, and knocking out genes associated with specific proteases [164, 171, 172]. Inhibition of protease activity with antisense RNA improved recombinant protein production fourfold in tobacco BY-2 cells, while suppression of protease activity using RNA interference resulted in an approximately twofold increase in protein production in transgenic rice cultures [172, 173].

Secreted proteins are subject to extracellular proteases and the unfavorable environment in the plant cell culture medium. Protease inhibitors added to the medium can reduce proteolytic activity [164]. If it is determined that the presence of protease inhibitors is a result of cell lysis, the addition of osmotic agents may prevent cell breakage. The addition of mannitol ($90\,g\,l^{-1}$) to transgenic tobacco suspension cultures resulted in a 12-fold increase in recombinant antibody production [174]. Protein adsorption to the plastic, glass, or metal vessel is a large contributor to protein loss. More than 90% of human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) in transgenic rice cultures was lost within 1 h as a result of surface adsorption [175]. Large proteins are more susceptible to adsorption because of their increased surface area and larger number of binding sites [164]. Stabilizing agents such as polyvinylpyrrolidone (PVP), dimethyl sulfoxide (DMSO), gelatin, albumin, mannitol, and salt effectively reduce protein adhesion by altering the surface environment or competing for adhesion sites [176, 177]. However, the addition of stabilizing agents may increase foam formation, complicate downstream processing, and cause damage to the cultures. Furthermore, the addition of animal-derived components such as albumins presents the risk of human pathogen contamination.

10.10 Case Studies

10.10.1 Protalix and the ProCellEx™ Platform

The year 2012 saw a landmark breakthrough for plant cell culture technology with the FDA approval of ELELYSO® (Taliglucerase alfa), the first plant-derived

recombinant protein to be approved for human use. ELELYSO®, developed by Protalix Biotherapeutics (Carmiel, Isreal) and produced via their novel ProCellEx® platform, is a recombinant enzyme for the treatment of Gaucher disease. The ProCellEx® platform combines heterologous protein production, disposable bioreactor technology, and horizontal process scale-up to produce novel therapeutics on a commercial scale in transgenic carrot and tobacco suspension cultures. Protalix particularly focuses on the development of recombinant therapeutics for the treatment of rare genetic diseases. The drug development pipeline at Protalix, supplied by the pioneering ProCellEx® platform, contains four clinical candidates, including an orally bioavailable therapeutic enzyme encapsulated in carrot cells that showcases the true potential of plant cell culture technology [7].

10.10.1.1 Background

Gaucher disease, a genetic disorder characterized by lack of a functional version of the enzyme glucocerebrosidase (GCD), is an ultimately fatal disease that afflicts less than 100 000 individuals worldwide [178]. Enzyme replacement therapy is the standard of care for the orphan disease and alleviates most of the associated symptoms; however, it comes at a high financial cost. Before FDA approval of ELELYSO®, the only options for enzyme replacement therapy were recombinant CHO-cell-derived Cerezyme (Genzyme) or the human cell-derived Vpriv (Shire) at a cost of US$250 000 per year [178].

In 2009, Genzyme reported viral contamination of its GCD production facility, prompting a warning letter from the FDA and precipitating significant global shortages of the enzyme [179–181]. To meet demands, the FDA granted Protalix the right to provide patients with their plant-derived recombinant glucocerebrosidase (prGCD) under an extended access program [182]. prGCD, being of plant origin, is inherently free of viral contaminants. The ability to meet worldwide enzyme demand, positive patient feedback, and success in clinical trials paved the way for ELELYSO's® eventual FDA approval [180, 181, 183]. Additionally, in December 2009 Pfizer purchased the worldwide (excluding Israel) rights to the drug for $115 million, marking the first time a large pharmaceutical company had committed to taking a plant-derived biologic in the late stages of clinical development to the market [182].

10.10.1.2 The ProCellEx® Platform

ELELYSO® is produced in transformed carrot root cells carrying the binary Ti plasmid vector bearing the acid β-glucocerebrosidase cDNA, the CaMV 35S promoter, the tobacco mosaic virus (TMV) omega translational enhancer element, and the octopine synthase terminator sequence from *Agrobacterium tumefaciens* [184, 185]. Kanamycin resistance was conferred via the neomycin phosphotransferase type II (*NPTII*) gene driven by the *nos* promoter [184].The recombinant protein product is directed to the vacuole via a C-terminal sorting signal [184]. In comparison to mammalian-cell-produced GCD, vacuole-directed prGCD naturally bears the high-mannose glycans necessary for therapeutic action. GCD must be taken up by the affected macrophages, a process mediated by the interaction

between mannose receptors on the cell's surface and the mannose residues on the enzyme's glycans [186]. Mammalian-cell-produced GCD must undergo extensive and expensive postprocessing as part of its formulation to be therapeutically active. An added benefit of targeting prGCD to the vacuole is the organelle's relatively low protease levels, limiting the amount of post-translational degradation.

Successfully transformed cells were selected by antibiotic resistance, and highly expressing clones were chosen based on protein expression levels in transgenic calli [122]. Elite cell lines were cryopreserved to establish cell banks for use as inocula in future bioprocesses and to meet requirements set forth by various regulatory agencies [23]. The desired cell line is scaled-up and eventually grown in Protalix's novel bioreactor system that is the work-horse of its proprietary ProCellEx™ platform. The system is comprised of large (400 l), flexible, polyethylene bags that are supplied with growth medium and air from a central system (Figure 10.4) [7, 187]. The disposable vessels are fitted with a reusable harvester complete with a flow controller that allows a portion of the cells and medium to be retained after harvest for use as inocula in a second round of production. An assembled framework around the plastic vessels provides structure to the flexible bags [22]. These pneumatic, disposable bioreactors provide proper mass and oxygen transfer without mechanically driven mixing and may be serially assembled for the growth of thousands of liters of plant cells to meet industrial needs [187]. This horizontal scale-up guarantees that the desired culture conditions are met for every cell, as opposed to traditional scale-up, or simply the use of larger bioreactors, where temperature and nutrient gradients change with increasing size and height.

The ProCellEx® platform utilizes conventional equipment, materials, and processes, is FDA-approved, and operated under cGMP. The use of disposable bioreactors eliminates the need for precycle sterilization, a time-intensive and costly process. Also, the initial capital investment for setting up the large-scale plant cell culture facility at Protalix was significantly less than that for an equivalent facility hosting CHO cells [178]. For these reasons, Protalix is able to provide ELELYSO® to patients at 20% of the cost of mammalian-cell-derived GCD [178].

10.10.1.3 Future Outlook

The drug development pipeline at Protalix currently contains four clinical candidates for the treatment of orphan diseases (Gaucher disease, Fabry disease, and cystic fibrosis) as well as immune and inflammatory conditions [7]. Protalix's pioneering work focuses on the oral administration of plant cells expressing biotherapeutic proteins. Phase II clinical trials have begun for PRX-112, an orally delivered prGCD for the treatment of Gaucher disease. Patients enrolled in the trials drink a slurry of lyophilized carrot cells expressing the recombinant enzyme (daily) as opposed to receiving purified GCD intravenously (every other week). The cellulose-rich cell wall of the plant cell protects the prGCD from degradation in the stomach. Once in the small intestine, the cell wall breaks down and the enzyme passes through the lining of the intestine and into the neighboring blood stream. Protalix previously demonstrated both the safety and efficacy of oral

Figure 10.4 Disposable pneumatic bioreactors that lie at the center of the ProCellEx® platform. Structures assembled around each reactor provide structurally integrity, allowing for cultivation at 400-l volumes [7].

prGCD in Phase I clinical trials. Healthy individuals administered oral prGCD exhibited continuous enzyme presence ~30 h after administration [7]. A once-daily oral dose of prGCD would revolutionize enzyme replacement therapy and dramatically increase the quality of life for patients with Gaucher disease. Oral administration of lyophilized cells also eliminates nearly all downstream processing, significantly reducing therapeutic production costs. The oral delivery of biotherapeutics is a grand challenge in biotechnology, and plant cell encapsulation offers a promising solution. Utilizing plant cells as both production factories and delivery vehicles eliminates nearly all downstream processing, offers a long-term storage solution of the particular biopharmaceutical, and would reduce the number of infections that arise yearly as a result of injections [188].

10.10.2 Phyton Biotech, Paclitaxel, and Plant Cell Fermentation (PCF™)

10.10.2.1 Background

Taxol® (Paclitaxel), currently approved for the treatment of numerous cancers, including, but not limited to breast, lung, ovarian and skin, was first isolated from the bark of the Pacific yew (*Taxus brevifolia*) tree in 1971 [189]. Today, more than 400 structurally similar compounds (i.e., taxanes or taxoids) have been isolated from the *Taxus* species (Figure 10.5 b) [190]. Paclitaxel and two synthetic analogs are currently approved for the treatment of a wide variety of cancers. The potent cytotoxicity exhibited by many members of the taxane family stems from their ability to bind and subsequently stabilize microtubules, inhibiting the progression of mitosis and eventually triggering apoptosis without cell division [191, 192]. Phyton Biotech® (Ahrensburg, Germany) operates the largest cGMP-compliant plant cell culture facility in the world and supplies nearly 500 kg of paclitaxel annually via Plant Cell Fermentation (PCF®) (Figure 10.5 a) [11]. Additionally, Phyton Biotech® is the only company in the world to produce docetaxel semisynthetically from 10-deacetylbaccatin derived from plant cell culture and not harvested from yew plantations [11].

10.10.2.2 Why Plant Cell Culture?

Paclitaxel clinical trials began in 1983, and initial quantities of the compound were supplied via natural harvest from the bark of the Pacific yew tree [112]. Once paclitaxel was proven effective in the treatment of the most serious forms of ovarian cancer, demand dramatically increased and the limitations of natural harvest became apparent. The bark of the Pacific yew tree contains approximately 0.0004% paclitaxel, and 38 000 full grown trees were required to supply 25 kg of paclitaxel required per year [5]. Bark-stripping is also fatal to the trees, which can take as long as 200 years to mature [112]. Low yields and staunch opposition from ardent environmentalists forced scientists to develop alternate production platforms.

Two total syntheses of paclitaxel were reported in 1994 [193, 194]; however, both required about 40 steps and had an overall yield of 2%, and were therefore not viable for paclitaxel synthesis on the desired kilogram scale. Chemical synthesis is complicated by the sophisticated structure of paclitaxel, comprised of 4 rings and 11 chiral centers. In fact, the very first paclitaxel total synthesis took 12 years, and to date only six total syntheses have been reported [193]. A semisynthetic approach from a paclitaxel precursor isolated from the leaves and stems of the European yew (*T. baccata*) was implemented in 1995. This approach afforded substantial improvements over natural harvest, as the precursor is present at about 0.1% in the leaves and twigs and harvest of plant material is not fatal to the tree [112]. Although economically viable and able to meet market demands, semisynthesis required 11 transformations, involved hazardous reagents and solvents, utilized significant amounts of energy, and produced toxic waste streams. Bristol-Myers Squibb (BMS), motivated by environmental health and safety concerns as well as a financial incentive for a green pharmaceutical process, turned to large-scale plant cell culture for the production of paclitaxel. The plant-cell-culture-based process developed by BMS in conjunction with Phyton Biotech®

was fully implemented in the production of paclitaxel in 2002 and earned BMS the 2004 Presidential Chemistry Challenge Award [112].

10.10.2.3 Plant Cell Fermentation (PCF™)

Preliminary screening performed during process development found that suspension cultures derived from the actively growing needles of *T. chinensis* yielded the largest quantities of paclitaxel [18]. Medium conditions and cell lines were chosen so as to maximize paclitaxel accumulation while minimizing the production of the unwanted analog cephalomanine, an incredibly elegant strategy to reduce the difficulty and cost of downstream processing [18]. Suspension cultures are initiated from cryopreserved stock and scaled-up in a cascade of bioreactors to a maximum volume of 75 000 l. The overall process utilizes a two-stage cultivation regime. Once significant biomass levels are obtained, the growth medium is exchanged for an optimized production medium containing silver ions (an inhibitor of the plant hormone ethylene), methyl jasmonate (a stress hormone that promotes specialized metabolite synthesis), and inhibitors of the phenylpropanoid pathway (e.g., 3,4-methylenedioxy-6-nitrocinnamic acid). After production, the whole culture medium, cells included, is subject to liquid–liquid extraction with a mixture of isobutyl acetate and isopropanol (both are Class III solvents, Section 8.1) [112]. The crude extract is redissolved in dichloromethane, solvent-exchanged into a mixture containing dimethylformamide (DMF) and formamide, and purified via column chromatography, with the final product obtained via crystallization.

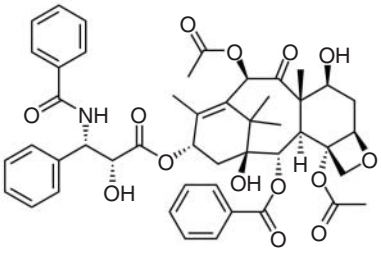
10.10.2.4 PCF™ Compared to Other Production Platforms

Phyton's PCF™ platform is an environmentally friendly and economically favorable platform for large-scale paclitaxel production. Both natural-harvest- and semisynthesis-based strategies produce large amounts of solid biomass waste. In contrast, the biomass harvested from bioreactors during PCF™ is processed by standard wastewater treatment facilities, after the addition of sodium hydroxide to deactivate any paclitaxel residues [112]. Compared to natural harvest, there is no need to destroy 100-year-old trees just for the bark. PCF™ can be performed year-round, whereas the semisynthesis approach relies on needles from *T. baccata* plantations that are only seasonably available. Phyton's approach uses 10 less organic solvents than semisynthesis as well as significantly less amounts of energy [112]. Most importantly, the costs associated with PCF™ are 20% that of natural harvest and 20% less than the semisynthesis-based approach [112] (Figure 10.5 c).

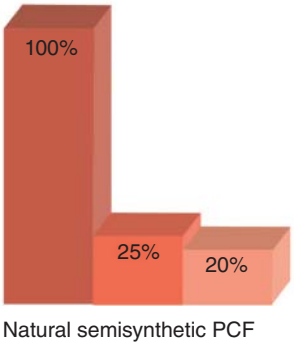
10.11 Conclusion

Plant cell suspension culture presents a renewable, economically favorable, and easily regulated platform for the production of specialized metabolites and recombinant proteins. Plant cell suspensions can be cultivated in a wide range of vessels utilizing the same process strategies applied to microbial and mammalian cell culture. Recent advances and knowledge in bioreactor design, cultivation strategies, cell aggregation, and product encapsulation have accelerated use

(a)



(b)



(c)

Figure 10.5 (a) Bioreactor facility at Phyton Biotech® [11]. (b) Paclitaxel (note the 4 rings and 11 chiral centers as well as the 6/8/6-membered ring system common to all taxanes). (c) Cost comparison of different paclitaxel production strategies. (From [112] with permission ©2010 Wiley-VCH Verlag GmbH & Co. KGaA.)

of plant cell culture technology. Emerging research in metabolic engineering, computational biology, cryopreservation/cold storage, and gene transformation techniques will enable further adoption of this versatile production platform. Plant cells are intrinsically safe and the only cell system that can serve both as a production host and delivery vehicle, offering a novel approach to drug synthesis and delivery.

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11
The Role of Bacteria in Phytoremediation

Zhaoyu Kong and Bernard R. Glick

11.1
The Problem

The widespread pollution of the biosphere by toxic metals and organic contaminants is a problem of environmental and toxicological concern worldwide. The primary sources of this pollution include industrial production, mining activities, combustion of fossil fuels, e-wastes, vehicular traffic, sewage, irrigation, and the extensive use of chemical fertilizers and pesticides. Contamination of both groundwater and soil by toxic compounds directly poses a significant threat to plants, which in turn, impacts negatively on human and environmental health. It has been estimated that more than 40 million tons of hazardous waste is produced each year in the United States, and 275 toxic compounds have been targeted as priority substances in the hazardous waste sites; these compounds are considered a serious threat to human health [1]. In Europe, 3.5 million sites, including industrial sites, landfills, energy production plants, and agricultural lands, are potentially contaminated, and thus soil contamination is considered an important issue in the European Community [2]. The estuarine and coastal ecosystems in China are now facing a serious threat of metal pollution [3]. It has been estimated by the Chinese government that 29 720 km^2 of coastal areas in China are heavily polluted [4]. China is one of the largest coastal countries in the world, and the coastal waters provide important habitats for marine organisms living in this region. Thus, urgent attention is required to address the pollution in the coastal environment of China [3]. In the past decades, considerable efforts have been made to remove toxic compounds from contaminated environments, including physical, chemical, and thermal processes resembling excavation and transportation; however, these methods are prohibitively expensive [5]. For example, remediation of all contaminated sites in the United States alone will cost approximately $1.7 trillion. Moreover, most of these conventional methods are not completely effective and do not offer permanent solutions for remediation [6]. Some methods may even lead to the generation of additional toxic wastes. Therefore, there is an urgent

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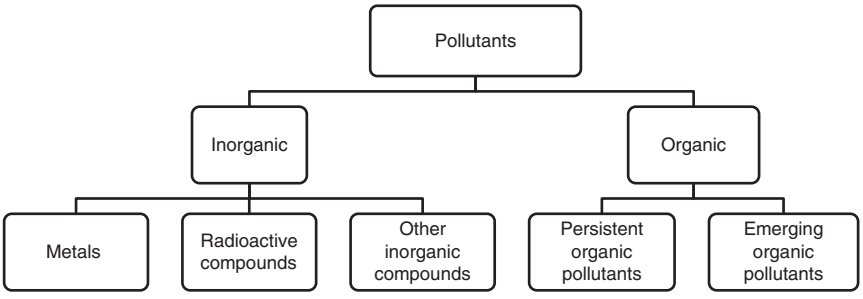

Figure 11.1 Categorization of pollutants in the environment.

need to search for alternative methods to clean up polluted environments in an inexpensive, efficient, safe, and environmentally friendly manner.

11.1.1 Metals and Organics in the Environment

Most contaminated environments contain heterogeneous mixtures of different metals as well as radioactive, inorganic, and organic compounds (Figure 11.1). For example, many soils are contaminated with one or more metals, including cadmium, copper, nickel, zinc, lead, chromium, cobalt, selenium, and mercury. The radioactive compounds may include cesium, strontium, and uranium, and the inorganic compounds may be ammonia, arsenic, sodium, nitrate, or phosphate [7]. In addition to the toxic metals, soils and water systems may also be contaminated by organic pollutants, which include persistent organic pollutants (POPs) and emerging organic pollutants. For example, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDT), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) are some of well-known POPs [8]. Typically, POPs would remain in the environment for years and decades, and bioaccumulate and biomagnify as they move through the food chain. Emerging organic pollutants include newly developed or discovered compounds in the environment and the compounds that have only recently been characterized as contaminants [9]. For example, many pharmaceuticals, pesticides, personal care products (PPCPs), and industrial products are categorized as emerging organic pollutants, which may adversely impact water bodies and human health [10–12].

11.1.2 Traditional Clean-up Procedures

In response to the urgent need to address environmental contamination, scientists and engineers have developed many remediation technologies to remove toxic compounds from the soil and wastewater. The traditional clean-up technologies of treating soil and groundwater contamination mainly rely upon the removal

or containment through physical or chemical methods. For example, thermal desorption, incineration, soil washing, chemical treatment, supercritical fluid oxidation, volatilization, steam extraction, encapsulation, and solidification are used for treating contaminated soil, and carbon adsorption, resin adsorption, wet air oxidation, supercritical fluid oxidation, chemical treatment, crystallization, and density separation have been used to remediate leachate or wastewater [6]. However, the disadvantages of these conventional methods, such as the very high cost, low efficiency, and secondary pollution, reduce their remediation potential and restrict their practical use.

11.2 Defining Phytoremediation and Its Components

Phytoremediation is an alternative method that offers the possibility to remove, destroy, or sequester hazardous substances from polluted environments using plants and associated soil microbes. Compared to the traditional methods, phytoremediation is a more promising and less expensive method for cleaning up contaminated soil and water. Phytoremediation of metals and organic compounds may involve different processes. Thus, for example, (i) phytovolatilization is the uptake of contaminants by plant roots, its conversion into a volatile form, and its release into the atmosphere; (ii) phytodegradation is the transformation or breakdown of organic contaminants by plant metabolic processes; (iii) phytoextraction is the absorption, concentration, and precipitation of toxic metals from contaminated soils into the plant biomass; (iv) phytotransformation is the enzymatic conversion of organic pollutants within plant tissues following plant uptake of those contaminants; (v) rhizodegradation is the breakdown of organic contaminants by rhizosphere microorganisms whose proliferation is facilitated by plant growth and metabolism; (vi) rhizofiltration is the absorption, concentration, and precipitation of contaminants from water by plant roots; and (vii) phytostabilization is the use of certain plants to reduce the mobility and bioavailability of soil and/or water contaminants.

Phytoremediation, representing a promising new strategy for the remediation of contaminated soil and water, has been highly touted [13–15]. A number of plant species have been identified to be hyperaccumulators of either heavy metals [16] or organic pollutants [14] and therefore may be useful in the development of highly effective phytoremediation protocols. However, hyperaccumulators of inorganic pollutants are often limited by their slow growth, low biomass, or the lack of suitable hyperaccumulating plants for some important trace metals [17]. Beyond those on hyperaccumulating plants, a number of studies also focus on the exploitation of plants that are not hyperaccumulators but have some potential in remediating heavy-metal-contaminated soils [18–20]. Nevertheless, the removal or degradation of contaminants is also limited to the surface area and the depth occupied by the plant roots. There are challenges when the contamination is in deeper soil than the plant's root zone. Furthermore, plant growth may be adversely

affected by extreme environmental conditions, toxicity, and the physicochemical conditions of the soil. It is therefore impossible to completely clean up contaminated environments with plant-based remediation systems, which by themselves cannot resolve all of the inherent problems of this approach [5]. However, one way to improve the usefulness of plants in phytoremediation is through the presence of specific soil bacteria along with the plants. These bacteria may interact with and affect the growth of plants in one or more ways, as discussed below.

11.3 Role of Bacteria in Phytoremediation

11.3.1 Biodegradative Bacteria

Many microorganisms are capable of degrading toxic organic compounds. A number of pollutant-degrading bacteria have been isolated and characterized from polluted soils, and it is assumed that such bacteria can be found in virtually all soils on earth [21]. Moreover, a variety of enzymes with a role in the degradation of organic compounds have been found in bacteria, including dehalogenases, dioxygenases, nitroreductases, and cytochrome P450 monooxygenases [14]. For example, *Pseudomonas* strains are excellent candidates for biodegrading various aromatic compounds, a process that typically requires dioxygenases as one of the key enzymes [22]. In microorganisms, the genes that code for the enzymes of degradative pathways are often located on plasmids [23–25]. Horizontal transfer of these degradative genes is a major mechanism for microorganisms to acquire new metabolic pathways and plays an important role in plant growth promotion and degradation of organic pollutants [26]. Because of the diversity and complexities of chemical organic compounds, there is probably a larger diversity in microbial members and their pollutant-degrading abilities [27–29]. In natural environments, a well coordinated microbial community is always required for transferring the pollutants and products in biodegradation, which is referred to as metabolic cooperation [30]. Although many attempts have been made to isolate and characterize biodegradative bacteria, microbial bioremediation approaches suffer from a number of limitations for their widespread application in field [31]. These include the poor capabilities of microbial communities in the field soil, low bioavailability of many contaminants, insufficient nutrients to support microbial growth in contaminated soils, and the high level of pollutants that inhibit microbial growth.

An integration of microbial bioremediation and phytoremediation is a more promising strategy for remediation of contaminants, particularly organic compounds. In the past 20 years, the combined use of plants and biodegradative bacteria, with both a specific single strain and a microbial consortium, has been exploited to remove organic contaminants under the laboratory, greenhouse, and field conditions (Table 11.1).

Table 11.1 Examples of contributions of degradative bacteria to phytoremediation of organic contaminants.

Microbes	Plants	Organic contaminants	Results	References
A microbial consortium composed of 10 bacterial strains and 3 fungal strains	*Cyperus laxus* Lam.	Mixed hydrocarbons	Improved the phenological characteristics of plants and the phytoremediation rate of total petroleum hydrocarbon (TPH) degradation	[32] (G)
Pseudomonas fluorescens strain TP13	*Lycopersicon esculentum*	Phenol	Significantly reduced the phenol content and increased plant biomass	[26] (F)
Sphingomonas paucimobilis, *Cunninghamella echinulata*	*Lolium multiflorum*	Petroleum hydrocarbons	Significantly increased leaf area, leaf and pseudostem dry mass; enhanced degradation of Arabian medium crude oil	[33] (G)
A microbial consortium composed of 11 bacterial strains and 6 fungal species	*Astragalus adsurgens*	Diesel fuel (alkanes 90.01% aromatics 0.82%, colloid and asphaltene 0.91% and others 8.26%)	Enhanced the removal of diesel fuel and its components by 13–30% after 2 years	[34] (G)
Burkholderia cepacia VM1468	Poplar (*Populus trichocarpa* × *deltoides* cv. Hoogvorst)	Toluene	Positive effect on plant growth; reduced the amount of toluene released through evapotranspiration	[24] (G)
Pseudomonas sp. GF3	Wheat (*Triticum aestivum* L. cv. Yangmai-158)	Phenanthrene	Improved the root and shoot growth of wheat; enhanced the dissipation of phenanthrene and polyphenol oxidase activities in the soil	[35] (G)
Enterobacter sp. 12J1	Wheat (*T. aestivum* L. cv. Ningmai-13);maize (*Zea mays* L. cv. Xiyu-3)	Pyrene	Improved the dry weights of plant roots and shoots; increased the rate of pyrene removal by 43–65%	[36] (G)
Microbacterium sp. F10a	Wheat (*T. aestivum* L. cv. Xumai-856)	Phenanthrene and pyrene	Significantly increased the growth of wheat and the removal rate of phenanthrene and pyrene in low-temperature soil	[37] (G)

Table 11.1 (*Continued*)

Microbes	Plants	Organic contaminants	Results	References
Pseudomonas putida VM1441 (pNAH7)	Pea (*Pisum sativum*)	Naphthalene	Enhanced seed germination and plant transpiration rates; increased naphthalene degradation rates	[38] (L)
Comamonas sp. strain CNB-1	Alfalfa (*Medicago sativa*)	4-Chloronitrobenzene (4CNB)	Completely removed 4CNB within 1 or 2 days; eliminated toxic effects of 4CNB on alfalfa	[39] (G)
Pseudomonas frdedriksbergensis	*Amaranthus caudate*, *Lactuca sativa*, *Nasturtium officinale*, and *Phaseolus vulgaris*	Pesticide residues (dimethoate and malathion)	Increased the plants' ability to degrade dimethoate and malathion	[40] (G)
Pseudomonas sp. ITRH25, *Pantoea* sp. BTRH79	Carpet grass (*Axonopus affinis*)	Diesel	Increased biomass production and phytoremediation activity of plants growing in diesel-contaminated soil	[41] (G)
Acinetobacter sp.	Ryegrass (*L. multiflorum*)	Phenanthrene and pyrene	Increased dissipation rates of phenanthrene and pyrene from polluted soil	[42] (G)
Staphylococcus sp. BJ06	*Alopecurus aequalis*	Pyrene	Significantly promoted plant growth and pyrene removal; reduced the pyrene concentrations in plant roots and shoots	[43] (G)
P. putida W619	Poplar [*Populus deltoides* × (*trichocarpa* × *deltoides*) cv. Grimminge]	Trichloroethylene (TCE)	Promoted plant growth and improved plant fitness during a short-term experiment; during a mid-term experiment, strongly reduced the amount of TCE in leaves and roots and reduced TCE evapotranspiration through leaves	[44] (L)
Burkholderia, *Variovorax*, *Bacillus*, *Pseudomonas*, and *Ralstonia* species	*Arabidopsis thaliana*	2,4-Dinitrotoluene (DNT)	Increased root length and root hairs of plants in the presence of 2,4-DNT	[45] (L)

Table 11.1 (*Continued*)

Microbes	Plants	Organic contaminants	Results	References
Mycobacterium sp. strain U1A	Soybean (*Glycine max*)	Phenanthrene	Improved plant growth and eliminated phytotoxicity effects of phenanthrene on plants	[46] (L)
Rhodococcus erythropolis ET54b, *Sphingomonas* sp. D4	*Cytisus striatus*	Hexachlorocyclohexane (HCH)	Inoculation with the combination of these two strains significantly enhanced HCH dissipation	[47] (G)
R. erythropolis ET54b, *Sphingomonas* sp. D4	*C. striatus*	HCH	Inoculation with both bacterial strains reduced HCH phytotoxicity and improved plant growth	[48] (G)
Pantoea sp. ITSI10, *Pantoea* sp. BTRH79 and *Pseudomonas* p. MixRI75	Italian ryegrass (*L. multiflorum*)	Diesel	Improved plant biomass production and hydrocarbon degradation	[49] (G)

Experimental conditions are designated (L) for more controlled laboratory conditions, (G) for greenhouse conditions, or (F) for field trials.

11.3.2 Plant-Growth-Promoting Bacteria

Bacteria that are beneficial to plants are generally referred to as plant-growth-promoting bacteria (PGPB), which improve plant growth and development by either direct promotion of plant growth or through their suppressive activity against phytopathogens, thereby indirectly stimulating plant growth [50]. PGPB may directly facilitate plant growth and development using one or more mechanisms, and a bacterium may utilize different mechanisms under different environmental conditions, including indole acetic acid (IAA) biosynthesis, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, nitrogen fixation, and siderophore production.

11.3.2.1 Role of IAA

The plant hormone IAA influences numerous cellular functions and, therefore, is considered to be an important regulator of plant growth and development. In addition to its synthesis in plant tissues, IAA production is widespread among plant-associated bacteria [51, 52] and provides bacteria with a mechanism to affect plant growth [53]. The level of IAA synthesized by the plant and the sensitivity of the plant to IAA are important in determining whether bacterial IAA stimulates

or inhibits plant growth. In plant roots, endogenous IAA may be suboptimal or optimal for supporting plant growth [54] and additional IAA synthesized by bacteria could alter the IAA level to either optimal or supraoptimal, resulting in either plant growth promotion or inhibition, respectively.

IAA synthesized by bacteria plays an important role in plant–bacterial interactions. One of the main effects of bacterial IAA is to increase the number of root hairs, the number of lateral roots, and the total root surface, leading to an enhancement of mineral uptake from the soil as well as root exudation. This latter effect may act to further stimulate root colonization by bacteria by increasing the concentration of plant metabolites that are available in the rhizosphere. The effect of IAA synthesized by the plant-growth-promoting bacterium *Pseudomonas putida* GR12-2 on the development of the canola roots has been studied following the construction of an IAA-deficient mutant of this strain [53]. Seed inoculation with wild-type GR12-2, which produces a relatively low level of IAA, induced the formation of roots that were 35–50% longer than the roots from seeds inoculated with the IAA-deficient mutant and the roots from non-inoculated seeds. On the other hand, inoculation of mung bean cuttings with an IAA-overproducing mutant of *P. putida* GR12-2 induced a greater number of shorter roots compared to the plants inoculated with the wild-type strain. This result was explained by the combined effects of IAA on root growth promotion and inhibition of root elongation by ethylene [55]. The bacterial IAA incorporated by the plant stimulated the transcription of the gene encoding the enzyme ACC synthase, resulting in increased synthesis of ACC and a subsequent increase in ethylene production that inhibited root elongation. In addition, although experiments comparing the behavior of wild-type and IAA-deficient mutants of PGPB have confirmed that IAA is involved in promoting root growth, most IAA knock-out mutants are still able to promote plant growth [56–58]. Therefore, IAA biosynthesis alone is not responsible for the effects of bacteria on plant growth promotion. In this regard, multiple mechanisms, including dinitrogen fixation, ACC deaminase activity, phosphate solubilization, and iron sequestration together with IAA biosynthesis, are responsible for the observed plant growth promotion and yield increase [59].

It has been suggested that PGPB producing IAA may alleviate some of the deleterious effects of environmental stresses. For example, bacterial IAA increased the length of the root and shoot of wheat seedlings in the presence of high levels of salt [60]. An increased salt tolerance was observed in *Medicago truncatula* plants when nodulated by the IAA-overproducing *Sinorhizobium meliloti* strain RD64 [61]. The plants inoculated with strain RD64 accumulated a high level of internal proline and showed enhanced activity of antioxidant enzymes compared to plants inoculated with the comparable wild-type strain. In addition to the effects of bacterial IAA on plant growth in the presence of high salt concentrations, a number of PGPB have been found to protect plants from heavy metal stress. *Bacillus subtilis* strain SJ-101, which shows nickel resistance and produces both IAA and phosphate-solubilizing activity, was used to facilitate the nickel accumulation in Indian mustard [62]. Inoculation of strain SJ-101 significantly improved plant

growth in the presence of high concentrations of nickel and showed enhanced metal accumulation. Our group used *Pseudomonas brassicacearum* strain Zy-2-1 as a co-inoculant with *S. meliloti* to promote the growth of *Medicago lupulina* under copper stress conditions. Moreover, strain Zy-2-1 was capable of producing IAA, siderophores, and ACC deaminase and showed the highest level of growth promotion in the presence of Cu^{2+} up to 2.0 mM (Kong *et al*., unpublished data). As expected, the presence of strain Zy-2-1 improved both plant growth and nodulation to a significant extent in the presence of excess copper. In addition, the total amount of copper taken up by the plants was significantly increased when the plants were co-inoculated with strain Zy-2-1 and *S. meliloti*, compared to *S. meliloti* inoculation alone. The results of these experiments suggested that the PGPB played an important role in improving metal phytoremediation. However, these studies did not provide definitive proof of the direct involvement of IAA. In this regard, it would be necessary to construct either IAA-deficient or IAA-overproducing mutant of these bacteria to test their efficacy in the presence of increasing concentrations of various metals.

11.3.2.2 **Role of Ethylene**

Ethylene, a key regulator in a large number of aspects of plant growth and development, is involved in plant responses to both biotic and abiotic stresses [51]. Ethylene that is synthesized as a response to various environmental stresses is called "stress ethylene" [63]. This increase in ethylene synthesis is associated with environmental stresses including drought, flooding, salt, freezing, high temperature, toxic metals, organic pollution, wounding, radiation, insect predation, and various pathogens including viruses, bacteria, and fungi [64]. The increased amount of ethylene in response to environmental stresses can either exacerbate the symptoms of stress or lead to responses that enhance plant survival under adverse conditions. This seemingly contradictory behavior has been explained by a two-phase model [65] wherein plants exposed to stress rapidly responded (a few hours after the onset of the stress) by producing a small peak of ethylene, which is thought to initiate a protective response by plants, for example, transcription of genes encoding defensive proteins [66, 67]. On the other hand, if the stress persists or becomes more intense, usually a few days later, a second much larger peak of ethylene occurs. The second ethylene peak is deleterious to plant growth and development, including processes such as senescence, chlorosis, and abscission.

PGPB that express ACC deaminase can facilitate plant growth and development through the conversion of the immediate ethylene precursor ACC into α-ketobutyrate and ammonia, thus decreasing ethylene levels that could otherwise inhibit plant growth under various environmental stresses, including flooding, organic toxicants, heavy metals, high salt concentrations, drought, and phytopathogenic microorganisms [68–70]. Several studies have shown that PGPB that produce ACC deaminase can facilitate metal phytoremediation by decreasing the level of stress ethylene that is induced by high concentrations of metals. The treatment of plants with bacteria that produce ACC deaminase allows the plants to grow larger and, as a consequence, take up more metal from

soil, indicating the benefit of using these organisms in metal phytoremediation strategies [71, 72]. Such enhancement of plant biomass and total copper uptake was observed when *M. lupulina* was nodulated by a genetically engineered *S. meliloti* strain overproducing ACC deaminase [73]. In addition, although this engineered strain promoted metal accumulation both in shoots and roots, the increase in roots was much higher than in shoots, thus decreasing the copper translocation factor. These findings emphasize the potential use of the legume–*Rhizobium* symbiosis in copper phytostabilization. Various studies that have included ACC deaminase minus mutants [74–78] or transgenic plants expressing a bacterial *acdS* gene also confirmed the positive effects of this enzyme on plant growth [79–81]. The main visible effects of seed or root inoculation with PGPB that synthesize ACC deaminase are the enhancement of plant root elongation, promotion of shoot growth, improvement in rhizobial nodulation and N, P, K uptake, as well as increased mycorrhizal colonization in various legumes and crops [68, 82–85].

11.3.2.3 Role of Nitrogen Fixation

Atmospheric nitrogen (N) can be converted into plant-utilizable forms of nitrogen by nitrogen-fixing microorganisms, which are widely distributed in nature, using the enzyme nitrogenase [86]. In addition to symbiotic N-fixing bacteria, a number of nonsymbiotic bacteria are also able to fix N and provide it to plants (Figure 11.2). Symbiotic N-fixing bacteria include members of the family Rhizobiaceae, which are able to form symbiosis with leguminous plants like rhizobia and non-leguminous trees such as *Frankia* [87]. Nonsymbiotic N-fixing bacteria including free-living, associative, and endophytic organisms like *Azotobacter* spp., *Bacillus* spp., *Beijerinckia* spp., and so on, can also benefit plants with fixed N [88], although it is generally believed that nonsymbiotic N-fixing bacteria provide only a small amount of the fixed N that the host plant requires [68]. Besides, nonsymbiotic N fixation basically occurs in free-living diazotrophics, belonging to genera such as *Azoarcus*, *Azospirillum*, *Burkholderia*, and *Gluconacetobacter* [89–92].

Recently, extensive concerns about environmental contamination have stimulated interest in the use of the legume–*Rhizobium* symbiosis as a tool for

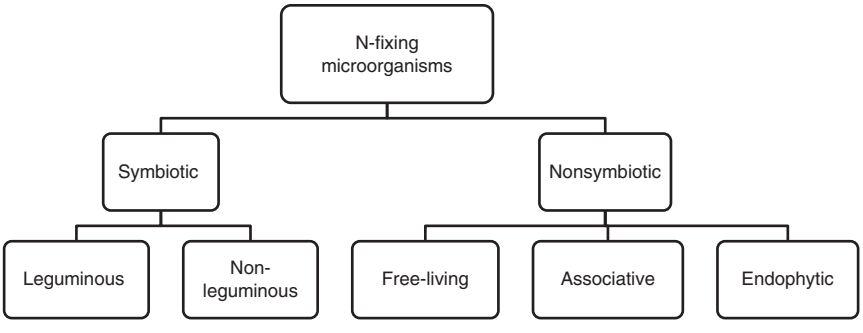

Figure 11.2 Categorization of nitrogen-fixing microorganisms.

Table 11.2 Rhizobial strains resistant to heavy metals and producing plant-growth-promoting substances.

Genus and species	Metal resistance	Plant-growth-promoting substances	References
Bradyrhizobium sp. RM8	Ni, Zn	IAA, siderophores, HCN, ammonia	[96]
Rhizobium sp. RP5	Ni, Zn	IAA, siderophores	[97]
Mesorhizobium sp. RC3	Cr	IAA, siderophores	[98]
Azorhizobium caulinodans	Cd	—	[99]
Bradyrhizobium sp. STM2464	Ni	—	[100]
Mesorhizobium metallidurans	Zn, Cd	—	[101]
Mesorhizobium sp. RC1, RC4	Cr	IAA, siderophores, HCN, ammonia	[102]
Rhizobium leguminosarum bv. viciae E20-8	Cd	—	[103]
Rhizobium sp. VMA301	$AsO4^3$	—	[104]
Sinorhizobium sp. MA11	AsO^{2-}	—	[105]
Sinorhizobium meliloti CCNWSX0020	Cu	IAA, siderophores, ACC deaminase	[106]
Rhizobium sp. RL9	Ni, Cd, Cr, Pb, Zn, Cu	IAA, siderophores, HCN, ammonia	[107]

—, not measured.

bioremediation of both heavy metals [73, 93, 94] and organic pollutants [95]. Nutrient deficiency, especially nitrogen and phosphorus, is often one of the main constraints that limit plant growth in soils [94]. The efficient use of rhizobial bacteria in the legume–*Rhizobium* symbiosis under stressful conditions not only facilitates the growth of legume plants but also increases soil fertility. In recent years, a great diversity of highly heavy metal-resistant rhizobial strains, including *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium*, have been isolated from industrial areas (Table 11.2). In addition to N fixation, a number of rhizobial bacteria also have positive effects on plant growth and development through other mechanisms, including IAA biosynthesis, siderophore production, and ACC deaminase activity. These rhizobia with both metal resistance and plant-growth-promoting abilities are of great interest for their potential use in phytoremediation of heavy metals. Rhizobial species isolated from legume nodules often show high resistance to one or more metals, and substantially improve the growth, symbiotic properties, and nutrient uptake of the plants grown in metal-containing soils [97, 98, 106, 108]. Although toxic metals are known to reduce the formation of root nodules as well as the N fixation efficiency, once nodulation is established, the effect of heavy metals on the N-fixing ability of legumes depends on the legume species and the type of metals [94]. Nitrogen content is generally known as an important

parameter used to evaluate the N fixation efficiency. A decrease in N content in legumes grown in heavy-metal-contaminated soils has been reported [98, 109]. However, *M. lupulina* plants inoculated with the Cu-resistant strain *S. meliloti* CCNWSX0020 showed a slight increase in N content in both shoots and roots [110]. Similar results have also been reported in other legume plants grown in heavy-metal-contaminated soils [97, 111]. Since no exogenous N was added to these plants, this means that these heavy-metal-resistant rhizobia are able to survive under the metal concentrations used and subsequently promote a normal level of plant nitrogen. Furthermore, a reduction in metal uptake by plant organs was observed in the inoculated plants, which in turn decreases the metal toxicity and consequently improves the overall performance of plants grown in metal-contaminated soils [96, 98, 108]. On the contrary, inoculation with the Cu-resistant strain *S. meliloti* CCNWSX0020 significantly increased the total amount of Cu uptake in both shoots and roots [110]. Moreover, the increase in roots was much higher than in shoots, thus decreasing the Cu translocation to shoots and facilitating phytostabilization, as previously shown for the *Medicago*–rhizobia symbiosis for Cu phytostabilization [112]. In metal phytoremediation, increasing or decreasing the amount of metal taken up by plant tissues is a function of the bacterium, the particular plant involved, the metal species, and the metal concentration [113].

In addition to N fixation, the plant-growth-promoting abilities expressed by metal-resistant rhizobia affect the metal solubility and bioavailability, both of which affect plant metal uptake [94]. Although the exact mechanisms of plant growth improvement by rhizobia in the presence of metals largely remain elusive, strains with multiple plant-growth-promoting activities are generally much more effective. For instance, the rhizobia containing ACC deaminase activity are thought to be valuable for use in phytoremediation due to their positive effects on the growth and nodulation of legumes. However, rhizobia typically produce a lower level of ACC deaminase compared to free-living PGPB. Although the endogenous rhizobial ACC deaminase is important in facilitating legume nodulation, it is probably not sufficient to protect plants against environmental stresses. In order to improve the performance of the *Rhizobium*–legume symbiosis under excess Cu conditions, an ACC deaminase-overproducing *S. meliloti* strain was constructed and its symbiotic performance tested in the presence of excess Cu [73]. The results showed that the engineered strain could improve the growth as well as the Cu tolerance of *M. lupulina*. Notwithstanding the apparent effectiveness of this approach, regulatory authorities in most countries worldwide are currently extremely reluctant to permit the release of genetically engineered bacteria into the environment. In this regard, several studies have already screened and tested metal-resistant PGPBs for their effects when co-inoculated with rhizobia on the growth and N fixation of legumes grown in metal-contaminated soils [93, 114]. Furthermore, under stressful conditions, the IAA synthesized by rhizobial strains and used to improve the root system of plants can also promote nodulation and, consequently, enhance N fixation. For example, positive effects of IAA overproduction in a derivative of the *S. meliloti* strain 1021 on the growth

and salt tolerance of *M. truncatula* have been demonstrated [61]. Moreover, it was recently shown that the growth of black locust trees (*Robinia pseudoacacia*) was greater when inoculated with *Agrobacterium tumefaciens* CCNWGS0286 than when inoculated with its mutant with lower IAA production under excess Zn conditions [115]. Thus, in addition to the rhizobial ability to fix N, other plant-growth-promoting abilities can work together and therefore improve phytoremediation by facilitating legume growth and affecting plant metal uptake, which becomes an important component of using the legume–*Rhizobium* symbiosis for metal phytoremediation.

11.3.2.4 Role of Siderophores

Despite the fact that iron is one of most abundant elements on earth, in the aerobic environment iron occurs principally as Fe^{3+} and usually is found in the form of insoluble hydroxides and oxyhydroxides, thus making it generally unavailable for assimilation by both plants and microorganisms [116]. To survive under such a Fe-limited conditions, bacteria synthesize low-molecular-mass iron chelators referred to as siderophores, which bind Fe with a very high affinity ($K_a > 10^{30}$), thereby facilitating iron uptake by microorganisms [117–119]. Bacterial siderophores stimulate plant growth by improving the Fe nutrition of the plants or by inhibiting the activity of plant pathogens [120] or other harmful microorganisms [121]. The improvement of plant Fe nutrition by soil bacteria is even more important when plants are exposed to heavy-metal pollution. In this case, bacterial siderophores help to alleviate the stresses imposed on plants by high levels of heavy metals in soils [122–124]. The effects of siderophore-producing bacteria on plant growth and their metal accumulation have been reported in various plant species [116]. For example, the plant- growth-promoting bacterium *Kluyvera ascorbata* SUD165, which is able to synthesize siderophores, could protect canola, Indian mustard, and tomato from metal (Ni, Pb, and Zn) toxicity [125, 126]. In addition, a siderophore-overproducing mutant of this bacterium showed greater protection, as indicated by enhanced biomass and chlorophyll content in plants grown in nickel-contaminated soil [126]. In another study, mung bean plants inoculated with the siderophore-producing bacterium *P. putida* KNP9 and grown in the presence of Pb or Cd showed greater biomass and enhanced chlorophyll content compared to uninoculated plants [127].

In addition to increasing the iron content of plants, bacterial siderophores can enhance metal mobility and bioavailability for uptake by plants [128]. For example, inoculation of maize with *Pseudomonas aeruginosa* increased shoot biomass and plant metal concentrations as well as translocation from roots to shoots in maize grown in an agricultural soil containing Cr and Pb [129], effects attributed to the siderophores produced by this strain that enhanced the bioavailability of Pb and Cr. Similarly, the bacterial culture filtrates containing hydroxamate siderophores produced by *Streptomyces tendae* F4 significantly increased Cd uptake by sunflower plants [130]. These results highlight the potential use of inoculating soils or plants with siderophore-producing bacteria

in metal phytoextraction. On the other hand, other workers have reported that the presence of siderophore-producing bacteria can reduce metal uptake by plants and thereby enhance plant biomass. For example, the concentrations of Pb and Cd in both roots and shoots of mung bean were significantly reduced in the presence of the siderophore-producing bacterium *P. putida* KNP9 [127]. Furthermore, the siderophores produced by *Pseudomonas* sp., *Serratia marcescens*, and *Streptomyces* sp. did not enhance Zn or Cd accumulation by willow [131]. These seemingly conflicting results of bacterial siderophore on metal uptake by plants suggested that the efficiency of siderophore-producing bacterium to either mobilize or immobilize heavy metals from soil depends on several factors, including soil composition, metal type and concentration, plant species, and the particular siderophore(s) [116].

Siderophores may also promote bacterial auxin synthesis, through a chelation reaction, which in turn increases the plant-growth-promoting effects of auxins [132]. However, the presence of metals, including Fe, generally inhibits microbial auxin synthesis. In order to enhance the plant-growth-promoting effects of auxin-producing bacteria in metal-polluted soils, siderophore and auxin-containing bacteria culture filtrates, rather than the bacteria that produce these compounds, were added to the metal-polluted soil [133]. Here, it was found that the addition of siderophore-containing culture filtrates of *Streptomyces acidiscabies* E13 alleviated the metal-induced oxidative stress in cowpea plants by lowering the formation of free radicals, protecting auxins from oxidative degradation and thereby improving plant growth and metal uptake.

11.3.3 Interaction with Mycorrhizae

Arbuscular mycorrhizal fungi (AMF), the ubiquitous rhizosphere fungi, form beneficial symbiosis with the root system of more than 90% of terrestrial plants [134]. In recent years, the arbuscular mycorrhizal association has received considerable attention due to its potential to promote plant growth, nutrition, degradation of organic pollutants, and heavy-metal accumulation by plants in contaminated soils. For example, Andrade *et al*. [135] assessed the effects of AMF on the growth of coffee seedlings in the presence of excess Cu and Zn. They observed that the mycorrhizal seedlings grew faster, had greater yields, and improved mineral nutrition than non-mycorrhizal seedlings. A greenhouse experiment was performed to determine the effects of three AMF inocula on biomass, metal uptake, and the microbial community structure in the rhizosphere of the native plant *Prosopis juliflora* [136]. After 2 months, mycorrhizal plants showed greater dry biomass and root length compared to non-mycorrhizal plants grown in compost-amended lead/zinc mine tailings. In addition, AMF inoculation had a significant effect on the composition of both the fungal and bacterial community structures in the rhizosphere. The synergistic effects of PGPR, combined with AMF, on plant growth have been reported in various plant species [137–141]. These studies revealed that co-inoculation improves

plant growth and nutrition compared to single or noninoculation. The benefits of PGPR and AMF consortia in the protection of host plants against environmental stresses have also been reported [137, 138, 142–144]. These findings suggested that the PGPR–AMF–plant tripartite relationship could be a promising approach to remediate polluted soil. Dong *et al*. [144] investigated the effects of PGPR inoculation combined with the AMF *Glomus intraradices* on the phytoremediation of petroleum-contaminated soil. The results of pot experiments showed that the degradation rate of total petroleum hydrocarbons with PGPR and AMF dual-inoculation treatment was up to 72.24%, which indicated that remediation efficiency of petroleum could be enhanced by this co-inoculation. In another study, co-inoculation with PGPR and AMF enhanced plant growth, soil quality, and the responses of plant antioxidant defense to hydrocarbon contaminants [143]. The extent of degradation of total petroleum hydrocarbon with PGPR and AMF co-inoculation treatment reached a maximum of 49.73% within 2 months. Similarly, a multicomponent phytoremediation system consisting of ryegrass, AMF, and PAH-degrading bacteria was performed to clean up PAH-contaminated soil [42]. The results highlighted the important role of both AMF and PAH-degrading bacteria in the phytoremediation of PAH-contaminated sites. Furthermore, ACC deaminase-containing *B. subtilis* not only alleviated ethylene stress but also enhanced mycorrhizal colonization and rhizobial nodulation in *Trigonella foenum-graecum* plants grown under drought stress, thereby improving nutrient uptake and plant growth [145]. Thus, metal-resistant PGPR in collaboration with AMF, displaying various plant-growth-promoting traits, can act synergistically to improve metal phytoremediation.

Under natural conditions, many important tree species form ectomycorrhizia (EM) between roots and ectomycorrhizal fungi (EMF). EMs are important in the nutrient uptake of forest trees, and are known to protect their host tree against various adverse environments, including heavy metals [146]. Studies conducted on the effects of EMF in metal uptake and translocation to plants revealed the mechanisms through EMF to restrict toxic metal uptake, including binding onto cell walls, extra- and intracellular chelation, compartmentation, and repair of damaged biomolecules [147–149]. It has been suggested that in functional EMs the mechanisms mediated by the fungal symbiont may sustain mineral nutrition and reduce metal uptake by the roots of host tree and thus enable the host tree to survive in metal-contaminated soils [150, 151]. For instance, seedlings of *Pinus sylvestris* inoculated with a Cu-adapted *Suillus luteus* isolate contained lower Cu concentrations in needles under high Cu concentrations (60 μM) than non-mycorrhizal trees [152]. Similarly, an earlier study demonstrated that two EMFs *Suillus bovinus* and *Thelephora terrestris* provided effective protection against Cu toxicity in seedlings of *P. sylvestris* exposed to high Cu concentrations [153]. A pot experiment was carried out to study the effects of Cu and Pb on the growth and chemical constituents of *Betula pendula* seedlings [154]. A reverse relationship was found between EMF colonization and heavy-metal levels in birch leaves, indicating the potential roles of EMF in protecting the host tree from excess Cu and Pb. Nevertheless, it is

suggested that the protection provided by EMF symbiosis highly depends on the fungal strain used and its tolerance to the metals [155, 156]. Therefore, it is important to select the appropriate fungal strains for tree inoculation, which ought to be considered in phytoremediation of heavy-metal-contaminated soils by woody species. Furthermore, in a recent study, the combined effects of the fungus *Paxillus involutus* and the rhizobial bacterium *Mesorhizobium* sp. on the growth *Betula pubescens* were determined in two different soils, a forest soil and an alkaline sediment [157]. However, an antagonistic interaction was detected between *P. involutus* and *Mesorhizobium* sp. in the alkaline sediment, which indicated that the response of white birch (*B. pubescens*) to inoculation with a mycorrhizal fungus and a PGPR is soil-dependent. Similarly, although it was previously demonstrated that co-inoculation of legumes with rhizobia and metal-resistant PGPBs is a way to overcome environmental limitations and improve plant growth [158–160], other results have suggested that co-inoculation might impair rhizobial colonization and subsequent nodulation [161–164]. Thus, in order to optimize the phytoremediation potential of a system under particular environmental conditions, it is necessary to pay detailed attention to the selection of the appropriate co-inoculation partners and the traits that they encode.

11.4 Examples of Phytoremediation in Action

Although the successes of laboratory and greenhouse experiments are often difficult to replicate in the field, a number of encouraging results from the field have been reported. For example, a 3-year field test of phytoremediation of total petroleum hydrocarbons was performed at a Southern Ontario site [165]. It showed that the amount of plant growth and the extent of oil remediation were consistently enhanced by PGPR. The potential use of ACC deaminase-containing transgenic canola and PGPB as a phytoremediation strategy was assessed at a nickel-contaminated field site [71]. The results showed that the addition of the PGPB *P. putida* HS-2 enhanced plant growth, resulting in a 10% increase in nickel accumulation per plant. In *in situ* bioremediation of 2,4,6-trinitrotoluene (TNT), 96% of the extractable TNT in rhizosphere soil associated with maize roots was removed within 2 months [166]. At a crude-oil-contaminated site, 3 years of phytoremediation management through a combination of vegetation establishment and fertilizer addition led to a decrease of crude oil contaminants [167]. In a 2-year field trail at a heavy-metal-contaminated site in Chang-Hwa City, *Jatropha curcas* (a bioenergycrop plant) showed high absorption capability for Cd, Ni, Cr, and Zn [168]. In a phytoremediation study at a former uranium leaching heap site near Ronneburg, Germany, the results suggested the potential use of *Sorghum bicolor* for metal phytoextraction, especially for Cd and Co, with microbial inoculation improving plant survival by alleviating the toxic effects of other metals, such as Ni, which often exist at anthropogenically contaminated

and metalliferous areas [169]. The compound ethylenediaminetetraacetic acid (EDTA) enhances metal phytoavailability in soil, subsequently improving metal uptake and translocation in plant aerial organs [170–172]. A recent field trial was conducted to evaluate the effectiveness of metal phytoremediation using three plant species and EDTA or ammonium addition to assist in the removal of heavy metals (Pb, Hg, and Cd) from contaminated farmland in northwestern Iran [173]. The results of this trial indicated that *Amaranthus retroflexus* showed great potential in phytoremediation of metal-contaminated soils. Moreover, the application of EDTA significantly enhanced the bioavailability of Pb, Hg, and Cd. Finally, a 3-year phytoremediation study at a hydrocarbon-contaminated lagoon in England recommended that long-term studies are needed for all ecologically sensitive sites such as this lagoon [174].

11.5 Summary and Perspectives

Bacterially assisted phytoremediation is emerging as a promising technology for remediating contaminated soils. In the past 15 years, scientists have developed a much better understanding of how various bacteria contribute to plant growth and subsequently improve the effectiveness of phytoremediation. The recent integration of stable isotope probing (SIP) with the rapidly advancing field of metagenomics provides scientists with a much deeper insight into microbial process and interactions, which may enable breakthroughs in the field of bioremediation [175]. The efficacy of phytoremediation approaches has been demonstrated under laboratory and greenhouse conditions. For organic contaminants, phytoremediation, particularly rhizoremediation, has been found to be cost efficient and effective in the field, and it is likely that its application will become widespread in the next few years. However, there are still many areas with poor understanding or scant information where further research is needed. In order to implement bacteria-assisted phytoremediation in the field, further research is needed to understand the diversity and ecology of plant-associated bacteria in contaminated soils. In metal phytoremediation studies, different bacteria affect plant growth and metal uptake in different ways, so that detailed attention must be paid to the selection of the appropriate bacteria and the traits that they encode. For this purpose, further research should be focused on how a plant's metal tolerance is affected by its associated bacteria. Moreover, a further understanding of the effect of heavy metals on plants and their associated bacteria are also required. Since the plant's heavy metal tolerance and uptake depend on various factors including the bacterium, soil composition, metal type, and concentration, the scientific community should strongly focus on the selection of the most suitable plants to overcome such environmental constrains. To implement bacteria-assisted phytoremediation on a larger scale in the environment, more field application of these approaches is needed. In this regard, regulatory acceptability, site assessment, and the potential ecological risks should be specially addressed.

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Part IV
Animal Cell Cultures

Applied Bioengineering: Innovations and Future Directions, First Edition. Edited by Toshiomi Yoshida.

12
Cell Line Development for Biomanufacturing Processes

Mugdha Gadgil and Wei-Shou Hu

12.1
Introduction

With the increasing reach of protein therapeutics in different regions of the world and the expanding manufacturing capacities in Asian countries in the past few years, there has been renewed interest in enhancing the productivity and streamlining cell line and process development. The core of the production of a recombinant protein molecule using animal cells is the establishment of a production cell line stably expressing the protein of interest at an acceptable volumetric productivity. This process can take anywhere between 4 and 12 months. Small amounts of protein for preclinical studies can, however, be expressed transiently in a shorter time without establishing a stable cell line [1]. Development of a stable protein-producing cell line for the production of therapeutic proteins begins with transfecting the gene encoding the protein of interest into a suitable host cell and selecting single-cell clones in which the transgene has integrated into the host cell genome. The selected clones are further screened to select the best clones with the highest volumetric productivity and with an acceptable growth rate, out of the heterogeneous population of transfected clones. The few best clones are further evaluated for stability in cell growth, productivity, and product quality, and the best "stable" clone is identified for use as a production clone. Figure 12.1 details the steps involved in stable cell line development, along with choices to be made by the user at each step (Figure 12.1). The chapter is organized into sections describing each step during cell line development beginning with the choice of the host cell, the DNA elements on the vector carrying the transgene which are then transfected into the host cell, followed by methods for selection of a stable single cell clone.

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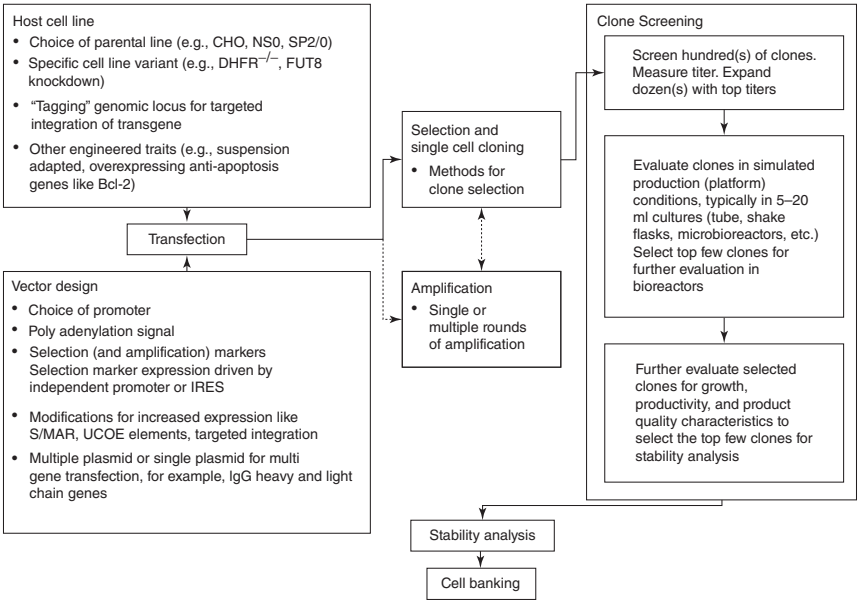

Figure 12.1 Steps involved in stable cell line development. Options available at each step are described in brief. The amplification step, when implemented, may be carried out either on a bulk selected pool or on expanded single-cell clones, as discussed in text.

12.2 Host Cell

In selecting a host cell for protein production, the regulatory history of the cell line is a major consideration. Cell lines that have been used in commercial manufacturing of biologics and have received previous regulatory clearance for production are often favored because of their lower risk of unforeseen safety concerns. Cell lines of rodent origin, including those from Chinese hamster ovary (CHO), NS0, Sp2/0, and baby hamster kidney (BHK), as well as those derived from human (HEK293), have been used in commercial production. Two cell lines in particular, CHO and NS0, account for over 70% – in terms of protein quantity and commercial value – of currently manufactured recombinant proteins in animal cells. In host cell selection, the profile of post-translation modification of the product, especially glycosylation, is another important factor for consideration. The profile of glycosylation on the protein molecules affects their therapeutic activity and immunogenicity. The N-linked glycan structures from CHO and NS0 contain the same basic oligosaccharide structures naturally occurring in human proteins with some differences [2]. For example, *N*-glycolylneuraminic acid (NGNA), not typically found in adult humans, is present in CHO and NS0 cultures. The ratio of *N*-acetylneuraminic acid (NANA) to NGNA is variable and depends on the cell line [3]. Cell lines of human origin such as PER.C6 and CAP have been more recently developed for commercial manufacturing [4]. The proteins produced in these lines will have human glycans, but to date a large majority of commercial products are not produced in human cell lines.

An ideal host cell line should also be easily cultured in large-scale bioreactors and not liable to conditions adverse to growth, productivity, or product quality. Since suspension growth is often preferred in large-scale operation, many host cells have been adapted to suspension growth if they were derived from adherent cells. Susceptibility of host cells to viral infection is an important safety concern. A compilation of previously reported CHO and BHK cell infections by viruses suggests that CHO cells are less susceptible to viral infection than BHK [5]. The number of viruses known to infect CHO cells is small. Wiebe *et al.* found only 7 out of 45 viruses from nine virus families were able to infect CHO-K1 cells [6]. An evaluation of 14 viruses from 12 families including a few known to have contaminated CHO cell-derived biologics in the past two decades found that viral infections of CHO cells are detectable either through the induction of a visible cytopathic effect or during routine adventitious agent testing demonstrating safety from viral contaminants [5].

12.2.1 Host Cell Engineering

A significant amount of effort is going on in genetic engineering of host cells to achieve more efficient growth, metabolism, and protein secretion or for engineering desirable product characteristics like specific glycosylation profiles. The

productivity of a clone results from a combination of its specific productivity, which can be increased by some of the strategies discussed in Sections 12.2–12.5, and the longevity of the culture. Cell engineering strategies used to increase to latter include approaches to suppress apoptosis [7] and manipulation of cellular metabolism to reduce waste metabolite formation [8], which eventually affects cell growth and viability, especially in fed-batch culture. Engineering of cell-cycle-related genes has been employed to induce cell-cycle arrest to increase productivity after initial cell growth [9]. Cell engineering has also been used to improve the post-translational capacity of the cell, with most effort being directed to the glycosylation pathway to engineer glycan features such as increased sialylation [10] or decreased fucosylation [9]. For a review on cell engineering strategies, the reader is referred to [11].

12.3 Vector Components

The gene encoding the protein of interest is invariably transfected into the host cell via a plasmid. The transgene sequence can be optimized for features like codon usage, GC content, and removal of secondary structure, which can result in an increase in recombinant protein expression [12]. The following elements are required on the plasmid, in addition to DNA encoding the protein of interest.

12.3.1 Promoter/Enhancer

The promoter region is the stretch of DNA upstream of the gene to be transcribed, generally before where transcription is initiated. It can be as short as 100 bp or up to 10 kbp in length, including regulatory elements. Different promoters have different strengths in initiating transcription and different dynamics in responding to its cues. Some promoters are constitutive, expressed at a relatively constant level all the time, whereas others are dependent on the cell cycle or other growth conditions. If the promoter is specific to a stage of the cell cycle, it influences the growth association of expression. For example, a promoter specific to the S phase of the cell cycle will result in protein expression being growth-associated, while a promoter specific to the G1 phase shows protein expression to be inversely related to growth [13–15]. Ludwig has summarized mammalian expression systems for antibody expression [16]. Expression of the recombinant protein product is driven by a strong promoter, while the expression of the selection marker is typically driven by a weaker promoter such as SV40 or the TK promoter. The most commonly used promoter for protein production is the constitutive cytomegalovirus (CMV) promoter; however, its strength varies considerably between cell lines [17].

An oncogene-activated production system was developed on the premise that oncogenes can enhance promoter activity [18]. It uses a combination of an effector

plasmid, which consists of an oncogene, and a reporter plasmid that carries the gene of interest. The product of the effector plasmid should stimulate the promoter of the reporter plasmid to enhance recombinant protein expression. Upon evaluation of several oncogenes and promoters, the best system was reported to comprise the oncogene *ras* and CMV promoter [18]. A hybrid system was further reported using the amplified *ras* oncogene to activate the CMV promoter, which was further amplified using methionine sulfoximine (MSX) (glutamine synthetase (GS) marker present along with transgene; the amplification is described in detail in Section 12.6), which showed higher clonal productivity as compared to only MSX amplification [19].

The presence of introns is also known to increase efficiency of transcription. The protein expression levels driven by the human CMV promoter in the presence or absence of intron A, the mouse and rat CMV promoters with intron A, and the mouse myeloproliferative virus (MPSV) Long Terminal Repeat (LTR) were evaluated in both CHO-K1 and HEK293EBNA cells [20]. The human CMV, in the presence of intron A, was reported to give the highest levels of protein expression during transient expression in both cell lines. However, the MPSV promoter resulted in the highest expression of four proteins evaluated in selected bulk pools in CHO-K1 cells, suggesting, not surprisingly, that promoter strength is not the only important factor for protein expression in stable cell clones. Under the hypothesis that the use of transcription control regions from a highly expressed gene in CHO cells to drive expression of the transgene might result in significantly increased protein expression, expression vectors containing 5′ and 3′ flanking sequences from the Chinese hamster EF-1α (CHEF1) gene were constructed. These vectors showed better reporter gene expression compared to its expression driven by CMV or the human EF-1α promoter [21]. Recently, the characterization and use of a core cold-inducible RNA-binding promoter for increasing protein expression was reported [22].

The promoter used for product protein expression can also influence the long-term stability of its expression level. CpG dinucleotides within the human CMV promoter/enhancer were reported to be methylated in recombinant CHO cell lines that have a decreased productivity after long-term culture [23]. Inducible promoters may be used for the expression of proteins that may be toxic to cells. These usually require the addition of an expression-inducing agent to the culture, which needs to be later removed during purification. Inducible promoters based on the principle of removal of an external agent have also been reported (US Patent 5,814,618).

In view of the importance of the promoter in driving protein expression, it is surprising that more attention has not been paid to this area. Increased molecular understanding of the functions of the different promoter elements can result in the synthetic design of a promoter region for high-level constitutive protein expression [24].

Enhancers are DNA sequences that increase gene transcription and may not necessarily be located close to the promoter sequence. Commonly used promoters such as the CMV or SV40 have associated enhancer sequences that can be

included along with the respective promoter in the vector construct. Among several promoter/enhancer combinations evaluated, the CMV promoter/enhancer was found to result in the highest levels of reporter gene expression [25, 26].

The Kozak sequence (CCA/GCC [27, 28]) can be placed in front of the AUG start codon of the gene of interest to improve the translational initiation of mRNA.

12.3.2 Intron

The incorporation of introns into the coding region of the gene can sometimes result in an increase in recombinant protein expression. The mechanism of how introns affect mRNA processing is reviewed in [29]. The addition of introns to the coding sequence of naturally intron-less genes can also result in increased transgene expression, as shown for the human interferon-β [30]. The expression level of human interferon-β from the intron-added construct, however, depended on the location of the inserted intron. A similar effect was shown where insertion of two introns derived from the mouse immunoglobulin heavy chains into the coding region of the green fluorescent protein (GFP) resulted in a fivefold increase in mean fluorescence intensity of clones generated with the intron-added construct compared to those without the introns [31]. Conversely, the removal of an intron from an antibody heavy chain coding sequence (CMV promoter driving expression of the heavy and light chain sequences continued to contribute an intron to the primary transcript) was shown to have no effect on the antibody productivity in CHO cells [12]. At the same time, gene optimization by a process designed to optimize codon usage, remove cryptic splice sites, optimize GC content, and remove direct repeats and secondary structure elements resulted in an increase in antibody expression. This reinforces that the rate-limiting step during protein expression for the particular cell line will decide whether a particular modification results in higher protein expression, thus making "generalizations" of efficacy difficult.

12.3.3 Poly-Adenylation Signal

The 3′ UTR (untranslated region) consists of the region between the nucleotide immediately following the stop codon and the polyA tail on the mRNA. It is important for proper termination of transcription, and also plays a role in mRNA stability and transport of the mRNA from nucleus to cytoplasm (reviewed in [32]). The 3′ UTR contains the polyA signal with the consensus AATAAA, and a GT-rich downstream sequence element. Because of the limited options currently available, the predominant polyA signals used in gene constructs for recombinant protein production are either SV40 or bovine growth hormone (BGH) polyA. A comparison between three polyA signals showed that the one from BGH gave three times higher recombinant protein expression compared to those from SV40 early and human collagen, irrespective of the promoter used [33].

The 3′ UTR of some genes can contain instability elements such as the AT-rich elements (ARE), which can reduce the stability of the nascent transcript and need to be removed before their use in recombinant protein production. However, there appears to be a complex relationship between the particular polyA signal used and the presence of ARE. For example, the deletion of ARE of the human granulocyte stimulating hormone cDNA resulted in increased rhG-CSF synthesis when using polyA signal from the rabbit β-globin gene but not from SV40 [34]. Attention should also be paid to the coding sequence and polyA signal sequence to avoid unintended consequences. For example, CHO-K1 cell lines expressing the recombinant complement activator blocking protein showed a large fraction of the mRNA for the transgene to be shorter than the expected length due to aberrant splicing from within coding sequence to a site within the 3′ UTR and due to premature polyadenylation at an AATAAA site within the coding region [35].

12.3.4 Selection Marker

A selection marker is required to select the presence of the transfected plasmid in cells. Selection markers include genes imparting resistance to drugs such as neomycin, geneticin, hygromycin, puromycin, blasticidin, and zeocin. In addition to these, enzymes essential for cell growth that are not expressed in the host cell can also be used for selection. Two such commonly used enzymes for selection are dihydrofolate reductase (DHFR) and GS.

DHFR catalyses the production of tetrahydrofolate by the reduction of dihydrofolate. Mutant cells lacking DHFR use the salvage pathway for nucleotide synthesis, which requires the supply of hypoxanthine and thymidine. In a medium without hypoxanthine and thymidine, those mutant cells cannot survive unless they acquire DHFR from the transfected plasmid. The GS systems functions similarly: GS catalyzes the formation of glutamine, an essential amino acid for cultured cells, from glutamate and acts as a selection marker in the absence of glutamine in the culture medium. These enzymatic markers also allow gene amplification, as discussed in Section 12.6.

Not all cells that received transfected plasmids and express the selection marker protein also express the gene of interest. The frequency of the false positives (those expressing the selection marker but not the protein of interest) varies with the selection marker used. These false positives arise through mutations that develop alternate mechanisms such as inactivation of transport of the selection marker into the cell. A comparison of clones expressing GFP obtained using different selection markers in two human cell lines showed that all zeocin-resistant clones exhibited GFP expression in both cell lines. However, only 79% of hygromycin B-resistant, 47% of neomycin-resistant, and 14% of puromycin-resistant clones expressed GFP [36]. Thus the choice of the selection marker can be important for reducing false-positive clones. The expression of the selection marker is usually

driven by a weaker promoter than the promoter driving the expression of the protein of interest. Sometimes a mutated selection marker with a lower activity to rescue the missing enzyme function is used [37]. With such "weakened" expression of selection marker, the integration site must be transcriptionally active and allow for a higher transcript level of the selection marker in order to counter the selection pressure. This may lead to increased expression of the protein of interest.

Selection marker can also be expressed in a dicistronic construct through an internal ribosome entry site (IRES) element [38, 39]. *Picornavirus* F2A and "2A-like" sequences are also reported for coexpression of multiple genes [40]. Split GFP fragments linked to IgG heavy and light chain genes resulting in the use of GFP as a selection marker have been used to identify high producers using fluorescence-activated cell sorting (FACS) [41]. Such approaches can be useful for high-throughput identification of optimal integration sites on the host cell genome. Incorporation of mRNA and protein destabilizing elements such as ARE [42], or the use of a selection marker with a start codon that confers attenuated translation initiation frequency [43], can also be used to reduce the strength of the selection marker to increase the probability that the selected clones have high productivity of the recombinant protein of interest.

12.3.5 Secretion Leader Sequence

Virtually all therapeutic proteins produced in mammalian cells are secreted. In their native forms, each has its own leader sequences for translocation into the endoplasmic reticulum during translation. These leader sequences can be possibly replaced with a stronger signal sequence to increase the efficiency of secretion [44]. Sixteen different secretion signal sequences from several species in mammals, fish, scorpions, snails, fungi, plants, viruses, and bacteria were fused to the antibody heavy chain and light chain genes to compare their ability to drive the secretion of the recombinant antibody [45]. The signal sequences derived from human albumin and human azurocidin gave the highest productivity. However, the signal sequence derived from human albumin had previously been reported to result in weak expression of a reporter protein [46], indicating the downstream protein sequence to the signal sequence has an effect on its performance.

Post-translational cleavage of the leader sequence at nonspecific sites or incomplete cleavage of the signal sequence has been reported, which can result in heterogeneity in the mature protein sequence at the N terminus [47, 48]. The efficiency of cleavage of the leader signal is thus an additional parameter to be considered during its selection.

12.3.6 Components for Plasmid Cloning in *E. coli*

These include the bacterial selection marker to select for presence of plasmid in *Escherichia coli* and the bacterial origin of replication for propagation in *E. coli*.

12.4 Transfection

The plasmid containing the transgene is transfected into the host cell. The efficiency of transfection is assessed by the percentage of cells subjected to transfection that have received the plasmid, regardless of how many copies of plasmids have entered the cell. High transfection efficiency may not be necessary for establishing stable cell lines, since as, the transfection step is followed by selection, only the cells expressing the gene imparting resistance to the selection pressure eventually survive. The transfection efficiency and the distribution of plasmid copy number per cell in the transfected population are affected by the concentrations of plasmids and cells, culture age, and the method of transfection used.

12.4.1 Method of Transfection

Transfection can be carried out by several means such as calcium phosphate precipitation [49], lipofection [50], polycation-mediated transfection [51], and electroporation [52]. The most commonly used methods are lipofection and electroporation. Calcium phosphate precipitation, lipofection, and polycation-mediated transfection are strongly affected by the medium composition. Chenuet *et al.* compared calcium phosphate precipitation and polycation-mediated transfection using linear 25-kDa polyethyleneimine (PEI) and observed that the efficiency of recombinant cell line recovery was higher for PEI transfection at one colony per 275 plated cells than $CaPO_4$ transfection at one colony per 1350 plated cells [53]. The copy number of integrated plasmids was higher in cells transfected with $CaPO_4$, and the average specific productivity of a recombinant antibody was about twofold higher for the $CaPO_4$-derived cell lines. This difference was, however, not seen for GFP expression levels for transfections by the two methods. No difference between the two transfection methods was observed in terms of the stability of protein production. Several liposomal formulations are available commercially that result in high transfection efficiencies for the host cells commonly used for protein production. The effectiveness of PEI and Lipofectamine in delivering a 150-kb bacterial artificial chromosome DNA to mouse and human cell lines (adherent and suspension) was evaluated. It was found that though a 22-kDa linear PEI was the most efficient method for delivery, Lipofectamine 2000 gave a higher proportion of stable clones with intact DNA [54].

Repeated transfection of the same cells has been shown to result in increased expression of the protein [55]; however, it remains to be seen whether such an approach has any effect on the productivity or stability of stable cell lines. However, repeated lipofection or electroporation may aggravate the cell damage caused by transfection. Overexpression of the anti-apoptosis protein Bcl-2 was recently reported to allow transfections at higher nitrogen/phosphate (N/P) molar ratios with PEI which would otherwise be severely toxic to the cells, and hence result in higher transfection efficiency [56].

12.4.2 Plasmid Conformation

Plasmid used for transfection can either be circular or linearized using a restriction enzyme whose recognition sequence lies outside the transgene and selection marker. Though circular plasmids have been used for transfection, linearized plasmid DNA is thought to result in better efficiency. An evaluation of the effect of linearization by two different restriction enzymes – of which one was cut at two locations and resulted in the loss of a small DNA fragment from the linearized plasmid – concluded that linearization of plasmid DNA prior to transfection can increase the efficiency of stable clone generation and protein expression compared to circular DNA, but is dependent on the site of linearization [57]. This could possibly explain dissimilar conclusions regarding the higher effectiveness of linearized DNA for transfection. Circular DNA was reported to be better than linearized DNA for transient protein expression [58].

12.5 Integration of Foreign DNA into Host Chromosome

After transfection, the number of plasmid molecules entering cytoplasm may be large, perhaps in thousands. Only a fraction of those enter the nucleus, while the rest are degraded. Note that the plasmid must enter the nucleus and be transcribed in order for the selection marker protein to be translated in the cytoplasm. Only a small number of plasmid molecules will be integrated into the genome. Integration of plasmid DNA into the host chromosome is a random event. Integration may occur at a single location in the genome or in multiple loci on the same or different chromosomes. Analysis of the karyotype and chromosomal integration site for 20 recombinant CHO DG44 clones transfected with GFP showed a single integration site in all clones irrespective of whether DNA was delivered by calcium phosphate–DNA coprecipitation or microinjection [59]. The copy number of the integrated gene varied between 1 and 51 for the 20 clones, but was not correlated to the strength of GFP expression. Random integration of the gene of interest can result either in integration in a condensed heterochromatin region that is relatively inaccessible to transcription or in unwanted loss/attenuation in expression of an essential gene in the vicinity of the site of integration. In both cases, the performance of resultant clone will be suboptimal. Targeting the vector to a pre-identified suitable site on the genome of the host cell can prevent such effects. The rapid development in technologies for targeting any genomic location, such as the CRISPR/Cas systems, will aid in this, once suitable sites are identified [60]. CRISPR/Cas system is a RNA-guided DNA endonuclease, which brings about DNA cleavage in a sequence specific manner, thus facilitating integration of the transgene at the targeted genomic location. The guide sequence in the crRNA provides sequence specificity to the Cas endonuclease [61]. The ease of design of CRISPR/Cas has greatly improved access to the targeted integration technology.

12.5.1 Site-Specific Integration

To target the gene of interest to a transcriptionally active site in the host genome, targeting using recombinase-mediated cassette exchange (RMCE) can be employed [62]. RMCE is based on the replacement of gene cassettes flanked by two recombination target sites. Cre and FLP are two commonly used Tyr recombinases, while the more recently used ΦC31 is a Ser-integrase.

Tyr recombinases use a catalytic tyrosine to attack a phosphodiester bond of target DNA, site while the Ser integrases use a conserved serine. Both result in a recombination, triggered by crossover of the recombinase sites (loxP, FRT, and attB and attP). Cre-loxP- or FLP–FRT-mediated recombination between two directly repeated recombination targeting sites excises all DNA sequences located within the two sites. The ΦC31 recombinase mediates the recombination between the heterotypic attB and attP sites and results in strictly unidirectional integration. The RMCE technique follows two steps [63]: (i) "Tagging" a genomic locus: introduction of recombination target sites into the host genome. This can be achieved by random insertion of a reporter gene like GFP flanked by the target sites followed by identification of a clone with the best expression of the reporter gene. The reporter gene is then assumed to be integrated into a transcriptionally active site which is "tagged" in this clone. (ii) "Targeting" of the tagged site: RMCE of the tagging cassette with the desired transgene cassette flanked by matching recombination target site. The RMCEs employed in mammalian cells are summarized in [63]. Recently, the "tagging" step was carried out using ΦC31 integrase to selectively direct GFP into the CHO genome at pseudo-attP sites, which may support sustained transcriptional activity [64]. This resulted in fewer clones being required to be screened to identify high GFP expressing clones, compared to when GFP was randomly integrated in the CHO genome. RCME was then further employed using Cre-loxP for rapid development of clones expressing $\sim 2\,g\,l^{-1}$ IgG.

12.5.2 Use of cis-Acting DNA Elements

Besides integration into a transcriptionally inactive region of the genome, the gene of interest may become transcriptionally less active or even inactive over a period of time due to silencing. Such silencing can be a result of causes such as DNA methylation at the gene's promoter and histone deacetylation in the region containing the gene [65]. An alternate approach to delivering the gene of interest to a transcriptionally active spot on the host genome has been proposed following the discovery of short DNA sequences in transcriptionally active parts of the chromatin [65].

Transcription of the gene of interest can be isolated from the effect of the integration site by employing such cis-acting DNA sequences (reviewed in [66]). Such sequences are used to flank the gene of interest in the vector and are thought to

result in a more accessible chromatin structure at the site of integration, irrespective of the status of the neighboring chromatin region. Several such sequences, typically a few thousand base pairs in length, have been identified. They are categorized on the basis of the specific experimental assays used to identify the sequence and are hence thought to have different mechanisms of action. The more popularly used among these include scaffold/matrix attachment regions (S/MAR), ubiquitous chromatin opening elements (UCOE), and stabilizing and antirepressor (STAR) elements, the technology associated with some of which is also commercially available. Insulators are DNA sequences that act as a barrier to the influence of the neighboring chromatin.

S/MAR are sequences in which the chromosome is attached to the nuclear matrix and hence separate euchromatin and heterochromatin. Consequently, flanking the gene of interest with these elements is thought to insulate the effect of the adjoining chromatin elements at the site of integration. However, the increase in transgene expression seen with the incorporation of S/MAR requires functional aspects of the S/MAR in addition to its ability to bind the nuclear matrix, as seen by the inability of subfragments of a chicken lysozyme gene 5′ MAR exhibiting nuclear matrix binding activity to enhance transgene expression [67]. Several S/MARs have been reported to be used for recombinant protein expression such as Chicken lysozyme MAR, human b-globin MAR, and human β-interferon gene associated MAR. The MAR element can be introduced either in cis on the same plasmid as the transgene or in trans on a separate plasmid, which also allows control of the relative number of MAR elements introduced by varying relative concentrations of the two plasmids [68].

UCOE are regulatory elements derived from ubiquitously expressed housekeeping genes which prevent heterochromatin formation and hence gene silencing. Expression vectors in which the CpG island from the *RNP* locus was combined with the CMV promoter/enhancer showed an increase in the level of expression and proportion of transfected cells expressing the recombinant protein in CHO [69]. The 1.5-kb RNP UCOE was also reported to perform better than the 3.2-kb mouse RPS3 fragment for expression of the B domain deleted factor VIII in BHK21 cells while evaluating different UCOE-promoter combinations in both attached and serum-free suspension adapted cells [70]. A comparison of the use of pFUSE expression vector and UCOE containing vector to express H1C2 mAb in NS0 cells and CHO cells, respectively, showed that incorporation of UCOE in CHO cells generated a higher number of high-producing stable clones [71].

STAR elements were identified based on their ability to block heterochromatin-associated repression. They also enhanced transgene expression in different cell lines in combination with different promoters. A 3.6-kb transgene expression enhancing sequence (expression augmenting sequence elements, EASE) included with the transgene on the vector was reported to result in higher protein expression, even after DHFR amplification [72].

The studies on such cis-acting DNA elements were carried out in different host cells, promoters, and other vector elements. The interaction between these cis-acting DNA elements and the vector and host cell has been recently shown to be

important. A DNA element effective in one host cell/vector system may not be equally effective in another [68, 71].

Transposons provide another means of introducing foreign genes into host cells. PiggyBac transposon, originally derived from *Trichoplusia ni*, has been used to deliver transgene for protein expression. The PiggyBac transposon consists of the PB transposase gene flanked by terminal repeats required for transposition and is thought to result in integration into a transcriptionally active region of the genome, which will result in increased protein expression. The transgene is flanked by the left and right terminal domain of the PB transposon, and is transfected along with a helper plasmid expressing the PB transposase. The use of the PiggyBac transposon was reported to yield increased and more stable protein expression in CHO cells [73, 74]. Multiplexed transfer of up to four transgenes has been reported using transposons with selection for a single marker transposon [75]. Simultaneous application of more than one of the above strategies may be beneficial in some cases but will need careful optimization, as demonstrated by Ley *et al.* who recently evaluated the effect of simultaneous use of MAR and transposon and found that the relative distance between MAR and transgene could modulate transposon activation [76].

12.6 Amplification

Gene amplification was discovered four decades ago through the investigation of the mechanism of resistance to chemotherapeutic drugs such as methotrexate (MTX). It has since become widely used for generating cell lines producing recombinant proteins. The most commonly used amplification systems in cell line development are the DHFR and GS genes. DHFR and GS are both selection marker genes and amplifier genes. The gene of interest is transfected along with the amplifier gene (DHFR or GS) in a single plasmid or on two different plasmids that are co-transfected. Co-transfected plasmids are usually co-integrated in the host genome [77], resulting in the transgene and DHFR being integrated at the same chromosomal locus. Amplification is usually carried out on the highest producing clones selected from the initial selection step. For amplification using DHFR, the selected cells are treated with MTX. MTX is a potent inhibitor of DHFR. Its presence suppresses DHFR activity, thus allowing only those cells expressing a high amount of DHFR and hence the transgene to overcome the inhibition. The amplification selection step is carried out in a single step with a fixed MTX concentration, or in several steps with increasing MTX concentration. The MTX concentration used also varies depending on the host cells and the medium used, ranging between 20 nM and 5 μM. The duration of MTX treatment ranges from 10 days to over 2 weeks. After each step of MTX amplification, a population of surviving cells is allowed to recover and expand before further increasing the MTX concentration. With stepwise amplification, the entire period can stretch to months.

After amplification, the copy number of DHFR gene and the transgene ranges from a couple hundred to a few thousand. Cells that overcome the MTX effect

by developing alternative mechanisms may grow faster than the DHFR-amplified cells and overtake the population. Some practices thus perform the amplification using isolated clones instead of the bulk pool of cells. However, each single-cell cloning takes at least 3 weeks. Repeated single-cell cloning after each amplification steps would pose a high time constraint.

A number of factors have been reported to influence the degree of DHFR gene amplification. The integration locus plays a role in amplification. Several cells lines with deregulated c-Myc expression showed DHFR gene amplification and ongoing rearrangements [78]. However, copy numbers of several other genes remained unaltered, indicating locus specific genome instability of DHFR in cells with deregulated c-Myc protein levels. It has been recently shown that the amplification rate of human adenosine deaminase (ada) varies widely, and heritably, between subclones [79]. Site-specific recombination was used to target a transgene and functional DHFR at the locus of ada integration for a high ada amplification candidate clone, which resulted in higher levels of gene amplification compared to randomly targeted DHFR gene. Presence of the DNA element HSAG-1 was also reported to promote gene amplification of DHFR transfected using a plasmid, though the exact mechanism of action of HSAG-1 is unknown [80]. More stringent selection was also achieved by incorporating the DHFR gene within splice donor and acceptor sites in a dicistronic construct [81].

GS catalyzes the formation of glutamine from glutamate and ammonia. The transfected cells are typically grown in a medium without glutamine to maintain selection pressure, thus resulting in low ammonia production. GS selection marker is widely used in a GS-negative cell line NS0. GS inhibitor MSX is used for gene amplification, in a way similar to MTX in the DHFR amplification system. In NS0 cells, 10–100 μM MSX is used to amplify GS to 4–10 copies per cell. CHO cells have endogenous GS activity, which can potentially reduce the stringency of selection by MSX. Recently, the endogenous GS in CHO-K1 was knocked out using zinc finger nucleases [82]. The IgG-producing GS^{-} cells derived with a lower level (25 μM) of MSX amplification had a higher frequency of high-producer clones than the parental CHO-K1 amplified with a higher level (50 μM) of MSX.

12.7 Single-Cell Cloning

Single-cell cloning is carried out before the final candidates of the producing cell line are selected. This is necessary to ensure that the producing line does not originate from a heterogeneous population in which some subpopulation may eventually outgrow others changing the characteristics of producing line. So, single-cell cloning must be carried out after amplification. It is also carried out after selection or in different stages of stepwise amplification, although this is not universally performed.

Adherent cells are generally cloned after transfection by plating at a sufficiently low density in Petri dishes to allow the individual cells that survived selection or amplification to grow into discernibly well-separated colonies, which can be

isolated using cloning rings or by simply scraping. A number of methods can be employed for single-cell cloning of suspension cells; some are amenable to high-throughput operations and are described in more detail in the next section. Suspension cells can be immobilized in semisolid media to separate individual cells that grow into colonies. Limiting dilution cloning is done by plating cells in 96-well plates such that, on average, less than 20% of wells have a surviving colony. At such a low average, the probability that one well has more than one surviving cell at the beginning is low. Clonality must, however, be confirmed by microscopic visualization, and sub-cloning is frequently required to guarantee clonality. Automated high-throughput imaging has been reported to evaluate deposition of single cells in well plates to ensure clonality [83]. Such methods can increase the throughput of the otherwise laborious manual microscopic visualization step. Wells in which surviving cells grow are visually ascertained.

Single-cell cloning may also be carried out after selection, thus allowing the transfected cell to grow into a "pool." This, however, has the risk of fast-growing cells that may not be high producers to be overrepresented in the population and decreasing the probability of isolating high-producing cells.

The single-cell cloning step is usually also coupled to a product titer assay so that higher producers can be isolated for further development. When well methods are used, typically the supernatant is withdrawn after the colony is formed for assays using enzyme-linked immunosorbent assay (ELISA) or other methods. In the case where colonies derived from single cells are fixed in a physical location, such as adherent cells or suspension cell grown in agar plates, a semiquantitative assay is often carried out by overlaying the plate with an agar that contains an antibody against the product protein. The low diffusion rate of the secreted protein allows it to accumulate in the vicinity of the clonal colony. The size of the halo zone that appears after incubation is an index of the product titer. The clones with higher titer are then withdrawn from the agar for further development.

12.7.1
Culture Medium for Single-Cell Cloning

Single-cell cloning steps are widely carried out in the presence of serum since single cells thrive better in serum-containing media. Regulatory requirements increasingly encourage serum-free processes, and this has led to the development of serum-free media that promote the growth of single cells. The medium is conditioned by supplementation for single-cell cloning and has been reported to provide growth-promoting and anti-apoptotic factors that help in survival and growth [84]. A 1 : 1 mixture of CD-CHO and DMEM/F12 supplemented with $1.5\,g\,l^{-1}$ of recombinant albumin was reported to allow single-cell cloning of CHO cells [85]. Another interesting approach involves the use of "omics" technologies for rational medium design [86]. By identifying the growth factors that were secreted into protein-free medium and supplementing them in the medium, an increased cloning efficiency was achieved [87]. Such approaches would facilitate the design of serum-free cloning medium composition.

Table 12.1 High-throughput clone selection methods and criterion for clone selection.

	High-throughput clone selection method	Parameter measured
1	Limiting dilution cloning	Volumetric productivity
2	FACS-based screening	
	a. Using coexpressed reporter like GFP	Surrogate of specific productivity
	b. Cell-surface staining with labeled antibody to the recombinant protein	Surrogate of specific productivity
	c. Gel microdrop technology	Volumetric productivity
3	Laser-enabled analysis and processing (LEAP)	Specific productivity
4	ClonePix (Genetix)	Volumetric productivity, surrogate of specific productivity
5	Microwell-based	Volumetric productivity

12.7.2 Automated High-Throughput Screening for High-Producer Clones

After selection and amplification, the resulting cells are very heterogeneous in terms of their growth and product secretion characteristics. The probability of isolating a clone with favorable characteristics increases with increasing number of cloned clones. Higher-throughput screening methods for high productivity are thus increasingly used in cell line development (reviewed in [88]). Table 12.1 lists the different methods used and the productivity-related parameter implicitly used by each method to rank clones.

For cell lines in which transgene expression is linked to DHFR expression, fluorescent MTX binding can be used as a measure of the level of DHFR expressed by the transfected cells [89]. The labeled cells are then sorted by FACS. FACS has also been used to screen transfected cells that were stained with fluorescently labeled antibody against the product protein. The antibody binding to the recombinant protein on the cell membrane as it gets secreted from the cell is used as an indicator of the protein secretion rate of the clone for sorting [90]. Another approach encapsulates cells within gel microdrops to retain the secreted protein molecules in the gel, which is then quantified by a fluorescently labeled antibody [91]. The gel microcapsules with high fluorescent intensity are then sorted for cell isolation.

Both methods rely on a fluorescently labeled antibody or ligand specific to the product protein. An alternative approach coexpresses GFP with the product protein and uses the GFP expression as the indicator of the product protein expression [92]. Another approach uses the coexpression of a cell-surface protein CD20 not normally expressed by CHO cells along with the protein of interest through the use of an internal ribosomal entry site [93]. Cells are then sorted with a fluorescent anti-CD20 antibody. A dual fluorescence approach employs GFP and YFP as reporters for the expression of the light chain and heavy chain, respectively

[94], thus facilitating the selective sorting of clones expressing both the heavy and light chain genes. These methods of employing reporter proteins, while useful in laboratory discovery, may not be suitable for cells destined for manufacturing. The reporter proteins are generally not secreted molecules but may be indicative of transcription and translation activities, so to be predictive for hyperproductivity, the reporter will also need to represent folding and secretory capacities. Overall, FACS-based methods have been used with varying degrees of success. Cell-surface staining may not be proportional to the protein secretion rate over a wide range of productivity. The expression of a trans-membrane anchor domain from a leaky stop codon introduced after the gene of interest on the vector, resulting in a part of the expressed protein of interest being displayed on the cell surface, has been reported to enable quantification of protein expression using techniques like FACS [95].

Single-cell cloning has also been carried out in microwell arrays in conjunction with fluorescent assay of the product protein titer. The product concentration in each well was then assayed by transferring the secreted protein to a cover glass coated with the binding antibody or ligand for analysis by fluorescence-based immunoassay. Cells with a high specific protein production rate were then retrieved from the microwells and expanded [96].

The semisolid medium system described above has also been developed into high-throughput systems. In the high-throughput version, usually a fluorescently labeled antibody against the secreted protein is used to facilitate quantification through the size of the fluorescent zone around the clone [97]. Automated imaging systems coupled with robotic systems are used to identify the best clones and transfer them into appropriate well plates for further expansion. Commercially available systems, such as the ClonePix system from Genetix (http://www.genetix.com) and the CellCelector™ from ALS Automated Lab Solutions (http://www.als-jena.de), work on this principle. Another approach uses a laser to kill the low-expressing clones, thus isolating the higher producers for expansion [98].

12.8 Selecting the Production Clone

12.8.1 Screening Platform

In the single-cell cloning stage, titer of hundreds of clones may be assayed, but only a dozen or two will be expanded and characterized further. It is in this stage, the probable performance of each clone in the production scale is to be evaluated, and the few best candidates as production cell lines are to be selected. Virtually all cell lines used for biopharmaceutical production are aneuploid. After selection, amplification, and one or more rounds of single-cell cloning, significant differences may arise among different clones. These differences are not only in the productivity but also their growth rate, propensity for apoptosis under adverse culture condition, nutritional and metabolic characteristics, and even the post-translation

modification patterns of the product protein. The optimal medium composition and cultural conditions for different clones may thus differ. However, developing the optimal medium and cultural conditions for each clone is a resources-intensive and prolonged process. Deferring the final selection of the producing line after process optimization for all clones is not feasible, and hence platform processes are used in practice. Many practitioners of cell line development are also industrial manufacturers of biologics. Through historical data of media and process development of other production lines derived from the same host, a mid-range of values of key process parameters can be established. Such a set of process parameters, although may not result in stellar performance, will have a high probability of delivering an acceptable process and product.

For the firms with established cell-culture development efforts, a platform process is thus used in the final production cell line selection to evaluate different candidates. For those who have not yet established a platform process, a somewhat iterative approach is taken. To facilitate simultaneous screening of clones and medium formulations, automated culture systems can facilitate in identifying the best available clone.

12.8.2 Adaptation

Most contemporary processes employ suspension cells. In cell line development, the host cells used are often already adapted for growth in suspension and other process conditions. For example, the host cells may have been adapted to a serum-free or chemically defined medium. Some cell line development practices employ adherent culture conditions either by starting with adherent host cells or, in case the host cells have been adapted to suspension growth, by adding serum to the medium to facilitate cell adhesion. Clones derived this way may need to be further adapted to suspension, serum-free, and other culture conditions.

Adaptation is usually carried out by gradually changing cell's culture conditions to the new environment, for instance, in the case of adaptation to cholesterol-free medium for NS0 cells, by gradually reducing cholesterol concentration. For adaptation to the serum-free medium, this is done by mixing the original medium with the serum-free medium at gradually decreasing proportions while passaging. For most industrially used cell lines like CHO, a range of medium formulations ranging from serum-free to chemically defined are commercially available and can be used as the starting point. The clones are assumed to be fully adapted to serum-free suspension conditions when their growth rate is stable at least for three passages. For adaptation to suspension growth, initially some additives such heparin and dextran sulfate, which help reduce cell aggregation, may be used [99].

12.8.3 Process and Product Attributes

During the various stages of cell line development, the product titer in the culture supernatant is typically monitored by ELISA. More recently, faster quantification

of titer is being enabled by measuring the binding kinetics of an analyte, such as a human IgG, to a ligand, such as protein A, immobilized on a biosensor, which can be used to calculate the analyte concentration using a standard curve in as little as 2 min [100, 101]. As the cells are expanded, further quantifications of other important product attributes are also carried out. These include biochemical activities (such as blood coagulation, T-cell activation), isoelectric point (for assessing sialic acid content of the product protein), glycoform, the fidelity of amino acid sequences or other chemical modification of product protein, and the degree of protein aggregation.

The important factors in cell line selection are not only related to the titer and quality attributes of the product. The growth and even metabolic characteristics are also points to be considered. Many high-producing cells are slow growers; however, slow growth rate will cause the expansion period to prolong. Many processes are operated as fed-batch culture; a somewhat hampered apoptosis after reaching a peak cell density, and thus a prolonged period of the production phase, can greatly increase the final product titer. Furthermore, the metabolic characteristics in the last stage of fed-batch culture, especially a decelerated glucose metabolism, have been correlated to a higher product titer. In the last period of cell line development of final cell line selection, growth and metabolism are carefully evaluated.

The choice of the parameter to assess the "goodness" of the clones is important for the eventual outcome of the clone screening process. In the early stage of cell screening, clones can be ranked by specific productivity, but for the final production-line candidate selection the product protein titer and the time to reach the target titer are usually used to rank clones.

12.8.4 Scale-Down Model

The majority of contemporary industrial manufacturing of protein biologics are operated as fed-batch culture. The determination of the final few production clone candidates is best based on the performance of those clones in culture conditions similar to those used in the manufacturing setting. Evaluation based on the results obtained in fed-batch mode yielded better screening outcomes compared to batch mode [102, 103]. If the performance evaluation under culture conditions mimicking the manufacturing scale can be carried out in a large number of clones selected from the earlier cell line screening, the probability of obtaining a hyperproducing clone and establishing a high productivity process will be also higher.

There have been several approaches and commercial culture systems to increase the capacity of process performance evaluation on a quasi-high-throughput fashion. Tissue culture flasks and shake flasks have been widely used. Larger volume multiwell plates agitated on an orbital shaker for more efficient mixing and gas transfer have also been employed for screening [104]. Another system uses aseptically ventilated conical bottom tubes for culturing a large number of clones [105]. However, all these suffer from the lack of environmental control of parameters such as pH. A hydrogel-based platform not requiring liquid handling technology

has been proposed to allow *in situ* continuous feeding and pH maintenance in the vicinity of the set point in shake-flask cultures [106].

In the last decade, several high-throughput, small-scale culture systems with control of environmental parameters such as pH have been proposed to enable screening under conditions mimicking a bioreactor and are available commercially. Tubes with a capacity up to 15 ml installed in a 24- or 48-well plate format have been commercially introduced and allow control of pH [107, 108]. A system for parallel use of up to 24 spinner flasks with pH and DO control is reported [109]. Such scale-down model systems mimicking bioreactor conditions can result in better screening outcomes during clone selection.

12.9 Clone Stability

A frozen vial of cells from a working cell bank, with a cell number in the vicinity of 10 million cells, will need to double its cell number ~20 times before the inoculation of a 10 000-l culture. The total culture duration between inoculation and end of production will be approximately 2–3 weeks for a fed-batch culture. Until production is completed, the production cell line must maintain its productivity. Thus the production clone is typically evaluated for stability (in terms of productivity) for a period of 6–8 weeks. This duration could be significantly higher for perfusion cultures.

Stability is evaluated by culturing the clone for the desired duration. Cell growth rate, productivity, and protein quality are assessed at regular intervals to ascertain stability. The exact molecular mechanism of instability is not always understood. Some causes such as gene silencing and loss of gene copies were discussed earlier. There may also be a threshold level of mRNA, depending on utilization of mRNA for translation, which defines stability or instability of protein production: above this level of recombinant mRNA expression, cell lines are stable, but below this level they show unstable protein expression [110]. In addition, metabolic changes during long-term culture [111] or changes in histone acetylation [112] have been suggested to cause instability.

12.10 Conclusion

Rapid development of cell lines with high volumetric productivity is essential for commercial production of recombinant proteins, especially recombinant antibodies, in animal cells. Several new technologies are increasingly being made available to enable higher recombinant gene expression and clone screening to enable faster development of high-producing clones. This, coupled with significant research activity in the directed genetic manipulation of host cells to provide more robust cells with better growth and metabolic characteristics for large-scale culture and

desired post-translational modifications, can be expected to result in better outcomes in shorter durations for cell line development programs.

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13
Medium Design, Culture Management, and the PAT Initiative

Ziomara P. Gerdtzen

13.1
Historical Perspective on Culture Medium

In vivo, mammalian cells constitute part of a whole organism, and receive all the nutrients they require from circulating blood and the extracellular matrix. In order to survive and proliferate *in vitro*, animal cells must be supplied with a balanced combination of nutrients. The first attempts to develop such solutions involved the use of balanced salt solutions (BSS), which provided water, inorganic anions for cell metabolism, osmotic balance, glucose as an energy source, and a buffering system to keep the pH at the physiological range [1]. These solutions allowed cells and organs to be maintained *ex vivo*. Popular BSS were described by John Hank (Hank's BSS) [2] and Renato Dulbecco (phosphate buffered saline, PBS) [3]. However, in order to get cells to proliferate *in vitro*, additional nutrients must be added to the media, including amino acids and vitamins, plus a supplement of undefined biological fluids or extracts such as plasma or serum from various sources, which provide growth factors, hormones, albumin, and transferrin [4]. The nutritional needs of cells in culture were identified in detail by Harry Eagle, resulting in the Eagle's Minimal Essential Medium (EMEM) and its modification by Dulbecco (DMEM) [5, 6], which is one of the most commonly used media formulations (see Table 13.1). Intracellular nutrient concentrations as well as concentrations found in the cellular environment are listed in Table 13.2. Basal media formulations provide some of these substances, but in order to support cell growth, additional supplementation is required. The first attempts to obtain a medium for animal cell culture involved the use of tissue extracts and biological fluids such as serum in order to mimic the extracellular conditions found *in vivo*. However, these fluids are a source of uncertainty in media formulation. A chemically defined synthetic medium, F12, was developed by Richard Ham to grow cells without this element of variability [7]. Later on, other factors such as insulin were identified as a key for growing cells in a serum-free condition [13] and supplements and protein mixtures were developed as an alternative to serum in culture.

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Table 13.1 Examples of commonly employed media.

Basal medium	Serum-free media formulations
BME (Basal Medium Eagle's) [6]	CMRL 1066
EMEM (Eagle's Minimal Essential Medium)	Ham's F10 and F12 [7]
DMEM (Dulbecco's Modified Eagle's Medium) [8]	IMDM (Iscove's modification of DMEM)
GMEM (Glasgow's Modified Eagle's Medium) RPMI 1630 and 1640 [9]	MCDB 301 [10] and 411 [11]

Table 13.2 Approximate concentrations in the cellular environment.

	Interstitial (mM)	Intracellular (mM)
Na^+	140	14
K^+	4.0	140
Ca^{2+}	1.2	0.01
Mg^{2+}	0.7	20
Cl^-	108	4
HCO^{3-}	28.3	10
HPO_4^{3-}, $H_2PO_4^{2-}$	2	11
SO_4^{3-}	0.5	1
Amino acids	2	8
Lactate	1.2	1.5
Glucose	56	
Protein	0.2	4

Source: Adapted from [12].

13.2 Cell Growth Environment

13.2.1 Natural Cellular Environment

13.2.1.1 The Role of Medium

A medium is required to provide cells with a suitable environment and all the nutrients cells cannot synthesize themselves, that are required for cell maintenance, growth, and protein production. The composition of cell culture media will affect metabolic activities and other biological capabilities of the cell. Therefore, they must be designed specifically to accomplish process goals such as enhanced productivity or product quality.

13.2.1.2 Medium Design

Medium can be designed for optimizing cell growth, sustaining differentiated properties, and improving productivity or enhancing a certain product quality.

The culture process involves a cell inoculum scale-up stage and a production stage, which should be addressed with different strategies. In all cases, it remains critical to provide cells with an optimal medium during the inoculum scale-up stage. Most cells share common basic nutritional requirements. However, their specific need in terms of some nutrients, growth factors, and cytokines may differ. For cell inoculum scale-up, a starting point should be the composition of the cells' natural chemical environment, namely the interstitial fluid, shown in Table 13.2. Even though the specific characteristics of this fluid may vary between different tissues, the low molecular weight solute composition and osmolarity are similar. Na^+ and Cl^- are the main contributors to osmolarity, followed by HCO_3^-. Other ions such as K^+, Mg^{2+}, and Ca^{2+} are present in lower concentrations. The intracellular concentrations of Na^+ and Cl^- are 10 times higher outside the cell, while the concentrations of K^+, Mg^{2+}, and PO_4^{3-} are lower outside the cell. These concentration differences are required to drive cellular processes using the electrochemical gradient associated with the transport of these ions [14].

Since cells spend most of their life span in growth, it is crucial to provide them with an optimal medium during their expansion stage. Media used for cell inoculum scale-up are required to safeguard the cell's long-term healthy state. For production, however, since cells are not required after the production process and long-term effects on the culture are not relevant, culture conditions that would normally be considered as harmful for the culture can be used. For the production stage, a design strategy based on cellular requirements measured during production, metabolic objectives, or the required cellular environment for correct product formation could be used [15–17]. Media must supply the basic physicochemical aspects of mammalian cell requirements. Under normal culture conditions, listed in Table 13.3, oxygen levels ranging from 35% to 45% saturation, temperature between 35 and 37 °C, osmolality ranging from 280 to 300 mOsm kg^{-1} and a pH level between 7.2 and 7.4 maintained using a

Table 13.3 Main constituents and physical characteristics of extracellular fluid.

	Normal range	Approximate nonlethal limits
Oxygen (mm Hg)	35–45	10–1000
Carbon dioxide (mm Hg)	35–45	5–80
Sodium ion (mmol l^{-1})	138–146	115–175
Potassium ion (mmol l^{-1})	3.8–5.0	1.5–9.0
Calcium ion (mmol l^{-1})	1.0–1.4	0.5–2.0
Chloride ion (mmol l^{-1})	103–112	70–130
Bicarbonate ion (mmol l^{-1})	24–32	8–45
Glucose (mg dl^{-1})	75–95	20–1500
Temperature	98–98.8 °F (37.0 °C)	65–110 °F (18.3–43.3 °C)
Osmolality (mOsm kg^{-1})	280–300	260–320 mOsm kg^{-1}
Acid–base (pH)	7.3–7.5	6.9–8.0

Source: Adapted from [12, 18].

CO_2–$NaHCO_3$ buffer, are considered. Although the normal range for medium composition is narrow, cells can tolerate a significant deviation from these growth conditions, for a limited period. Since at this point the objective is not to provide the cells with the best environment for growth but to generate one that will allow obtaining of the highest therapeutic protein titer while maintaining an acceptable viability, culture strategies that deviate significantly from normal conditions can be applied.

13.3 Media Types

Based on their composition, four categories of media types can be identified: serum-containing media, serum-free media, chemically defined media, and protein-free media. The shift from serum-containing into animal-free, protein-free, and chemically defined formulations, which has been observed for the therapeutic protein manufacture industry over the past decades, has been driven initially by patient safety concerns associated with the use of animal-derived medium components and later by the search of a more consistent cell culture performance [19].

Basal medium provides the environment and basic requirements for cell maintenance and growth. Balanced salt concentrations and osmolarity ensure a suitable environment for cell growth. Basal medium considers all small molecular weight components including sugar, amino acids, vitamins, and salts. Sugar is used as an energy source and carbon source for cell mass and product formation. Basal medium can be supplemented to provide additional factors to enhance a biological process, such as growth factors, phospholipids, soy hydrolysate, and serum, leading to a complex medium. These factors promote cell growth through stimulation of specific signaling pathways or supply special nutritional needs. They can also be employed to direct cell differentiation or maintain cells at a particular differentiation state. There are different commercial formulations to be used with or without serum supplementation (see Table 13.1).

Traditional cell culture medium contains up to 15% animal serum in addition to the basal medium. Serum is a highly complex fluid in terms of its chemical composition, which is not fully known and can have a degree of variation from batch to batch. This *serum-containing medium*, with its chemical composition not fully defined, is called a complex medium. Other supplements such as soy-meal hydrolysate, peptone or beef hydrolysate, or other plant hydrolysates are often used in serum-free media formulations, but also make the chemical composition of the media undefined [20–22]. Nucleosides are also often included in small quantities in serum-free medium formulations.

A *chemically defined medium* contains only components whose chemical composition is known and characterized, and the concentrations and identities of all its chemical species are specified. It contains ultrapure inorganic and

organic ingredients, and may also contain pure protein additives, such as growth factors. Unlike serum-containing formulations, it does not contain any mixture of components with unknown or varying composition. Its constituents are produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, specific amino acids, and fatty acids [23]. This type of medium is manufactured with reagent-grade water and highly purified supplements in order to minimize the presence of impurities. A specific type, *protein-free medium,* contains no protein. Most protein-free media are also chemically defined. A chemically defined medium can contain growth factors, cytokines, or carrier proteins, but these components are known and their concentrations defined.

Serum-free formulations of chemically defined media consist of a nutritionally complete basal medium supplemented with an empirically determined mixture of hormones, growth factors, attachment factors, attachment proteins, and binding proteins of known composition.

Cells that are able to grow well in a chemically defined medium are likely to be highly adapted or transformed to synthesize specific metabolites in order to eliminate their dependence on the extracellular medium to provide them directly.

Cells can be adapted to serum-free conditions by lowering serum concentrations to select cells that are able to survive at decreased serum levels. This is a gradual process that involves progressive adaptation to lower serum concentrations until serum-free conditions are reached. The adaptation process requires cells to be in the exponential growth phase with high viability. Several serum-free adaptation strategies have been described, including sequential serum reduction after cell passaging, the use of conditioned media, and maintaining confluent cells under serum-free conditions for adaptation [24]. In addition, albumin, protein hydrolysates, and lipid supplements can be added to the media as serum substitutes.

13.4 Medium Components

Basal medium components can be categorized based on their role in cell growth and maintenance. Cells do not consume environmental components, but the latter are required in given concentrations to provide suitable conditions for the cells to be maintained and perform their basic biologic functions. Some of these components are bulk ions such as sodium and chloride, and proteins such as albumin and growth factors. Other components can be identified as nutrients, such as glucose, amino acids, vitamins, nucleotides, lipids, fatty acid, growth factors, and some salts that are consumed by cells as they grow with a stoichiometry related to cell composition and are used as structural components for energy, biosynthesis, or as catalysts in metabolic processes.

13.4.1 Growth-Associated, Unconsumed, and Catalytic Components

13.4.1.1 Growth-Associated Components

These are the components that are consumed by cells as they divide and synthesize products. Most of the components found in cell culture media, such as glucose, amino acids, lipids, and vitamins, are consumed following their relative proportion in cellular composition. As cells grow and more product is synthesized, more of these components are consumed. Growth-associated components must be supplied in sufficient amounts to reach the desired cell density and product concentrations. For higher biomass or product levels, they would become limiting, so additional growth-associated components should be supplemented.

13.4.1.2 Unconsumed Components

These components provide a chemical environment in which cells can be maintained and grow. Some of these components are actually consumed, but in such small amounts that change in their concentration may not be measurable. Examples of unconsumed medium components include chloride ions, sodium ions, and phosphate ions. Since the role of these unconsumed components is to provide a favorable environment for cells, it is important to maintain their concentration throughout the culture. However, it must be taken into account that under high-density cultivation conditions like those found in fed-batch cultures, unconsumed medium components such as phosphate ions and trace metals may be consumed in significant amounts so they would need replenishment.

13.4.1.3 Catalytic Macromolecular Components

Medium also contains macromolecules such as insulin, fibroblast growth factor (FGF) and serum albumin among others. These components play a role as carrier proteins, growth factors, and so on as discussed in later sections. Many macromolecules are internalized by the cells, but some are degraded or consumed like insulin, while others such as transferrin are used and recycled. As some of these molecules are taken up by cells and degraded or consumed, they would also need to be replenished in the media.

13.4.2 Water in Media Preparation

Water quality is crucial in media preparation, as mammalian cells are highly sensitive to the quality of water used. Many contaminants can be found in water that affect cell culture. On one hand, there are inorganic compounds such as heavy metals, iron, calcium, chloride, and organic compounds such as detergents and products of plants decay, all of which can affect the concentration of key components in culture media, thereby affecting cell growth and perturbing other cellular functions. On the other hand, particulate material, colloids, and

bacterial contaminants such as endotoxins and pyrogens can have a detrimental effect on cells in culture, altering cell growth and protein production [25, 26]. In order to ensure that these contaminants are not present in the medium, water quality assurance is key in cell culture. Multistage distillation systems, deionization, microfiltration, and reverse osmosis systems are required to process water and reduce conductivity to $>20\,M\Omega\,cm$. In addition, continuous monitoring is essential in order to maintain these contaminants within the required threshold [27]. Some cell therapy applications would require water for injection (WFI), which has higher quality standards. It is prepared by distillation at low evaporation rates, in order to avoid the entry of any pyrogenic contaminants. The standards for the quality of purified water and WFI for pharmaceutical use based on American and European regulation are provided in Table 13.4. Endotoxin-free water can also be used in pharmaceutical applications, but that would increase the production cost significantly and it can be of limited availability.

Table 13.4 Quality standards for purified water (PW) and water for injection (WFI) for pharmaceutical use.

Parameter	Unit	USP[a)]		Ph Eur[b)]	
		PW	WFI	PW	WFI
Acidity/alkalinity	$mg\,l^{-1}$			1	2
Ammonium	$mg\,l^{-1}$	0.3	0.3	0.2	0.2
Bacterial endotoxin	$EU\,ml^{-1}$		0.25		0.25
Calcium	$mg\,l^{-1}$	0.5	0.5	1	1
Carbon dioxide	$mg\,l^{-1}$	4	4		
Chloride	$mg\,l^{-1}$	0.5	0.5	0.5	0.5
Conductivity	$\mu S\,cm^{-1}$	1.3 at 25 °C	1.3 at 25 °C	4.5 at 20 °C	1.1 at 20 °C
Heavy metals (Pb)	$mg\,l^{-1}$	0.5	0.5	0.1 ppm	0.1 ppm
Magnesium	$mg\,l^{-1}$	—	—	0.6	0.6
Nitrates	$mg\,l^{-1}$	—	—	0.2 ppm	0.2 ppm
Oxidizable substances	$mg\,l^{-1}$	5	5	5	10
pH		5–7	5–7	—	—
Pyrogens	$EU\,ml^{-1}$		0.25	—	0.25
Sulfates	$mg\,l^{-1}$	0.5	0.5	0.5	0.5
Total organic carbon	$mg\,l^{-1}$	0.5	0.5	0.5	0.5
Total solids	%	0.001	0.001	0.001	0.003
Total bacteria count	$CFU\,ml^{-1}$	100	10	100	10
Production method		Suitable process	Distillation	Distillation, ion exchange, or any other suitable method	Distillation or RO/UF

a) Current U.S. Pharmacopeia.
b) Current European Pharmacopoeia.
EU, endotoxin units.

13.4.3 Sugars and Amino Acids

13.4.3.1 Sugars as the Main Carbon Source

Glucose and glutamine present in the medium are the main energy sources for cells in culture. In the natural cellular environment, glucose concentration ranges from ~0.8 $g\,l^{-1}$ in blood to up to 10 $g\,l^{-1}$ in the interstitial fluid. In culture, concentrations ranging from 1 $g\,l^{-1}$ (5.5 mM) to 5 $g\,l^{-1}$ (27.5 mM) are used to support cell growth. During production stages concentrations up to 15 $g\,l^{-1}$ can be used. At these levels, glucose becomes a large contributor to osmolarity in the medium [12].

Glucose is metabolized mainly through glycolysis, leading to pyruvate formation, which can enter the tricarboxylic acid (TCA) cycle as acetyl-CoA or be converted into lactate and secreted into the medium. Lactate accumulation can cause acidification of the medium, and has an inhibitory effect on cell growth [28]. Alternative carbon sources such as mannose, fructose, and galactose have been used to replace glucose. The Glut1 transporter, which is present in most cells, can transport both galactose and glucose. The substitution of glucose by galactose has been reported to reduce cell growth in Chinese hamster ovary (CHO) cell cultures without altering the specific production rate of the recombinant protein [29, 30]. Some cells are not able to grow in galactose alone, and a mixed carbon source is required [31]. Fructose requires a specific transporter, Glut5, which is not expressed by all cells. As a result, cells may not be able to use fructose efficiently [32]. In general, the alternative sugar is often taken up by cells at a slower rate, leading to a reduction in lactate production while supporting cell growth and maintenance. However, total substitution of the main carbon source may have an effect on the glycosylation pattern of the product. Pyruvate and ribose are sometimes supplied in small quantities as supplements, but they are insufficient to cover all of the cells' energy needs. Lactate supplementation has also been hypothesized to be a required substrate at certain stages of culture and a tool for reducing ammonia accumulation [33].

13.4.3.2 Amino Acids

Amino acids are required by the cells for protein, nucleotide, and lipid synthesis, and, as in the case of glutamine, they might also be used as an energy source. Cells lack the biosynthetic pathways for making essential amino acids and rely on exogenous supply to meet growth needs. Early formulations of cell culture media contained the 13 essential amino acids (shown in Table 13.5), and serum as a supplement. Media designed nowadays for serum-free culture include all amino acids. In addition, these can be supplied through protein hydrolysates. Salts and hormones may also be introduced to increase medium osmolarity in order to activate System A, the major regulated Na^+-dependent amino acid transporter system [34].

Table 13.5 Essential and nonessential amino acids.

Essential amino acids	Nonessential amino acids
L-Arginine[a)]	L-Alanine
L-Cysteine[a)]	L-Asparagine
L-Histidine	L-Aspartic acid
L-Isoleucine	L-Glutamic acid
L-Leucine	L-Glycine
L-Lysine	L-Proline
L-Methionine	L-Serine
L-Phenylalanine	
L-Threonine	
L-Tryptophan	
L-Tyrosine[a)]	
L-Valine	
L-Glutamine[a)]	

a) Essential for cells in culture, not for animals.
Source: Adapted from [12].

Glutamine Glutamine is a nonessential amino acid for animals. It is synthesized from glutamic acid through glutamine synthase. The expression level of glutamine synthase, however, decreases when cells are cultured *in vitro*; hence, it is an essential amino acid for cells in culture. It acts as a carbon, nitrogen, and energy source, supplying the amino group for nucleotide biosynthesis and the carbon backbone for TCA cycle intermediates. It can also be converted enzymatically to glutamic acid, leucine, and isoleucine [35]. It contains one atom of nitrogen as an amide and another atom of nitrogen as an amine, delivering nitrogen to cells in quantities that would be toxic as free ammonium. The amide nitrogen is used in the synthesis of nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP), purine nucleotides, cytidine 5'-triphosphate (CTP) from uridine 5'-triphosphate (UTP) and asparagine and to produce carbamyl phosphate for the synthesis of pyrimidines. Glutamine is also a precursor of glutamate, a key amino acid used for the transamination of α-keto acids to form other α-amino acids. In addition, when glucose levels are low and energy demands are high due to high protein synthesis rates or rapidly dividing cells, it is metabolized for use as an energy source leading to ammonia and lactate production. In the TCA cycle, glucose-derived pyruvate is transaminated with glutamate to form α-ketoglutarate and alanine, which are released into the medium [36].

Glutamine is present in culture media in high concentrations ranging from 1 to 5 mM. However, it shows low stability in solution, where it spontaneously degrades, undergoing cyclization to pyrrolidone carboxylic acid and ammonia [37]. Glutamine's half-life for different media formulations (IMDM, RPMI-1640, DMEM, and OPTI-MEM) ranges from 5 to 6.7 days at pH 7.7, and becomes significantly higher at lower pH values. Besides pH, osmolarity, and the presence

of glutaminase in serum also influence the dynamics of glutamine degradation observed in culture [38]. Because of this, in biomanufacturing it is added to the base medium at time of use, or supplied in more stable dipeptide forms such as alanyl-L-glutamine and glycyl-L-glutamine. Glutamine has a high uptake rate, of ~10–20% the specific consumption rate of glucose. Still, care should be taken in glutamine addition to the medium, since glutamine feeding at a high rate can lead to ammonia build-up if it degrades at a faster rate than it is consumed by the cells [38], and ammonia has been reported to be toxic and inhibitory for mammalian cell growth at high concentrations [39, 40]. Tracer experiments have also shown that the metabolism of glutamine through the central carbon pathway can contribute significantly to lactate production in culture in some cell lines [41–43].

Other Amino Acids Amino acids are required and taken up at different rates and levels depending on the cells' specific nutritional needs, metabolic patterns, the concentrations of amino acids, and the levels of other nutrients present in the medium. Usually glutamine, arginine, leucine, and isoleucine are consumed at higher levels, followed by lysine, valine, and phenylalanine [35]. Common by-products of their metabolism are glycine and ammonia. Nonessential amino acids, listed in Table 13.5, can be synthesized from other amino acids or keto acids via transamination. They are precursors for protein, nucleotide, and lipid synthesis and are provided in the medium in concentrations ranging from 0.1 to 1 mM. Some nonessential amino acids are also produced and transported into the culture medium as a result of the cells' metabolism. Alanine accumulation in the medium, and often asparagine and proline accumulation as well, is observed as the culture progresses. Even though proline is not an essential amino acid, it is required by mutant CHO cells. Early experiments showed that asparagine, glycine, and serine are also required for cell growth in the case of some tumor-derived cell lines [44–46]. These requirements can be satisfied by the addition of protein hydrolysates.

13.4.4 Vitamins, Nucleosides, Fatty Acids, and Lipids

13.4.4.1 Vitamins' Role

Vitamins play very diverse biological roles *in vivo*, and hence have diverse physical and chemical characteristics. Many vitamins are essential for the growth and proliferation of cells. Even though they are required only in trace amounts compared to glucose and amino acids, they are essential for cells in culture because they act as enzyme cofactors. Vitamins cannot be synthesized in sufficient quantities by cells and are therefore important supplements required in tissue culture. The B-group vitamins are most commonly added for growth stimulation. They are present in serum and yeast extract supplements. Commonly used vitamins for cells in culture are listed in Table 13.6.

Table 13.6 Vitamins for cells in culture.

Water soluble	Fat soluble
Biotin[a)]	Vitamin A
Folic acid[a)]	Vitamin D
Niacinamide (or nicotinic acid)[a)]	Vitamin E
Pantothenic acid[a)]	Vitamin K
Pyridoxine[a)]	
Riboflavin[a)]	
Thiamin[a)]	
Vitamin B-12[a)]	
Ascorbic acid (vitamin C)	
Lipoic acid	

a) Required by cells.

Water-soluble vitamins are required by all cells and include biotin, thiamine pyrophosphate, riboflavin, and cobalamin. Biotin is a coenzyme for carboxylase enzymes, and it is necessarily involved in the synthesis of fatty acids and in the metabolism of amino acids such as isoleucine, and valine, and in gluconeogenesis. Thiamine pyrophosphate acts as a coenzyme in many enzymatic reactions. It catalyzes the oxidative decarboxylation of pyruvate to form acetyl-coenzyme A, α-ketoglutarate, and branched-chain α-keto acids derived from certain amino acids. It is also acts as a coenzyme for transketolase and transaldolase. Cobalamin (B12) is involved in free-radical reactions of intramolecular C – C bond rearrangement, methylation, and conversion of ribonucleotides to deoxyribonucleotides. Other vitamins are required only by specific differentiated cell types, and include vitamins D, K, A, and C. Vitamin D is involved in Ca^{+2} regulation and, unlike other vitamins, it can be synthesized by mammalian cells and is highly toxic when present in excess. Vitamin K is required for γ-carboxylation and correct post-translational processing of a variety of vitamin K-dependent proteins. Vitamin A can have a pronounced effect on growth and differentiation of some cell types, as it plays a role in gene transcription. Ascorbic acid is a water-soluble antioxidant that protects esterified and non-esterified unsaturated fatty acids from peroxidation; it is required for collagen synthesis. Although vitamins act mainly as catalysts and are not consumed in reactions, they need to be replenished in cell culture media because of their turnover.

13.4.4.2 Fatty Acids and Lipids

This category of biomolecules refers to water-insoluble biomolecules, biosynthetically or functionally related to fatty acids and their derivatives. Major categories of biological lipids include fatty acids and glycerophospholipids (e.g., phosphatidyl choline), sterols (e.g., cholesterol in animals and phytosterols in plants), sphingolipids (e.g., ceramides), triacylglycerols, and various lipoprotein

Table 13.7 Approximate lipid content of bovine serum [47].

Lipid class	Content ($\mu g\ \mu l^{-1}$)
Phospholipid	1123
Cholesterol	167
Free fatty acid	183
Triglyceride	347
Cholesteryl ester	937

complexes, but exclude steroid hormones, fat-soluble vitamins, and petroleum products. Mammalian cells can synthesize most of the lipids and fatty acids they require for growth, except for auxotrophic mutants. Cells can make fatty acids of all different carbon lengths in membranes, including most unsaturated fatty acids, which constitute almost half of the fatty acids in phospholipids.

Lipids function in three roles. They serve as energy stores, as structural components of cellular and organelle membranes, and in transport. Many signaling systems also rely upon lipid-containing complexes. Ethanolamine, choline, and inositol are precursors for phosphatidyl, phosphatidyl-choline, and phosphatidyl-inositol biosynthesis. Lipids such as fatty acids, phospholipids, and cholesterol are essential for cell growth as cellular membrane components. Cholesterol is involved in membrane fluidity and mechanical resistance of the cell, and is required by a few cell lines such as NS0 myeloma cells for proliferation. When becoming a limiting factor, its addition can also have an effect on the specific production of the culture.

Lipids are provided by serum, at the concentration shown in Table 13.7. Animal serum, the original vehicle for providing lipids to cells in culture, uses proteins as carriers of every lipid required by mammalian systems. Triglycerides and phospholipids represent the highest consumption of lipids from serum [48]. Their limited solubility in media, however, makes delivering sufficient quantities to the cells a challenge. A decreased availability of lipids in the medium is difficult to assess and may not be immediately observable. In the long run, it can result in changes in membrane composition, in turn altering membrane fluidity and causing a reduction in protein secretion.

Supplementation of cell culture systems with nonessential fatty acids, phospholipids, and sterols significantly improves culture performance. Cells can synthesize most of the dozens of lipids they require for primary cellular functions. For mammalian cells though, two fatty acids, linoleic acid (18 : 2 *cis*-Δ9, Δ12) and linolenic acid (18 : 3 *cis*-Δ9, Δ12, Δ15), have been proven to be essential for most cell lines in culture. This is due to the fact that mammals do not introduce double bonds beyond C9 into fatty acids. Providing cells with appropriate preformed lipids, even when not essential, reduces the need for their biosynthesis by the cell, resulting in a more efficient metabolism. Hence, a complete medium for serum-free culture usually contains oleic acid, linoleic acid, sometimes also arachidonic acid [12].

Lipids also can be beneficial for cells in culture, since some substances absorbed by the cells need to be solubilized in lipids, or in some cases the toxicity of compounds may be reduced by the formation of complexes with lipids.

13.4.5 Bulk Ions and Trace Elements

Mammalian cells have low concentrations of sodium and calcium and large concentrations of potassium ions compared to the extracellular medium. These differences are critical for homeostasis and are required for the regulation of metabolism. This distribution is due to the membrane's permeability to ions and ion pumps that are built into these membranes. Because of passive permeability, the cell has a tendency to equalize concentrations, while pumps work to maintain a concentration gradient [49]. The addition of ions in the form of salts is required to maintain osmotic balance in the medium. In addition, they are required by the cells as enzyme cofactors. The most important ions are Na^+, K^+, Ca^{+2}, Cl^-, Mg^{+2}, $SO_4{}^{-2}$, $PO_4{}^{-2}$, and $HCO_3{}^-$. The concentration of these ions in the basal medium is shown in Table 13.8. These ions are mainly responsible for the osmolality in a fresh medium, which should be kept in an optimal range between 280 and 300 mOsm kg^{-1}.

Ionic gradients and electric potential across the plasma membrane drive many biological processes, such as the transport of amino acids and other molecules against their concentration gradient. Sodium and potassium, as well as chloride and calcium, are required for the regulation and maintenance of these membrane potentials through ion transport [50]. Magnesium is conjugated to adenosine triphosphate (ATP). Calcium and magnesium are required for cell–cell and cell–substrate adhesion processes. Therefore their concentration should be kept low to prevent cell aggregation. Calcium is also involved in many cell signaling processes, including excitability, exocytosis, motility, transcription, apoptosis, and the cellular response to oxidative stress [51, 52]. Copper is an essential element for cells in culture. It is involved in iron homeostasis, as it is involved

Table 13.8 Concentration of bulk ions in basal medium.

(mM)	DMEM/F12	William's E	DMEM	F12
Na^+	150.31	143.71	155.12	144.03
K^+	4.18	5.37	5.37	3.00
Mg^{2+}	0.71	0.81	0.81	0.60
Ca^{2+}	1.05	1.80	1.80	0.30
Cl^-	126.66	125.33	118.48	134.83
$PO_4{}^{3-}$	1.02	1.17	0.78	1.17
$HCO_3{}^-$	29.02	26.19	44.04	14.00
$SO_4{}^{2-}$	0.41	0.81	0.81	0
Total	313	302	327	288

Source: Adapted from [12].

in the transfer from ferritin to transferrin. It also plays a key role in electron transfer complexes and the reduction of oxidative damage, cell signaling, and gene expression [53, 54]. Its use at appropriate concentrations, not necessarily equal to physiologic levels, can result in higher productivity and reduced lactate production in culture [55]. In culture media supplemented with serum, copper is provided at 50–100 ng ml^{-1}. Since this element is essential for cells in culture, it must be added to serum-free formulations. Iron is required for iron-containing proteins such as cytochromes, which are a group of heme-containing proteins located in the mitochondria that transfer electrons along the respiratory chain. Iron exists in a non-heme form as part of enzymes such as succinate dehydrogenase, which catalyzes the formation of fumarate. It exists as a heme component of enzymes such as catalase and peroxidase that catalyze the conversion of peroxides to water. As an enzyme cofactor, iron protects cells from oxidative damage. It is supplied to the media by iron salts. SO_4^{-2}, PO_4^{-2}, and HCO_3^{-} are required for the synthesis of macromolecules and used by the cells to regulate intracellular charge. Phosphate is specifically required for nucleotides and nucleic acids.

Zinc is not included in basal media formulations (shown in Table 13.8). However, it is essential in cell culture, as it is involved in amino acid and nucleic acid metabolism as part of regulatory proteins in transcription and as an antioxidant. In serum-free formulations, it is provided conjugated with insulin. Selenium participates in oxidation/reduction and free-radical reactions. These metals, along with iron, manganese, vanadium, copper, and molybdenum, are usually added to the culture medium at low concentrations, especially if the medium is not supplemented with animal serum [56].

13.4.6 Non-Nutritional Medium Components

Non-nutritional medium components are additives that, even if not taken up by cells, can minimize operational deviations and facilitate culture operation. These include buffer systems for pH regulation, antioxidants, and pH indicators.

13.4.6.1 Phenol Red

Phenol red is used as a pH indicator in most commercially available culture media. As cells grow, metabolites such as lactate are released into the medium, causing a color change due to the decrease in pH. At pH 7.4, it looks bright red. At low pH it is yellow, while at higher pH it is purple.

13.4.6.2 Sodium Bicarbonate Buffer

Mammalian cells can survive over a wide pH range (6.6–7.8), but the optimal growth of cells is obtained at pH 7.2–7.4. It is undesirable to allow the pH to deviate outside the limits of pH 6.8 and 7.6 during culture, so buffering systems are used. However, none of these systems is capable of holding the pH constant in a system in which acids or bases are being produced. Buffers only slow the rate of

pH change since cells in culture produce acidic products, which lower the pH of the medium as the culture progresses.

The sodium bicarbonate buffering system acts similar to the main buffer system in blood *in vivo*. It is low cost and nontoxic, and is based on the equilibrium with soluble carbon dioxide. Hence, it requires CO_2 in the gas phase to be in contact with the medium [57]. This system has a pK_a of 6.1 at 37 °C. The concentration of bicarbonate in the medium (14 mM in F12, 44 mM in DMEM) maintains equilibrium with CO_2 in the gas phase inside the incubator (5–10%). Based on Le Chatellier's principle for the equilibrium between CO_2, bicarbonate, and carbonic acid [$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$], for a given bicarbonate concentration, the higher the CO_2 in the gas phase, the lower the pH. A typical cell culture medium contains 14–44 mM of $NaHCO_3$, which requires 10% CO_2 to keep the pH at 7.4 at equilibrium. When removed from the incubator, the medium becomes alkaline. As the pH decreases as a result of lactate production, the CO_2 level in the gas phase can be reduced to take the proton to the left-hand side of the equilibrium equation in order to maintain pH. Conversely, as cells begin to consume lactate in the late stage of fed-batch culture CO_2 concentration in the gas phase is increased to maintain pH.

13.4.6.3 **Alternative Buffers**

Under some conditions, bicarbonate buffer may not be ideal for maintaining pH: for instance, when culture must be performed outside a CO_2 incubator. There are other buffers available with pK_a in the vicinity of 7.0 that are even more suitable for sustaining neutral pH than bicarbonate. However, these buffers have a toxic effect on cells, and so they have a lower maximum concentration that can be used without a negative effect on cell growth; therefore, they provide a limited buffering capacity.

Zwitterions have both acidic and basic groups in the same molecule, which makes them good components for buffer solutions, as they can resist changes to the pH of a solution by selective ionization. In the presence of acids, zwitterions accept hydrogen ions, removing them from the solution. In the presence of bases, zwitterions donate hydrogen ions to the solution, again balancing the pH. HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane) acts as a zwitterion and has many properties that make it ideal as a buffer to tissue culture media. It was first described in [58], and it is often employed as a substitute or supplement for the bicarbonate buffer. It has a superior buffering capacity in the 7.2–7.4 pH range, and, unlike bicarbonate, it does not require an enriched atmosphere to maintain the correct pH. On the down side, it is more expensive. It is often used in the range of 10–50 mM in culture, but it can be growth-inhibitory or toxic at concentrations greater than 40 mM or even 25 mM for some cell types [59]. Its use requires balance of osmolality by adjusting bulk salt levels while maintaining the Na^+/K^+ ratio (30 : 1). It can interfere with protein assays and, even though it does not bind metal ions, it forms radicals, so it is not suitable for redox studies.

It must be considered that since CO_2 is a metabolite, is a medium component for the buffer, and is also a nutrient required for some biochemical reactions,

such as the carboxylation reaction in fatty acid synthesis, using a bicarbonate-free medium may lead to CO_2 becoming limiting for supporting cell growth as it is stripped away with airflow. To avoid this, an air supply supplemented with CO_2 (0.2–0.5%) can be used.

13.4.6.4 Antioxidants

Reactive oxygen species (ROS) that result from cellular biochemical reactions cause the transient presence of superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). Free iron ions, copper ions, and many other transition-metal ions present in media formulations such as DMEM induce oxidative stress. Ascorbate, flavonoids, many other polyphenolic compounds, and thiols are unstable in commonly used cell culture media, undergoing rapid oxidation,which also generates H_2O_2 and other ROS [60]. The reaction of these two compounds with lipids, proteins, and DNA trigger damage to cells and other media components. Cellular oxidative stress can cause senescence, cell death, or adaptation. Physiologically, cells deal with ROS through reducing reactions using cellular reducing agents such as glutathione. When cultivating cells under stress conditions, the presence of antioxidants can delay the onset of irreparable cellular damage. If the culture medium is deficient in antioxidants, the addition of vitamin E, taurine, β-carotene, transferrin, ceruloplasmin, catalase, superoxide dismutase, reduced glutathione, or β-mercaptoethanol can show an enhancement of cellular protection against oxidative stress. Besides mercaptoethanol, a few other antioxidants are also routinely used in cell culture. Addition of selenium to selenium-deficient cultures allows the activation of selenium-dependent antioxidant systems. These include thioredoxin reductase and the glutathione peroxidase family.

13.4.6.5 Mechanical-Damage-Protective Agents

Protective agents such as polyethylene glycol or carboxymethyl cellulose, among others, have been used as media supplements to modulate the physiochemical environment of the cell since, besides osmolarity, viscosity was regarded as a relevant factor for serum-free cell growth.

Sodium carboxymethyl cellulose has been shown to reduce mechanical damage to cells caused by shear forces associated to the stirrer impeller, forced aeration, or perfusion. Pluronic F68 is a non-ionic surfactant consisting of a block copolymer of poly(oxyethylene) and poly(oxypropylene). The larger the polyoxyethylene group, the more hydrophilic the molecule and the greater its detergent-like activity and cell cushioning effects. The larger the polyoxypropylene group, the greater the toxicity and the antifoaming ability of the molecule. This polymer protects cells from damage due to agitation and aeration, reduces foaming when serum is present in the medium, and decreases the attachment of cells to glass. Other examples of mechanical-damage-protective agents used in cell culture are listed in Table 13.9.

For fed-batch cultures, the consumed components must be fed in a highly concentrated medium in order to reduce dilution of the culture. Introducing a surfactant such as polysorbate into chemically defined feed media (CDFM) facilitates

Table 13.9 Synthetic protective agents and surfactants used in cell culture.

Common name	Chemical identity
Pluronic F68 or F88	Block copolymer glycols of poly(oxyethylene) and poly(oxypropylene) (MW ~8350)
PEG	Poly(oxyethylene) glycol (or polyethylene glycol) (MW ~20 000)
PVA	Polyvinyl alcohol (MW 20 000)
MC	Methylcelluloses (15 cps methocel) 0.1–0.2%
CMC, Edifas B50	Sodium carboxymethylcellulose
HES	Hydroxyethyl starch
PVP	Polyvinylpyrrolidone
PS	Polysorbate
Haemaccel	Modified gelatin

Source: Adapted from [12].

media components remaining in solution for a longer time, effectively allowing the use of concentrated feed media [61].

13.4.6.6 **Antibiotics**

Antibiotics are added to culture media in order to prevent microbial growth. The most commonly used antibiotics in cell culture such as penicillin, streptomycin, and amphotericin B among others, as well as their recommended concentrations, are listed in Table 13.10. Their utilization, however, is not a substitute to aseptic manipulation. In manufacturing processes, antibiotics are rarely used, since they can be cytotoxic, especially in serum-free formulations, and they are only moderately more inhibitory to bacteria than to cultured cells. In addition, their continuous use can lead to the selection of resistant microorganisms and chronic contamination, and can cause hypersensitivity reactions in patients. In all cases, toxicity testing is necessary. Neomycin, polymyxin B, streptomycin, and gentamicin are sometimes used in vaccine production. For the cultivation of primary cells, gentamicin reduces the rate of contamination significantly.

Table 13.10 Antibiotics for cell culture.

Antibiotic	Recommended	Antibiotic spectrum
Amphotericin B	2.5 mg l^{-1}	Fungi and yeasts
Chlortetracycline	5 mg l^{-1}	Gram-positive/negative bacteria
Gentamicin sulfate	50 mg l^{-1}	Gram-positive/negative bacteria and mycoplasma
Nystatin	50 mg l^{-1}	Fungi and yeasts
Penicillin G	100 U ml^{-1}	Gram-positive bacteria
Spectinomycin	20 mg l^{-1}	Gram-positive/negative bacteria
Streptomycin	100 mg l^{-1}	Gram-positive/negative bacteria

Source: Adapted from [12].

13.5 High Molecular Weight and Complex Supplements

Some cells are capable of rapid growth using only the basal medium without any further supplementation (NS0, hybridoma/myeloma cells and HepG2 cells). However, most cells in culture require the supplementation of at least a number of growth factors and carrier proteins. Many stem cells and other normal diploid cell strains still need a high concentration of serum to grow.

Many industrial protein processes have moved to serum-free and animal-free formulations to minimize contamination. For viral vaccine production, bovine serum is still often used. In processes involving the cultivation of primary differentiated cells, the elimination of animal serum from the culture medium is not an easy task, so when isolating cells from tissue, serum is commonly used at least in the early stage of cell cultivation. In order to be able to supplement the culture medium to reduce or eliminate serum dependence, one must examine in detail the role of this supplement and its components in mammalian cell culture.

13.5.1 Serum

Serum is the fluid obtained from blood after the coagulation and removal of blood cells and most coagulation proteins. It is a complex mixture of amino acids, growth factors, vitamins, metabolites, hormones, lipids, minerals, plasma proteins, substances released from damaged cells, antibody molecules, and infectious agents. Fetal bovine serum (FBS) is the most widely used serum in animal cell culture since it has higher concentrations of growth-stimulatory factors and lesser concentrations of growth-inhibitory factors. It is often from a bovine source, although equine and even human serum can also be used. It is added in proportions of 5–10%, and in some cases up to 20% v/v. Given its complex composition, serum plays many different roles in culture, such as providing nutrients, factors for cell–substrate attachment, and modulating the physiological properties of medium.

13.5.1.1 Functions of Serum in Cell Culture Medium

The main functions of serum are to stimulate growth and other cellular activities through hormones and growth factors, to increase cell adhesion through specific proteins, and to supply proteins for the transport of hormones, minerals, and lipids [62]. FBS is the most commonly used, as it contains low immunoglobulin and high growth factor concentrations. There are a number of globulins present in serum that have different effects on cells in culture. α-Globulins are mainly protease inhibitors and carrier proteins. These protease inhibitors can help neutralize proteases used in trypsinization or produced by cells. Carrier proteins bind and transport low molecular weight substances and nutrients that dissolve poorly, thereby increasing nutrient availability for cells in culture. Transferrin is an important β-globulin for cells in culture. It complexes iron and transports it, and is

taken up by the cells via specific receptors, releasing iron intracellularly in a pH-dependent manner.

The ferric ion carrier, transferrin, is also present in serum. It releases the ferric ion in the cell lysosome, and then it is recycled to the culture broth. As long as ferric ions are replenished, the transferrin supplied by FBS can continue to transport iron inside the cells.

Because of the presence of albumin and other large proteins and lipoproteins, serum acts like a tampon for toxic substances, as these proteins bind or neutralize compounds that are toxic when present in excessive amounts and release them slowly back into the media.

Detailed studies have been performed regarding the role of serum in cell attachment [4]. Fetuin, fibronectin, and hydrocortisone have been shown to be involved in cell attachment processes. Most cells can rapidly attach to a clean plastic glass or soft glass surface. This attachment does not require the presence of divalent cations and is temporary in nature. Serum promotes cell attachment in a more permanent manner; however, this process is slower in nature and does require the presence of the divalent cations found in serum. Serum has also been shown to play a role in cell stretching after attachment.

Serum also provides polypeptide hormones and proteins with specific mitogenic or growth-stimulating activities, such as the nerve growth factor, epidermal growth factor, erythropoietin, and colony-stimulating factor. One of the key factors present in serum, and identified as key to stimulate cell multiplication in several culture systems, is insulin, which is present in a small amount, along with other insulin-like growth factors (IGF) that cause the multiplication-stimulating activity of serum.

The main advantage of serum use is the simplification of media formulation, since in the presence of serum many nutrients, hormones, proteins, and growth factors are present in the medium, and the same formulation can be used for different cell lines.

13.5.1.2 **Disadvantages of Serum in Cell Culture Medium**

For serum production, blood is collected by cardiac puncture or umbilical blood collection, and then it is slowly clotted and centrifuged at low temperatures. It is a low-yield process since each fetus yields 0.2 – 0.5 l of blood, and only 50% of that corresponds to serum. Because of the nature of its production process, there is high biochemical variability in the types and concentrations of serum components from one lot to another. This variability could affect the reproducibility of culture performance and prevent the complete characterization of culture media. In order to minimize such variability, serum is normally purchased in large lot sizes, which involves an additional cost due to storage. As a result, high-quality serum is a high-cost supplement with limited availability, so one of the most expensive media components, and an expense that can be avoided.

Even though production regulation requires that the animal must be free of infectious diseases, such as cattle plague, foot-and-mouth disease, and bovine spongiform encephalopathy, and the blood is to be tested for virus,

bacteriophages, bacteria, mycoplasmas, and fungi, serum can still be a source of contamination risk due to the presence of animal viruses, prions, and endotoxins. In addition, serum contains high concentrations of proteins, which makes product purification a more difficult task, increasing the complexity of downstream processing and characterization of the final product. The albumin present in serum can also cause allergenic and immunogenic reactions in humans.

The use of defined components is desirable in culture from a regulatory standpoint and also for the evaluation of stoichiometric or kinetic nutritional requirements for cells in the culture. The replacement of serum, however, increases the complexity of the medium, analytical requirements, and time for medium development since the effect on cell growth, lag phase, productivity, and protein glycosylation must be analyzed for the different serum substitute formulations. The use of serum substitutes has been reported to cause a potential increase of 20–30% in specific productivity, with no major effects on protein glycosylation [63].

13.5.2
Insulin and Insulin-Like Growth Factors

Insulin is a growth factor commonly used in culture. It plays a key role in the regulation of glucose uptake by adipocyte and other cell types. Insulin and insulin-like growth factor (IGF) can have a mitogenic effect in culture, enhancing cellular metabolism and promoting cell growth. This effect is cell-line-specific and can change depending on culture conditions, as the number of insulin and IGF-1 receptors fluctuates with growth conditions and is different for different cell lines [64]. It has been shown that in some cases the specific growth rates are similar in cultures with or without insulin, with a higher specific glucose consumption rate with insulin [65]. IGF-1 has been shown to increase cellular uptake of glucose and amino acids and stimulate glycogen and protein synthesis. The mitogenic effect of insulin would occur through an overlapping signaling pathway with IGF-1 through the insulin receptor and IGF-1 receptor, as insulin is able to signal through the IGF-1 receptor at supra-physiological concentrations [66]. In culture, insulin is used in the 1–10 $\mu g\,ml^{-1}$ range. Normal blood insulin concentration is $\sim$1.3 $\mu g\,ml^{-1}$. IGF-1 concentration is in the 100–200 $ng\,ml^{-1}$ range, so it can be used to replace insulin in culture at much lower concentrations.

Insulin binds to the insulin receptor and triggers a signaling event that does not involve its own internalization. However, when present at high concentrations (higher than standard blood concentrations), insulin is rapidly internalized by some cells such as hepatocytes and degraded. Therefore, even though as a growth factor it is not consumed, its concentration can decrease at high levels.

13.5.3
Transferrin

Iron is indispensable for some metabolic processes, including ATP synthesis, the production of deoxyribonucleotides, and cell proliferation. Intracellularly, iron is

Table 13.11 Iron chelators used as transferrin replacements.

Compound	Concentration range
Ferric citrate	0.05–5 mM
Ferric iminodiacetic acid	0.001 μM
Ferric ammonium citrate (FAC)	2–10 μM
Tropolone and FAC	5 and 0.4 μM

Source: Adapted from [12].

found as ferrous ion Fe^{+2}, bound to heme and other proteins with iron cores. Transferrin is the predominant iron carrier in mammalian cells, and it has recently become available in its recombinant form. Human transferrin is used in culture at concentrations ranging from 1 to 30 μg ml^{-1}. It is an 80-kDa glycoprotein with a large capacity as an iron carrier due to the presence of homologous N-terminal and C-terminal iron-binding domains. It binds Fe^{+3} very strongly with a k_d 1022 M^{-1}. It has low interspecific potency, so specific concentration requirements for each cell line need to be determined. Iron chelating agents such as tropolone and ferric ammonium citrate (see Table 13.11) are sometimes added to the medium in replacement of transferrin or for high-density culture or applications that stress on the potency of the medium [67].

Many mammalian cells possess transferrin-specific receptors that capture transferrin/Fe^{3+} complexes. Transferrin is taken up by the cells and translocated to the lysosome, where it releases iron in a pH-dependent manner. The iron-free protein is recycled back from the cell and released into the culture broth for reutilization. Therefore, if transferrin is supplied in the medium, the level of transferrin receptors is the major factor that determines the rate of iron uptake [68].

13.5.4 Serum Albumin and Other Carrier Proteins

Albumin is the most abundant protein in serum, present typically at ~50 mg ml^{-1}, where it makes up ~60% of the total protein. It plays many roles in culture medium, but most of the physiological actions and the molecular mechanisms involved in these roles are not well understood. It acts as a carrier protein, binding and transporting physiologically relevant ligands, including lipids, metal ions, amino acids, and other factors, as well as compounds with low solubility in aqueous solutions, such as vitamins, hormones, or other micronutrients. It transports fatty acids, which in their free form can be toxic in high concentrations. Albumin can act as a blocking agent because of its binding properties. It binds toxins to avoid toxic effects, excessive proteins to act as a buffer, and hormones and growth peptides to keep them stable.

Table 13.12 Transport and carrier proteins.

Transport proteins	Source	Structure	Effects
Serum albumin	Plasma	1-Chain (MW = 68 000)	Supplies free fatty acids, detoxifier, contains trace elements
Transferrin	Plasma	1-Chain (MW = 77 000)	Supplies iron, detoxifier
High-density lipoprotein (HDL)	Plasma	Particle (multiple protein subunit)	Accepts and transports cholesterol and cholesterol esters
Low-density lipoprotein (LDL)	Plasma	Particle (ApoB)	Transports cholesterol and cholesterol esters
Transcobalamin	Plasma		Binds vitamin B12

Source: Adapted from [12].

Albumin also binds free radicals to reduce damage to cells. The antioxidant potential of albumin is associated with the sulfur-containing amino acids cysteine and methionine. There are 35 cysteine residues in albumin with all but one, Cys-34, involved in disulfide linkages. The antioxidant capacity of albumin relates largely to its ability to bind metal ions and to scavenge free radicals as a substitute substrate, nucleic acids, or lipid moieties. The antioxidant effect of Cys-34 works through its action as a scavenger of free radicals, while Met acts mainly as a metal chelator to reduce the subsequent generation of ROS [69].

In serum-free media, serum albumin is often used in place of FBS. Its addition to cell culture media at concentrations ranging from 0.1 to 5 mg ml^{-1} improves the performance of a wide range of cell types including stem cells, primary cells, and CHO cells. Serum albumin as a cell culture supplement can be obtained from bovine or human blood, but these sources carry safety concerns including risk of viral or prion contamination and problems with variation across product lots. These concerns can be overcome with the use of recombinant albumin, which can provide all the benefits of serum albumin without lot-to-lot variation or safety concerns. The complete chemical properties of recombinant albumin, however, can vary in terms of fatty acid composition and content depending on the preparation method. Most defined media use fatty-acid-free human recombinant albumin coupled to oleic acid or linoleic acid.

Albumin is a suitable replacement for serum in cryopreservation and cell culture for animal-free biomanufacturing processes [70, 71]. It also acts as a buffering or detoxifying agent by binding to endotoxin and pyrogens.

Other carrier proteins, shown in Table 13.12, are present in serum in addition to transferrin and serum albumin. These are more rarely used in culture.

13.5.5 Cell Adhesion Molecules

Anchorage-dependent cells are used in applications such as the vaccine industry and stem cell and cell therapy-based technologies. To culture these cells, adhesion can be enhanced by using serum-containing media or by coating the culture

Table 13.13 Adhesion molecules used for cell culture.

Proteins	Source	Structure	Effects
Fibronectin	Plasma, cell lines	Dimer MW = 220 000	Promotes attachment and growth, mesenchymal
Laminin	Extracellular matrix	Two subunits MW = 900 000	Promotes attachment and growth, ecto/endodermal
Collagens	Skin, E.M., placenta	1–3 Subunits	Promotes attachment and growth
Vitronectin	Plasma	MW = 70 000	Promotes attachment and growth of a variety of cell types
Fetuin	FBS	Alpha 1-globulin	Promotes attachment of cells to glass and plastic
Poly-D-lysine	Synthetic	Polymer MW = 30 000–70 000	Promotes attachment of many cell types (even with serum)

Source: Adapted from [12].

surfaces with adhesion molecules that promote the adhesion of various cell types but do not attach well to standard tissue culture flask surfaces. The most commonly used adhesion molecules are shown in Table 13.13, and include biological molecules (fibronectin, laminin), extracellular matrix extract (matrigel, rich in laminin), and synthetic molecules (poly-L-lysine or arginine, glycine, and aspartic acid (RGD) tripeptide), which are also used for adhesion of macromolecules in other applications.

Methodologies such as cell cloning require cells to be grown under adherent conditions so that cells can form colonies on the culture dish surface, which can be easily identified and isolated. Adhesion molecules can help revert suspension cells to adherence for cell cloning.

For studies that aim at understanding *in vivo* structure, function, and signaling of cell adhesion in 3D migratory environments, studies have shown that 3D matrices derived from tissues or cell culture differ in cytoskeletal components and tyrosine phosphorylation of focal adhesion kinase (FAK) compared to traditional adhesion molecules and 2D substrates [72].

In cases where adhesion to the culture surface needs to be prevented, for instance, in cell adaptation to suspension culture, cell clumping and cell adhesion to vessel surfaces can be reduced with the presence of the glycosaminoglycans heparin (10–100 $\mu g\,ml^{-1}$) or heparan sulfate in the culture medium, or by the addition of the surfactant Pluronic F68 (0.01–0.1%).

13.5.6 Protein Hydrolysates

Different types of hydrolysate or extracts from animal and plant tissues have been used to reduce serum dependence in culture. They are produced by enzymatic digestion of different protein sources. Animal extracts derived from the digestion

of animal tissues (tryptose, Primatone RL) are an excellent source of phospholipids [21], but their use has decreased in culture, to be replaced by hydrolysates from casein, albumin, lactalbumin, yeast cells (Yeastolate) [63], and plant tissues such as soybean (HySoy, NZ-Soy, Soy peptone) [20, 22], cottonseed, wheat, and rice (Hy Pep), and peas (Martone B-1, Veg peptone). Protein hydrolysates are complex mixtures containing amino acids, small peptides, inorganic ions, carbohydrates, and vitamins, which provide nutrients, growth factors, and antioxidants to the cells in culture. Amino acids could be present in the form of oligopeptides, which may bind various cell-surface receptors in a nonspecific manner, mimicking signaling molecules that have a growth-promoting or apoptosis-retarding effect. Small peptides present in these mixtures can serve the same function as amino acids, with an added advantage that they can be more stable and transported more easily than free amino acids. These hydrolysates are not fully characterized and can have beneficial effects on product yield, and detrimental effects associated with unknown components.

Given their nature, there is high lot-to-lot variability in these supplements. The utilization of hydrolysates from animal sources is reduced because of regulatory constraints and potential immunogenic reactions in humans. Soybean hydrolysates and peptone are most commonly used, and are often ultra-filtered to reduce endotoxin levels. Other complex components such as lipid emulsions are also used to supplement media.

13.5.7 Lipid Supplements

Lipids are often used as supplements in serum-free formulations. They are commonly provided to cells associated with animal serum or carrier proteins, since free lipids in the medium can be toxic for the cells in high concentrations. Supplementation considers cholesterol, cod liver oil, soybean oil and oleic, linoleic, and palmitic acids. Reconstituted lipid supplements with defined composition of phospholipids are commercially available for use in chemically defined media. A suitable lipid supplement must disperse selected lipids such that they are nontoxic, are taken up by the cells in a controlled fashion, can be micro-filtered, and remain stable upon storage. This is achieved by adsorbing the lipid to a soluble carrier molecule, devising a formula that drives lipid self-assembly to the required particle size, or dispersing and stabilizing a lipid mixture to a particle of sufficient transient size and stability [73]. Bovine serum albumin (BSA) is a common vehicle for lipids that contains high levels of serum lipids, especially fatty acids and lecithin. It also has the capacity to adsorb a significant amount of added lipids of choice. Cyclodextrins are naturally occurring circular polymers of glucopyranose that encase or chelate lipids in a more water-soluble molecule and thereby increase the lipids' aqueous solubility, and have been successfully utilized to solubilize cholesterol and fatty acids for supplementation of cell culture media. Liposomes, emulsions, and microemulsions are forms of lipid dispersions used in media supplementation. Phosphatidyl choline, phosphatidyl ethanolamine, and

sphingomyelin can be added to the medium in the form of liposomes or may be dissolved in dimethyl sulfoxide (DMSO).

13.6 Medium for Industrial Production

Commercial and industrial processes are nowadays directed toward serum-free media, and preferentially to chemically defined media. Serum-free formulations supplemented with specific proteins or purified extracts of known composition represent an advantage in terms of process reproducibility and quality control, facilitating product purification and reducing contamination risks.

The key features to consider when designing or selecting a serum-free medium formulation are that the chosen medium should support higher growth rates and culture densities, promote culture longevity, suppress apoptosis, support clone stability in continued passage, promote high product yield, maintain critical product quality attributes, reduce product and process related impurities, reduce regulatory consideration, risk, and cost, and be friendly to downstream process [74]. For most culture platforms currently available, optimized serum-free media can now support cell division rates comparable to those obtained in serum-containing media, with yields $>10^6$ viable cells per milliliter in batch culture and $>20^6$ viable cells per milliliter in fed batch, with expected product yields of at least $2-3\,g\,l^{-1}$ and reported yields up to $20\,g\,l^{-1}$.

The recommendation for developing a serum-free medium formulation is to start with an appropriate basal medium supplemented with insulin–transferrin–selenium (ITS). This supplementation has been successfully used in numerous studies with many different cell types including stem cells, primary cells, cell-based vaccine lines, and in antibody production lines like CHO, hybridoma, and HEK293. If using glutamine, it should be added at a concentration of 2–4 mM; otherwise, a dipeptide supplement can be added. Specific growth factors, hormones, vitamins, trace elements, and lipids must be added as required by the specific cell type. Osmolarity should be closely monitored. Essential fatty acids may need to be added if not present in the basal medium. A shear protection agent may be added for bioreactors and perfusion cultures [24].

The factors that are required in nearly all cell lines for serum-free formulations include insulin, transferring, and selenium. For certain cell lines, ethanolamine may also be critical, and in some cases β-mercaptoethanol; attachment factors such as fibronectin, laminin, vitronectin, growth factors, and lipid mixtures may also be beneficial. Insulin and transferrin supplements have been formulated to include selenium and sometimes ethanolamine. Each of these supplement components carries out an important role in the culture. Insulin is a growth factor that helps cells utilize glucose and amino acids; it has been known to be essential in cell culture from 1924 and is now the most commonly used hormone in culture media [75]. Transferrin is a universal iron carrier that provides iron and also

helps the cells maintain homeostasis by regulating the amount of iron uptake [76]. Selenium is required for proper functioning of glutathione peroxidase, thioredoxin reductase, and other antioxidant enzymes [77]. Ethanolamine is required for phospholipid synthesis in some cell types. Depending on the formulation and the manufacturer, insulin and transferrin supplements are also called ITSE, SITE, or ITS. Since product formulation may be recombinant or animal-free, or could be derived from animal tissue or from donor blood. There are a few recombinant and completely animal-free insulin and transferrin supplements that can be used for serum-free and animal-free media formulations. Alternatively, complex supplements such as BSA and protein hydrolysates or animal tissue extracts can be used. However, these compounds do not have a defined chemical composition, and contain components of animal origin, which regulatory agencies recommend to avoid in the production of biopharmaceuticals for human use.

The purpose of medium design is to identify the conditions that can be imposed on cells in order to maximize their productivity while taking into account the cost factor. The development of a culture medium requires testing all the individual components in order to determine their optimum concentrations and comparison against serum-containing media in terms of growth and productivity. Since media contain many components whose effects on culture performance are not independent, statistical analysis is required. Some strategies to achieve this include univariate analysis, factorial experimental design, or the variation of the concentration of only a set of nutrients [78, 79]. The nutrients that are not under study should be supplied in adequate amounts to avoid them from becoming limiting. An adaptation stage should be considered when developing a new media. This can be carried out through subsequent passages in increasing concentrations of the new media formulations. It can be done in culture flasks, or better in suspension cultures where there is better oxygenation and cells can be subcultured with less damage and stress.

The most challenging part of media development is to identify which supplement each cell line requires. For studies on specific media formulations, knowledge of the cell metabolism is required in order to develop new formulations as well as feed media for fed-batch or continuous culture strategies. In addition, cells must be stable and standardized in terms of their metabolic features, dynamic behavior, and genetic characteristics. The most commonly employed media formulations are listed in Table 13.1. The evaluation of cell performance in different media and at different stages of the culture is both laborious and expensive. It requires the characterization of total and viable cell concentrations over several passages, cell doubling time, and duration of the growth phase. To characterize media utilization, the initial and residual nutrient concentrations (e.g., amino acids), metabolites and their concentrations (e.g., lactate and ammonium), substrate consumption (e.g., glucose and glutamine), and product synthesis rates must be determined. Specific media formulations can be designed for the different stages of culture: cell inoculum scale-up, where the objective is to optimize cell growth and maintain viability; and production, where the objective is to rapidly achieve an adequate cell density for production,

sustain viability, and productivity [12]. In addition, the study of different feeding strategies may be required.

For the production of recombinant proteins, viruses and other non-cell products, a more extreme strategy in media design, which favors productivity using certain components at concentrations that can be toxic to cells or decrease viability, can be used, as high cell viability at the end of the production process is not required. These strategies include the use of high glucose concentrations (up to 15 $g\,l^{-1}$) at the beginning of the production stage, which leads to high osmolarity in the medium. This high osmolarity condition affects the growth rate of most cell types. In addition, during the production stage in fed-batch cultures, the feed medium is added with additional salts to maintain amino acids in solution, thereby further increasing osmolarity, reaching up to 400 mOsm by the end of the culture. Recombinant protein production enhancers such as sodium butyrate [80] and valproic acid [81] may also be added during the production stage to increase productivity, even though they might reduce the culture viability.

13.6.1 Medium Design and the PAT Initiative

In 2004, the FDA issued a guidance that encourages industry to take up new technologies that modernize manufacturing operations and enhance process control [82]. This guidance describes a regulatory framework, called process analytical technology (PAT), which promotes the implementation of innovative pharmaceutical development, manufacturing, and quality assurance in order to help the pharmaceutical industry address anticipated technical and regulatory issues. These recommendations are suggested but not required, and do not establish legally enforceable responsibilities. They are intended to facilitate progress of pharmaceutical manufacturing and regulation to a state where product quality is ensured through the design of the manufacturing processes. Both product and process specifications are based on a detailed mechanistic understanding of how formulation and process factors affect product performance, assuring quality on a real-time basis. The FDA's PAT initiative encourages drug manufacturers to apply innovative ideas to better understand their processes, to improve the online monitoring of manufacturing processes, and facilitate control and correction during a campaign rather than testing the product against specifications after manufacture.

PAT was aligned with the quality by design (QbD) initiative, which aimed to prevent production errors as far as possible by building quality control into process development. There are many challenges to applying these techniques to monitor mammalian cell culture bioreactors for biologics manufacturing. Regarding media composition, the ability to monitor multiple components in complex medium formulations noninvasively and inline is required. This has been shown to be feasible by using Raman spectroscopy for monitoring a mammalian cell culture bioreactor and obtaining simultaneous prediction of culture parameters including glutamine, glutamate, glucose, lactate, ammonium, viable cell density, and total

cell density [83]. The development of sensor and monitoring technologies like this one compatible with single-use facilities is required in order to facilitate the integration of process analytical technologies. Noninvasive sensor technologies that can monitor the health and performance of cell cultures are required. These could include metabolic sensors added to the media to indicate changes in cell metabolism and technologies that allow monitoring processes from external measurements.

The formulation of a basal medium is the first stage of cell culture where PAT approaches can be applied to define the quantity and quality of the product. In order to do this, not only process monitoring tools, control strategies, and instrumentation need to be implemented in order to ensure predefined final product quality, but also detailed knowledge of the process is required in order to be able to anticipate how variations in process variables and perturbations in raw material composition will affect process performance and product quality. Design-of-experiment studies of media formulation can reveal previously unobserved interactions between media components and the culture. Such media optimization experiments can be expensive at the manufacturing scale, so historical data from previous production runs can be retrospectively analyzed to reveal variability within raw material sources and to guide advanced parameter control. To gain this understanding, sensor enabled, disposable, scale-down bioreactors can be used as a cost-effective alternative during the research phase of bioprocess design. Empirical scale-down process information and historical data can be fed into a mathematical model to predict process outcomes based on online process data [84].

The ideal technologies for biopharmaceutical processes should operate *in situ*, be noninvasive, and generate online information about multiple key bioprocess and/or metabolic variables. This monitoring and control of mammalian cell processes can provide detailed physical and chemical representation of the process, which allows the construction of models that can handle the simultaneous prediction of different process parameters [85]. Mathematical models can consolidate and provide scientific understanding of product and process, essential for monitoring and controlling bioprocess. However, the implementation of good mathematical models that can actually capture the characteristics of the process requires the correct identification of critical process parameters [86]. A fully automated analytical system that combines exhaust gas analysis, inline analytics, and a corresponding algorithm was developed to determine culture parameters such as oxygen uptake rate, CO_2 evolution and transfer rate, and respiratory quotient, and was tested with two different cell lines (hybridoma and DG44 cells), providing data that could be used to draw conclusions with respect to the batch-to-batch reproducibility of mammalian cell cultivation processes in real time, which is a relevant factor under the PAT initiative [87]. Recently, models based on multivariate culture parameter measurements have been reported for real-time *in situ* monitoring of multiple process variables within cell culture bioreactors. These models can be applied to various products, media, and cell lines based on CHO host cells, and

are scalable to large pilot and manufacturing scales and are capable of predicting process parameters. These models allow monitoring of multiple key bioprocess metabolic variables, and hence can be utilized as an important enabling tool for PAT implementation [88].

Regarding the effect of media in the biomanufacturing process, mathematical models can help determine the variability and robustness of the process associated with changes in media composition, allowing process correction before the end of the culture run, based on online process measurements. A deep understanding of the role that each media component plays in the culture process is also required in order to implement and interpret these findings. As a result, we can expect that the implementation of the PAT initiative and the need to show control over all aspects of the production process will stimulate the development of chemically defined media formulations.

13.7 Conclusions

A detailed discussion on the classification of media for mammalian cell culture based on its characteristics, as well as a detailed review of all of its components, was provided. The fundamental role of each component in biochemical processes, cell growth, and production was discussed in detail.

There have been a large number of developments in media design, concerning media supplementation and the elaboration of specific serum-free media formulations for cells with distinct metabolic types. No standard universal culture medium formulation useful for all cell types has been proposed, given that different cell lines can present different metabolic profiles, requiring specific culture media compositions. Media formulations are dependent on the cell line, culture system, conditions, and culture objectives.

The utilization of medium components and supplements in an industrial setting, as well as the implications of the PAT initiative, were also addressed. There has been a paradigm shift in recent years in which media design is no longer oriented to provide cells with an environment that mimics its natural environment, but rather is aimed at identifying the conditions that can be imposed on the cells in order to maximize cell growth or productivity, in a cost-effective manner that complies with regulatory restrictions. The implementation of the PAT initiative and the need to monitor and control all aspects of the production process will likely stimulate the development of chemically defined media formulations. Standard media design concepts, such as providing cells with stoichiometric components in sufficient quantities and ensuring the presence of catalytic and unconsumed components, still need to be applied for these formulations. Empirical information obtained from scaled-down bioreactor settings and historical process data can be used to determine the effect of raw material composition on product quality and quantity for PAT implementation.

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Advanced Bioprocess Engineering: Fed-Batch and Perfusion Processes

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Mammalian cells are the preferred hosts to produce a number of recombinant biologics, especially proteins that require complex post-translational modifications for their *in vivo* efficacy. The hosts range from the most widely used Chinese hamster ovary (CHO) cells to the less commonly used baby hamster kidney (BHK), HeLa, and NS0 cells. Current bioprocesses are mostly based on suspension-adapted cells, which allow scalability and industrial culturing in bioreactors to achieve high titers by achieving high cell density, longevity of the culture, and high productivity. Although different types of bioreactors are used for culturing mammalian cells [1], stirred-tank bioreactors are the most common. In this review, we provide a comprehensive description of the different modes of bioreactor operation and the problems associated with their implementation in large-scale manufacturing. We will primarily concentrate on the fed-batch and the perfusion modes of operation, the two primary modes used in industry for culturing suspension cells. The growing importance of disposables has found its way into commercial biologics production. The use of disposables for large-scale bioprocessing using fed-batch and perfusion models will also be discussed. Advances in technologies to monitor metabolites in culture allow online control of bioprocesses. Some of these are also summarized.

14.1
Primary Modes of Bioreactor Operation

Bioprocess optimization has played an important role in increasing the productivity of recombinant therapeutics such as monoclonal antibodies (mAB) [2]. A bioreactor can be operated in four primary modes (Figure 14.1). The simplest method is a batch mode of operation where cells are seeded along with the required medium and allowed to grow. No further addition or removal of the medium occurs subsequently. A typical batch process for CHO cells lasts for 7–8 days, after which the cell viability drops to below 30%. The drop in viability is due to a combination of nutrient limitation and also build-up of toxic metabolites

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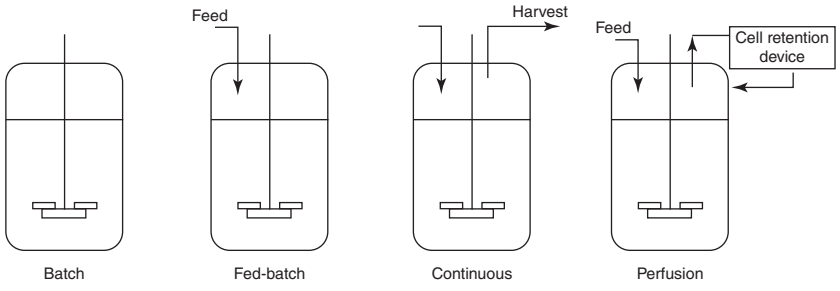

Figure 14.1 Schematic of the different modes of a bioreactor operation.

including lactate and ammonia. This is the most common and simplest bioprocess method and is widely used in initial process optimization. Other modes of operation are modifications of the batch mode, with each offering its own set of advantages and limitations. In the fed-batch mode of operation, medium is fed to the bioreactor at regular intervals based on a control strategy. In the continuous stirred-tank reactor, the medium is fed continuously at a fixed flow rate, while simultaneously the cells and supernatant are withdrawn from the reactor. A constant volume of the reactor is maintained, and a given growth rate can be maintained for hours to days. A continuous mode of operation suffers from a major limitation that dilution rates greater than the growth rate lead to washout. An alternative is a reactor with a recycling operation, called as the perfusion reactor. Here, cells are separated from the output stream and retained within the reactor. Retention of cells allows the reactor to be operated at dilution rates greater than the growth rate. Moreover, the cell density in the reactor is higher than that obtained in a continuous bioreactor. Figure 14.2 shows a comparison

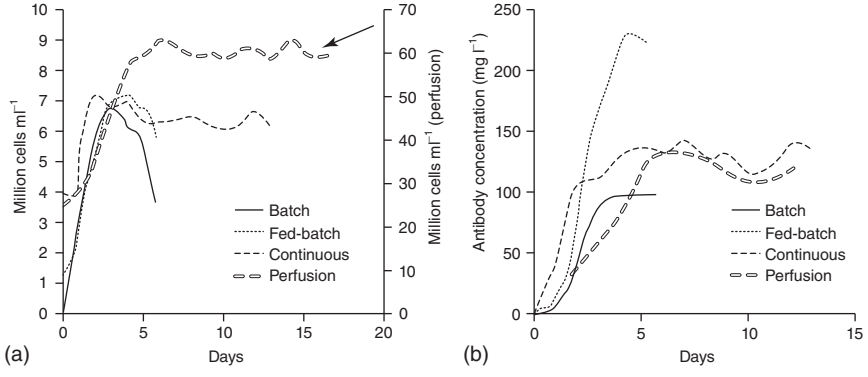

Figure 14.2 Comparison of the different modes of a bioreactor operation: (a) Growth profile for batch, fed-batch, continuous, and perfusion cultures. For perfusion, cell density data is plotted only till day 16. The culture actually lasts for 2–3 months. (b) Antibody profiles for batch, fed-batch, continuous, and perfusion cultures. For perfusion, data has been shown only till day 13. Note that while titers (mg l^{-1}) of antibody are lower in the perfusion culture, cumulative product amounts (mg) are much higher [3].

of cell densities and product titers in a batch, a fed-batch, and a perfusion bioreactor based on data from an immunoglobulin G (IgG)-producing rat hybridoma cell line cultivated in a 200-l reactor [3]. The fed-batch and perfusion modes of operation are most common for the production of recombinant therapeutics in mammalian cells. In this review, we will discuss these two modes of animal cell cultivation in detail.

14.2 Fed-Batch Mode of Operation

Fed-batch is the method of choice of bioreactor operation for large-scale biopharmaceutical production. Its operational simplicity with a number of feeding strategies provides a better platform for process development. Fed-batch operations are touted to have lesser impact on product stability while still achieving higher product titers. In fed-batch operations, the cells are supplemented with key nutrients such as glucose, glutamine, vitamins, and amino acids at periodic intervals in order to maintain the cells at high productivity conditions. A vast majority of recombinant proteins are produced using the fed-batch process [4].

Fed-batch operations involve a detailed design of the feed medium, process control algorithm, mode and frequency of feeding, and adjustments based on feedback. An optimum feed is essential for maintaining proper growth and productivity of the cells. The control algorithm provides an estimate for feeding, which in turn controls the feeding frequency and feed volume. Various metabolites measured either online or offline provide a feedback control to prevent under/over-feeding. The various steps involved in designing a fed-batch process are summarized in Figure 14.3. These are discussed in detail in the following sections.

14.2.1 Design of Feed Composition

The primary aim of feed medium design is to supply nutrients that get exhausted in the culture so as to prolong the longevity of the culture. However, mammalian cells have complex nutritional requirements, where, in addition to an energy source such as glucose and/or glutamine, many amino acids, vitamins, lipids, growth factors such as insulin, and inorganic salts need to be provided. Design of the feed medium should therefore be based on stoichiometric principles so as to maximize the resources while avoiding deleterious effects of feeding excess nutrients [5].

Glucose is the major source of energy responsible for the bulk of adenosine triphosphate (ATP) production. It is directly involved in the formation of cell biomass. Complete oxidation of glucose, under aerobic conditions, generates 30–32 molecules of ATP per mole of glucose. However, mammalian cells in culture usually undergo wasteful metabolism by directing part of the glucose toward lactate. Under these conditions, the cells generate only two ATP molecules per

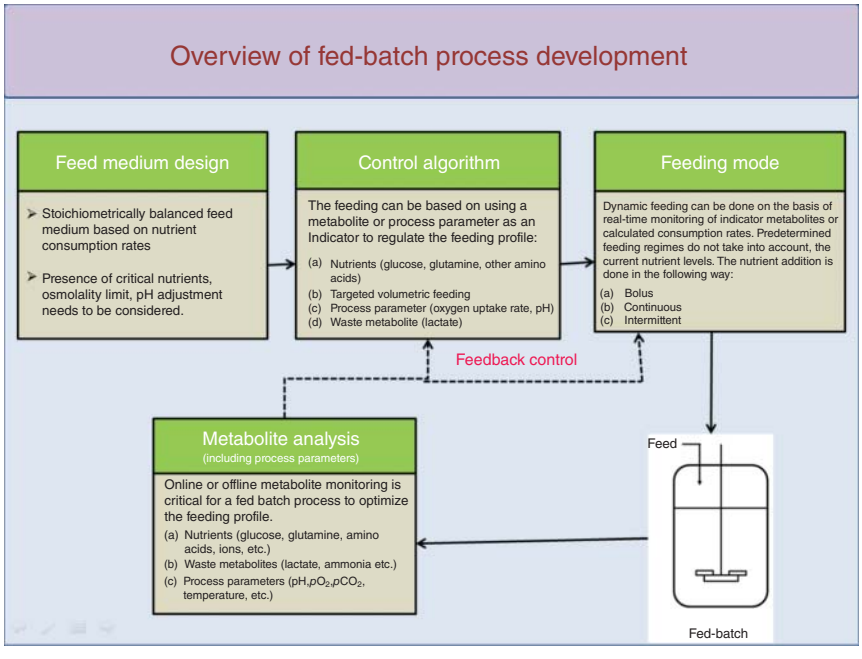

Figure 14.3 Overview of fed-batch process development.

glucose molecule, which is converted to lactic acid. Lactate is a toxic metabolite that reduces the culture pH, leading to significant reduction in culture viability. Because of this limitation, it is advisable to maintain low levels of glucose in the culture and feed according to need. It has been observed that there is a significant decrease in glucose consumption [6] and lactate production at lower temperatures and this can be further reduced by culturing at a lower pH [7]. Under certain conditions, such as the stationary phase in fed-batch cultures, cells have also been observed to consume lactate [8, 9].

Glutamine is one of the major amino acids needed in a cell culture process because of its involvement in multiple processes. It provides the amide group needed for nucleotide synthesis. It is involved in the synthesis of important amino acids such as asparagine, serine, alanine, and aspartate. In an essential resource conservation step, glutamine, after double deamidation, is utilized in the TCA cycle as α-keto-glutarate. This is then utilized for ATP synthesis. However, one of the major disadvantages of higher glutamine consumption is the production of ammonia, which hinders cell growth by increasing the culture pH. The metabolism and growth rate of the cells are affected only at very low levels of glutamine in the culture because of its low K_m value. Ammonia formation due to excess glutamine and amino acid has a detrimental effect on cell growth as well as the product glycosylation profile [10].

The medium for the culture of mammalian cells is complex, where, in addition to glucose and glutamine as the primary nutrients, other amino acids are also

required. Thirteen essential amino acids need to be supplemented, as cell growth is severely affected in their absence [11, 12]. Generally, the nonessential amino acids are also supplied in the medium, though these can be synthesized from other amino acids. In addition, the medium contains various vitamins including nicotinamide, riboflavin, thiamine, biotin, and others. The medium is also generally supplemented with lipids, salts, and essential fatty acids such as arachidonic acid, oleic acid, and linoleic acid. It has been a general practice to supplement cells with phospholipids and cholesterol, even though their absence will not result in the immediate suppression of growth.

Glucose and amino acids are used at much higher rates compared to vitamins and growth factors and therefore are the first ones to deplete. The feed medium is therefore designed primarily based on their requirements. Typically, glucose or glutamine is used as a reference metabolite, and stoichiometric ratios of the other metabolites such as amino acids are calculated from batch culture data.

The basal cell culture medium is designed with the optimum levels of different nutrients to support growth [13]. The concentrated feed medium can be developed from the same basal medium provided that the molar ratios of the components remain similar. In a study on JJ1 cells [14], a linear relation between the consumption of amino acids or glucose and yield (mAb productivity) established the following ground rule for feeding based on the consumption ratio of metabolite *i* with respect to glucose:

$$A_{i/\mathrm{Glu}} = \frac{q_i}{q_{\mathrm{Glu}}} = \frac{dC_i}{dC_{\mathrm{Glu}}} = \frac{(\mathrm{C}_{i,t} - \mathrm{C}_{i,t-1})}{(\mathrm{C}_{\mathrm{Glu},t} - \mathrm{C}_{\mathrm{Glu},t-1})} \tag{14.1}$$

Based on this, the concentration of metabolite *i* in the feed medium was calculated.

$$C_{\mathrm{feed},i} = A_{i/\mathrm{Glu}} C_{\mathrm{feed,Glu}} \tag{14.2}$$

However, a much better estimate of the proportionality can be obtained if data from multiple previous time points are available. In a study by Hu *et al*., the stoichiometric ratio between glucose, glutamine, other amino acids, lactate, and ammonia was determined. It was found that above a critical concentration of glucose, the stoichiometric ratio remained constant over many different cell lines and culture conditions [15].

It has been suggested that the amino acid requirement for growth and production are different [16], necessitating the estimation of stoichiometric ratios through the course of the culture. The stoichiometric ratios can be estimated online by measuring the metabolite concentrations over past few time points. The cumulative consumption of the nutrient can be plotted versus the cumulative consumption of reference nutrient such as glucose or glutamine. The slope of the line then gives an estimate of the stoichiometric ratio.

14.2.2 Feeding Strategies for Fed-Batch Culture

To design a fed-batch process, it is important to decide the composition of the feed medium (whether it is similar to the basal medium), when to feed the extra nutrients, how much to feed, and whether the feed should be added all at once. In the previous section we discussed how the composition of the feed medium is usually decided. Next we discuss the different philosophies that guide the design of a fed-batch process.

The most straightforward method of feeding is one in which a fixed percentage of the volume is fed at regular times. However, a rational design of feeding requires one or more critical parameters to be measured in real time, which are subsequently used to derive an optimum feed rate. This method is known as dynamic feeding. Continuous or periodic online measurement tracks the concentration of the indicator metabolite, and the culture is fed either manually or automatically to attain the set-point value. Parameters that can be measured in real time include viable cell density (VCD), oxygen uptake rates (OUR), and concentrations of the key metabolites including glucose, lactate, glutamine and other amino acids, and ammonia. A target value (mostly critical value) is used as a set point, and the feeding strategy is designed to achieve the set-point concentration.

Alternatively, the consumption or production rates of key metabolites and toxic by-products are used as indicators for feeding. The feeding strategy may be dependent on the glucose-to-lactate ratio or the specific consumption/production rates of the metabolites and not directly dependent on the concentrations.

The design of a feeding strategy can also be based on either a corrective action, referred to as supplementation approach, or a preventive action. In the supplementation method, the culture is supplemented with one or more nutrients based on deviation from a target value. The critical concentration of an indicator metabolite is used as the set point to regulate the feeding. The metabolite chosen should be involved in multiple pathways in order to establish a direct correlation with other metabolites in the medium or obtain a ***universal nutrient consumption rate*** based on which the concentration of other nutrients in the feed medium will be stoichiometrically balanced. The general choices of the indicator metabolites include glucose (target: $1-3\,g\,l^{-1}$ as baseline value), glutamine (target concentration of 1–3 mM), and dissolved oxygen (DO; saturation set at 40% (30–60%)). Other amino acids including asparagine (1–3 mM), tryptophan, cysteine, and methionine have also been used as indictor metabolites [17].

In contrast, the preventive approach is aimed at minimizing the accumulation of waste metabolites. Performance of a fed-batch system can be enhanced significantly based on "spent media analysis" or online monitoring of wasteful metabolites. In adherence with the viability data, the waste metabolite production rate can be used to design feed aimed at optimum utilization of all the resources without wasteful production. The two key toxic metabolites lactate and ammonia are produced in the culture as a result of utilization of glucose and amino acids, respectively. A lactate/glucose ratio of 1.6–1.9 suggests a wasteful metabolism

due to O_2 limitation or due to the presence of excess glucose. The different control strategies used in a fed-batch process are discussed in detail with specific case studies.

14.2.2.1 Culture Working Volume as Control

In this strategy, feeding is based on a fixed feed volume control. For example, the culture is fed with 30% of the total volume. The feed medium is designed on the basis of the consumption rates of the different nutrients and is different from the basal medium. In a comparison of this strategy with that based on a set target of glucose, 17–30% increase in titers was seen in cultures fed with 30–40% volume of the culture. In contrast, no increase in titers was observed in cultures maintained at ~3 g/l [18].

14.2.2.2 Concentration of Indicator Metabolite as Control

In this strategy, a key metabolite is chosen as an indicator metabolite, whose concentration in the bioreactor defines the feeding profile of the culture. Metabolites such as glucose or glutamine, which are utilized in or channeled across different metabolic pathways, are better suited for this strategy as they indirectly depict the metabolic state of the cells. The volume of the feed can be calculated on the basis of the deviation from the set point or target value of the indicator metabolite, as shown in Equation 14.3. Feed pumping or manual feeding is initiated once the concentration drops below the setpoint.

$$V_{\mathrm{F}} = \frac{(C_{i,\mathrm{setpoint}} - C_i(t))V_{\mathrm{r}}}{C_i^{\mathrm{F}}} \tag{14.3}$$

The above equation does not account for the consumption of the metabolite until the next feeding. To account for this, the volume of the feed can be calculated as follows, where current consumption rate is used along with predicted cell density to estimate additional requirement of nutrients:

$$V_{\mathrm{F}} = \frac{(q_i * X_{t+1} + C_{i,\mathrm{setpoint}} - C_i(t))V_{\mathrm{r}}}{C_i^{\mathrm{F}}} \tag{14.4}$$

Glucose and glutamine are the most commonly used indicator metabolites, and we next discuss specific case studies involving either of these nutrients. Attempts have also been made to use alternate sugars and other essential amino acids to design the fed-batch strategy.

Glucose as an Indicator Metabolite Glucose has been widely used as a critical indicator for feeding a variety of cell lines including hybridoma, CHO, and NS0 [18–22]. A critical concentration of glucose, generally in the range of $1-3\,\mathrm{g\,l^{-1}}$, is used as the set point to regulate feeding. Numerous examples are available in the literature that demonstrate fed-batch processes based on the maintenance of glucose concentration. One of the problems associated with glucose feeding is the build-up of lactate in the culture, which leads to toxicity. Thus, glucose is generally controlled at a critical low concentration to minimize wasteful metabolism.

In an interesting comparison between different modes of feeding [19], IgG-producing CHO cells were either fed every 3 days using a fixed volume method or based on the decrease in glucose concentration below the critical value (3 g l^{-1}) every 6 h. The feed mixture was designed such that all components were stoichiometrically balanced with glucose. The titers for glucose-fed cultures (8.2 g l^{-1}) were more than twice that for bolus feeding (4.1 g l^{-1}), suggesting that continuous availability of nutrients at a constant concentration leads to robust productivity. Further improvement in titers (9.1 g l^{-1}) could be obtained when the fed-batch was controlled dynamically with online measurement of glucose every 6 h. In another study, CHO (GS) – antithrombin (rAT) was cultured at a low pH of 6.8 [20] with daily continuous feeding of target glucose 2 g l^{-1} along with balanced amino acid feeding. This led to an increase in rAT activity by 30% with decreased glucose consumption and lactate production at later stages of the culture. This suggests that glucose remains an ideal choice for designing the medium and feeding profile of a fed-batch process.

It is important to determine the optimum concentration of glucose. In a fed-batch culture, NS0 cells were grown at different target glucose levels (2–8 g l^{-1}) after day 2 [23]. The feed volume was calculated according to Equation 14.3. The cultures that were fed with the target 2–8 g l^{-1} glucose showed a lower specific antibody production rate of 10 mg/10^9 cells day^{-1} as compared to 13 mg/10^9 cells day^{-1} seen in the culture maintained at target value of 4 or 6 g l^{-1}. In addition, there is a critical limit of underfeeding of glucose and glutamine, especially for myeloma-based cell lines such as hybridomas. Hybridomas are characterized by rapid growth, which leads to higher rate of glutaminolysis and glycolysis [24]; limitation of key nutrients such as glucose and glutamine may lead to apoptosis in these cells [25, 26]. In a CRL1606 hybridoma cell line [27], 2–8 h of deficient conditions led to activation of caspases and triggering of apoptosis.

Controlling the glucose at low concentrations (<1 mM) has also been shown to induce a metabolic shift in the cells toward a lactate-consuming state [28]. For certain cell lines, two consecutive fed batches were required to effect this metabolic shift [29].

Glutamine as an Indicator Metabolite Glutamine is an important metabolite for animal cell culture and consumed at high rates. However, excessive glutamine can lead to the formation of ammonia, which is toxic to cells beyond 5 mM [30]. In addition, ammonia has also been shown to have a detrimental effect on glycosylation [31]. Therefore, many fed-batch studies have used glutamine as an indicator metabolite to control its concentration in the reactor.

In a study on 293-HEK cells lines, Lee *et al*. [32] demonstrated that significant increase in viable cell densities and adenovirus production was possible by controlling the process at low glutamine levels. Automatic sampling was performed every 1.5 h to calculate the levels of glutamine, which was used to dynamically regulate its levels in the culture. Cultures inoculated at 0.3 mM glutamine and maintained at 0.1 mM glutamine were able to achieve 1.6-fold higher VCD and 10-fold higher adenovirus levels as compared to control maintained at 4 mM of

glutamine at inoculation. This strategy of maintaining low glutamine also resulted in either lower or similar levels of lactate and ammonia as compared to control culture in different media formulations (SFMII and DMEM/F-12).

In another study on CHO cells producing interferon gamma [33], a glutamine set-point concentration of 0.3 mM was found to be optimal with higher cell densities and culture longevity and significant reduction in ammonia and lactate formation. In this study, glucose was fed at 5 : 1 molar ratio. When glucose was independently controlled, the yields dropped by almost 50%. In another study, a high and a low glutamine feed was compared in IgG-producing CHO cells [34]. The feed was added every 2 days starting from day 2. The excess feeding of glutamine led to a significant increase in the level of IVCC (integrated viable cell count), which in turn resulted in higher titers, but the per-cell productivity levels were very low. On the other hand, the cultures fed with lower amounts of glutamine were better able to utilize their resources, resulting in very high per-cell productivity and comparable titers to high-glutamine fed-batch cultures.

The quality of the bioproduct was also shown to be effected in a fed-batch process. In a study on interferon gamma (IFN-γ)-producing CHO cells, a low concentration-based feeding strategy was used where glutamine concentrations was kept at 0.3 mM to maintain both the N-glycosylation macro and microheterogeneity of IFN-γ [35].

Dual Control Using Glucose and Glutamine as Indicators Glucose and glutamine are the main nutrients for cell culture. An excess of either of them leads to toxic metabolites, lactate, and ammonia. Therefore, it would be useful to control the levels of both of these nutrients in a fed-batch process. This strategy was successfully demonstrated in 1994, when simultaneous control of glucose and glutamine at 0.2 g l^{-1} (1.11 mM) and 0.1 g l^{-1} (0.68 mM) levels, respectively, led to a twofold increase in cell density and antibody titers as compared to when glucose alone was controlled at 2 g l^{-1} (11.1 mM) in mouse – mouse hybridoma cells (16-3F) [36]. In another study on hybridoma cells, cultures maintained at less than 1 mM glucose and glutamine had 1.4-fold and 2.3-fold increase in titers when compared with glutamine-limited and glucose-limited cultures, respectively [37]. Specific production rates have also been seen to double compared to when glucose alone was controlled [36]. Many other studies have demonstrated reduction in lactate and ammonia production by controlling both, the levels of culture glucose and glutamine [28, 38].

Alternate Sugars and Amino Acids as Indicator Metabolites The common problem arising out of the use of glucose is the accumulation of lactate in the culture, which leads to a decrease in viability. An alternative to glucose as primary carbon source is other hexoses. In a study on t-PA-producing CHO cells [21, 39], alternative carbon sources including mannose, fructose, and galactose, at 20 mM concentration, were compared with glucose feeding. The cultures fed with glucose and mannose reached higher cells densities, but severe lactate build-up (13 mM) was seen. The cells fed with fructose and galactose had relatively low cell densities

compared to the other two hexoses, but the lactate levels (~1 mM) were much lower [40]. In these cultures, the slow metabolism of hexoses was compensated by significantly higher consumption of glutamine (7 mM), which led to ammonia build-up. An ideal carbon–nitrogen pair source is glucose and glutamine, as the cells fed with this combination outperformed the cells fed with combinations of glucose/galactose–glutamine/glutamate in terms of culture longevity and titers even though the same culture had maximum lactate-ammonia build-up. An optimum balance needs to be maintained between the glucose and glutamine consumption by controlled feeding in order to achieve higher titers.

Recent studies have shown better utilization of carbon fluxes toward acetyl CoA formation via overexpressing levels of fructose transporters involved in carbon metabolism [41]. In a study on IgG-producing CHO cells, Wlaschin and Hu were able to achieve higher cell densities and lower lactate levels by overexpressing the GLUT5 transporter to increase the uptake of fructose into cells [42], suggesting the importance of modulating carbon fluxes. In a study on 293-2 cells expressing E1A and E1B proteins, the effect of feeding essential and non-essential amino acids was studied while maintaining glutamine at minimum concentration [43]. The fed batch with glutamine led to a maximum cell density of 2.4×10^6 cells ml^{-1} compared to 1.8×10^6 cells ml^{-1} achieved in the batch mode. Owing to higher glutamine consumption, 2.5 mM ammonia was produced in the fed-batch mode as compared to only 1 mM in the batch culture. A fed batch with essential amino acids without glutamine showed further increase in VCD to 3×10^6 cells ml^{-1} and reduced ammonia production below 1 mM. Flux analysis showed that the cells could adapt to a medium with low glutamine by increasing the amino acid fluxes towards the Kreb's cycle. Adding non-essential amino acids during this feeding strategy did not improve the growth further and these amino acids accumulated in the medium.

14.2.2.3 Nutrient Consumption Rate as Control

Another strategy to design the feed is to keep up with the demand for the nutrients. Thus, the requirement of a key nutrient until the next feeding is calculated on the basis of the consumption rate and the average cell density over this period. The volume of feed medium to be added in the time interval t_{i+1} to t_i can be estimated as follows:

$$V_{\text{feed}} = \frac{q_i \int_{t_i}^{t_{i+1}} X_v \, dt}{C_i^{\text{F}}} V_c \quad \text{or} \quad V_{\text{feed}} = \frac{q_i[(X_v)_{t(i+1)} + (X_v)_{t(i)}][t_{i+1} - t_i][V_c]}{2C_i^{\text{F}}} \tag{14.5}$$

The consumption rate can either be estimated online based on the previous data points or can be a fixed value based on earlier batch cultures.

$$q_i = [C_{(0)} - C_{(t)}] \times \left[\frac{\mu}{10^{-3}\left(X_{v0}\left[\exp(\mu t) - 1\right]\right)} \right] \tag{14.6}$$

Similarly, the integral cell density can either be estimated based on an earlier computed growth rate [44] or, if online measurement of cell density is possible [45], future cell density can be predicted. Generally, glucose is the most preferred nutrient.

In a study with NS0 cells, the feeding strategy was based on constant specific nutrient consumption rates (previously determined by Robinson *et al*. [46]) and the predicted IVCC. Cells were able to achieve much higher final IVCC (1.6×10^{12} cells h^{-1} l^{-1}) and titers (2.7 g l^{-1}) [44]. These cells were fed with nutrients showing rapid exhaustion (proprietary), amino acids, and cholesterol-containing lipid emulsion daily. The nutrients that were consumed at lower rates, including low-density lipoprotein, bovine serum albumin, transferrin, insulin, nucleosides, and vitamins, were fed every 2–4 days. The feeding amounts of each solution (V_{feed}) in between the time intervals ($t_{i+1} - t_i$) were estimated using Equation 14.5. This simple feeding strategy was effectively able to extend the cell growth phase and culture lifetime by delaying the starvation-induced apoptosis phase. Similarly, in another study, the GS-NS0 and GS-CHO cell lines constitutively producing mAb against CD25 and the dimeric fusion protein, respectively [47], were fed with well-defined, stoichiometrically balanced feed where the feeding rate matched with the consumption rate, with glucose as the reference point. This resulted in 8.2- and 5.7-fold increase in IVCC and 7.53- and 4.4-fold increase in titers in GS-NS0 and GS-CHO cells, respectively. Interestingly, this strategy led to a lower specific lactate production rate (2.41–3.99 mmol/10^9 cells day^{-1}) than in a batch culture (6.11–6.22 mmol/10^9cell day^{-1}).

14.2.2.4 Predicted Growth Rate as Control

Very few studies are reported in the literature where the feeding is based entirely on maintaining the growth rate. In a dynamic fed-batch study [17], asparagine concentration was used as a measure of the decrease in growth rate. The feeding was therefore based on maintaining the consumption rate of asparagine. However, measurement of the growth rate is important to accurately determine the nutrient requirement [44], and therefore a variety of probes are used to correlate the optical density to cell count [45, 48].

14.2.2.5 Culture pH as Control

Another useful and straightforward strategy to design a fed-batch process is to couple the feed to the drop in pH, as mammalian cells are sensitive to the pH change. The drop in pH can be estimated from the amount of base added to the reactor, and a proportional feed can be added to the culture. This strategy can also help maintain a constant glucose concentration, as the pH drop is primarily due to lactic acid production, which is stoichiometrically related to glucose consumption.

pH-based fed-batch control was first demonstrated in recombinant human kidney cells-293 in 1995 [49], where adding a concentrated feed was coupled to pH control stoichiometrically. The drop in pH was estimated from the amount of base added to the reactor to maintain a constant pH. For every mM of NaOH added, 0.205 g (1.14 mM) of glucose was fed to the reactor, resulting in 10-fold increase

in protein C titer and twofold increase in cell density as compared to the batch process.

In another recent study [50], a pH-controlled feeding strategy, referred to as HIPDOG (HI-End pH-Controlled Delivery of Glucose), was implemented on a panel of IgG-producing CHO-K1 cells. The glucose pump was controlled by the change in pH of the culture. HIPDOG cultures maintained comparatively very low levels of lactate, all below 3 g l^{-1} (33.3 mM), throughout both the growth phase and the production phase. When the glucose level in culture reached a low level (generally below 1 mM), cells began to take up lactic acid from the culture medium, resulting in a rise in pH above the set point. At this point, the glucose pump started automatically at a slow rate, resulting in a shift from lactate consumption to glucose consumption, resulting in a decrease of pH. When the pH went below the set point, the glucose feeding was stopped. This pH based glucose-lactate feedback regulation resulted in 1.83-fold higher IVCC and 3.9-fold higher titers as compared to control on day 12. Using this technology, the fed batch could be scaled up to 2500 l. On the contrary, the levels of ammonium in the HIPDOG cultures were nearly two-fold higher than those in conventional fed-batch cultures. The positive effect seen due to low lactate levels masks the inhibition effect of the excess ammonium.

14.2.2.6 Oxygen Uptake Rate as Control

OUR is a critical parameter used to monitor the health of the culture. A higher OUR suggests a higher metabolic activity, which can be correlated to higher titers or biomass formation or waste generation. The OUR rate is calculated by measuring the rate in drop of pO_2 after the O_2 is cut off. Oxygen uptake is very sensitive to changes in metabolic rates, and therefore can be easily used to control nutrient levels in a fed-batch culture.

Once airflow is interrupted in the bioreactor, the accumulation of oxygen in the liquid phase is a balance between the oxygen transfer rate due to desorption and the OUR of cells. The oxygen balance can thus be given by

$$\mathrm{OUR} = \frac{C_{O_2}(t_0) - C_{O_2}(t_f)}{t_f - t_0} + \frac{\int_{t_0}^{t_f} (-k_L a C_{O_2}(t))dt}{t_f - t_0} \tag{14.7}$$

OUR gives a sense about the oxygen consumption and availability in the culture. OUR can be also used to accurately predict the beginning of the death phase, which can be used to prolong the exponential phase or keep the cells between the log and stationary phases in order to achieve maximum productivity. The coupling of nutrient consumption rate with OUR can help in tightly regulating the culture parameters near a set point. This method of feeding is more suitable in the case of dynamic feeding, as it will sense even minor fluctuations.

OUR is generally used at the reactor scale, with an automatic feeding strategy. The hybridoma cell line IV F 19.23, producing IgG1, was cultivated in a 2-l bioreactor (Bioengineering, Switzerland) in which a feeding algorithm based on OUR was used [22]. The time point at which significant drop in the OUR of the culture;

revealed a very low levels of glucose (0.36 g/l) and glutamine (0.2 mM) left in the culture. This correlation between OUR and the nutrient uptake rate was used to control the feeding profile.

In another study on mouse hybridoma cells (MAK), OUR was used to control the fed-batch process [51, 52]. OUR was stoichiometrically correlated with glucose consumption rates, and accordingly the feeding rate was dynamically adjusted to maintain a glucose concentration of 0.1 g l^{-1}. The stoichiometric relation between glucose and oxygen was determined online. This strategy led to a significant reduction in the level of lactate, and the cells reached a very high concentration of 1.36×10^7 cells ml^{-1}. Under glucose limitation, increase in OUR could also be correlated to glutamine consumption [53]. In a novel approach, a closed-loop control algorithm was designed to control OUR by varying the glutamine feed rate [54].

14.2.3 Mode and Frequency of Feeding

The frequency of feeding as well as the mode of addition of the feed influences the performance of a fed-batch culture. There are primarily two modes of feeding (Figure 14.4). In the bolus mode of feeding, the cells are fed with a concentrated feed medium in a very short interval of time in a periodic manner. This high-concentration feeding is sufficient for the cells for few days. This mode of feeding is preferred in large-scale mammalian cell cultures for the production of mAbs, as the osmolatic shock coupled with decrease in temperature significantly boosts the overall titers [55–57]. One of the major disadvantages of this mode is the sudden increase in the overall osmolality of the culture, which can be detrimental for some cell lines. The bolus mode of feeding has been employed in many cell lines to achieve higher product titers. A few examples from the literature are presented in Table 14.1. For example, HEK293 cells producing the human recombinant IFNα2b were fed with 1 ml of EfficientFeed (Invitrogen) medium daily, whereas glutamine was fed separately to maintain a desired set point (2.5 mM) from day 2 onward [58]. This resulted in twofold increase in cell density and threefold increase (160 mg l^{-1}) in the IgG titer.

Alternatively, the feeding can be done continuously, referred to as continuous feed. Based on the stoichiometric analysis of the earlier batch culture data, the nutrient consumption rates and growth rates are calculated. These data are then used to predict an optimum feeding rate that accounts for dilution, which is maintained throughout the culture duration. The feeding rate can be adjusted on the basis of offline or online measurements. This method is most suited for the cultures in which an optimized uniform feed is used rather than feeding the individual components. The nutrient levels in the culture post initiation of feeding remain close to the target levels during the course of feeding. This is maintained by dynamically adjusting the feed flow rate (F) according to the online measurements and accounting for the deviation from the set point;

$$F = \frac{V_{\text{feed}}}{f} \tag{14.8}$$

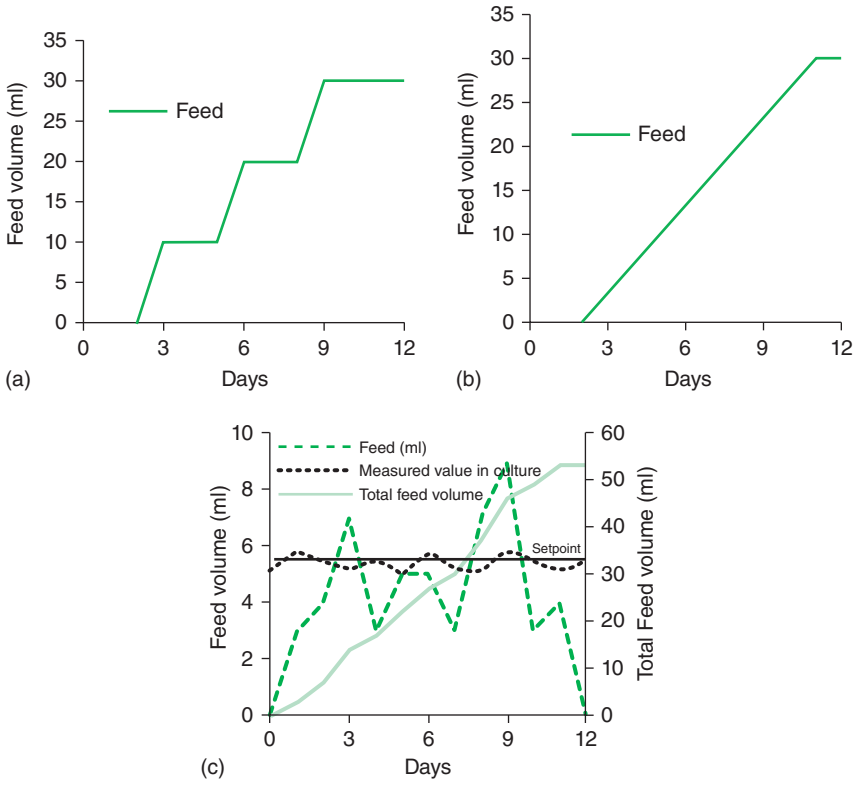

Figure 14.4 Different modes of feeding. (a) Bolus, (b) continuous, and (c) dynamic.

14.2.4 Challenges in Fed-Batch Culture and Future Directions

One of the major challenges of fed-batch culture is the reduction in the accumulation of toxic metabolites, namely lactate and ammonia, in the culture. A number of process-based strategies that have been successful in achieving this objective have been discussed earlier in the review. The development of serum-free medium as well as the optimization of feeding strategies has led to 100-fold increase in titers of rCHO cultures over the last two decades [59]. Compared to the batch culture in the 1980s, optimized fed-batch process has allowed the culture duration to be extended by a factor of 3 while simultaneously increasing the maximum cell densities by more than 10–15-folds. Specific productivity has also increased 5–10 times, while 20–50 fold increase in volumetric productivities has been possible. As a result, titers of more than $5\,g\,l^{-1}$ are now routinely achievable in fed-batch cultures.

Owing to the strong competition in biopharmaceutical production market, most of the details of process development in biopharmaceutical companies are out of bounds for the general public [60]. However, process modifications alone

Table 14.1 Different fed batch strategies in cell culture bioprocesses.

Feeding strategy	Mode of addition, feeding Frequency	Sampling	Target set point	Feed composition	Cell line/ product	Scale/reactor	Cell density and titers	References
Fixed volume fed based on glucose measurements	Bolus, Every 3 days from day 3 onward	Autosampler	Fed from media containing 133 $g\,l^{-1}$ glucose	Varying chemically defined feeds with different amino acids, osmolality, and pH	CHO-IgG	3l bioreactor (Applikon), production reactors	4.1 $g\,l^{-1}$, 8.5 million cells ml^{-1} on day 12	[19]
Target nutrient Glucose	Dynamic, Every 4–6 h	Autosampler	3 $g\,l^{-1}$	Chemically defined media	CHO-IgG	3l bioreactor (Applikon), production reactors	9.1 $g\,l^{-1}$, 13 million cells ml^{-1} on day 19	[19]
Target nutrient Glucose	Continuous, After day 2	Manual sampling by syringe	2 $g\,l^{-1}$	Protein-free chemically defined feed media developed in house	CHO (GS) – antithrombin	2-l (Bio Master D type; Able, Tokyo) fed-batch culture	Increase in rAT activity by 30%	[20]
Target nutrients Glucose/ glucose + galactose	Bolus, Every 12 h	Manual sampling	FB1 Target Glucose = 2–3 mM, FB2 target galactose = 13–20 mM	10× (amino acids as compared to basal BIOPRO1 medium), 4× vitamins, 8× lipids,	CHO TF 70R	250 ml spinner flask (Techne) with a working volume of 150 ml	Batch – 4.2 $mg\,l^{-1}$, FB1 (glucose) 5.2 $mg\,l^{-1}$, FB2 (glucose + galactose) 5.6 $mg\,l^{-1}$	[21]

(*continued overleaf*)

Table 14.1 (*Continued*)

Feeding strategy	Mode of addition, feeding Frequency	Sampling	Target set point	Feed composition	Cell line/ product	Scale/reactor	Cell density and titers	References
Model-based Adaptive OLFO (open-loop feedback optimal) controller	Dynamic, Periodic feeding after day 2	Not mentioned (manual)	Glucose = 1 mM, Glutamine = 2 mM	Similar to 10× IMDM/Ham's F-12 (1 : 1) without salts	Hybridoma cell line IV F 19.23	2-l polyamide-foil stirred bioreactor (Bioengineering, Switzerland)	Maximum VCD: Batch – 2×10^6 cells ml^{-1}, OFLO – 2.7×10^6 cell ml^{-1}, 2.9-fold increase in titer vs batch	[22]
Glucose linked to lactate and pH	Bolus, (2–3% of reactor volume per day starting on day 4)	Not mentioned (Manual)	Details not mentioned	In-house feed (Pfizer)	Serum free CHOK1 suspension cells	1 or 2 l bioreactor (Applikon)	Day 12 – control (IVCC = 192 million cells ml^{-1}), HIPDOG (IVCC = 352), 3.9-fold increase in IgG titers	[50]
Target nutrients: Glucose and glutamine	Bolus, daily	Manual sampling	Glucose = not mentioned, Glutamine = 2.5 mM	Feed B media (Invitrogen)	HEK293 – human recombinant IFNα2b and expressing the PYC	125 ml (20 ml W.V.) shake flask scale, 2 l stirred bioreactor	Threefold increase in final product titer (160 mgl^{-1}), twofold increase in maximum cell density (up to 10.7×10^6 cells ml^{-1})	[58]

Low glutamine	Dynamic	Not mentioned (Manual)	Glutamine = 0.3 mM, glucose maintained at 0.35 or 0.7 mM Glucose/ glutamine = 5 : 1	Formulated concentrate of DMEM/F-12 with soybean protein hydrolysate and other additives	CHO (IFN-gamma)	5.0 l bioreactor (B. Braun, Melsungen, Germany)	10-fold improvement in IFN-gamma yields	[33]
Online capacitance to measure growth	Bolus, Every 4–8 h	Glucose always above 0.25 mM		Chemically defined media	Murine hybridoma cell line, JJ-1, mAbs against human tissue factor (hTF)	125 ml shaker flasks, Biostat M stirred-tank bioreactor	Batch : VCD (1.37×10^6/ml), mAb (136 mg l^{-1}); Fed-batch: VCD ($3.31-7.04 \times 10^6$ ml^{-1}), mAb (304–707 mg l^{-1})	[14]
Online capacitance to monitor VCC	Dynamic	Fed when glucose went below 3 g l^{-1}, every 6 h		Chemically defined media	CHO-IgG	3 l bioreactor (Aplikon), production reactors	Bolus – 4.1 g l^{-1}, Dynamic (VCC) – 6.5 g l^{-1}	[19]
OUR was coupled to glucose consumption	Continuous	Glucose levels maintained between 0.9 and 1.2 mM		CHO-S-SFMII	KB26.5 murine hybridoma cell line, IgG3 mAb against antigen A1 of the RBCs	Biostat MCD2 bioreactor equipped with a 2 l cylindrical vessel	Batch: VCD (2×10^6/ml), $Y_{Lac/Glc} = 1.7$; Fed-batch: VCD ($4.1-5.2 \times 10^6$ ml^{-1}), $Y_{Lac/Glc} = 1.21-1.4$	[214]
Stoichiometric feeding based on OUR	Dynamic, Feeding started on day 2	(cGln = 0.2 mM, cGlc = 2 mM)		similar to 10× IMDM/Ham's F-12 (1 : 1) without salts	Hybridoma cell line IV F 19.23	2-l polyamide-foil stirred bioreactor	Maximum VCD: Batch – 2×10^6 cells ml^{-1}, OFLO – 2.8×10^6 cell ml^{-1}, 1.5-fold increase in titer vs batch	[22]

may not advance the longevity of fed-batch processes. With recent advances in cell engineering approaches, development of better clones with reduced lactate and ammonia production has resulted in much higher titers [42, 61, 62]. Small interfering RNA (siRNA)-mediated knockdown of lactate dehydrogenase A (LDHa) and pyruvate dehydrogenase kinases (PDHKs) in CHO cells expressing a therapeutic mAb reduced lactate production by 90% and increased the specific productivity and volumetric antibody production by approximately 75% and 68%, respectively [61]. To reduce the ammonia produced from glutamine, a less ammoniagenic substrate, namely glutamate, was used. rCHO cells engineered with the glutamate synthetase (GS) system, when cultured with glutamate, showed lower ammonia production [62]. GS catalyzes glutamate with ammonia to yield glutamine. A major target of the cell engineering technique has been the apoptotic pathway with an aim of prolonging the culture duration. The overexpression of the anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, or downregulation of pro-apoptotic proteins such as Bak and Bax in rCHO cells, resulted in increased cell viability and protein production [26, 63].

Vector engineering is an emerging alternative to gene amplification strategies to increase productivity owing to its less time and labor requirements. Using the current vector engineering techniques, gene targeting by site-specific integration [64–67], and a cis-acting element to augment gene expression [68, 69], high producing stable rCHO cell lines were established. The CRISPR/Cas9 genome editing system has been successfully used to generate isogenic cell lines with consistent protein production, owing to its ability to mediate targeted gene integration into site-specific loci in CHO cells [70]. Cell proliferation engineering approaches have targeted the cell-cycle pathway to achieve cells with better growth and productivity [71–74]. Protein secretion pathway remains a major bottleneck in achieving higher productivities [75]. To overcome this challenge, various cell engineering techniques have aimed at increasing/decreasing the expression of chaperones [76–78], proteins assisting in secretion [79–81], and unfolded protein response pathway proteins [82–84]. The limited success of these cell engineering techniques can be attributed to the unavailability of rCHO genetic information for a long time. Finding of hotspots and neighboring sequences based on available genome of CHOK1 [85] will lead to better targeted cell line engineering approaches to identify high producers.

Another major challenge in a fed-batch process is scalability. The scaling-up process is intensive and prone to batch-to-batch variations. The full potential of the fed-batch remains unutilized owing to the limitation of the bioreactor volume. The maximum achievable cell density in a fed-batch culture is less than in perfusion cultures by 10–100-folds, which ultimately limits its maximum titers. Moreover, the continuously changing environment in the bioreactor can lead to alteration of cellular metabolism and genetic reprogramming of cells, which can hinder cell growth and make the product more liable to aggregation and fragmentation [86].

14.3 Perfusion Mode of Bioreactor Operation

An alternative to fed-batch process is a continuous reactor with recycling, referred to as perfusion. In this mode, the reactor is continuously fed with the medium. Simultaneously, cells are harvested using cell retention devices and re-fed into the reactor. Thus, higher cell densities can be obtained in a relatively small culture volume.

A perfusion bioprocess offers many advantages over a fed-batch process. Continuous removal of toxic metabolites such as lactate and ammonia assists cellular growth, with cell densities higher by at least an order of magnitude. As the reactor is operated at a constant growth rate, it provides a uniform environment for the cells, leading to more reliable and reproducible runs with high consistency in the product quality. A perfusion culture can be maintained continuously for many days and months, leading to higher product levels compared to fed-batch or batch processes. As an example, SEAP, a model protein, was produced in Sf9 insect cells in batch, fed-batch, and perfusion modes with an acoustic-settler-based retention device. While the cell densities in the fed-batch and perfusion system were similar, and higher product titers in the fed-batch, the overall product levels were 8 times higher in the perfusion system, as the culture could be maintained for 43 days as compared to 16 days in the fed-batch culture [87].

Perfusion bioprocess is the natural choice for commercial production of unstable biologics, as the residence time for the product is much lower than in a fed-batch process. Factor VIII is produced commercially by Bayer (Kogenate), Pfizer (Refacto), and Novo Nordisk (Novoseven). Perfusion-based upstream process coupled with continuous downstream platform can significantly enhance the overall titers of such unstable products [88]. Other molecules that are produced using perfusion systems include Protein C (Xigris, Eli Lilly) and mAbs such as Simulect (Novartis), Reopro (Jansen), and Remicade (Jansen) [89].

14.3.1 Types of Perfusion Devices

The key components of a perfusion system are the retention devices that continuously separate cells and spent medium from the bioreactors. Retention devices exploit a physical property of cells to separate them from the supernatant. Sedimentation-based devices use cell density, whereas in filtration-based devices the primary mode of separation is cell size. Other perfusion devices employ centrifugal force or acoustic waves for separation. A variety of these devices are used for the commercial production of recombinant proteins [89]. Table 14.2 lists some of the retention devices currently used with their application in culturing animal cells.

14.3.1.1 Gravity Settlers

Gravity settlers are the simplest devices to retain cells based on cell density (Figure 14.5a,b). These systems are used to culture shear-sensitive cells, and

Table 14.2 Cell retention devices and their application in perfusion bioprocess.

Type	Retention device	Cell line	Product	Max. VCD (million cells ml^{-1})	Max. PFR (day^{-1})	Max. days	References
Sedimentation	Sedimentation	Hybridoma, CHO	IgG2a, SEAP	23	0.89	36	[215, 216]
	Inclined settler	CHO, BHK21	mAb, FVIII	20	1	34	[217–219]
Acoustic	Acoustic cell-separation (BioSep)	Hybridoma, CHO	IgG1, tPA	100	10	110	[145, 148, 220–222]
	Ultrasonic resonance field device	Hybridoma	IgG1, IgG	50	72	58	[143, 147]
Filtration	Spin filter	NS0, Myeloma, CHO	IgG4, IgM, IgG	10	1.2	34	[119, 120, 125]
	Tangential cross-flow filter	Sf9	VP6	15			[99]
	Filtration	Hybridoma	mAb	20	2.9		[223]
	Floating filter	CHO, S2	mAb	104	9.9	16	[224–226]
	Hollow fiber	CHO, 293, Sf21	IgG1, GFP	55	6	27	[227, 228]
	Depth filter	CHO	IgG	0.35	6	20	[229]
	Dynamic rotating disc filter	CHO	IgG	10	2	16	[230]
	Polyethene membrane filter	Hybridoma	IgG2a	20	0.15	10	[231]
	Porous ceramic filter	Hybridoma	IgG2a	35	1	10	[232]
	Ultrasonic filter	Sf9	Chitinase	27	10	12	[150]
	Vortex flow filtration	NS0, SP20	mAb, IgM	14.7	1.57	18	[233, 234]
Centrifugation	Centritech Lab centrifuge	Hybridoma, CHO	IgM, IgG	16	4	15	[133, 134, 235]
	SorvalCentritech Lab cell II centrifuge	HEK293	Beta secretase	30	0.5, 1.0, 2.0	21	[236]
Others	CS10 cell gravity settler	NS0	mAb	8.3	3	53	[237]
	A BTS A90 sampling valve	BHK	NA	10	NA	102	[238]
	Cell culture cap	A549					[239]
	Cell entrapment alginate	Hybridoma	IgE	12	NA	33	[240]
	PET fibrous matrices	HT-29 cells	EGFP	50	NA	5	[241]

retention rate of viable cells is usually above 90%. Two different working modes are available using gravity settlers: vertical counter current flow and inclined surface flow. Most of the studies on gravity settling are through external retention loops, although some of them have tried internal retention zones [90–95].

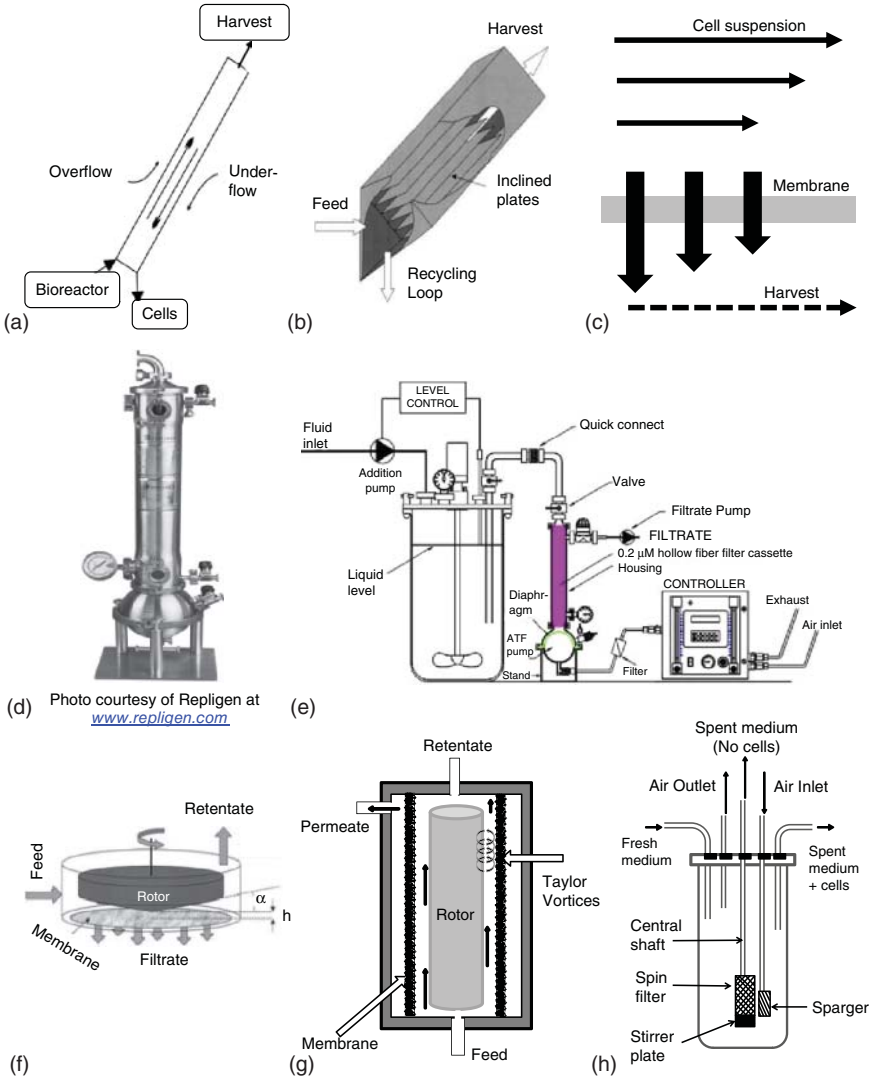

Figure 14.5 Sedimentation- and filtration-based retention devices for perfusion process. (a) Principle of gravity settlers. (b) Compact inclined settler ([211] with permission from Wiley ©Wiley 2003). (c) Principle of cross-flow filtration. (d) Repligen ATF system [108]. (e) Schematic of ATF system [108]. (f) Schematic of experimental laboratory-scale CSF ([114] with permission from Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany). (g) Schematic of the vortex flow filter [211]. (h) Schematic of a spin filter bioreactor.

14.3.1.2 Filtration

Filtration is the most widely used method in a retention device. A physical barrier separates the cells and spent medium from the cultures. The major disadvantage of using this type of retention device is that it creates fouling and clogging of the filter by cells [96–98]. Its inability to distinguish viable and dead cells is another major bottle neck for large-scale bioprocessing. A number of variants of this device are available in the market offering efficient separation of cells from the media. The following sections describe a few of them in detail with their industrial-scale setup.

Cross-Flow Filtration (CFF) or Tangential Flow Filtration (TFF) In a cross-flow filtration system, the cell suspension is continuously passed through a membrane held tangentially, thereby allowing retention of cells at the bottom of the bioreactor (Figure 14.5c). The most widely used membranes are polyvinylidene fluoride (PVDF), Nucleopore, and polycarbonate cross-flow membrane filters [99–101]. With minor adjustments of the flow rate in the retention loop, fouling could be avoided in these filters at the laboratory scale. A major limitation of the cross-flow filtration is the damage to cells due to shear stress. Both the magnitude of shear stress and the time of exposure to stress contribute to the loss in viability of the cells [102]. This has also been observed experimentally, where a shear-stress-induced decrease in the growth rate was seen in Sf9 cells when perfused through cross-flow filtration units [99]. Cross-flow-filtration-based devices are difficult to scale up because of the high surface area requirements and problems of fouling. However, there are many examples of using such devices for purification of proteins from large-scale cultures. High recovery rates were obtained using six cross flow filtration units at 5000 l scale with no change in the product quality [103]. There have been many studies on the use of tangential flow filtration in mammalian cultures such as hybridoma cells [104] and FM-3A cells [101].

Hollow Fiber Hollow fibers provide a large surface area for cross-flow filtration. On the basis of a study with a microfiltration system using polysulfone membranes, a pore size of 0.45 μm was found to be more effective at reducing pore blockage than a smaller pore size of 0.20 μm [105]. In addition, a feed flow rate corresponding to the wall shear rate of 4000 s^{-1} was found to be optimum.

For commercial manufacture of biologics, a number of large-scale hollow fiber filtration devices are available on the market. GE presents a hollow fiber membrane filter with an operational capacity of 2000 l. Alternatively, installing multiple hollow fiber filtration devices has also been tried at large-scale manufacturing sites [103]. Filtration devices such as the single-pass tangential flow filtration (TFF) [106] have been modified, where multiple membranes are put together such that a single unit provides multiple filtrations and avoids recirculation. However, this has primarily been used in downstream processing to concentrate the antibodies and not in a perfusion culture. Using a hollow fiber bioreactor, a hybridoma cell line producing IgM was maintained in culture for 40 days with higher antibody yields as compared to a cross-flow-filtration-based perfusion system [107].

Alternating Tangential Flow The alternating tangential flow (ATF), a patented technology by Repligen (Waltham, MA), is widely used in industrial applications [108].

This system uses an external hollow fiber (Figure 14.5d) module through which the medium is exchanged using diaphragm pumps. However, the cell culture feed flows along the membrane, while the filtrate flows across it. The ATF pump is specifically designed with a diaphragm so as to maintain the flow in and out of the reactor smoothly. Pressurized air is used to force the diaphragm upwards, leading to the flow of cells and the medium along the hollow fibers. The filtrate flows out into the reactor. Removal of air forces the diaphragm back down. This rapid flow also reduces membrane fouling. With a range of pore sizes and filter materials, different models of the ATF system are available, which can be used according to the requirement of the bioprocess. Flow inside the ATF system is controlled by reversing the fluid flow through the hollow fibers. This allows low residence time for the cells outside the reactor (Figure 14.5e). Note that the pore size is to be selected according to the material to be retained by the fiber. Five different setups of ATF (ATF-2, -4, -6, -8, and -10) are available on the market for large-scale bioprocessing. The selection of the model depends on the volume of the culture reactor. ATF-10 is used for culturing cells in a 5000-l reactor. Typical surface area of an ATF-4 system is 0.42 m^2 with a minimum filtration capacity of 30 l day^{-1}.

Of the different cell retention systems, ATF has the advantage of linear scalability. In case of failure of the ATF filter, a new system can be easily installed without compromising the sterility of the bioreactor. Table 14.3 compares the different retention devices and illustrates the advantage of using ATF over the other systems. Shear stress on the cells is reduced in an ATF compared to a TFF

Table 14.3 Comparison of perfusion devices.

Properties	Spin filter	ATF	Hollow fiber	Centrifuges	Acoustic settler	Inclined settler
Fouling	− −	+	− −	+	+	−
Ease with operation	+	+ +	+ +	−	+	+
Optimization of culture parameters		+ +	+ +	−	−	− −
Sterility maintenance	+ +	+ +	+ +	+	+ +	+
Scalability		+ +		+	−	
Re-sterilization	Yes, if external	Yes	Yes	Yes	Yes, if external	Yes, if external
Residence time of cells in separator and connection tubing	Zero, if internal	1–2 min	≅10 s	2–9 min	3–14 min	10–20 min
Purchase cost	+	+	+	−	+	+

++ Very good
+ Good
− Poor
−− Very poor

system because of the use of a diaphragm pump in former. In high-cell-density cultures, other retention devices fail to operate because of higher rate of clogging. ATF system has a dynamic flow pattern with an inherent flow cleaning capacity, which prevents clogging. ATF continuously cleans the filters periodically using the back-flush mechanism. However, fouling is seen in some cultures. Kelly *et al*. showed through scanning electron microscopy that fouling is due to the combined effect of biological material and accumulation of antifoam micelles on the surface of the membrane [109].

Using the ATF-9 model in a 70-l bioreactor, the highest VCD of 10^7 cells ml^{-1} for adenovirus production in HeLa-S3 cells has been reported [110]. Cell densities as high as 10^8 cells ml^{-1} have been reported in a wave bioreactor coupled with the ATF system [111]. The ATF system has also found widespread acceptance in industrial processes. Jansen Biotech uses the ATF system at a maximum operational volume of 1000 l to commercially produce mAbs. A 20 % increase in cost of goods (COG) savings has been reported when continuous perfusion batches are run compared to a fed-batch process at the 1000-l scale [112]. However, at the 500-l scale, continuous perfusion processes are not profitable compared to a fed-batch run. Biovolutions report the use of ATF-6 perfusion system connected to a 50 SUB unit to manufacture 1 – 2 kg of antibodies in a span of 2 – 3 weeks [113].

Controlled Shear Filtration (CSF) To avoid clogging of the membrane, a high shear force needs to be applied at the membrane. However, high shear rates lead to loss of cell viability and accumulation of cell debris in the reactor. The controlled shear filtration (CSF) design was proposed to determine an optimal shear rate with low residence time to minimize the detrimental effect on cells. In a shear-filtration-based device, a conical rotor is fitted on the top of a membrane in a chamber (Figure 14.5f). Depending on the angle of the cone, the rotor speed, and the gap between the rotor and the membrane, the shear stress can be altered [114]. In addition, the transmembrane pressure and the residence time of the cells can also be altered [114]. In a study with BHK cells, five-fold higher flux could be obtained with CSF as compared to conventional filtration. In a proof-of-concept demonstration of integrated processing, a CSF device coupled with an affinity membrane (CASF) used for the purification of t-PA from a 1-l CHO cell culture [115]. Adding an affinity chromatographic unit to this system allowed protein capture and purification directly from the cell culture fluids. Using computational fluid dynamics (CFD), an optimal rotor design was identified for the CASF system with a 60% decrease in shear stress as opposed to the model developed by Vogel. The rotor diameter in this model could be scaled up to 2.5 times, leading to higher volumetric throughput [116]. The scalability of the CSAF was also demonstrated using CFD at scales greater than 1000 l in the batch, fed-batch, and perfusion modes [117]. However, there is no published commercial application of this type of filtration device.

Vortex Flow Filters The vortex flow filter (VFF) was proposed in 1987 to overcome the disadvantage of high shear stress in tangential filtration devices [118]. These systems have three concentric cylinders, with the intermediate cylinder supporting the filtration membrane (Figure 14.5g). Rotation of the inner cylinder leads

to the formation of Taylor vortices between the inner and intermediate cylinders, thus avoiding fouling of the membrane. Comparison of VFF with the traditional cross-flow filtration suggested longevity of VFF compared to cross-flow filtration (CFF) in hybridoma cultures [98]. Another advantage of this device is that the entire module is completely autoclavable. Addition of hydrophilized polysufone membranes, stainless steel wire screens, and increase in membrane surface area are most important modifications to this unit that further reduced fouling and clogging of cells.

Spin Filters Spin filter technology was introduced in 1969 [119]. This consists of a rotating cylindrical filter with pore size slightly smaller than the cell size such that the cells are retained on the outside of the spin filter whereas spent medium is on the inside (Figure 14.5h). Because of the steric effect, particles are retained on the screen of the spin filter. The advantage of using this system is that the pore size of the filter can be modified to fit the size of particle that needs to be retained. Spin filters are available either as an internal part of a reactor or an external part of the reactor. The filters are often fixed on a hollow shaft. A variety of materials have been used for the spin filter mesh design, including stainless steel, polymers, and porcelain, to avoid clogging [120, 121]. In a recent study, a silk-based screen was used for the spin filter, achieving 30% higher productivity of urokinase compared to the stainless-steel-based filter [122].

The most important factor in the design of a spin filter is the pore size. Studies have demonstrated the variation in pore size over a large range from 1 to 120 μm (for cultures where cells form aggregates). While pore sizes smaller than the cell size will potentially lead to effective separation of cell from the spent medium, this often leads to clogging. For example, a 5 μm filter clogged within 7 days. Pore sizes slightly larger than the cell size were found to be effective. Scale-up up to 175 l was demonstrated by using a pore size of 25 μm, slightly larger than the cell size [123].

The screen tangential speed is another important factor for operating spin filters. Rates as high as 1700 cm min^{-1} have been reported to avoid clogging of cells in perfusion systems using spin filters. Another important parameter that affects cell retention is the agitator speed. In a study by Yabannavar *et al.* [124], cell leakage doubled within 4 h when the rotation speed was increased from 70 to 90 rpm. Higher spin filter speeds and an increase in screen surface area per reactor volume also seem to reduce clogging [120]. A number of applications of spin filters have been reported for mammalian and insect cells [125–127]. Infliximab mAb from Centacor is produced commercially using a spin-filter-based perfusion process. A number of commercial spin filters are available; for example, bioreactors up to 750 l supplied by INFORS are equipped with spin filters [128].

14.3.1.3 Centrifuges

Particles settle depending on their settling velocity. Applying a centrifugal force to enhance this settling velocity reduces the time taken for settling. Sedimentation centrifuges employ a centrifugal field for sedimentation, which later retains or separates the cells based on their size (Figure 14.6a). Owing to their operational stability and easy scalability, they find widespread applications in biopharmaceutical manufacturing. In an early study with hybridoma cells, a

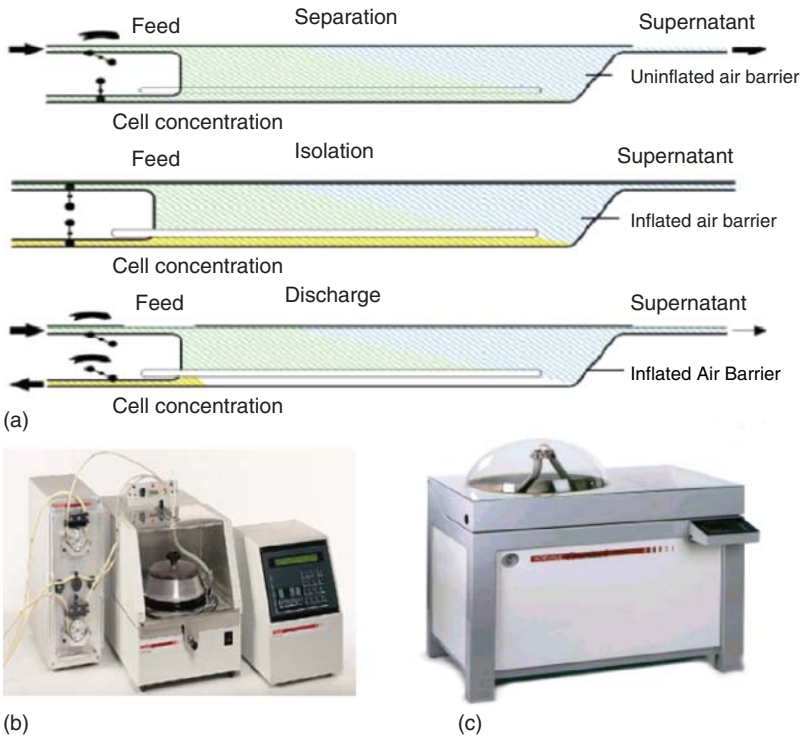

Figure 14.6 Centrifugation-based retention devices for perfusion bioreactor. (a) Schematic of separation cycle. (b) Centritech Lab III. (c) Centritech Cell II [212].

continuous centrifuge was used in a perfusion system to obtain high cell densities of 10^7 cells day^{-1} and high specific productivities over a substantial portion of the culture. No detrimental effect was observed on the growth of cells due to centrifugal forces up to 100*g* when applied either intermittently or continuously [129, 130]. Introduction of multiple settling devices improved the length of the perfusion culture. Multilayered centrifuges with settling zones, developed by Tokashiki's group, have higher operational area with no clogging issues [131]. A 2–3-fold increase in cell densities has been observed using this recycling technique compared to the one without recycling [132]. Centrifugal force up to 500*g* has been tried out in culturing CHO cells, which resulted in a 50% decrease in the total product titers [131]. Although laboratory centrifuges have shown promising results for small-scale perfusion runs, their deleterious effects on product quality and cell morphology still persist [133–135]. Addition of Pluronic F-68 did not improve cell culture performance using Centritech Lab centrifuges [133]. Industrial-scale centrifuges from Centritech can handle up to 120 l h^{-1} perfusion rates (Figure 14.6b,c).

14.3.1.4 Hydrocyclones

A hydrocyclone (Figure 14.7) uses the centrifugal force generated by the tangential flow of the cell suspension into it for separation of cells from the medium. The short residence time of the cells inside the hydrocyclone (fraction of a second), as well as the lack of moving parts, makes them an attractive option for separation. While the larger cells are concentrated in the underflow as a result of the acceleration of the spinning primary vortex toward the bottom, cell debris is removed via the overflow with the flow direction in the inner secondary vortex in the opposite direction.

Although higher cell retention rates are observed in these systems, a drop in culture viability makes them ineffective in large-scale cell culture processes. CHO cells showed a drop in the viability by 11% while using hydrocyclones.

Experimental and computational studies show that hydrocyclones of less than 10 mm diameter operate with higher separation efficiency [136]. Six different designs of hydrocyclones were tested during perfusion cultures of both CHO-K1 and a recombinant cell line in order to maximize culture viability [137]. The separation efficiency was more than 97% using hydrocyclones. The hydrocyclone has also been tested on the recombinant mouse lymphoid cell line SP 2/0 with separation efficiency up to 95% based on operating pressure. Using CHO, BHK, and HeLa cells, a detailed investigation of the effect of pressure and flow rates on cell viability and efficiency was carried out [138, 139]. At high pressures (>1 bar), separation up to 97% could be achieved without any loss in viability. Hydrocyclones are commercially available from Sartorius [140].

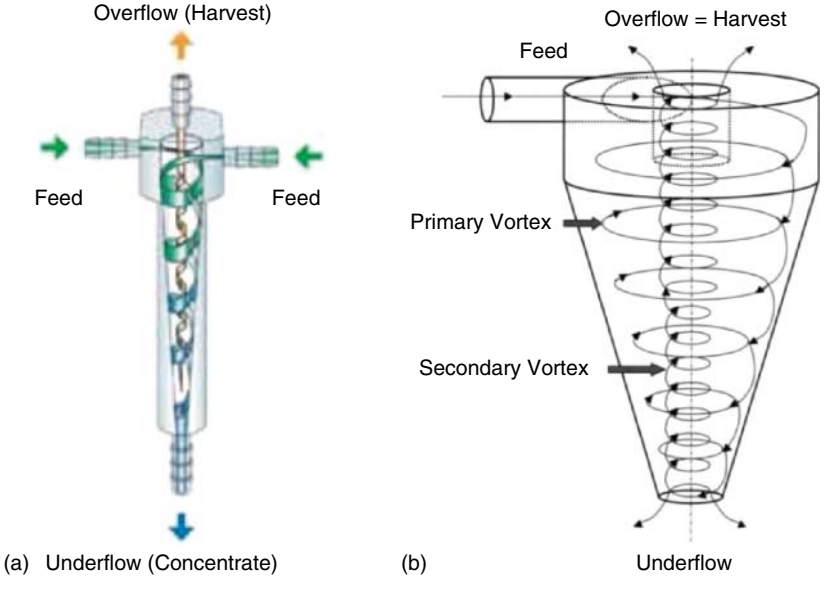

Figure 14.7 Hydrocyclones. (a) Sartorius hydroclone [140]. (b) Scheme and working principle of the hydroclone [211].

14.3.1.5 Acoustic Settlers

Application of acoustic waves to enhance settling velocity forms the basis for acoustic settlers as retention devices. Acoustic resonance generates radiation and Bernoulli forces to separate cells from the spent medium. The separation efficiency increases at high cell densities but higher power is required to operate the system. However, cell aggregation and clumping issues have been reported while using acoustics-based separation devices. On being subjected to ultrasonic standing waves of megahertz frequency, cells concentrate in certain regions, generally separated by half a wavelength/submillimeter length [141]. Depending on the acoustic aggregation of cells by the resonator, ultrasonic retention devices separate cells on the basis of size, density, and compressibility differences from smaller particles and the medium [142]. Thus viable cells can be selectively retained with separation efficiencies as high as 99% at low flow rates of 0.5 mm s^{-1} [143]. The disadvantage of using this system is its high power consumption and scalability issues. Larger bioreactors require large resonators, which could result in different acoustic patterns leading to undesired or unknown effects on cell culture performance. Using a modular chamber design, the perfusion rate could be increased to up to 200 l day^{-1} from the earlier observed 50 l day^{-1} with >95% separation efficiency [144]. However, further increase of the perfusion rate to 400 l day^{-1} led to a drastic reduction in separation efficiency to less than 60% [145].

Exposure of mammalian cells to acoustic wave fields had no impact on the cell culture performance [146]. Trampler *et al*. [147] reported a 70-fold increase in volumetric productivity using perfusion cultures. High cell densities, as high as 25 million cells ml^{-1}, were reported in CHO cell perfusion systems using an acoustic retention device with a overall separation efficiency of 90% [148]. The application of acoustic filter devices for long-term perfusion cultures at the industrial scale is promising, and the critical operating parameters have been reviewed earlier [149].

Applikon's BioSep (Figure 14.8) was applied in commercial manufacturing at a perfusion rate of 1000 l day^{-1}. Cell densities as high as 30 million cells ml^{-1} with a viability of above 90% were achieved throughout the Sf9 perfusion culture for

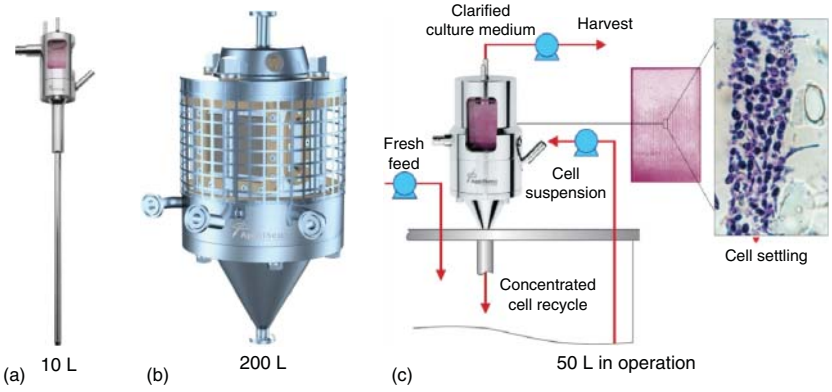

Figure 14.8 Acoustic settler-based perfusion device. (a) Biosep 10 l. (b) Biosep 200 l. (c) Typical configuration of the 50-l Biosep acoustic cell retention system [213].

the production of viral proteins [150]. A similar observation was made in recombinant CHO cells secreting HCG and Aryl sulfatase B (ASB) using the BioSep acoustic separation device [151, 152]. Metabolic flux analysis of HEK293 cells producing adenoviral vectors showed higher metabolic activity in the perfusion mode using an BioSep acoustic device [153]. In another application of the BioSep, a perfusion culture was performed at low temperatures, which resulted in increased productivity and deceased product aggregation in CHO cells secreting recombinant human IFN-β [154].

14.3.2 Feeding Strategies for Perfusion Cultures

An optimal feeding strategy is essential for a perfusion system so as to obtain higher product titers with minimal effect on the product quality and the physiology of cells. Optimization of perfusion rates, defined as the culture volume day^{-1}, depends on a number of factors, including the specific productivity and growth rate of cells. Suboptimal perfusion rates might interfere with the growth of cells leading to decrease in product titer and quality. Hence, an optimal perfusion strategy is to employ a low perfusion rate with a good control over cell growth and productivity. Performing metabolomic and transcriptomic studies on cells cultured with low perfusion rates should provide information on the physiology of these cells, thereby enabling a molecular basis to perfusion strategies. In general, perfusion reactors are fed based on two different strategies. The cell-based strategy depends on the maintenance of the total cell density in the culture, while the metabolite-based strategy depends on maintaining a particular concentration of the metabolite in the system. Although both systems are fed using fresh media, the component used to maintain the perfusion rate is different.

14.3.2.1 Cell-Density-Based Feeding

Most perfusion processes are divided into a growth phase and a production phase. In the growth phase, the culture is allowed to reach the maximum possible cell density ($10^7 - 10^8$ cells ml^{-1}), while in the production phase this cell density is maintained to maximize the product yields. Thus, precise control of cell densities in the production phase is desirable. A simpler way to moderate perfusion rates is to monitor the cell densities. Based on the cell densities, the perfusion rate is decided so as to maintain a stable growth rate. Cell-density-based alteration of perfusion rates in CHO cells cultured in a wave bioreactor equipped with an ATF-based retention system increased the density to as high as 2.14×10^8 cells ml^{-1} [111]. An alternative approach is to maintain a constant cell specific perfusion rate (CSPR, defined as perfusion rate per cell). This can be achieved by altering the feeding rates based on online measurements of viable cell concentration [155].

14.3.2.2 Metabolite-Based Feeding

Indirect maintenance of perfusion rates is also done using a relationship derived from cell-specific growth rates and metabolite concentrations. Although the

method is cell-line- and process-dependant, offline measurements are possible. For example, OURs were used to moderate the perfusion rates in a recombinant 293 cell line cultured using an internal hollow fiber retention device [156]. In another study, glucose and lactate concentrations were monitored in real time and subsequently maintained at the setpoint of 1 and 1.5 $g\,l^{-1}$, respectively, by adjusting the perfusion rates [157]. In a controlled-fed perfusion culture with hybridoma cells, a twofold increase in mAb titers as well as an increase in cell density was achieved in comparison to uncontrolled perfusion [158]. In the controlled perfusion cultures, the cell density was maintained constant and the nutrient levels were maintained at the minimum setpoint.

14.3.3 Challenges in Perfusion Culture and Future Directions

Although perfusion culture has distinct advantages, its widespread application is limited because of the technical complexity of retention devices and the lack of appropriate in-house expertise. A successful perfusion culture can be maintained for long periods (up to 6 months), but it increases the risk of contamination. High-cell-density perfusions may also be limited with respect to DO concentration and mass transfer [159]. Aggregation of cells leads to heterogeneity in the culture and may also further reduce the DO concentration. Another major challenge is the scalability of the culture, as many retention devices are not linearly scalable. Spin filters, which were the most commonly used retention device until a few years ago, are limited in terms of their capacity to handle large-scale commercial manufacturing. However, the ATF system offers a higher degree of scalability. The acoustic settler is also showing great promise.

A pertinent challenge in a perfusion culture is the stability of cell lines in long-term cultures. A number of recent studies have employed genomics-, proteomics-, and metabolomics-based approaches to understand cellular physiology in perfusion cultures [160, 161, 163]. Shift in metabolism and growth kinetics is inevitable in perfusion cultures owing to the continuous feeding and retaining of cells inside the bioreactor. As the cells are subjected to fresh medium inside the bioreactor, a shift in their physiology leading to altered metabolic activity takes place in perfusion cultures. However, transcriptomics- or proteomics-based studies are not available to substantiate the shift in their metabolic activity.

Aggregation of cells is a common phenomenon in perfusion cultures due to cell retention. Not only does this lead to heterogeneity of culture but it also affects the estimation of the VCD. Precise control of cell density in a perfusion reactor is critical for the maintenance of a constant environment in the reactor. In a microarray analysis on the aggregated BHK cells due to perfusion, the extracellular matrix (ECM) membrane pathway genes were found to be differentially expressed, causing aggregation [162, 163]. High-throughput omics studies in perfusion cultures would provide meaningful insights for better optimization of perfusion rates and for reducing the accumulation of other detrimental metabolites.

14.4
Use of Disposables in Cell Culture Bioprocesses

Disposables have become indispensable in upstream processes of biopharmaceutical production. Traditional glass and stainless steel have been replaced by polymers such as polyethylene (PE), polypropylene (PP), polystyrene, and so on. These polymers are approved by the U.S. Food and Drug Administration (FDA) for the commercial production of biologics. The advantage of using disposables is that it helps in maintaining higher levels of sterility and flexibility with reduced risk of contamination. It also reduces the time incurred in preparing the vessel for culturing. Another major advantage of using disposables is that it reduces the cumulative labor costs incurred in preparing a bioreactor. With burgeoning advances in sensor technology, development of such sensors for bioprocessing has enhanced the use of disposable bioreactors. Further, the use of disposables may lead to next-generation continuous manufacturing processes with completely robust and disposable technology from vial thaw to the finished drug. The use of disposable sensors has made cultivation of cells using these bioreactors easier. According to a press release by Amgen in 2014, its biomanufacturing facility in Singapore is equipped with completely disposable bioreactors with modular design, and the company estimates a record 60% decrease in cost per gram of protein produced [164].

Accumulation of solid waste is one of the major disadvantages of using disposables. An unresolved issue in the use of disposable bioreactors is the detection of chemicals arising from leaching of the plastics, which may have an impact on the product quality and safety. Although the use of disposables is not limited to upstream processes, we limit our discussion only to the disposables used to culture cells based on the scope of this review.

Plastic roller bottles were one of the first disposable culture dishes to be used for the production of biopharmaceuticals such as vaccines. Later, multitray systems such as Cell Cubes® (Corning) and Cell Factory™ (Nunclon 10 Chamber) were found suitable for current good manufacturing practice (cGMP) manufacture of biologics.

The use of disposable "cell bags" has made large-scale bioprocessing of cultured cells easier [165]. With similar mixing and aeration patterns, these systems allow limited variations during process transfer from pilot to large-scale studies to culture a number of cell lines including insect cells [166], BHK21 cells for vaccine production [167], and adherent cells on microcarriers [168].

Wave bioreactors (Figure 14.9) were introduced in 1998, in which a rocking motion was used instead of mechanical mixing to achieve mass transfer in a disposable bag bioreactor [165]. These bioreactors have been found to be promising in cultivating different cell lines and viruses for the production of a number of biologics including mAbs [165, 167–171]. The wave bioreactor has also been operated in a variety of culturing modes including batch, fed-batch, and perfusion modes. Recently, a modified version of the wave bioreactor, known as the BaySHAKE®, was designed with low-shear agitation that can handle high cell

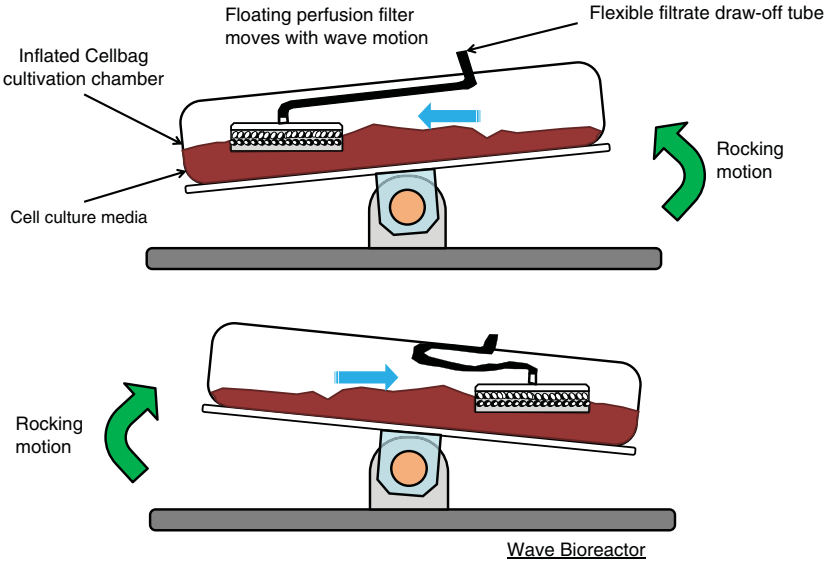

Figure 14.9 Schematic of wave bioreactor.

densities observed in fed-batch and perfusion processes [172]. The cube-shaped bag is designed to oscillate around its vertical axis [173]. The bioreactor was compared with the traditional stainless steel reactors up to the 1000-l scale and found to be equivalent. Other versions of the bag bioreactors such as the PBS bioreactor, SBB (slug bubble bioreactor) from Nestle, and the CellMaker (Regular and Plus) system from Cellexus employ pneumatic and mechanical power input for mixing [174]. Examples of fed-batch and perfusion-based bioprocesses in wave-type bioreactors are presented in Table 14.4.

Oxygen transfer limitations in wave-mixed bioreactors make them unsuitable for large-scale cultivation of high cell densities. Adapting the working principle of conventional stirred-tank bioreactors to single use technology, stirred-tank disposable bags were designed. Today, stirred-bag systems hold a huge market share in the bioprocessing industry. Equipped with top (Hyclone-Thermofisher SUB) or bottom (Xcellerex-GE) axial flow impellers and spargers for aeration, the culture characteristics of these cylindrical bags are reported to be comparable with those of stirred-tank bioreactors [175–177]. Commercially available BIOSTAT CultiBag STR is equipped with an axial and radial flow impeller with a microsparger. This model can be connected to the SCADA software to gain complete control over the culture process [178]. Cube-shaped Nucleo Bioreactors with dynamic sparging equipped with microcarriers provided a minimum of 30% increase in product titers compared to conventional stirred-tank bioreactors. Both fed-batch and perfusion modes have been tried using this model [179, 180].

Mini bioreactors in the form of multiwell plates were the first set of disposables to be used to screen, isolate, and expand clones for biopharmaceutical production [181]. Recently, the introduction of miniaturized, disposable, stirred

Table 14.4 Examples of disposable bioreactors in cell culture bioprocessing.

Name	Cell line	Volume	Mode of operation	Analytical measurement	VCD max. (10^6 cells ml^{-1})	References
WAVE bioreactor	CHO, Schneider S2, Hybridoma, Vero, MDCK, Activated cells, E-FL	2–500 l	Batch, perfusion	pH, DO, temp.	214 (ATF mode), 132 (TFF mode)	[111, 167–171, 224–226, 231, 242–244] GE Healthcare
Cell Bag	Sf21, NS0, Sf9	10–20 l	Batch, fed-batch	pH, DO, temp.	10	[166, 245]
BIOSTAT CultiBag RM	Sf9	300 l	Batch	pH, DO, temp.	12	[178, 246, 247]
CellCube (Corning), Roller Cell	Retroviral vectors, Phoenix Frape-1	20 l	Batch	pH, DO, CO_2	NA	[248, 249]
Cell Factory (Nunclon)	2T47D-V and 3SKBR3-7, HEK293, packaging cell lines, 3T6	10 stacks	Batch	NA	1.1	[151, 250–252]
SupperSpinner D1000	Sf9	1 l	Batch	NA	16.6	[174]
Nucleo Bioreactor		1000 l	Batch, perfusion	NA		[108, 179, 180, 253]

micro-bioreactors (~10 ml volume) has been a great advantage to process development scientists to mimic large-scale culturing conditions at scaled-down levels. This is especially useful for rapid screening of clones to identify high-productivity clones. Systems such as the AMBR (TAP Biosystems, Sartorius Stedim Biotech GmbH), the SimCell bioreactor (BioProcessors Corporation), the μ24 microbioreactor (Pall corporation), and Sensolux (Sartorius Stedim Biotech GmbH) are equipped with fully controllable process setups, enabling tight control over culturing parameters such as pH, temperature, and DO [182]. Fed-batch culture of a CHO cell line was successfully performed in a miniaturized bioreactor μ24 system with cell densities higher than in shake flasks [183], primarily because of better control of the process parameters including pH, temperature, and DO. The major disadvantage in using these bioreactors is that they may not be useful for high-cell-density cultivations. Tube spin bioreactors such as the **C**ultiflask 50 with vented caps and the disposable Super Spinner D1000 flasks are specifically designed for high-cell-density cultivations to achieve high

product titers [184–186]. Note that the cell densities obtained from these systems are comparable with those of wave bioreactors and other stirred bioreactors. A large number of disposable bioreactors are currently on the market, and the reader is referred to a review for a detailed discussion [174].

14.5 Analytical Methods to Monitor Key Metabolites and Parameters

For precise control of fed-batch and perfusion-based bioprocesses, online and fast monitoring of key metabolites and process parameters is critical. In this section, we describe a variety of tools that are currently being used or under development to measure the concentrations of various bioprocess parameters. The most common set of assays are enzymatic that can be performed both offline and online. A variety of spectroscopic techniques are also gaining popularity. Many of these are capable of noninvasively measuring multiple variables simultaneously. An overview of the various methods is provided next, and specific applications are summarized in Table 14.5.

14.5.1 Enzymatic Assays

Most metabolites involved in a cell culture process can be analyzed offline by enzymatic assays. These sensitive assays are used to quantify metabolites such as glucose (GOD-PAP assay) [187], L-glutamine (glutamine assay) [188], lactate (LDH assay) [189], ammonia [190], and so on. Sandwich enzyme-linked immunosorbent assay (ELISA) [191] is used to accurately quantify the levels of secreted IgG. These enzymatic assays with some instrument modifications are also used in online determination of metabolite concentrations. The most commonly used instruments are biochemical analyzers (NOVA, YSI). An enzyme specific for the substrate of interest is immobilized between the two membrane layers of polycarbonate and cellulose acetate. The substrate is oxidized as it enters the enzyme layer, producing hydrogen peroxide, which passes through cellulose acetate to a platinum electrode where the hydrogen peroxide is reduced. The resulting current is proportional to the concentration of the substrate. These biochemical analyzers can also be connected to the bioreactor to give a reading online. In an interesting development, Applikon Biosenz uses the heat produced during enzymatic oxidation of glucose to gluconic acid as a measure of glucose concentration in a bioreactor [192]. Flow injection analysis is also used for online measurement of glucose, lactate, IgG, and so on [193–195]. Substrate-specific enzymes are immobilized covalently onto activated controlled pore glass beads and integrated into the flow injection analysis system. The cell-free supernatant is passed through this sensor, and the generated signal is compared with a reference compound (e.g., H_2O_2) to accurately determine glucose or IgG concentrations.

Table 14.5 Overview of methods for quantification of metabolites and process parameters.

Metabolite/parameter	Mode of measurement	Response time	References
Nutrient and waste metabolite measurements			
Glucose, glutamine, amino acids, lactate, ammonia	Enzymatic assays (offline)	30 min to 2 h	[187–190]
Glucose, glutamate, glutamine	Near-infrared spectroscopy (online)	<10 min	[196–199]
Glucose, lactate	Appikon heat flux method	<30 min	[254]
Glucose and other carbon metabolites	HPLC-RID (Offline + online)	<10 min	[203]
Glucose, glutamine, glutamate, lactate, IgG	Enzyme-based flow injection analysis (FIA) (online)	<10 min	[193–195]
Glucose, glutamine, lactate, ammonia, salts	Biochemical analyzer (YSI, NOVA, Hitachi) (offline + online)	<10 min	[19, 255]
Amino acids	RP-HPLC (offline + online)	20–30 min	[204, 256]
Product level measurements			
Cell density	Hemocytometer (offline)	<10 min	[46]
	Cell counters (Beckman coulter, Invitrogen) (offline + online)	<10 min	
	Dielectric spectroscopy (online)	<10 min	[196, 257, 258]
	2D fluorometry (online)	<10 min	[205–207]
	Laser turbidometer	<10 min	[45, 259]
Product titers	ELISA (IgG) (offline)	5–6 h	[191, 260]
	Protein A/G chromatography (offline + online)	<10 min	[261]
Process parameter measurements			
pH	pH probe, microneedle probes	<10 min	
Dissolved oxygen	Photovoltaic probes (luminescence), microneedle and electrodes (polarographic, galvanic)	<10 min	[201]
	Noninvasive DO sensor patches	<10 min	[202]
Exit gas analysis	Electric nose (online)	<10 min	[196, 209]
	Paramagnetic analyzer (O_2)	<10 min	[262]
	Long-path infrared analyzers (CO_2)	<10 min	
	Gas chromatography-flame ionization detector (volatile compounds)	<10 min	[263, 264]
	Mass spectrometry	Variable (up to 1 h)	[200]
Osmolality	Osmometer (offline)	<10 min	[210]

14.5.2 Spectroscopy-Based Methods

The different properties of light are used to determine various metabolites and process parameters. Near-infrared spectroscopy (NIRS) is used to quantify multiple metabolites at a time. In this, the molecules are able to absorb energy and rotate or vibrate when excited at defined frequencies. The transmitted light reveals how much energy is absorbed at each wavelength, providing details about the molecular structure of the mixture [196–199]. A mass spectrometer is used to detect oxygen uptake and carbon dioxide production rates from animal cell cultivations (generally quadruple MS) [200]. The instrument is sensitive to the low levels of O_2/CO_2, generally seen in animal cells. Alternatively, infrared gas analyzers can be used to measure trace gases such as CO_2. Optical oxygen sensors work according to the principle of dynamic fluorescence quenching. The sensors contain a fluorescent dye that is excited by light of a certain wavelength. Depending on the amount of oxygen molecules present, the fluorescence response of the optical sensor varies. A polymer optical fiber transmits the excitation light of the sensor and at the same time also transmits the fluorescence response of the sensor to the measurement device [201]. As a modification, these are available in the form of sensor patches that can be attached to the transparent surface of a glass plate [202]. Laser probes are also employed to measure turbidity of cultures and correlate it to cell density [45]. Refractive index detectors coupled to HPLC are used to detect substances with limited or no UV absorption [203]. These chemical components included alcohols, sugars, fatty acids, polymers, and carbohydrates.

14.5.3 Chromatography-Based Methods

Chromatographic techniques are used to quantify the levels of amino acids and IgG in the culture. These are generally used in the offline mode at small scales and integrated into the process at large scales. The amino acids are derivatized using *ortho*-phthalaldehyde (OPA) or Phenyl isothiocyanate (PITC) followed by separation in a C-18 column using HPLC [204]. The high affinity of Protein A, isolated from *Staphylococcus aureus* for IgG subclass, is exploited in affinity purification, which has become the gold standard for IgG purification and quantification. The IgG molecule binds to the Protein A column, whereas the HCP and HCN (host cell protein and nucleic acids) are flushed out; IgG is eluted by changing the pH of mobile phase.

14.5.4 Microscopy-Based Methods

Direct visualization of cells gives an indication of cell health and size. Various nuclear staining dyes such as propidium iodide are used to stain the nucleus of dead cells, and thus quantify the dead cell population. In another unique

approach, 2-D fluorometry is used to correlate cell density to the fluorescence of the naturally fluorescent amino acids in culture media, such as tryptophan, as these are incorporated into the cell [205–207]. An automated flow cytometer connected to the bioreactor can be used to accurately and frequently assess cell proliferation and death kinetics. This information has been used to predict the end of the exponential phase in the culture and to take proper corrective actions [208].

14.5.5 Electrochemical Methods

Electrochemical methods are generally used to monitor process parameters such as pH, pO_2, and exit gases. pH is measured using an online probe or offline analysis using a pH meter. The online pH is corrected based on the offline pH as the recalibration of online probe is restricted during the culture period. DO in a reactor is continuously monitored using galvanic or amperometric probes. An "electric nose" containing a MOSFET (metal–oxide–semiconductor field-effect transistor) sensor is used to accurately determine the composition of the gases exiting the reactor [196, 209]. The electrical capacitance of cells measured in a reactor is used to indirectly determine the cell density in the culture during the exponential phase [19]. Osmolality is measured by calculating the relative lowering in freezing point due to presence of solutes [210]. The osmolality of the culture is maintained below 500 mOsm to avoid hypertonic conditions.

14.6 Concluding Remarks

Application of engineering principles to bioprocess design has led to improvements in titers of recombinant proteins from mammalian cells by many folds. Stoichiometric principles-based design of the feed media along with dynamic control strategies has been instrumental in enhancing the longevity and cell density with product yields surpassing $5\,g\,l^{-1}$ in a fed-batch culture. In contrast, perfusion processes are technically complex but can potentially yield higher cumulative product at lower costs and in reduced space. With the advent of biosimilars and increasing demand for therapeutic proteins and mAbs, there is renewed debate on whether fed-batch or perfusion will be the process of future.

With recent advancements in instrumentation for online sampling and metabolite analysis, high-throughput screening of different feeding profiles can be performed to arrive at optimum scale-up conditions. However, advances in process alone may not be enough to achieve further increase in yields, especially in fed-batch processes. The next phase of improvements of a fed-batch culture will depend on the use of cell engineering approaches to better understand cellular physiology and thereby develop clones that are superior in both productivity and utilization of nutrients with minimum production of toxic metabolites such as lactate and ammonia. Similarly, for perfusion processes, it is important

to demonstrate the stability of clones over long-term culture, for example by using next-generation sequencing approaches. The choice between fed-batch and perfusion is usually dictated by the availability of technical competence and in-house experience because of the possibility of high failure rates of latter. However, with recent advances in retention devices coupled with increasing use of disposables, perfusion processes may be more widely adopted in future. It remains to be seen whether future biomanufacturing will produce recombinant proteins using fed-batch or perfusion processes.

What to Choose, Fed-Batch or Perfusion?

The following hypothetical example illustrates the comparison of a fed-batch and perfusion process. Consider the sample data for annual operations of fed-batch- and ATF-based perfusion cultures.

Culture parameter	Unit	Fed-batch	Perfusion (ATF)
Culture time	Days	12	60
Bioreactor volume	Liters	200	14
Perfusion rate	(vv day^{-1})	-	3
Annual batches	No.	25	5
Final or daily titers	(g l^{-1})	10	4

Fed-batch culture:

Annual medium consumption

= Reactor volume × annual number of batches

$= 200\,\mathrm{l} \times 25 = 5000\,\mathrm{l}$

Total annual protein production

= Final titers × annual medium consumption

$= 10\,\mathrm{g\,l^{-1}} \times 5000\,\mathrm{l} = 50\,\mathrm{kg}$

ATF based perfusion culture:

Annual medium consumption

= Daily perfusion volume × annual number of batches × culture duration

$= (14\ \mathrm{l} \times 3) \times 5 \times 60 = 12\ \ 600\ \mathrm{l}$

Total annual protein production

= Daily titers × annual medium consumption

$= 4\,\mathrm{g\,l^{-1}} \times 12\ \ 600\mathrm{l} = 50\,\mathrm{kg}$

Perfusion offers an advantage of achieving high levels of protein production at a small scale. For 50 kg of annual protein production, a fed-batch process requires a 200-l reactor, whereas a perfusion reactor will require a working volume of 14 l. Fed-batch mode operations are simpler and require less instrumentation.

However, at complete medium replacement, a perfusion process requires higher quantities of the medium. Perfusion offers stringent process control, as the cell density and perfusion rate can be effectively modulated to achieve a steady state.

It is also suitable for less stable products as they are daily removed from the culture.

Nomenclature

$A_{i/\mathrm{Glu}}$	consumption ratio of metabolite i with respect to glucose
q_i	consumption rate of metabolite i
q_{Glu}	consumption rate of glucose
C_i	concentration of indicator metabolite
$C_{i,ti}$, $C_{i,t-1}$	concentration of indicator metabolite at time t and $(t-1)$, respectively
$C_{\mathrm{Glu},ti}$, $C_{\mathrm{Glu},t-1}$	concentration of glucose at time t and $(t-1)$, respectively.
V_{F}, V_{feed}	volume of feed
$C_{\mathrm{feed},i}$	concentration of indicator metabolite in the feed
$C_{\mathrm{feed,Glu}}$	concentration of glucose in the feed
F	feed rate
f	frequency of sampling
$C_{i,\mathrm{setpoint}}$	set point concentration of metabolite i
$C_{i(t)}$	indicator concentration at time t
V_{r}, V_{c}	volume of reactor
C_i^{F}	concentration of metabolite i in feed
X_{v0}	viable cell density at inoculation or time = 0
X_{t+1}, $X_{v(t+1)}$	predicted viable cell density at time $(t+1)$
$C_{(0)}$, $C_{(t)}$	concentration at time $t=0$ and t, respectively
μ	growth rate
OUR	oxygen uptake rate
$k_{\mathrm{L}}a$	volumetric mass transfer coefficient
$C_{\mathrm{O}_2}(t_0)$	initial oxygen concentration
$C_{\mathrm{O}_2}(t_{\mathrm{f}})$	final oxygen concentration
μ_{OUR}	growth rate based on oxygen uptake rate
$\mathrm{Glc}_{\mathrm{pred},t}$	predicted glucose concentration for next time interval
Y_{O_2}	yield of oxygen (mM) per glucose (mM)

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Part V
Environmental Bioengineering

Applied Bioengineering: Innovations and Future Directions, First Edition. Edited by Toshiomi Yoshida.

15
Treatment of Industrial and Municipal Wastewater: An Overview about Basic and Advanced Concepts

Jyoti K. Kumar, Parag R. Gogate, and Aniruddha B. Pandit

15.1
Types of Wastewater

Wastewater can be categorized into two types based on the source of origin. One that results on account of human activities is called *sewage* and the other that is a result of industrialization is called *effluent*. The former falls under municipal wastewater and the latter under industrial wastewater. Municipal and industrial wastewater treatment is of paramount importance given the fact that both the streams are essentially released into our environment and result in polluting it. The consequences of such pollution are manifold. Air, water, and soil are affected thereby leading to health hazards to humans and lethality to plants and animals. This has had a profound effect on mankind for centuries and will continue to do so if appropriate control measures are not taken. Preventive measures have limited scope in this arena owing to increasing global population and rampant growth of industries worldwide. The only way out is to have effective treatment methods for the wastewater and find useful applications of the treated water that will take care of the water crunch that the world is facing today. This strategy is not new and has been employed to some extent globally. The focus in future will be to make the existing techniques much more effective and also bring in novel methods.

Physical, chemical, and biological methods have been routinely used to treat municipal and industrial wastewater. This section deals with the biological methods. An attempt is made to explain the conventional biological methods, and current research in terms of modifications wherever applicable are also incorporated.

15.2
Biological Treatment

"Biological treatment" implies the use of naturally present microorganisms for treating wastewater. The microorganisms utilize the organic carbon present in the wastewater as their source of energy for growth and result in byproducts that are less toxic. Thus, it can be said that the effluent is treated by the microorganisms

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based on consumption as food. The inception of this method occurred somewhere in the early twentieth century. Although the entire process appears simple, the treatment process requires monitoring due to complexities such as varying composition of the influent waste stream, temperature, pH, and most importantly, the microflora may also differ [1]. It is important to note that not all compounds are broken down by the microorganisms [2]. Some recalcitrant chemicals may remain. Moreover, the products formed during the treatment could be toxic to the microorganism thereby hindering microbial growth and may eventually lead to death of the culture. Scenarios such as these are not rare and it often necessitates the emptying of the treatment tank to discard the dead bacteria and for the introduction of new seed inoculums. Generally, municipal wastewater (sewage) contains large proportion of solids at least about 60% along with proteins and amino acids. Wastewater from each industry also differs in its composition including the solid content [3]. It is interesting to note that wastewater from brewing and paper and pulp industry contain no nitrogen and phosphates. In such cases, nitrogen and phosphates are added before treatment to enhance the effluent treatment. Monitoring the efficacy of wastewater treatment necessitates the performance of certain tests on a routine basis. The important ones are described here.

1) *Effluent toxicity:* Although effective treatment techniques are employed, it is mandatory that the toxicity of the treated effluent is checked on representative microorganisms of the receiving water bodies. In Europe this is called the direct toxicity assessment (DTA) and described as the whole effluent toxicity (WET) test in the United States. This test quantifies the aggregate toxic effect on aquatic organisms from all pollutants present in the wastewater. It essentially measures the wastewater's effects on specific organisms' ability to survive and grow by exposing living organisms (plant, vertebrates, and invertebrates) to various concentrations of pollutants in the effluent stream [4].
2) *TOC:* For wastewater treatment, it is necessary to ascertain the organic load of the influent stream. This is referred to as the total organic carbon demand (TOC). This can be determined by subjecting the sample to combustion thereby resulting in the evolution of carbon dioxide that can be measured to know the total carbon present. The major limitation here is that the carbon compounds that cannot be biologically treated can also be converted to carbon dioxide. Thus, TOC is divided into carbon that cannot be oxidized at all and that can be oxidized, often referred as chemical oxygen demand (COD). The latter further consists of carbon that is not biologically oxidized and that is oxidized by microbes called biological oxygen demand (BOD), which in turn can be hard BOD (difficult to degrade) and soft BOD (easy to degrade).
3) *BOD:* The BOD is an important parameter in wastewater analysis and is often quantified using a 5-day BOD test. It is essentially the amount of oxygen required for bio-decomposition of organic solids under aerobic conditions at a standardized temperature in a given interval of time. Theoretically, it takes a very long time to complete biological degradation of organic matter and, therefore, a 5-day period is considered for the BOD test since a

large amount of organic matter is successfully degraded by the end of 5 days. Since this is also a considerably long period, short-term BOD estimated over short time intervals between 30 min to several hours is also routinely performed. It is important to note that BOD is generally less than COD because microorganisms cannot degrade some organic compounds that are oxidized by strong chemicals as oxidizing agents. Moreover, some carbon compounds are not oxidized by microbes but are converted to biomass.

4) *COD:* COD is the amount of oxygen required to chemically oxidize organic compounds in the sample. Potassium dichromate is employed as the oxidizing agent and the sample is subjected to heating in the presence of sulfuric acid. The dichromate consumed is proportional to the carbon present in the sample that can be oxidized chemically. A limitation of this method is that some carbon compounds broken by microorganisms only partly are totally broken down by chemicals. In addition, recalcitrant organic carbon compounds are also included in COD, which cannot be actually broken readily. Thus, COD overestimates the carbon that can be removed by microbes and hence not a true representative of the capacity of microorganisms to oxidize.

15.3 Wastewater Regulations

Treated wastewater may either be discharged to the environment or used for specific applications as the case maybe. Stringent regulations are required to monitor the quality of the treated effluent for both cases. Regulatory bodies are in place in different countries to ensure that the prescribed limits for every type of pollutant is met and safety is maintained always. In Europe, the Urban Wastewater Treatment Directive was established in 1991 and Water Framework Directive in 2000 to monitor the quality. Similar works are carried out by the environmental protection agency (EPA) in the United States and the industry-wise guidelines for wastewater can be accessed from the EPA website [4]. Similar regulations in Canada and England are also available [4].

15.4 Biological Treatment Processes

Conventional wastewater treatment process usually involves three steps [5], that is, primary, secondary, and tertiary treatment as shown in Figure 15.1. The primary treatment is essentially the physical removal of 20–30% BOD that is present in particulate form by employing processes such as settling, screening, coagulation, and so on. The resultant solid material is termed as *sludge*. Major portion of the BOD is removed during the secondary treatment where biological removal of the dissolved organic matter occurs. Secondary wastewater treatment process can be either an aerobic or an anaerobic process. An aerobic process is essentially carried out in the presence of air and employs aerobes (microbes that require

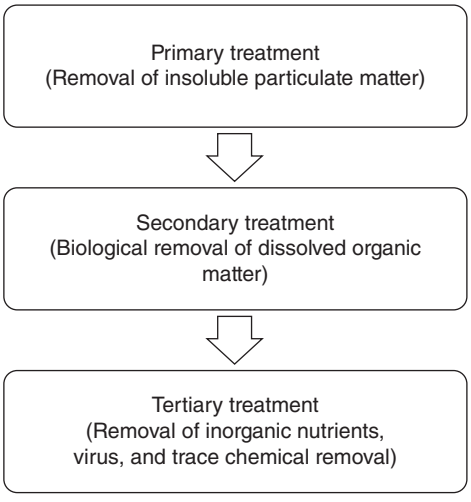

Figure 15.1 Conventional wastewater treatment.

Primary treatment: initial step in wastewater treatment that usually employs coagulation and settling.
Secondary treatment: second stage of wastewater treatment involving aerobic or anaerobic microorganisms.
Tertiary treatment: final stage where virus and trace contamination is removed.

oxygen/air for their growth and degradation of organic matter) such as *Bacillus*, *Nocardia*, *Enterobacteriaceae*, *Streptococcus*, and *Staphylococcus* species to name some. In such cases, air has to be provided by means of suitable aeration techniques [6]. The organic waste is converted into biomass, carbon dioxide, and water. On the other hand, anaerobic wastewater treatment does not require air or oxygen and use anaerobes (microbes which do not require oxygen/air for their growth and degradation of organic matter) such as *Escherichia coli*, *Clostridium*, *Actinomycetes*, and *Klebsiella* species. Here, aeration is not necessary and organic wastes after biodegradation result in methane, carbon dioxide, and biomass. Generally, aerobic processes are employed to treat wastewater with low to medium content of organic impurities and for wastewaters that are difficult to biodegrade such as municipal sewage [7]. Wastewater with medium to high concentrations of biodegradable mattter and food and beverage wastewater that are rich in sugars and starches and alcohols are better treated using anaerobic processes. The kinetics of aerobic treatment is fast and yield high quantum of sludge as compared to anaerobic processes. Both these processes have been described in the following sections.

Tertiary treatment purifies the wastewater further. It is particularly important to remove phosphorus and nitrogen that can lead to eutrophication. Activated carbon filters are used to remove final traces of organic pollutants. Virus inactivation is also done at this final stage.

15.5
Aerobic Techniques

15.5.1
Mathematical Modeling

Mathematical models are tools that simplify the complexity of any process. Biological wastewater treatment is a complex process that requires mathematical modeling to optimize designs or to troubleshoot problems that arise at treatment plants. Mathematical models are divided into different types based on the type of microbial growth, that is, attached or suspended growth or a mixed type.

Substrate removal and growth of microorganisms are closely connected in biological wastewater treatment processes. Monod's equations have been used to describe substrate removal for decades [8].

$$r_s = -\frac{kX_v S}{K + S} \tag{15.1}$$

$$r_s = -\frac{kS}{K + S} \tag{15.2}$$

where, the rate of substrate removal in $ML^{-3}\,T^{-1}$ is denoted by r_s, X_v is the concentration of volatile suspended solids (VSS), k and K are the maximum and half-velocity constants respectively, and S is the concentration of the substrate.

At low substrate concentrations, Equations 15.1 and 15.2 are reduced to the following first order kinetic equations:

$$r_s = -kX_v S \tag{15.3}$$

$$r_s = -kS \tag{15.4}$$

Rate of substrate removal is dependent on the substrate concentration and both are directly proportional to each other. Therefore, when the value of the substrate concentration decreases, the rate of substrate removal also decreases.

VSS are used instead of active biomass (Xa) since the measurement of the former is convenient and practical whereas the active biomass may not only contain viable microorganisms but it also constitutes enzymes and other catalytic agents giving errors in measurements.

The Arrhenius equation is used to describe the temperature dependence of the maximum velocity coefficient k in Equations 15.1–15.4, and is given as follows:

$$k_t = k_{20}\theta^{(T-20)} \tag{15.5}$$

where θ is a constant with a range of 1.0–1.8 [9] and k_t and k_{20} are rate coefficients at temperature T and 20 °C respectively.

Substrate utilization is accompanied by growth of microorganisms and production of biological sludge. Generally, production of biomass is described by the following equation:

$$r_{Xp} = -\,Y\ r_s \tag{15.6}$$

where r_{Xp} is the production of biomass from the substrate removal in $ML^{-3}\,T^{-1}$ and Y is the yield factor that represents the mass of biomass produced per mass of substrate consumed. Endogenous decay of biomass attributed to death of microorganisms in a substrate-limited condition can be depicted by the following equation:

$$r_{Xc} = -K_c X_v \tag{15.7}$$

where, r_{Xc} is the rate of decrease of VSS due to endogenous decay and k_c is the rate constant. Therefore, the net growth rate of the biomass is the summation of Equations 15.5 and 15.6, which can be stated as:

$$r_x = -Y\ r_s - K_c X_v \tag{15.8}$$

15.5.2 Types of Aerobic Treatment

15.5.2.1 Activated Sludge Process (ASP)

This is one of the oldest and most commonly used municipal and industrial wastewater treatment process. Developed in England in the early 1900s, the activated sludge (AS) process was not employed in the United States until the 1940s. Slowly with time, the process was adopted globally and at present, along with the traditional activated sludge process (ASP), several variants have also been developed. It is considered one of the most popular biological treatment processes that produce high quality treated effluent economically due to its low construction cost and small land requirement suited aptly for both small and large communities [10]. The process is very simple and is shown in Figure 15.2. Wastewater after primary treatment is subjected to the ASP. The entire process essentially consists of four parts (i) aeration tank, (ii) mode of aeration, (iii) settling tank or clarifier, and (iv) recycle of AS or generation of waste sludge. The wastewater post primary treatment enters the aeration tank that can be either a plug flow type or a completely mixed variant [11]. Specific concentration of biomass is maintained in the tank and is generally denoted as mixed liquor suspended solids (MLSS) or mixed liquor volatile suspended solids (MLVSS) along with a sufficient dissolved oxygen (DO) concentration to enable biological degradation of organic impurities. The MLSS concentration is usually measured by filtering a small sample of mixed liquor through fiber glass filter paper. The organic fraction is usually analyzed by burning off the organic fraction of the filtered sample as suggested by its name, MLVSS. The aeration tank is provided with a suitable means of aeration that ensures that required levels of oxygen are provided to the process. This is very crucial given the fact that the process is aerobic and mass transfer of oxygen into the wastewater is often a rate-limiting step. This requirement is usually met by mechanical surface aerators. The aerated mixed liquor flows into the clarifier unit where the biomass separates out and the clarified treated water is subjected to further treatment. Lastly, the separated

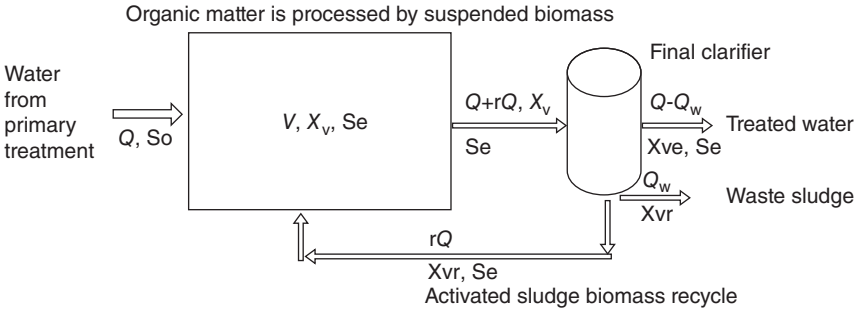

Figure 15.2 Activated sludge process (ASP).

Q = volumetric flow rate
So = influent substrate concentration
V = volume, X_v = volatile suspended solids (VSS)
Se = soluble substrate concentration
Xve = VSS in clarified effluent
Xvr = VSS in waste sludge or in recycled underflow
Q_w = waste sludge flow rate.

biomass is either recycled back into the aeration tank in the form of return activated sludge (RAS) or the excess biomass that is produced as a result of the biodegradation process is sent to the waste handling or dewatering facility as waste activated sludge (WAS). The purpose of RAS is to maintain an adequate number of microorganisms in the aeration tank, which is required to treat the load entering the treatment plant [12]. The RAS contains microorganisms that have the ability to feed on the organic matter of the wastewater. For large treatment plants, the RAS flow rates are adjusted between 50% and 100% of the treated wastewater flow rates and for smaller plants, around 150% of treated wastewater flow rates. The RAS flow rate denoted as Q_R is calculated in cubic meter per day as follows [13]:

$$Q_R = \frac{\text{Settled sludge volume (ml l}^{-1}) \times Q\,(\text{m}^3 \text{ per day})}{1000 \text{ (ml l}^{-1}) - \text{Settled sludge volume(ml l}^{-1})} \tag{15.9}$$

The AS system is controlled by WAS. This is done to ensure the required balance of microbial population in the tank. Control over the microbial population is achieved by maintaining a constant food to microorganism ratio (F/M) or a constant sludge age. The F/M ratio is defined as the ratio of the food entering the plant to the ratio of microorganisms in the tank (Volume of tank as Va) expressed as per the following equation [13]:

$$\frac{\text{F}}{\text{M}} = \frac{\text{kg BOD per day}}{\text{kg MLSS}} \tag{15.10}$$

where, kg BOD per day $= \frac{\text{BOD(mg l}^{-1}) \times Q}{1000}$ and kg MLSS $= \frac{\text{MLSS} \times V_a}{1000}$.

The amount of sludge wasted from the system can be calculated as follows [13]:

Actual kg MLSS − Desired kg MLSS = mass of sludge solids to be wasted, per kilogram. Based on this the WAS flow rate (Q_w) is determined as follows:

$$Q_w\ (\mathrm{m^3\ per\ day}) = \frac{\text{Mass of sludge solids wasted (kg per day)} \times 1000}{S_w(\mathrm{mg\ l^{-1}})} \tag{15.11}$$

Sludge age is defined as the total mass of sludge contained in the aeration tank divided by the total mass of sludge wasted daily, including the suspended solids discharged in the outflow. This is expressed in the equation given below [13]:

$$\text{Sludge age} = \left(\frac{\text{MLSS}\ \left(V_a + V_c\right)}{Q_w \cdot S_w + (Q - Q_w)S_e} \right) \tag{15.12}$$

The efficiency of the treatment is usually monitored by performing tests such as the jar test to check the floc and settleability performance, microbiological analysis to ascertain the types of bacteria that predominate, and certain chemical analysis that includes alkalinity, BOD, COD, total dissolved solids (TDS), nitrogen content, and phosphorus content to name a few. The treatment environments that affect the process are manifold. DO, organic load, TDS, temperature, pH, age of the sludge and its type, presence of toxins, are some of the factors that can have a critical impact on the success of the treatment. The AS usually consists of bacteria in majority (95%) with a few higher organisms such as fungi, protozoa, and higher invertebrates (5%) [14]. The sludge volume index (SVI) is used to assess the sludge settling quality. It can be measured by filling a liter graduated cylinder with mixed liquor from the tank and allowing it to settle for 30 min. The SVI is computed as follows [13]:

$$\text{SVI} = \frac{\text{Volume of settled sludge}(\mathrm{ml\ l^{-1}}) \times 1000}{\text{MLSS}\ (\mathrm{mg\ l^{-1}})} \tag{15.13}$$

A value between 50 and 80 ml g^{-1} indicates good settling of the sludge.

Typical variants of the conventional ASP are now discussed below:

1) *Extended aeration*: This process as the name suggests holds the wastewater for 18 h or more. Aeration is carried out either by mechanical or diffused methods and mixing is done either by aeration or mechanical means [15]. It is generally operated at high solids retention time (SRT) and, therefore, the microbes compete for remaining food which often results in highly treated effluent with low solid waste product. It is considered a stable process due to light food to microorganism loading ideally suited for small communities [16]. Added advantages are its ability to accept intermittent loading without upsetting the system and very less waste sludge generation.
2) *Sequencing batch reactors* (*SBRs*): This is also commonly referred as the fill and draw ASP. The special feature of this method is the use of a single tank where equalization, aeration, and clarification occur one after the other. The process is considered suitable when the influent flow is typically 5 million

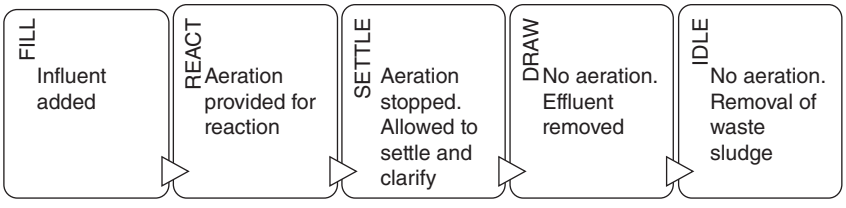

Figure 15.3 Sequencing batch reactor (one cycle).

FILL: wastewater to be treated is added
REACT: aeration is provided by suitable means such as mechanical aerators for initiating the reaction
SETTLE: aeration is stopped after reaction and contents are allowed to settle, followed by clarification
DRAW: treated wastewater is removed at this stage, and
IDLE: no reaction occurs and generally waste sludge is removed at this stage.

gallons per day or lower [17]. The influent is added to the tank (fill) and then allowed to react with ample aeration followed by settling and clarifying without aeration and subsequent decanting of the treated effluent (draw). This step is also carried out without aeration. Finally, the system is kept idle and waste sludge is removed [18]. This process is depicted in a flow diagram shown in Figure 15.3. The major advantage of this process is the low land requirement and its economical feasibility.

3) *Oxidation ditches*: It essentially consist of a ring or oval channel with suitable aeration device. They operate in an extended aeration mode as depicted in Figure 15.4. Generally, this process is designed to achieve specific objectives such as nitrification, denitrification, phosphorus removal, and so on [19]. It is important to note that in this process no effluent surge occurs due to the constant water level and continuous discharge [20]. Main advantages are its energy efficiency and suitability for small communities. On the other hand, requirements of large areas of land makes it less economical as compared to other techniques.

AS and its variants as discussed earlier are types of treatment where the microorganisms are suspended. Therefore, they are also generally referred to as *suspended growth options*. Under this category, we can have another classification based on the aeration used.

1) *Diffused aeration:* This may use either fine bubble diffused aeration system of varying sizes generally made of ceramic or membrane or coarse bubble diffusion systems that are generally made of corrosion-resistant stainless steel components [21].
2) *Jet aeration:* This type of aeration provides air and also aids in mixing without the requirement of separate mixing equipment. When aeration

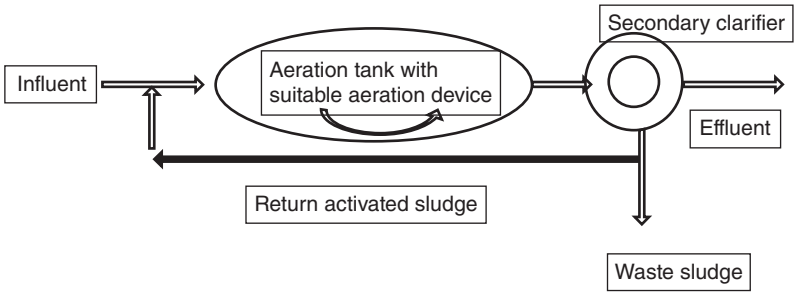

Figure 15.4 Oxidation ditch.

Influent: raw wastewater that enters a reactor for treatment
Aeration tank with suitable aeration device: Wastewater reactor equipped with aeration device
Secondary clarifier: settling tank where coagulated material is allowed to clarify
Return activated sludge: some amount of waste sludge is recycled back as a source of inoculum
Effluent: treated wastewater
Waste sludge: the remains of solid impurities along with microorganisms after complete treatment.

requirement decreases during the treatment process reducing the energy input by jets, pumps provide required mixing to enhance process control and dissipate required energy. Lower quantum of volatile organic compounds (VOCs) are released by using this type of aerators and since no moving parts are required, it results in long life of the system [22].

3) *Surface aeration*: In this case, high and low speed floating aerators provide pumping action that result in transfer of oxygen by breaking up wastewater into sprays of fine droplets. These systems may use horizontal mounted aeration disks or rotors (also used in oxidation ditches). Generally, disk and rotor surface aerators are very efficient in giving higher extents of removal of COD and BOD and are also easy to replace. It is interesting to note that reactors in a vertical loop configuration are also suitable as surface aeration setups. These are essentially oxidation ditches flipped on their side. In such setups the upper and lower compartment are separated by a horizontal baffle that runs through the length of the tank [23].

Aeration devices are usually compared by the amount of oxygen transferred per unit of energy introduced to the wastewater under standard operating conditions, which is expressed as kg O_2/kg Wh. This can be determined by removing the residual oxygen from the liquid being tested with subsequent addition of sodium sulfite and a catalyst such as cobalt chloride. Aeration is started and the rate of oxygenation of the liquid is measured with the help of DO recordings at regular intervals. The oxygen transfer coefficient, $K_L a$, can be determined as follows [13]:

$$K_L a\,(T) = K_L a(20^\circ\text{C})\theta^{(T-20)} \tag{15.14}$$

The solubility of oxygen in water is temperature dependent, hence the above equation is used.

θ is the temperature correction factor (usually considered as 1.024) and T is the temperature at which the test is carried out. The actual oxygen transfer rate is calculated using the following equation:

$$\mathrm{OTR_f} = \mathrm{SOTR}\left(\frac{\beta C_s - C_w}{C_{s20}}\right)\theta^{(T-20)} \cdot \alpha \tag{15.15}$$

where,

$\mathrm{OTR_f}$ = actual oxygen transfer rate under field operating conditions (kg O_2/kg Wh)
SOTR = oxygen transfer rate under test conditions at 20 °C (kg O_2/kg Wh)
C_s = oxygen saturation concentration in tap water ν (mg l^{-1})
C_w = operating oxygen concentration in wastewater (mg l^{-1})
C_{s20} = oxygen saturation concentration in tap water at 20 °C
α = ratio of $K_L a$ of wastewater to that of tap water
β = ratio of saturation concentration of oxygen at 20 °C for wastewater and tap water
θ = temperature correction factor (usually considered as 1.024).

The following equation gives the total oxygen demand in an AS system [13]:

Total demand = Carbonaceous oxygen demand + Endogenous oxygen demand + Nitrification oxygen demand + Oxygen derived from denitrification in an anoxic zone

$$\mathrm{OD} = 0.75Q(\mathrm{BODi} - \mathrm{BODe}) + 2V_A \cdot \mathrm{MLSS} + 4.3Q(\mathrm{Ammi} - \mathrm{Amme}) - 2.83Q[(\mathrm{Ammi} - \mathrm{Amme}) - \mathrm{Ne}] \tag{15.16}$$

where,

OD = mass of oxygen required (g h^{-1})
BODi = inflow BOD (mg l^{-1})
BODe = outflow BOD (mg l^{-1})
Ammi = inflow ammoniacal nitrogen (mg l^{-1})
Amme = outflow ammoniacal nitrogen (mg l^{-1})
Ne = outflow nitrate nitrogen (mg l^{-1}).

15.5.2.2 Trickling Filters

This is yet another type of aerobic wastewater treatment process that is also commonly referred to as *biotowers*. The schematic representation of the biotowers has been given in Figure 15.5. The distinctive feature of this method is that the microorganisms are attached to a suitable medium unlike the suspended growth seen in the ASP. Therefore, they are also classified under fixed film type of wastewater treatment. The microbes form a film or a slimy layer on the medium, which is generally around 0.1–0.2 mm thick. As the wastewater flows into the system, the microbes from the influent get attached to the medium to form a film.

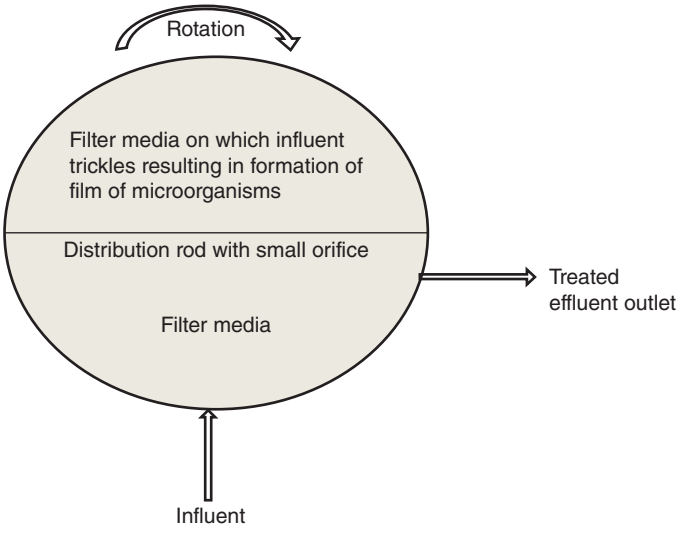

Figure 15.5 Trickling filter.

Rotation: slow movement of filter to ensure complete oxidation and uniform treatment
Distribution rod with small orifice: for uniform distribution of the influent over the filter media
Filter media: support on which microbes grow forming a film
Influent: raw wastewater that enters a reactor for treatment.
Treated effluent outlet: opening for release of treated water.

Microbes, especially the ones on the outer part of the film, are responsible for the degradation of organic pollutants aerobically [24]. However, the film thickness increases with time leading to difficulty in oxygen penetration into the film and often results in anaerobic conditions toward the inner part of film. Sometimes a portion from the film fall off into the wastewater from the filter and this is called *sloughing*. This portion can be clarified and filtered. The medium usually employed in trickling filters is rock, slag, or plastic that is suitably designed in terms of shape and arrangement [25, 26]. Aeration is an important parameter that is addressed by using natural draft and/or forced circulation. The void area in the medium and the ventilation ports at the bottom of the filters aid in the aeration process. The waste influent is dispersed over the trickling filter with the aid of distributors that are driven mechanically. The distributors are either rotating arms or fixed nozzles. An under-drain system that collects solids and filtrate, also serves as a source of air for the microbes on the filter. After treatment in the trickling filters, the material flows into a settling tank where the solids are separated. A part of the treated liquid is recirculated to the filter in order to improve wetting and flushing of the filter media. Trickling filters are generally classified into low-, intermediate-, and high-rate filters based on the amount of BOD they handle [27]. Low-rate filters can handle less than 40, intermediate ones handle up to 64, and high-rate handles between 64 and 160 kg BOD_5/1000 cu ft

per day. Besides this, roughing filters are designed to handle soluble BOD in the range of 160–480 kg BOD_5/1000 cu ft per day [4]. In general, trickling filters are less efficient for BOD and COD removal. On the other hand, the advantage is that they produce very little sludge and have potential for stripping VOCs which is an advantage for environmental reasons [28–30].

Two approaches have been used to design trickling filters [31]. The first approach, called the *NRC* equation is based upon the performance of similar units as follows:

$$E = \frac{1}{1 + 0.014\left(\frac{w}{V_m R_f}\right)^{0.5}} \tag{15.17}$$

where, E = fraction of BOD removed, w = loading, kg BOD per day, V_m = total volume of filter media, and R_f = recycle factor defined by

$$R_f = \frac{1 + r}{(1 + (1 - p)R_r)2} \tag{15.18}$$

where, p = a weighting factor typically equal to 0.9 and R_r = recycle ratio defined as ratio of the recirculation flow to the filter influent flow. The second approach is based on kinetic equations based on BOD removal:

$$\frac{dC}{dt} = -K_d C \tag{15.19}$$

where, C is the concentration of the biologically degradable substrate as determined by the BOD test and K_d is a rate constant [32].

15.5.2.3 **Rotating Biological Contactors (RBCs)**

This is yet another variant of the fixed film type of wastewater treatment methods [33]. It essentially consists of a vertically arranged plastic medium on a horizontal rotating shaft that rotates at a speed of around 1–1.5 rpm and remains submerged up to 40% in the medium. Microorganisms are coated on the rotating plates and are exposed to the wastewater and atmospheric oxygen alternately as depicted in Figure 15.6. These types of setups are generally preferred in petroleum facilities as they are adapted to recover quickly from upset conditions [34–36]. Moreover, they are easy to expand and enclose for VOC containment.

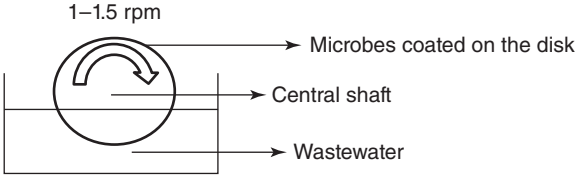

Figure 15.6 Rotating biological contactors.

Microbes coated on the disk: essentially a biofilm on the drum that treats wastewater
Central shaft: for easy rotation of the contactor
Wastewater: inlet water that is subjected to treatment.

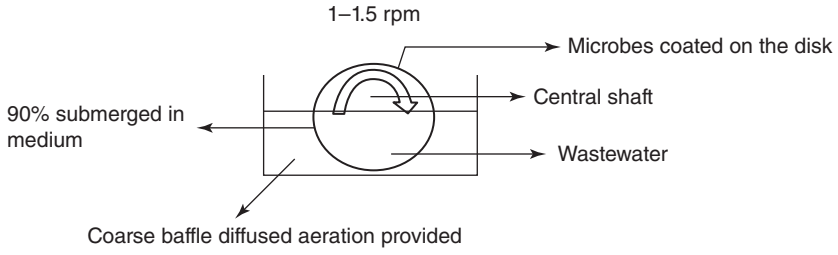

Figure 15.7 Submerged biological contactors.

90% submerged in medium: in this type of contactors, the treatment drum is submerged to a greater extent
Coarse baffle diffused aeration provided: baffles have larger openings through which aeration is induced at higher rate
Microbes coated on the disk: essentially a biofilm on the drum that treats wastewater
Central shaft: for easy rotation of the contactor
Wastewater: inlet water that is subjected to treatment.

15.5.2.4 **Submerged Biological Contactors (SBCs)**

These are almost similar to the rotating biological contactors (RBCs) except that they operate at 90% submergence in the medium and employ coarse baffle diffused aeration as shown in Figure 15.7. Since the submergence is more, the load on the shaft is much less than in RBCs and it has at least three times the surface area of a conventional RBC/ft/shaft length. Very good odor and VOC containment removal is possible with this setup if enclosed. It can be expanded and as it is driven completely by air, maintenance is much lower [37, 38].

15.5.2.5 **Powdered Activated Carbon Treatment (PACT) Systems**

These are conventional biological treatment systems to which powdered activated charcoal is added to increase the efficiency of the process as shown in Figure 15.8 [39]. This allows both physical adsorption and biological assimilation to occur simultaneously and can operate either aerobically or anaerobically [40]. Main advantages include toxicity removal, increase in nitrification efficiency, decrease in VOCs, and improved COD removal [41–43].

15.5.2.6 **Membrane Bioreactors**

They are unique setups used for wastewater treatment and are very similar to the conventional ASPs in that both have mixed liquor solids in suspension in an aeration tank. However, in membrane bioreactors (MBRs), the bio-solids are separated by using a polymeric membrane that is generally based on micro- or ultrafiltration. They are basically aerobic or anaerobic processes with integrated membrane system [44]. In this method, the wastewater is initially screened to remove coarse particles to avoid clogging of membranes especially as membranes are expensive [45, 46]. The screened wastewater generally enters a treatment tank that incorporates a submerged membrane that harbors the microorganisms. In aerated systems, suitable aeration is provided for maintaining the biomass and aiding the

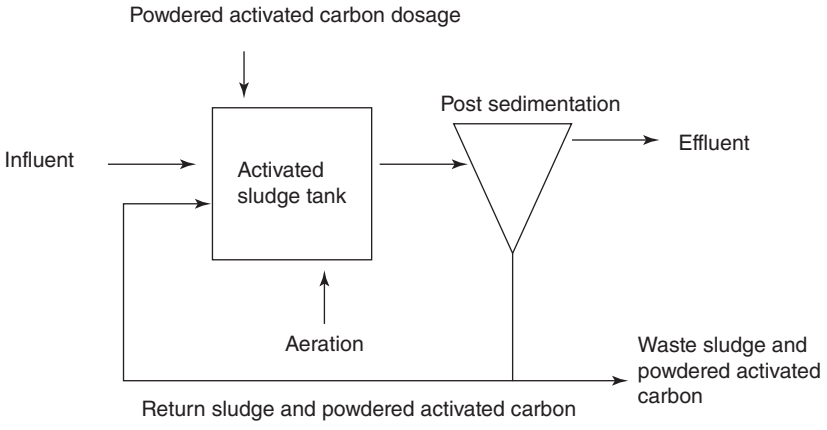

Figure 15.8 ASP combined with powdered activated carbon treatment (PACT).

Powdered activated carbon dosage: amount of activated carbon that is added for treating wastewater
Influent: raw wastewater that enters a reactor for treatment
Activated sludge tank: main area where wastewater treatment occurs
Aeration: suitable means of providing air for reaction
Post sedimentation: treatment after sedimentation process
Effluent: treated wastewater
Return sludge and powdered activated carbon: contents that are recycled back into the reactor
Waste sludge and powdered activated carbon: waste generated after treatment.

process. These systems can retain active biomass in the process by way of submerged membranes rather than using clarifiers thus eliminating sludge settling issues. A typical system is represented in Figure 15.9. MBRs are generally operated for longer periods of around 20–100 days. They are stable with complete nitrification being possible and also yield less biosolids [47, 48]. The advantages of membrane filtration are manifold. It provides an excellent barrier to suspended bio-solids thus preventing its loss and abating conditions of sludge-bulking in the clarifier that is often encountered in the ASP. The aeration tank size in MBRs are much less and compact in order to accommodate the membrane especially in the case of submerged systems. The effluent quality obtained is better than that obtained in ASPs and does not require subsequent post treatment [49].

15.5.2.7 **Biological Aerated Filters (BAFs)**

These are essentially biofilm systems that are used either for secondary or tertiary wastewater treatment process [50]. The first report of this setup dates back to around the early 1900s where aeration tanks containing layers of slate supporting a biofilm was used for sewage treatment [51]. Such a system encompasses a three phase structure comprising a solid phase that includes a support for the growing microorganisms, liquid phase in which the solid is submerged, and gas phase created by input of air in a reactor. The media used can either be

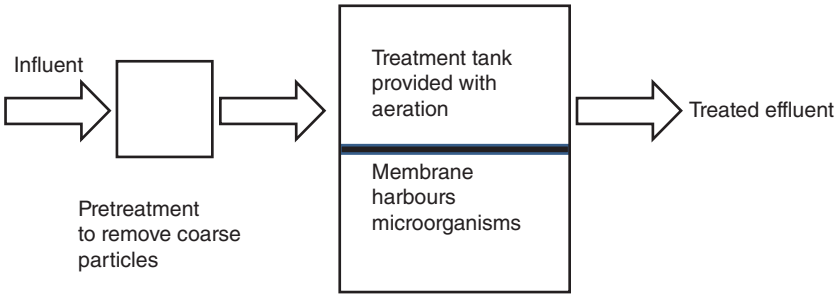

Figure 15.9 Membrane bioreactors for aerobic wastewater treatment.

Influent: raw wastewater that enters a reactor for treatment
Pretreatment to remove coarse particles: initial screening to eliminate impurities that are larger in size
Treatment tank provided with aeration: main wastewater treatment reactor
Membrane harbors microorganisms: microbes grow and form a film on the surface of the membrane
Treated effluent: treated wastewater.

structured or granular. The latter is generally known to be capable of removing organic matter and suspended solids at the same time. Therefore, there is no requirement for a subsequent sedimentation step. Biological aeration filters can be of two varieties, namely, upflow BAF and downflow BAF as shown in Figure 15.10a,b. In the upflow BAF, air and wastewater flow in a concurrent manner. These types of systems can easily cope up with increasing influent flow rates. Here, more effective oxygenation occurs due to bubbles not coalescing and retaining their optimal surface/volume ratio. An added advantage of using this system is less odor production due to air stripping of volatile compounds because ambient air is only in contact with treated effluent. Media used are myriad such as expanded clay [52], porous stone [53], and polypropylene [54] for upflow BAF and media such as anthracite for downflow BAF. BAFs have been widely used for treatment of domestic wastewater containing hydrocarbons with concentration up to 20 mg l^{-1} with almost 95% efficiency [55]. It has also been successfully employed to treat industrial wastewater [56], wastewater from paper mill [57], and distillery wastewater [53]. Small footprint and adaptability allows them to be used in upgrading established works especially where land costs are high. Moreover, optimum requirements especially with regard to media type, backwashing rates, aeration, and so on, are conditioned for less power consumption.

15.5.2.8 Hybrid Processes-Integrated Fixed Film Activated Sludge System

It is well established that hybrid processes are generally superior to individual techniques and a wastewater treatment is no exception. Among the several hybrid techniques in place, the Integrated Fixed Film Activated Sludge System has found widespread application [58–60]. This technique combines the conventional ASP

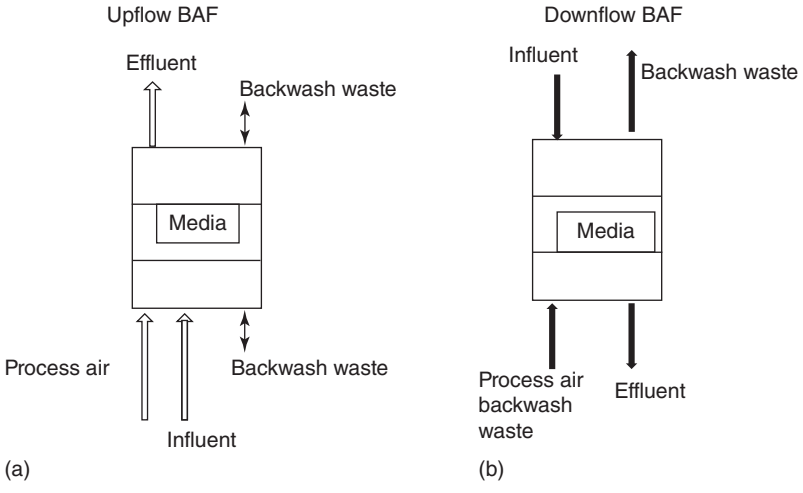

Figure 15.10 (a,b) Biological aerated filters (BAFs).

Upflow BAF: the air and wastewater flow in a concurrent manner
Influent: raw wastewater that enters a reactor for treatment
Effluent: treated wastewater
Process air: aeration provided for reaction
Backwash waste: dislodged solids and microorganisms that are generated during backwashing of the filter usually carried out by air (air scouring) or water
Media: suitable filter media such as expanded clay, porous stone, polypropylene, and anthracite
Downflow BAF: the air and wastewater flow in a counter current manner with downwards flow for wastewater.

with trickling filter with a subsequent clarifier. Yet another modification of this system is the moving bed bioreactor that is typically a fluidized bed system followed by an ASP and this combined approach is also described as the integrated hybrid method. Additional surface area of the film allowing biofilm formation either in fixed form or suspended form, better overall efficiency, effective nitrification [61], smaller foot-print and lesser sludge waste generated, and lower capital and operating costs are some of the merits of the hybrid method as compared to the conventional ASP [62].

15.5.2.9 Use of Ultrasound to Improve the Sludge Characteristics

A main drawback of the conventional aerobic processes commonly used in wastewater treatment applications is the production of large amount of excess AS. The sludge in these processes contains biomass, extracellular polymeric substances (EPSs) and large amounts of water (generally more than 95%). EPS is an important material that tries to combine the biomass and water into a matrix. A typical purification plant for the wastewater produces sludge quantum anywhere between 5% and 25% of the total volume of treated water. The sludge generated from municipal waste treatment plants can be converted into useful

fertilizers, but the sludge generated from industrial waste treatment plants is difficult to dispose as it contains large amounts of noxious chemical substances. The process of conversion to fertilizers is likely to be more effective if the water content in the sludge is reduced. Any improvements in reducing the quantity of sludge by virtue of efficient dewatering are also beneficial to the overall wastewater treatment process due to lower disposal costs. Ultrasonic irradiation can be effective in dewatering of suspensions such as slurries and sludge due to the cavitation events which can bring about structural changes. The changes in the structure and properties of sludge influence the efficiency of the dewatering process. Ultrasonic irradiations can also be combined with chemicals such as polyelectrolyte or alkali and the combined process is likely to be more effective. Use of ultrasound (US) changes the inner structure of polyelectrolytes, which helps in intensifying the polyelectrolyte activity on the sewage sludge.

15.6 Anaerobic Techniques

Since the early 1970s, anaerobic treatment of industrial wastewaters has gained a lot of importance. This is a result of increased knowledge of the microbiology of anaerobic purification, and development of improved reactors with high biomass concentrations. Anaerobic processes are an attractive alternative to treat wastewater given the fact that aerobic techniques are energy intensive due to high aeration requirements, yield large quantity of sludge (~0.4 g dry weight/g COD removed), and are able to treat efficiently only low strength wastewater like municipal wastewater that has less than 1000 mg COD l^{-1}. Anaerobic processes are, inexpensive compared to their aerobic counterparts and their use has increased over the past decade [63]. Anaerobic digestion consists of several sequential steps where the products from one group of microorganisms serve as the substrate for another group of microbes. Hydrolysis/liquefaction, acidogenesis, acetogenesis, and methanogenesis are the four phases that typically occur in an anaerobic digestion process. It has been reported that generally two biological kingdoms, the bacteria and the archaea, are actively involved in the process [64].

Anaerobic processes have the advantage of high efficiency, simplicity, flexibility, low space requirements, low energy consumption, low sludge production, and low nutrient and chemical requirements to name some. On the other hand, low pathogen and nutrient removal requiring post treatment, long start-up, odor generation due to production of hydrogen sulfide are some drawbacks of anaerobic treatments, which require special attention [65].

Anaerobic digestors are generally classified as low-rate and high-rate. The former are conventional and consist of anaerobic ponds and septic tanks. The latter are designed to operate at short hydraulic retention times (HRT) and long SRT for high activity biomass and better efficiency [66]. Some of the high rate digestors are described in the following sections. High rate anaerobic biological reactors may be classified into three types based on the mechanism of biomass retention.

These are suspended type, fixed film type, and hybrid. Among the plethora of full-scale installations globally, 67% are upflow anaerobic sludge blanket (UASB) reactors with or without suspended growth [67], 12% continuous stirred tank reactors (CSTRs), 7% anaerobic filters with or without fixed film [68], and 14% comprising other variants.

15.6.1 Types of Anaerobic Treatment Systems

15.6.1.1 Upflow Anaerobic Sludge Blanket (UASB)

The UASB reactors are widely used for anaerobic treatment of wastewater. They are considered to be very robust high-rate reactors especially for sewage treatment. It has been reported that more than 1000 such reactors are installed globally [69]. A typical setup is shown in Figure 15.11. The reactor consists of two parts, a cylindrical column and a gas liquid solid separator [70]. The reactor is seeded initially. The sludge enters from the bottom and the light particles are washed out leaving the heavier ones behind resulting in the formation of granules containing

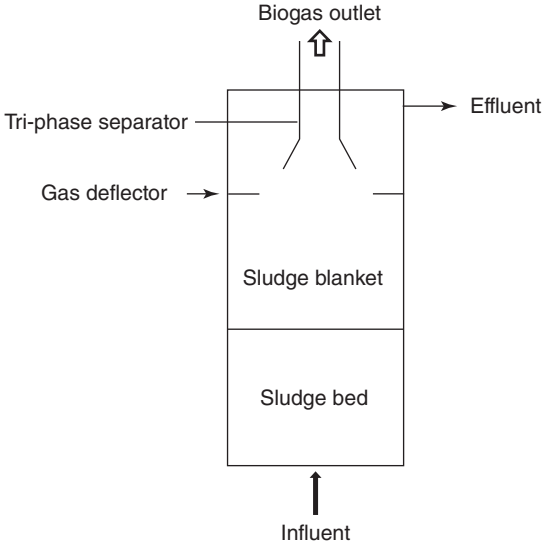

Figure 15.11 Upflow anaerobic sludge basket (UASB).

Influent: raw wastewater that enters a reactor for treatment
Effluent: treated wastewater
Sludge bed: a dense zone of sludge that develops as the microbes grow
Sludge blanket: zone comprising a much diffused growth and lower settling velocity located above the sludge bed
Biogas outlet: opening to release the biogas produced during treatment
Tri-phase separator: gas liquid and solid (GLS) separator that helps in the separation of the biogas and the sludge that has moved upward from the treated wastewater
Gas deflector: baffles below the GLS separator that prevent the wash-out of viable bacteria by pushing the material back into the reactor.

inert organic, inorganic matters, and bacterial aggregates in the seed sludge. Over a certain period of time a dense sludge bed develops above which there exists a sludge blanket zone which displays a much diffused growth and lower settling velocities [71]. The biological reactions occur in both the zones and the soluble organic compounds in the influent are converted to biogas comprising mainly methane and carbon dioxide. The gas–liquid–solid separator helps in the separation of the biogas and the sludge that has moved upward. The baffles in the separator prevent the wash-out of viable bacteria by pushing the material back into the reactor [72].

15.6.1.2 **Anaerobic Baffled Reactors (ABR)**

These reactors, as their name suggest, use a series of baffles to force the wastewater to flow under and over the baffles as it passes from the inlet to the outlet. A schematic representation of these types of reactors has been shown in Figure 15.12. The flow characteristics in the reactor coupled with the gas production cause the bacteria to gently rise and settle within the reactor. The most significant advantage of the anaerobic baffled reactors (ABRs) is its ability to separate acidogenesis and methanogenesis longitudinally down the reactor. Thus, the reactor behaves like a two-phase system without the requirement of elaborate control systems and the associated expense [73]. Simple design without moving parts, no mechanical mixing, lower cost of construction, reduced clogging, low sludge generation, low HRT, and protection from toxic materials in the influent are some major advantages of the ABRs that make it an attractive option for wastewater treatment. A lot of research has been devoted to enhance the performance of the ABRs especially by way of improving the existing design such as

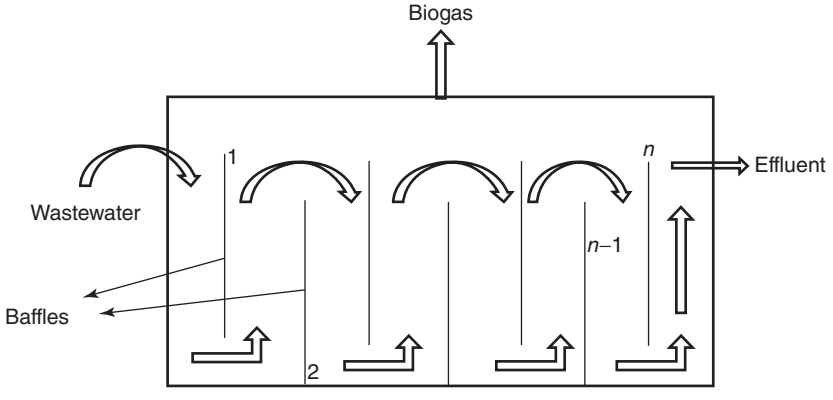

Figure 15.12 Anaerobic baffled reactors.

Wastewater: influent to be treated
Effluent: treated wastewater
Baffles: several baffles employed to force the wastewater to flow under and over the baffles as it passes from the inlet to the outlet
Biogas: gas (usually methane and carbon dioxide) generated as a result of treatment.

addition of vertical baffles to a plug flow reactor that allows higher solid retention and better substrate accessibility to methanogens [74]. Yet another modification was the narrowing of the downflow chambers so that cell retention is increased in the upflow chambers and slanted edges on the baffles of around 40–45°, which leads to the flow toward the center of the reactor and enhances mixing [75]. Similarly, settling chambers to improve solids retention, positioning packing at the top of each chamber to prevent washout of solids [76], and enlargement of the first chamber for better treatment capability [77] are some more variations in the conventional design which give efficient operation.

15.6.1.3 **Anaerobic Fluidized Bed Reactors**

These reactors offer successful designs for anaerobic wastewater treatment that has found widespread application since the early 1980s. Although the initial systems operated on a smaller scale, by 1984 many full-scale fluidized bed reactors were installed worldwide. Fluidized bed reactors are compact systems that require very little space. It essentially consists of a vertical long chamber (reactor) with biolayer covered inert particles that are maintained in a fluidized state by an upward directed water flow as depicted in Figure 15.13. The role of the

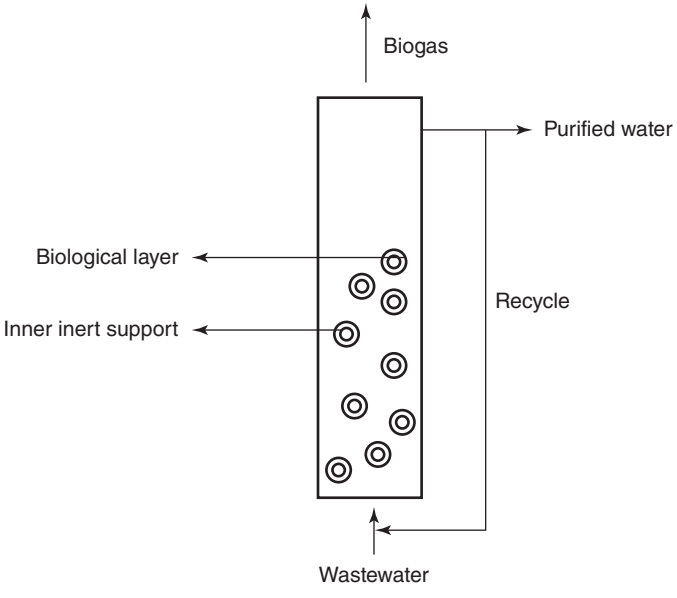

Figure 15.13 Anaerobic fluidized bed reactors.

Wastewater: influent to be treated
Biogas: gas (usually methane and carbon dioxide) generated as a result of treatment
Recycle: part of treated water and sludge that is sent back again for further treatment and as a fresh inoculum source
Purified water: treated wastewater
Inner inert support: suitable media present inside that does not participate in the reaction
Biological layer: film of microorganisms that grows on the support.

inert particles (carrier) is to provide a good support for the microbes to grow on them and form a biolayer. Sand is one of the most commonly used inert carriers apart from Al_2O_3, activated carbon, and synthetic resins. The characteristic of the inert carrier has been found to influence the whole process. For instance, smaller the size of the particles, lower is the liquid shear experienced. Overall, a diameter of 0.3–0.5 mm for sand particles has been reported to give a very good biofilm formation followed by an efficient wastewater treatment in anaerobic fluidized bed reactors. These reactors have many advantages over other anaerobic treatment methods such as high settling velocities of the particles, which enable high liquid velocities in the reactor. This also ensures that the inert sediment does not accumulate in the reactor and, therefore, sludge activity is very high. Moreover, the biomass concentrations are high due to high adherence to the large surface area available on the small inert particles. Higher purification and no problems of sludge retention compared to UASB reactors, no clogging as seen in filters, and small area requirements are additional intrinsic positive aspects of this system. As with any system, the fluidized bed reactors have some inbuilt disadvantages. Longer start-up times due to the requirement of biolayer formation on the inert particles, high energy requirement owing to high liquid recirculation ratio, and requirement of expensive liquid distributors for fluidization especially on a large scale are some of the problems associated with the system. Several research papers report the extensive work carried out on the design and optimization of the fluidized bed reactor and there are no general guidelines available as different wastewater will have to be treated in a different manner because of the varying composition of the wastewater. Optimization based on lab and pilot scale studies is a key requirement as some reports indicate that COD removal is very low (1–50%) [78–81]; on the other hand, higher COD removal (40–90%) has also been reported especially in the case of expanded bed at low velocity [82–84].

15.6.1.4 Expanded Granule Sludge Blanket (EGSB) Reactor

This reactor is very similar to a traditional UASB reactor that has a characteristically high recycle ratio. The distinctive feature of this reactor is that the upflow of this reactor is generally maintained higher and thus enables the reactor to contact the granules with the wastewater. A typical diagram is shown in Figure 15.14. This reactor design contributes to the separation of the dispersed sludge from the granules and helps withdrawing it out of the reactor. Very high velocities result in expansion of granules with greater mixing and higher contact with the wastewater. The expanded granule sludge blanket (EGSB) reactors are reported to handle low (1000–2000 mg l^{-1}) as well as high (30 kg m^{-3} per day) COD [85, 86]. Not many reports exist for modification of these reactors [87]. Although there are no major differences between this and the UASB, the hydraulic flow pattern in the case of EGSB can be between completely mixed and dispersed plug flow.

15.6.1.5 Anaerobic Membrane Reactors

These systems have only recently emerged mainly from the observed success from the aerobic membrane processes. They are known to accomplish very high COD removal up to the extent of 98% and often operate either as an external membrane

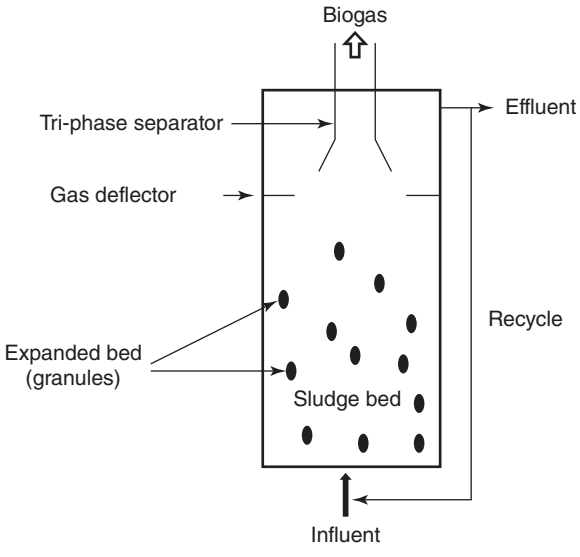

Figure 15.14 Expanded granule sludge blanket reactor.

Influent: raw wastewater that enters a reactor for treatment
Effluent: treated wastewater
Biogas: gas (usually methane and carbon dioxide) generated as a result of treatment
Recycle: part of treated water and sludge that is sent back again for further treatment and as a fresh inoculum source
Sludge bed: a dense zone of sludge that develops as the microbes grow
Expanded bed (granules): microbe coated support that gets expanded due to high flow velocities of wastewater
Gas deflector: baffles below the GLS separator that prevent the wash-out of viable bacteria by pushing the material back into the reactor
Tri-phase separator: gas liquid and solid (GLS) separator that helps in the separation of the biogas and the sludge that has moved upward from the treated wastewater.

or a membrane submerged within the reactor as shown in Figure 15.15. These membrane systems prevent washout of the biomass enabling higher efficiency of treatment. Although fouling can be an important demerit, it can be often circumvented by appropriate means. There are different ways by which the anaerobic bioreactors and membranes can be combined. The first kind of membrane reactor houses the membrane outside the reactor and the biomass is circulated at high velocities using an external pump. Although this ensures that there is no fouling, energy costs may be high. The second method of operating these reactors is to employ vacuum to draw the effluent through the membrane. Here, the membrane can be submerged in the reactor or in a separate reactor, which requires a pump but the flow is not through the module. The obvious advantage of a submerged membrane reactor is the elimination of energy requirement for pumping [88]. Moreover, the biomass is less stressed. Yet another membrane configuration reported consists of sequential membrane reactors where the effluents from one

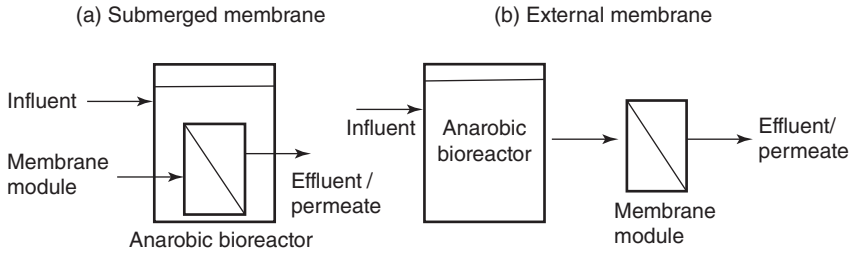

Figure 15.15 Anaerobic membrane bioreactor.

Submerged membrane: the membrane system is immersed in the wastewater to be treated (inside the reactor)
Influent: raw wastewater that enters a reactor for treatment
Effluent/permeate: treated wastewater that flows out of the membrane
Membrane module: unit (device) containing the membrane essentially on a support
Anaerobic bioreactor: a wastewater reactor that works anaerobically (without aeration)
External membrane: membrane system is housed outside the reactor and the biomass is pumped at high velocities from the reactor into the membrane system.

reactor with a large pore size are treated by another membrane reactor of a smaller pore size [89].

15.6.2 Improvements for Sludge Management

In the case of biological wastewater treatment, large quantities of biosolids (sewage sludge) are also produced that leads to severe problems in management due to higher transport costs as well as also lack of enough storage facilities. The sludge is highly susceptible to decay. Therefore, the generated sludge is generally stabilized by anaerobic digestion in order to enable environmentally safe utilization and disposal. Anaerobic digestion is achieved through several stages such as hydrolysis, acidogenesis, and methanogenesis. Due to the rate-limiting step of biological sludge hydrolysis, the anaerobic degradation is a very slow process, and large fermenters (digesters) are necessary. Typical digestion times are more than 30–40 days. One of the improvements for anaerobic digestion that has been successfully applied in commercial scale applications is based on the use of ultrasonic reactors. Ultrasonic reactors can be effectively used to improve the sludge hydrolysis, resulting in a significant reduction in the digestion time as well as a significant increase in the biogas generation. The intensification has been attributed to floc size reduction and cell lysis. Shear forces generated by low-frequency ultrasound are effective in disintegrating bacterial cells in sewage sludge. Thus, the quantity of dissolved organic substrate is increased and, consequently, the degradation rate and the biodegradability of organic biosolids mass are improved. Nickel and Neis [90] reported the use of a pilot-scale ultrasound reactor (maximum power consumption: 3.6 kW) for biosolids treatment. The reactor was with a capacity of 1.3 l and provided with 12 piezoceramic flat transducers fixed at each of the four sidewalls. The operating frequency was fixed at 31 kHz and the area of transducers

was adjusted in such a way that the acoustic intensities vary within the range of 5–18 W cm^{-2}. It has been reported that the use of ultrasonic biosolid disintegration resulted in a significant improvement in the overall process by virtue of increased volatile solid degradation rate (about 40%), increased biogas production (by almost 100% depending on the operating conditions), and a reduction of the nondegradable organic matter that exists in each type from 60% to 52%. The work has established that ultrasonic disintegration is a promising method to reduce the required volume of new biosolids digesters and also enable the operators to maintain undisturbed biosolids digestion rate, which is a very important requirement especially for the overloaded systems. In addition, there is a significant increase in the biogas production, which leads to overall economical operation and also might avoid installations of new digestors for enhancing the production.

15.7
Aerobic–Anaerobic Processes

Previous sections described various aerobic and anaerobic wastewater treatment processes. For any process, combination technology always works better than any individual method owing to the merits of two or more methods, which invariably offset the disadvantages of an individual technique. Biological wastewater techniques are no exception and both aerobic and anaerobic processes have been employed together successfully giving better treatment efficiencies [91, 92]. The conventional hybrid aerobic–anaerobic systems usually consist of large ponds connected in series and are frequently characterized by long HRT, low organic loading rate, high energy constraint, and problems related to requirement of vast area of land and lack of economic feasibility [93]. These issues can be overcome to a large extent by using high-rate bioreactors such as the UASB, fluidized bed reactor, and membrane reactors in order to provide a treatment technique that is technically sound and economically viable [94, 95]. Continued research along these lines has led to the development of integrated processes that have aerobic and anaerobic oxidation zones in the same bioreactor [96–98] generally classified into four types:

1) Integrated bioreactors with physical separation of the aerobic–anaerobic zone
2) Integrated bioreactors without physical separation of the aerobic–anaerobic zone
3) SBR based on temporal separation of aerobic and anaerobic phase
4) Combined aerobic–anaerobic system based on limited oxygen diffusion in microbial biofilms.

It is expected that these integrated processes will be able to meet the strict constraints of space, odor, and lesser sludge production and also be a cost-effective alternative to the conventional treatment technologies already in place. However, not many such integrated processes have been reported till now on a large scale. Moreover, these systems also warrant better optimization of the process and design [3].

15.8 Modified Biological Processes

The efficiency of the biological oxidation techniques is often hampered by the presence of bio-refractory materials as these cannot be readily degraded by the microorganisms. Under such conditions, it becomes imperative that appropriate pretreatment approaches are used so that the biorefractory molecules can be converted into lower chain compounds that can be more readily oxidized by the biological methods. The efficiency of the pretreatment process can be quantified in terms of the biodegradability index (BI) which is nothing but the ratio of biological oxygen demand established over 5 days to the chemical oxygen demand (BOD_5/COD) of the effluent. Advanced oxidation processes offer considerable promise as the pretreatment method and can be used with success to enhance the BI of the effluent stream to the desired range. Such a combined approach would be very useful as not only the overall efficiency of the treatment approach would increase leading to better discharge streams but the required treatment time can also be significantly reduced. In addition, although advanced oxidation processes offer potential for degradation of almost all the contaminants, their use is hampered by the lack of knowledge for the design and efficient operation of the large-scale reactors as well as by the cost of treatment that is considerably higher. The different advanced oxidation processes that have been used in the recent past as pretreatment approaches include cavitation, Fenton chemistry, ozonation, and photocatalytic oxidation. We now discuss the basic aspects of these processes followed by some specific cases studies for better understanding.

15.8.1 Cavitation

Cavitation can be used effectively for the destruction of the contaminants in water because of the localized high concentrations of the oxidizing species such as hydroxyl radicals and hydrogen peroxide, higher magnitudes of localized temperatures and pressures, and the formation of the transient supercritical water. Generation of conditions of intense turbulence and acoustic streaming also helps in enhancing the rates of processes limited by mass transfer and hence cavitation can be very effective when combined with other advanced oxidation processes such as Fenton processes and photocatalytic oxidation. Cavitation can be useful in altering the molecular structures of the pollutant leading to the enhanced biodegradability and hence higher rates of biological oxidation. Thus, cavitation can be a useful pretreatment to the conventional biological oxidation and the combined process would be more effective. In addition, the use of cavitation during the biological oxidation using a flow loop in the bioreactor can give rise to enhanced surface areas being generated due to the disintegration of the flocs and, consequently, the rate of oxidation increases.

Cavitation is classified into four types based on the mode of generation, namely, acoustic (use of ultrasound), hydrodynamic (use of changes in the flow geometry),

optic (use of laser), and particle (use of beam of elementary particles) but only acoustic and hydrodynamic cavitation (HC) have been found to be efficient in bringing about the desired chemical changes whereas optic and particle cavitation are typically used for single bubble cavitation which fails to induce chemical change in the bulk solution. The destruction/oxidation of pollutants using cavitation is usually described by two approaches, namely, free radical attack and pyrolysis. The controlling mechanism for the destruction is usually dependent on the pollutant in question as well as on the degree of cavitation intensity, which in turn depends on the operating conditions of the sonochemical reactor. The type of the pollutants in the effluent stream affects the extent of intensification in rates of the degradation obtained due to the cavitating conditions. The hydrophobic compounds react with $OH^{\bullet}$ and $H^{\bullet}$ at the hydrophobic gas/liquid interface, while the hydrophilic species react to a greater extent with the $OH^{\bullet}$ radicals in the bulk aqueous phase. Optimization of organic compound degradation rates can be achieved by adjusting the energy density, the energy intensity, and the nature and properties of the saturating gas in solution. The variety of chemicals that have been degraded using cavitational reactors, although in different equipments and over a wide range of operating scales, are *p*-nitrophenol, rhodamine B, trichloroethane, parathion, pentachlorophenate, phenol, CFC 11 and CFC 113, *o*-dichlorobenzene and dichloromethane, potassium iodide, sodium cyanide, and carbon tetrachloride among many others. Excellent reviews are available in the literature covering different aspects related to wastewater treatment applications of cavitational reactors [99, 100].

The similarity between the mechanism of destruction and some of the common optimum operating conditions in the case of different advanced oxidation techniques point toward the synergism between these methods and the fact that combination of these advanced oxidation processes should give better results as compared to individual techniques [101]. Generally, combination of two or more advanced oxidation processes such as cavitation/ozone, cavitation/H_2O_2, sonophotocatalytic oxidation, and so on, leads to enhanced generation of the hydroxyl radicals, which eventually results in higher oxidation rates. The efficacy of the process and the extent of synergism depend not only on the enhancement in the number of free radicals but also on the alteration of the reactor conditions or configuration leading to a better contact of the generated free radicals with the pollutant molecules and also better utilization of the oxidants and catalytic activity. More details about combined techniques and its applicability for different wastewater streams can be obtained from published literature [101].

Ultrasonic horns are the most commonly used reactor designs among the sonochemical reactors, although the cavitational effects are only observed close to the vibrating surface. Thus, the efficacy of the horn type system with larger scales of operation is poor compared to systems based on multiple transducers because ultrasonic horns cannot effectively transmit the acoustic energy throughout a large process fluid volume. Typically, these reactors are recommended for laboratory scale characterization studies or for larger scale continuous operations where lower residence times are sufficient to bring about the desired change.

Reactors based on the use of multiple transducers irradiating identical or different frequencies seem to be a logical approach for efficient scale up. The use of multiple transducers also results in lower operating intensities at similar levels of power dissipation, and hence, problems of cavitational blocking, erosion, and particle shedding at the delivery surface are reduced. The position of the transducers can also be easily modified so that the wave patterns generated by the individual transducers overlap, resulting in an acoustic pattern that is spatially uniform and noncoherent above the cavitational threshold throughout the reactor working volume. Arrangements such as triangular pitch in the case of ultrasonic baths, tubular reactors with either two ends irradiated with transducers or one end with a transducer and other with a reflector, parallel plate reactors with each plate irradiated with transducers having identical or different frequencies, and hexagonal flow cells are possible [102, 103]. The schematic representations of some of these novel configurations have been shown in Figure 15.16. The vessels can be operated in a batch mode or, for larger-scale work, in a continuous mode where multiple units can be combined in a sequential manner, which also increases residence time. In

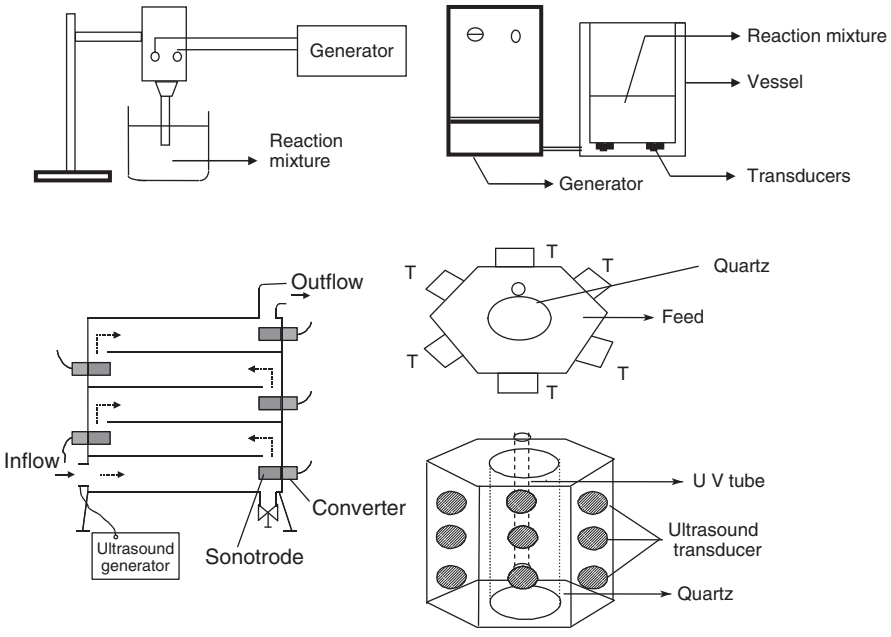

Figure 15.16 Schematic representations of different sonochemical reactor configurations.

A simple ultrasonic horn suitable for laboratory-scale characterization
A ultrasonic bath that can be of variable capacity depending on the number of transducers
A flow loop based on the use of ultrasonic horn, which can be used at a larger scale of operation and in a continuous manner
A hexagonal flow cell with a provision for simultaneous irradiation of ultrasound and ultraviolet.

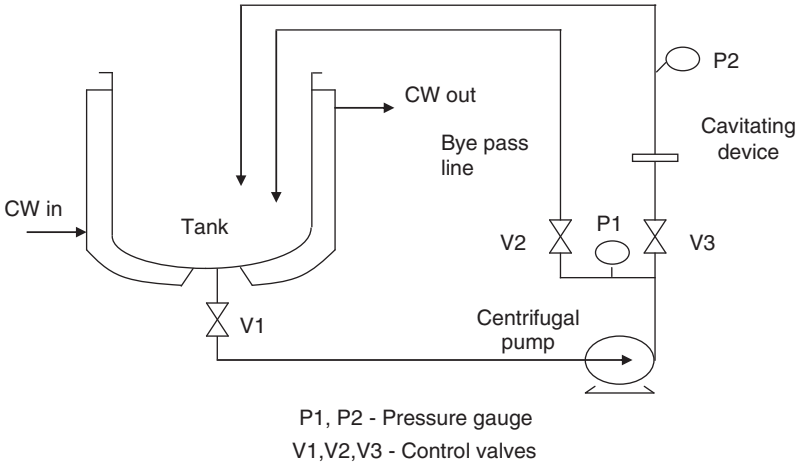

Figure 15.17 Schematic representation of hydrodynamic cavitation setup based on a flow loop housing a cavitation chamber.

The cavitating device can be of different geometries such as orifice, venturi, throttling valve, and so on

Capacity of the tank also can be varied with a corresponding variation in the capacity of centrifugal pump to maintain desired flow rates.

summary, a plurality of low electrical and acoustic power ($1-3\ \mathrm{W\ cm^{-2}}$) transducers produce $25-150\ \mathrm{W\ l^{-1}}$, with an ideal range of $40-80\ \mathrm{W\ l^{-1}}$. The power can be applied continuously or in a pulsed mode.

HC can simply be generated by using a constriction such as an orifice plate, venturi, or throttling valve in a liquid flow [104]. A commonly used device based on HC phenomena is the high pressure or the high speed homogenizer. Cavitating conditions will be generated above a critical pressure or the speed of rotation but these reactors are more energy-consuming options for generation of HC. In reactors based on the use of orifice plates (Figure 15.17), the flow through the main line passes through a constriction where the local velocities suddenly rise due to the reduction in the flow area, resulting in lower pressures that may even decrease to below the vapor pressure of liquid medium. Choosing a correct flow arrangement in the HC reactor is of paramount importance to maximize the effects of cavitation in the most desired and cost-effective manner. The constriction can be a venturi, a single hole or multiple holes in an orifice plate. Use of multiple-hole orifice plates helps to achieve different intensities of cavitation. Additionally, the number of cavitational events generated in the reactor varies. Thus, the orifice plate setup offers tremendous flexibility in terms of the operating (control of the inlet pressure, inlet flow rate, temperature) and geometric conditions (different arrangements of holes on the orifice plates, such as circular, triangular pitch, and so on, and also the geometry of the hole itself, which alters the resultant fluid shear, leading to different cavitational intensities).

15.8.2 Fenton Chemistry

The oxidation system based on the Fenton's reagent (hydrogen peroxide in the presence of a ferrous salt) has been used for the treatment of both organic and inorganic substances under laboratory conditions as well as for the treatment of real effluents from different industries such as chemical manufacturers, refinery and fuel terminals, engine and metal cleaning, and so on [105, 106]. Fenton chemistry can be a useful pretreatment to biological oxidation especially if the pollutant stream consists of biorefractory compounds. Fenton chemistry can convert the biorefractory compounds into easily biodegradable compounds, which can be quantified in terms of the BI. Optimization of the operating parameters for the Fenton oxidation can help in increasing the effectiveness of the overall combined treatment scheme involving Fenton chemistry and biological oxidation.

The mechanism of the oxidation is based on the formation of reactive oxidizing species that are able to efficiently degrade the pollutants present in the wastewater stream but the nature of these oxidizing species is still not well understood. In the literature, three main reactive radical species have been contemplated with two of them involving the presence of hydroxyl radicals (classical Fenton's chemistry) in either "free" or "caged" form, whereas a third oxidant has been postulated to be aquo- or organocomplexes of the high valence iron (Fe^{3+}). The rate of oxidation is strongly dependent on the presence of radical scavengers such as *t*-butanol or carbonate ions but in some cases substantial decrease may not been observed even at high concentrations of these species. This observation has been attributed to the presence of additional oxidant species, which can be confirmed based on the analysis of product distribution and electron paramagnetic resonance (EPR)-spin trapping techniques. It is also important to note here that both hydroxyl as well as ferryl complexes coexist in Fenton's mechanism and depending on the operating conditions (substrate nature, metal-peroxide ratio, presence of scavengers, etc.), one of these mechanisms will be dominant. The oxidation system can be effectively used for the destruction of toxic wastes and nonbiodegradable effluents to render them more suitable for a secondary biological treatment. More information on the basics of Fenton chemistry as well as its application to wastewater treatment can be obtained from open literature [105–107].

The typical reactors used for Fenton oxidation will be mainly stirred reactors as uniform mixing is essentially required. A batch Fenton reactor will essentially consist of a nonpressurized vessel equipped with agitators and metering pumps for acid, base, ferrous sulfate catalyst solution, and industrial strength (35–50%) hydrogen peroxide. It is recommended that the reactor vessel be coated with an acid-resistant material because the Fenton reagent is very aggressive and corrosion can be a serious problem in the operation. The pH of the solution must be adjusted for maintaining the stability of the catalyst as at pH value of 6, usually iron hydroxide is formed. For oxidation of many chemicals, an ideal pH for the Fenton

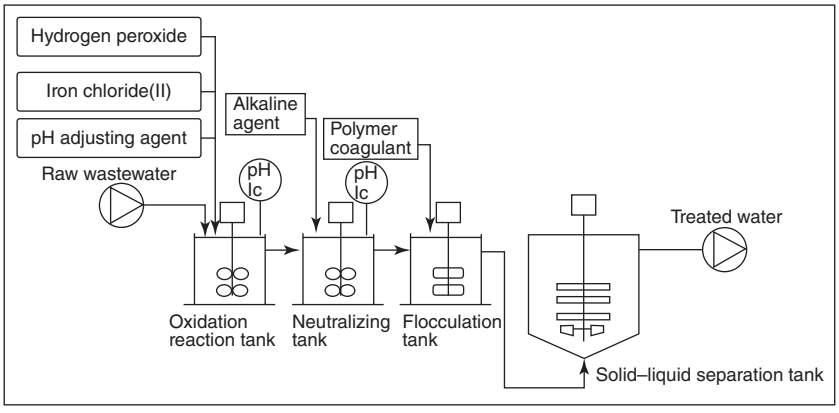

Figure 15.18 Treatment flow sheet for Fenton oxidation.

Typical flow loop for Fenton oxidation
Oxidation reaction tank is used for addition of all the oxidants and proper mixing
Neutralization tank is used for adjusting the pH of effluent stream that is required since Fenton chemistry is best operated under acidic conditions
Flocculation tank is essential for addition of agents that will result in solid agglomeration giving easy separation
Solid liquid separation tank is essential for separation of sludge generated in the process giving clear effluent stream.

reaction is between 3 and 4 and the optimum catalyst to peroxide ratio is usually 1 : 5 wt/wt. As the wastewater compositions are highly changeable depending on the specific facility, a Fenton reactor must need some design considerations to give flexibility in terms of the operating parameters such as the temperature, oxidant ratio, pH and also combination with other oxidation approaches. The discharge from the Fenton reactor has to be typically fed into a neutralizing tank for adjusting the pH of the stream followed by a flocculation tank and a solid – liquid separation tank for adjusting the TDS content of the effluent stream. A schematic representation of the Fenton oxidation treatment has been shown in Figure 15.18.

15.8.3 Ozonation

Ozone is a very powerful oxidizing agent ($E° = +2.07$ V) that can react with most species containing multiple bonds (such as C=C, C=N, N=N, etc.) and can be a useful option for the treatment of complex chemicals. Ozone, however, does not react with compounds containing single bonds such as C–C, C–O, O–H, and so on, at high rates as compared with the ones containing double bonds, which can be attributed to the fact that there is no easy chemical pathway for the oxidation. However, ozone does react with simple oxidizable ions such as S^{2-}, to form oxyanions such as SO_3^{2-} and SO_4^{2-}. These oxidations are simple and the mechanisms only require contact of ozone with the compounds. It is important to understand

that although the thermodynamics for ozone-induced oxidation may be favorable (due to ozone's high reduction potential), kinetic factors will most often dictate whether ozone will oxidize a pollutant in a reasonable time frame. Ozonation is often hampered by higher costs of operation but can help in partial oxidation of the compounds, which can be effectively applied for the case of biorefractory compounds. Similar to the case of Fenton chemistry, the use of ozonation can aid in increasing the biodegradability and hence can be an effective pretreatment to biological oxidation.

A number of devices can be used to transfer the ozone into water such as countercurrent bubble column, packed and plate columns, static mixers, jet reactors, and agitated vessels [108, 109]. Some of the configurations typically used for ozonation have been given in Figure 15.19. As the process is mass transfer limited, ozone transfer efficiency should be maximized by increasing the

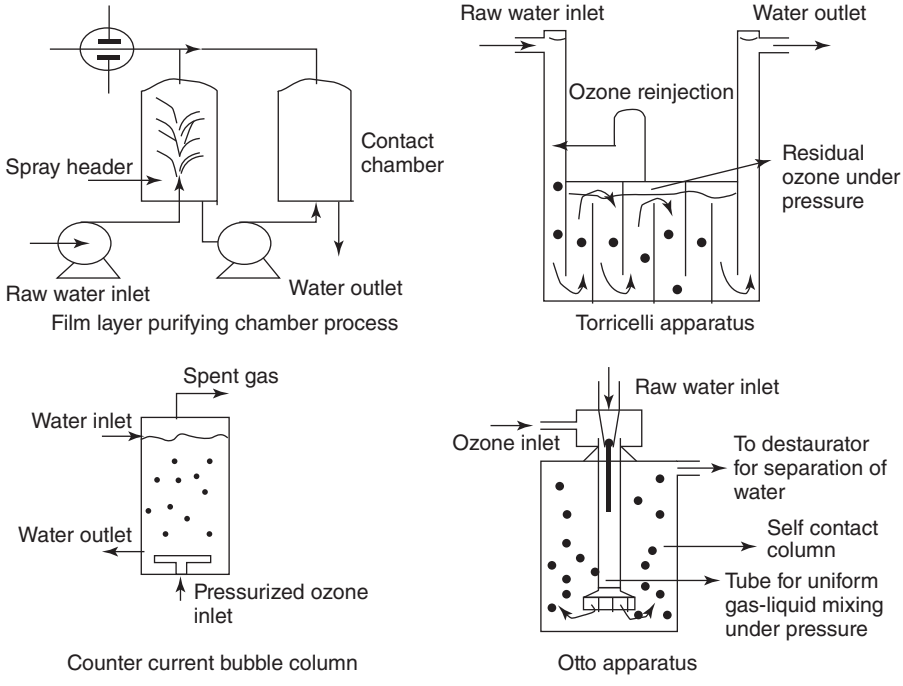

Figure 15.19 Schematic representation of equipments used for ozonation.

Different types of equipments demonstrated based on varying gas introduction options and utilization of the ozone

Film layer purifying chamber process typically works on the principle of generating fine spray of liquid with an objective of increasing the contact area

Torricelli apparatus involves reinjection of gas, which helps in increasing the utilization

Counter current bubble column is the simplest apparatus where the bubble size will depend strongly on the sparger dimensions

Otto apparatus has modified ozone injection facility to give better contact of gas and liquid.

interfacial area for contact, which can be achieved by reducing the bubble size by using small size ozone diffusers such as porous disks, porous glass diffusers, and ceramic membranes, and/or increasing the contact time between the gas and effluent. A major disadvantage of the use of ozone diffusers in the form of porous disks or rods is that the presence of suspended solids and oxidized precipitates may result in plugging of the pores and hence reduce the effective transfer of ozone. Side stream injectors can be used to avoid plugging. This approach also gives additional advantage of operation at higher ratio of gas flow rate to liquid flow rate and higher mixing efficiency without bubble channeling problems. Some drawbacks of side injection are lower contact times, bumping, and corrosion.

Static mixers offer excellent effectiveness for dissolving ozone in water and can also be used at a larger scale. Martin and Galey [110] reported the use of static mixers for the efficient transfer of ozone with studies related to the dependency of mass transfer rates on the operating parameters. It has been established that the mass transfer coefficient increased with a decrease in the water flow rate and increase in the gas flow rate. Although information on the use of static mixers in the ozonation of wastewaters for improving the biodegradability is lacking in the open literature, credence to the effectiveness can be established based on the references dealing with the use of static mixers for water disinfection applications. Because the use of static mixers for actual wastewater treatment applications especially as pretreatment to biological oxidation is lacking, more studies are required in this direction most importantly to calculate the energy requirements as pressure drop will be higher due to the presence of solids and different physical properties of the effluent stream as compared to water containing microorganisms mean different solubility and reactivity for the introduced ozone.

15.8.4 Photocatalysis

The photocatalytic or photochemical degradation processes are also gaining significant importance in the area of wastewater treatment in recent years due to the operation at mild conditions of temperature and pressure. The photo-activated chemical reactions are characterized by a free radical mechanism initiated by the interaction of photons of a proper energy level with the molecules of chemical species present in the solution with or without the presence of the catalyst. The radicals can be easily produced using UV radiation by the homogeneous photochemical degradation of oxidizing compounds such as hydrogen peroxide [111]. An alternative way to obtain free radicals is by the photocatalytic mechanism occurring at the surface of semiconductors (such as titanium dioxide or zinc oxide) and this indeed substantially enhances the rate of generation of free radicals and thereby the rates of degradation [112]. A major advantage of the photocatalytic oxidation based processes is the possibility to effectively use sunlight or near UV light for irradiation, which should result in considerable economic savings especially for large-scale operations and easy integration with the biological oxidation techniques. Various chalcogenides (oxides such as TiO_2, ZnO, ZrO_2, CeO_2, etc.

or sulfides such as CdS, ZnS, etc.) have been used as photocatalysts so far in different studies reported in the literature [112, 113]. As adsorption of pollutants on the catalyst surface is the rate-controlling step, the surface area and the number of active sites offered by the catalyst for the adsorption of pollutants play an important role in deciding the overall rates of degradation. It has been reported [112, 113] that the best photocatalytic performances with maximum quantum yields have been always with titania and also that Degussa P-25 type of titania is the most active form (hybrid mixture of rutile (~70%) and anatase).

The photocatalytic process can be simply carried out in a reactor where the slurry of the fine particles of the solid semiconductor is uniformly dispersed in the liquid phase irradiated with UV light, either directly or indirectly. The proper dispersion of catalyst in the liquid phase can be achieved using mechanical agitation and the design should be such that the liquid flow generation is dominant as compared to turbulence. Aeration should be usually maintained for scavenging the electrons ($HO_2^{\bullet}$ radicals are formed in the valence bonds) to prevent electron/hole charge recombination and this also helps in achieving good dispersion of the catalyst. The extent of dispersion can also be increased by sonication of the slurry at low frequency (e.g., 20 kHz) but the effects should be weighed against increased energy consumption. However, in the case of slurry reactors, the performance of the reactor might be severely affected by the low irradiation efficiency due to the opacity of the slurry. Further, after the oxidation treatment the solid catalyst needs to be separated from the liquid, which is not so easy with small sizes of the catalyst particles, which is important due to the offered higher surface area. Thus, the application of slurry reactors for the photocatalytic treatment on a large scale seems to be quite problematic currently.

An alternative to the use of catalyst in the suspended form is the use of supported photocatalysts. The key advantages would be the stability of the catalyst layer with no problems of separation and the possibility to obtain an active crystalline structure. The production of the supported catalyst and operational stability are the two important factors to be looked at while selecting these types of reactors. Films obtained by wash coating using a suspension of commercial products with a well-known photocatalytic activity gives an active crystalline structure, but can be very sensitive to erosion by the flowing liquid. On the other hand, durable supported films can be obtained by different techniques such as physical and chemical vapor deposition, but here the crystalline structure of the final product and, consequently, the catalytic activity might be difficult to control. Thermal treatment at 500 °C can be used to increase the extent of the crystalline nature of the catalyst obtained by physical or chemical vapor deposition. Scouring is another problem associated with films comprising immobilized powders of TiO_2, and reduced catalyst area to volume ratio of the immobilized photocatalyst due to the problem of scouring is likely to cause mass transfer problems, although this can be avoided using modified fabrication technique (cost to benefit analysis must be performed before final modifications).

Overall, the immobilized or supported catalytic reactors offer many advantages; most important being avoiding the separation problems as the supported catalyst is not allowed to mix with the fluid. Various reactor configurations have

been developed such as TiO_2 coated tubular photoreactor, annular and spiral photoreactors, falling-film photoreactors, and two commercial scale versions are also available (Matrix photocatalytic Inc., Ontario Canada and Purifies Environmental Technologies Inc., London, Ont. Canada). It should be noted that any configuration can be selected but the most important point is to achieve uniform irradiation of the whole active surface.

15.8.5 Overview of Literature Dealing with Combined Processes

After providing the basics about different processes that can be used as a pretreatment for biological oxidation to improve the biodegradability and, hence, the overall effectiveness of the biological oxidation, we now provide some representative overview of the literature for better understanding.

Beltran *et al.* [114] studied the use of ozonation or combined ozonation with hydrogen peroxide/UV light as a pretreatment for the improvement of the biological oxidation of the olive oil industry wastewaters. The reported study is very important as it has dealt with real industrial effluents unlike many studies reporting the use of simulated effluents. It has been reported that individual use of ozone resulted in a decrease in the COD by 50% but there was no significant improvement in the bio-degradability. On the other hand, the use of ozone in combination with hydrogen peroxide (typically at low concentrations) resulted in significant improvement in the bio-degradability as indicated by an increase in the BOD/COD ratio. The observed results can be attributed to the production of enhanced free radicals in the system resulting in oxidation products based on hydroxyl radical attack, which are easily biodegradable. Gulyas *et al.* [115] have also reported that the use of ozone resulted in only marginal enhancement in the biodegradability of real effluent from the paper-mill, whereas Kitis *et al.* [116] reported that the combined ozone/hydrogen peroxide oxidation technique resulted in significant increase in the biodegradability for the case of effluent stream containing ethylene oxide/propylene oxide block copolymer, alkylphenol ethoxylate, linear secondary alcohol ethoxylate, and polypropylene glycol. Ito *et al.* [117] have reported that even ozone/hydrogen peroxide combination technique didn't improve the biodegradability significantly and use of additional oxidant in terms of UV light was required to give best results for the effluents containing trihalomethanes. Thus, it indicates that certain minimum level of selective oxidation has to be carried out in the pretreatment stage so as to really benefit in terms of the enhanced biodegradability from the use of advanced oxidation process. It is also important to note here that addition of chemical oxidants can be detrimental to the microorganisms and, hence, the loadings must be adjusted in such a way that these are consumed completely in the pretreatment. Hence, it is necessary to perform an optimization exercise on the specific effluent under question for deciding on the optimized combination approach as well as the optimum oxidant dose so that only beneficial effects of increased biodegradability are obtained. Ledakowicz and Gonera [118] have performed such optimization exercises for the textile industry effluent considering various

oxidation techniques and reported that $UV/O_3/H_2O_2$ (optimum dose as 1 h UV irradiation with 0.6 g ozone/lit of the effluent and 10 ml of commercial grade hydrogen peroxide per liter of the effluent) is the best oxidation approach. It should be again noted here that these optimum dosages should not be taken as generalized recommendations but are likely to be dependent on the type of the effluent stream in question and other operating conditions. The work of Ledakowicz and Gonera [118] can be referred as an important guideline for the optimization exercise.

Hess *et al.* [119] investigated the approach of combined photocatalytic oxidation and fungal treatment for the degradation of 2,4,6-trinitrotoluene (TNT). It has been reported that even though no significant mineralization, as indicated by change in COD levels, was obtained in the photocatalytic process, there was significant improvement in the biodegradability leading to much better results for the combined treatment approach. The combined treatment scheme was reported to give 32% TNT mineralization as against only 14% for the biological treatment alone and negligible change for the photocatalytic oxidation operated individually. The observed results can be attributed to the fact that there is a considerable rearrangement in the molecules giving the formation of intermediates that are easily oxidizable by the microorganisms. The obtained extent of mineralization (32%) can be easily enhanced by a detailed optimization exercise for the selection of best set of operating parameters (TiO_2 catalyst concentration, operating pH, temperature, and incident light intensity), which can aid in maximizing the oxidation rates in the pretreatment.

Cavitation can also be used as a supplementary technique to conventional biological oxidation with an objective of reducing the toxicity of the effluent or, in other words, to increase the biodegradability. Again, with the use of cavitational reactors, there might not be a significant change in the COD of the effluent but definitely there will be breakage of complex structures into simpler compounds leading to an increase in the overall biodegradability. Mastin *et al.* [120] investigated the approach of combining the cavitation reactors with the biological treatment (constructed wetland type of reactors) for the treatment of chlorinated hydrocarbons such as trichloroethylene (TCE), perchloroethylene (PCE) and petroleum fractions in aqueous solutions. The reactor used in the investigation for the pretreatment was a typical ultrasonic horn with power dissipation of 137 W and operating frequency of 20 kHz. The degradation of TCE using cavitation was reported to be in the range of 40–80% depending on the initial concentration of TCE with the important observation that the residual concentration at the end of cavitation treatment was always below the toxicity levels for the biological oxidation. However, for the petroleum fractions dissolved in aqueous solutions, no measurable change in the TOC was observed due to sonication alone although there was some improvement in the biodegradability. Sangave *et al.* [121] investigated different approaches to increase the overall efficiency of the treatment process of distillery spent wash, which is a significant problem in developing countries like India, using a combination of different treatment techniques. Initially, the effluent samples were subjected to thermal pretreatment characterized as thermally pretreated distillery spent wash (TPT-DW) and anaerobic treatment characterized

as anaerobically pretreated distillery spent wash (ANA-DW). Advanced oxidation techniques based on the use of ultrasound (US) and ozone were then used for further COD reduction/rearrangement of the complex molecules considered as second stage pretreatment to the conventional aerobic oxidation based on the use of mixed microbial consortium. It has been reported that pretreatment of TPT-DW with US and Ozone (as individually operated techniques) enhanced the subsequent aerobic oxidation rate. For US-treated sample, a maximum of 13% COD reduction was attained at the end of 48 h of aerobic oxidation, while for the ozone-treated effluent a maximum of 45.6% COD reduction was obtained as compared to mere 1.8% COD reduction for the control (TPT-DW directly subjected to aerobic oxidation) indicating a 25 times increase in the rate of aerobic biodegradation for the ozonated sample. Comparison indicates that use of ozone as a pretreatment offers better efficacy as compared to the use of ultrasound.

15.8.6
A Typical Case Study of Biodegradability Enhancement of Distillery Wastewater Using Hydrodynamic Cavitation

Distilleries are considered as one of the highly polluting industries worldwide. The wastewater generated from a distillery unit has a typical obnoxious color (dark brown) and has a significant COD (110 000 – 190 000 ppm) as well as the high BOD (50 000 – 60 000 ppm). The quantum of the inorganic substances such as nitrogen, potassium, phosphates, calcium, sulfates, and so on, present in the wastewater is also very high. The conventional anaerobic digestion is not able to successfully treat the effluent as the treated effluent, described as biomethanated distillery wastewater still retains around 40 000 mg l^{-1} of COD and significant color. A major drawback of this treatment approach is that the treated effluent becomes recalcitrant (BI of around 0.14) to further treatment by conventional methods. With development of the stringent rules and effective regulations imposed by governments, distillery industries have been forced to look for more effective treatment technologies. Such technologies should not only be beneficial to the environment, but should also be cost effective as the quantum of effluent generated by the industries is very high.

We now present a case study based on an earlier work [122], where HC was applied as a pretreatment option for the effective treatment of the complex/recalcitrant biomethanated distillery wastewater. The typical setup used for HC reactor consists of a holding tank with a capacity of 15 l, a reciprocating pump of power rating 1.1 kW, flanges to accommodate the cavitating device, control valves, a main line, and a bypass line. The main line consists of a flange that houses the venturi, which is a cavitating device. The liquid flow through the main line is controlled by flow through the bypass line. The suction side of the pump is connected to the bottom of the tank and discharge from the pump branches into two lines (main line and bypass line). The effect of various process parameters such as inlet pressure, dilution, and the reaction time on reduction of COD/TOC, and enhancement in the BI (BOD_5:COD ratio) of the wastewater was investigated. The distillery wastewater treated in a conventional anaerobic

Table 15.1 Characteristics of complex wastewater (biomethanated distillery wastewater) used in the experimental work [122].

Parameters	Value
pH	7.61
Color	Brown
COD (mg l^{-1})	35 000
BOD (mg l^{-1})	5 000
TOC (mg l^{-1})	10 000
Total solids (mg l^{-1})	31 000
Total suspended solids (mg l^{-1})	1 600
Biomass (%)	1
BOD:COD ratio	0.168

Table 15.2 Effect of cavitation pretreatment on biodegradability index of biomethanated distillery wastewater [122].

Reaction condition	Time (min)	COD (mg l^{-1})	BOD (mg l^{-1})	BI: BOD_5/COD
Pressure = 5 bar, no dilution	0	34 391.00	4 853.00	0.14
	50	23 723.00	5 120.00	0.22
	100	23 442.00	5 500.00	0.23
	150	23 302.00	5 500.00	0.24
Pressure = 5 bar, 25% dilution	0	28 208.00	3 666.00	0.13
	50	19 539.00	4 170.00	0.21
	100	18 300.00	4 250.00	0.23
	150	18 163.00	4 500.00	0.25
Pressure = 13 bar, no dilution	0	33 973.00	4 756.00	0.14
	50	24 128.00	5 830.00	0.24
	100	22 325.00	6 400.00	0.29
	150	22 325.00	6 400.00	0.29
Pressure = 13 bar, 25% dilution	0	28 754.00	3 738.00	0.13
	50	18 795.00	6 000.00	0.32
	100	18 374.00	5 910.00	0.32
	150	18 363.00	5 800.00	0.32

digester was obtained from a distillery near Nagpur, India (source not given due to confidentiality issues) as a gift sample. The physicochemical properties of this waste as characterized in the work are given in Table 15.1 [122].

The wastewater was subjected to HC pretreatment for which, 6 l of wastewater was taken in a cavitation reactor. The experiments were conducted at two different inlet pressures 5 bar and 13 bar and at different dilutions of the biomethanated distillery wastewater over the time range of 50 – 150 min. At the end of each predefined time interval, samples were withdrawn from the reactor through a sampling port, centrifuged and analyzed for pH, COD, BOD, and TOC. The obtained results are reproduced in Table 15.2 [122].

From the results presented in Table 15.2, it can be established that HC is capable of reducing the COD and TOC of the wastewater by about 34% and 33%, respectively, at 5 bar inlet pressure and no further improvement is obtained with an increase in the pressure from 5 to 13 bar. It can also be seen from the values of COD and TOC that the dilution has no significant effect on the mineralization of distillery wastewater. Although the percentage reduction is marginally higher at 50% dilution, the net quantum (milligram of COD/TOC per unit volume) of COD and TOC reduction is lower at 25% dilution as compared to the undiluted wastewater. The obtained results in terms of the enhancement in the biodegradability were however different and were found to be dependent on the operating pressure and the levels of dilution. At lower operating pressure (5 bar), the increase in the ratio (BOD/COD) was only marginal under conditions of zero dilution and dilution of 25% confirming that the dilution does not aid BOD enhancement significantly. At higher pressure (13 bar), the increase in the biodegradability ratio was higher as compared to the lower pressures and also the dilution was found to be favorable (final BI of 0.32 for dilution as against a value of 0.29 at zero dilution). From the obtained results, it can be inferred that higher inlet pressure (13 bar) is more suitable for obtaining the enhanced biodegradability (higher BI). The work has clearly confirmed that HC is capable of reducing the toxicity of distillery wastewater as confirmed by enhanced values of biodegradability.

The actual effect of changes in the biodegradability of the cavitationally pretreated wastewater on the subsequent biological oxidation was evaluated by subjecting pretreated effluent to the conventional anaerobic treatment process. The amount of methane generated and reduction in the COD obtained for the cavitationally pretreated sample was compared with that obtained for the untreated wastewater. It has been reported that in the case of cavitationally pretreated sample (13 bar, 25% dilution, 50 min, BI: 0.32), 400 ml of gas volume was generated after a total duration of 40 days (including the lag period of 6 days), along with net COD reduction as 70%. On the other hand, for the untreated system (BI: 0.168), the gas volume was observed to be only 60 ml with around 12% COD reduction under similar experimental conditions.

Overall, it can be established that due to the HC pretreatment, the efficiency of the conventional biological process increased by almost sixfold in terms of COD removal and biogas formation. The study clearly confirmed that the HC is capable of enhancing the efficiency of conventional biological processes in terms of giving enhanced reduction of toxicity as well as increase in biogas generation, along with a significantly net higher reduction in COD and color.

15.8.7 Short Case Study of Intensification of Biological Oxidation Using Acoustic Cavitation/Fenton Chemistry

In a recent study performed by our group [123], the improved biological oxidation process based on the pretreatment using Fenton chemistry or combined approach of Fenton and ultrasound has been demonstrated for the treatment of

wastewater containing benzene, toluene, naphthalene, and *o*-xylene. The work has also investigated the effect of type of sludge in the case of biological oxidation. Ultrasonic horn procured from M/s Dakshin, India, operating at a fixed frequency of 22 kHz with a rated output power of 120 W has been used for the pretreatment based on the use of ultrasound. Fenton oxidation was performed in a glass reactor equipped with stirrer (pitched blade turbine type with standard geometric configuration) at a working volume of 1 l. The subsequent biological treatment was performed in an AS reactor, which is basically a jacketed glass vessel equipped with a pitched blade impeller. The reactor has a maximum capacity of 3 l whereas the working volume used in the work was 2 l. The main objective of the work was to optimize the pretreatment conditions such that maximum beneficial effects can be obtained in the case of biological oxidation. Different approaches such as only H_2O_2, only Ultrasound (US), US/H_2O_2, Fenton and US/Fenton have been used as pretreatment to partially remove BTNXs from wastewater and also obtain the desired change in the biodegradability. The different types of sludge investigated in the work include primary activated sludge (PAS) collected from Common Effluent Treatment facility, AS prepared using a selected strain of *Pseudomonas Putida* obtained as pure culture, and modified prepared activated sludge (MPAS) specifically prepared in the work using the combined cultures obtained from the industrial as well as municipal treatment units.

The optimum operating conditions for the pretreatment have been established as initial pH of 3–3.5, Fe^{2+} dosage of 2.0 g l^{-1} and H_2O_2 dosage of 1.0 g l^{-1}. Approximately, 80–95% reduction in the COD was obtained under optimum conditions and BOD_5/COD (BI) value after pretreatment was found to be in the range of 0.32–0.40 depending on the specific compound investigated in the work. The BI which is a measure of the extent to which a waste is amenable to biodegradation was quantified for the different approaches used in the work. It was observed that the BI ratio increased to about 0.17–0.22 at 20 min treatment and to about 0.30–0.40 at 40 min indicating that the effective change in the complexity of molecules to render them biodegradable was obtained in 40 min. The BOD_5/COD ratio for BTNXs improved from the initial value of 0.176 to final value of 0.37 after US/Fenton/Stirring pretreatment and also the extent of COD removal was the maximum as 84%. It was also reported that the BOD_5/COD value varied over the range of 0.30–0.37 for Fenton and US/Fenton, although longer treatment times and lower extents of COD reduction in the pretreatment have been observed.

In the case of biological treatment, maximum extent of COD removal was obtained for the case of MPAS sludge. Use of MPAS sludge was found to be 20–30% more effective as compared to the pure culture inoculated sludge (AS) as well as PAS, which are more commonly used in biological oxidation. The pretreated samples resulted in higher biomass yield and better conversion ability as compared to the untreated samples being directly subjected to biological treatment. According to the kinetic analysis for the combined approach of pretreatment followed by aerobic oxidation, it was observed that the oxidation rate constant was the maximum for the pretreated effluent for all the compounds

investigated in the work. The maximum oxidation rate constant was obtained for US/Fenton/stirring as the pretreatment and use of MPAS sludge in the aerobic oxidation. The profile for the evolution of biomass confirmed the lag-log-stationary-decay pathway. Overall, the best pretreatment approach among all the different approaches was ultrasound-assisted Fenton process and the work has clearly established the significance of US/Fenton oxidation as pretreatment and the use of mixed culture in the case of aerobic oxidation.

15.8.8 Summary of Pretreatment Approaches

It can be said that the use of advanced oxidation processes in conjunction with biological oxidation has been a successful innovation in the treatment strategies for the wastewater management for both industrial and municipal sectors. The work of Ledakowicz and Gonera [118] have also given the detailed procedure for the optimization of the pretreatment stage and it is important to understand that the degree of benefit that can be obtained would be indeed dependent on the intensity and mechanism of the oxidation processes used as pretreatment. The oxidant dosage must be so adjusted that it is completely utilized in the pretreatment stage itself, as some of the oxidants such as hydrogen peroxide may show toxicity to the microorganisms. It is also important to note that a detailed analysis of the oxidation products must also be done, as sometimes it may happen that the intermediates formed might be more toxic toward the microorganisms. However, the situation may not be that worse always, as the intermediates are usually susceptible to degradation by the advanced oxidation techniques and the effect on the microorganisms will be only felt if they accumulate in large concentrations and have large residence time in the reactor.

15.9 Overall Conclusions

The chapter has presented an elaborate overview on the industrial effluent and municipal wastewater treatment in terms of some well-established and emerging techniques, which are likely to be industrially important and relevant. It clearly defines the basic biological concepts used in various treatments and also the various terms used in the description for characterization of treatment. The general treatment trends have been described along with the regulations for direct comparison. In the first part, the fundamentals of the biological treatments such as aerobic and anaerobic treatments have been presented along with mathematical and conceptual representation. In addition, the discussion about suitability for different types of municipal and industrial wastewaters has been presented. The limitations of the conventional treatment schemes have also been highlighted pressing the need for hybrid treatment schemes.

The second part of the chapter has presented the discussion on the hybrid schemes clearly highlighting the improvements obtained in the biological oxidation schemes. The concept of hybrid schemes is based on exploiting the advantages of the individual treatment approaches and equipments in a synergistic manner leading to beneficial effects in terms of enhanced degradation. The approach can be used either in sequential manner or simultaneously. Some fundamental modifications in the conventional biological treatment options have also been proposed with proven benefits, which can possibly point toward the direction for the adaption of hybrid schemes. The emerging techniques such as cavitation, ozonation, Fenton chemistry, and photocatalysis have been elaborated and their possible successful combinations with the biological oxidation have been discussed. The identification of the operating window for synergism has also been identified and this has been validated with the help of a case study, which clearly confirms the success of the hybrid schemes. Overall, it can be said that properly designed hybrid treatment schemes need to be developed for effective treatment of both industrial and municipal wastewater leading to significant process intensification benefits as well as help in achieving a greener environment.

List of Acronyms/Abbreviations

DTA	direct toxicity assessment
WET	whole effluent toxicity test
TOC	total organic carbon
COD	chemical oxygen demand
BOD	biological oxygen demand
EPA	Environmental Protection Agency
VSS	volatile suspended solids
ASP	activated sludge process
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
RAS	return activated sludge
WAS	waste activated sludge
TDS	total dissolved solids
SVI	sludge volume index
VOCs	volatile organic compounds
DO	dissolved oxygen
SOTR	saturated oxygen transfer rate
OTR	oxygen transfer rate
RBCs	rotating biological contactors
SBCs	submerged biological contactors
PACT	powdered activated carbon treatment
MBRs	membrane bioreactors
BAFs	biological aerated filters
EPSs	extracellular polymeric substances
SRT	solid retention time

HRT	hydraulic retention time
UASB	upflow anaerobic sludge blanket
CSTRs	continuous stirred tank reactors
ABRs	anaerobic baffled reactors
EGSB	expanded granule sludge blanket
UV	ultraviolet
TNT	trinitrotoluene
PCE	perchloroethylene
TCE	trichloroethylene
US	ultrasound
TPT-DW	thermally pretreated distillery spent wash
ANA-DW	anaerobically pretreated distillery spent wash
HC	hydrodynamic cavitation
BI	biodegradability index
EPR	electron paramagnetic resonance
BOD_5	biological oxygen demand over 5 days

List of Variables and Coefficients

r_s	rate of substrate removal ($mg\,l^{-1}\,s^{-1}$)
X_v	concentration of volatile suspended solids (VSS) ($mg\,l^{-1}$)
k	maximum velocity constant
K	half-velocity constant
S	concentration of substrate ($mg\,l^{-1}$)
θ	a constant with a range of 1.0–1.8
k_t	rate coefficients at temperature T
k_{20}	rate coefficients at temperature 20 °C
r_{Xp}	the production of biomass from the substrate removal ($mg\,l^{-1}\,s^{-1}$)
Y	yield factor that represents the mass of biomass produced per mass of substrate consumed
r_{Xc}	rate of decrease of volatile suspended solids due to endogenous decay ($mg\,l^{-1}\,s^{-1}$)
k_c	rate constant
Q_R	the return activated sludge flow rate (m^3 per day)
Q	flow rate, (m^3 per day)
F/M	food to microorganism ratio
BOD	biological oxygen demand ($mg\,l^{-1}$)
MLSS	mixed liquor suspended solids ($mg\,l^{-1}$)
V_a	aeration tank volume (m^3)
Q_w	WAS flow rate (m^3 per day)
S_w	waste activated sludge suspended solids ($mg\,l^{-1}$)
V_c	secondary settlement tank volume (m^3)
S_e	outflow suspended solids ($mg\,l^{-1}$)
SVI	sludge volume index ($ml\,mg^{-1}$)
K_La	oxygen transfer coefficient
T	temperature (°C)

OTRf	actual oxygen transfer rate under field operating conditions ($kg\,O_2/kg\,Wh$)
SOTR	saturated oxygen transfer rate under test conditions at 20 °C ($kg\,O_2/kg\,Wh$)
C_s	oxygen saturation concentration with tap water v ($mg\,l^{-1}$)
C_w	operating oxygen concentration in wastewater ($mg\,l^{-1}$)
C_{s20}	oxygen saturation concentration with tap water at 20 °C
α	ratio of K_La of wastewater and that of tap water
β	ratio of saturation concentration of oxygen at 20 °C for wastewater and that for tap water
θ	temperature correction factor (usually considered as 1.024)
OD	mass of oxygen required ($g\,h^{-1}$)
BODi	inflow BOD ($mg\,l^{-1}$)
BODe	outflow BOD ($mg\,l^{-1}$)
Ammi	inflow ammoniacal nitrogen ($mg\,l^{-1}$)
Amme	outflow ammoniacal nitrogen ($mg\,l^{-1}$)
Ne	outflow nitrate nitrogen ($mg\,l^{-1}$)
E	fraction of BOD removed
w	loading (kg BOD per day)
V_m	total volume of filter media
R_f	recycle factor
p	a weighting factor typically equal to 0.9
R_r	recycle ratio defined as ratio of the recirculation flow to the filter influent flow
C	concentration of the biologically degradable substrate as determined by the BOD test
K_d	rate constant

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16
Treatment of Solid Waste

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Municipal solid waste (MSW) is a mixture of different waste fractions: organic waste (compare box for terminology), recyclables, and residual waste coming either from households or from other establishments that municipalities collect together along with household waste. Depending on many factors (country, climate, population density, urbanization, etc.) this waste mixture has different characteristics and compositions for each municipality. However, with 30–40% (in extremes it can range from 18% to 60%), the biodegradable waste stream is one of the main components of MSW. According to a Working Document from the European Commission, the EU produces 118 to 138 Mt bio-waste yearly of which around 88 Mt originate from MSW and 30–50 Mt from industrial sources. The bio-waste fraction of the MSW consists of two major streams: green waste (from parks, gardens, etc.) containing 50–60% water and more wood (lignocellulosic) and kitchen waste containing no wood and up to 80% water [1].

The most common practice for disposal of MSW in Europe was landfilling. Without any treatment, the biodegradable fraction in landfills leads to leachate production and gas and odor emissions, causing in many cases soil and water pollution and contributing to greenhouse gases. Due to increasing waste amounts, lack of disposal space, shortage of resources, and the environmental constraints arising from untreated biodegradable waste, MSW management systems are moving from landfill-based to resource recovery-based solutions. Biological waste treatment options are an important means of integrated MSW management solutions.

The biological waste treatment options differ not only in the technology applied and its purpose but also in the input material: mixed waste or separately collected biodegradable waste. For the treatment of separately collected biodegradable waste, *composting* and *anaerobic digestion* (AD) can be applied. While *composting* aims for the production of a humus-like product, the main purpose of the AD process is to produce energy in the form of biogas. *Mechanical–Biological Treatment* (MBT) can treat mixed MSW and aims on the one hand to produce inert, stabilized waste to reduce the waste amount to be disposed and save landfill space

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and the recovery of materials on the other. Common to all options is, however, the goal of reducing environmental constraints arising from biodegradable materials.

Agricultural waste is not included in MSW but since it is also biodegradable waste the different possibilities for handling source segregated MSW are also applicable for agricultural waste.

16.1 Biological Treatment of Source Segregated Bio-Waste

Only separately collected organic waste has the quality to produce a good, harmless product, free of contaminants. It is usually not possible to produce a useable compost or digestate out of mixed waste.

16.1.1 Composting

Composting refers to the aerobic biological decomposition of separately collected organic waste to produce a biologically stable product. The processes occurring in composting differ from the natural biological decomposition so far, as in composting the process is controlled to optimize the composting time, emissions, and compost quality. To understand the bio-chemical processes of composting is, therefore, essential to control and adjust the process in the different composting technologies.

16.1.1.1 Composting Process

Organic waste as input material for composting is to a large extent from plant origin [2–5]. Therefore, the main substrates for the biological process are structural components of plants. With 40–70%, cellulose is the major component. Other important plants compounds are: lignin, hemicelluloses, murein, and chitin (detailed information about substrates compounds can be found in [2]). Since the exact composition of the input material cannot be defined, only a general equation for the composting process can be given:

$$\text{Organic matter} + O_2 \Rightarrow CO_2 + H_2O + ([N] + [S] + [P]) + \text{stabilized organic matter} + \text{thermal energy}$$

During the biological process in composting, microorganisms decompose the plant compounds converting them to carbon dioxide and water, mineral salts, compost, and energy. In a well-managed composting system, around 50% of the organic matter is lost as it is transformed into CO_2, H_2O, mineral salts, and energy. This loss of organic matter during the composting process can range from 30% to 60%.

The energy ($-2.875\,\text{kJ mol}^{-1}$) produced by the microbial oxidation of carbon during the process is released as heat. This self-heating occurs spontaneously,

when the mass of organic material is sufficient for insulation, and determines the decomposition speed as usually microbial activity rises with increasing temperature. As it will be explained later in Section "Phases of the Composting Process," this temperature development is essential for the hygienization of the material.

Microorganisms There is a wide array of microorganisms attacking organic waste, most of them acting in a special temperature range. Therefore, the different species are replaced by others in short intervals. Bacteria, fungi, protozoa, and actinomycea are the most common microorganisms during the composting process but also protozoa and algae can be found (further information of the different species of microbial population involved in composting can be found in [2, 3], their metabolism is described in more detail in [4]). From all these organisms, bacteria are usually the major microbial species responsible for the degradation process. Although the bacterial population changes during composting, bacteria are present in large numbers throughout the whole composting process.

Phases of the Composting Process With regard to the temperature and microbial activity development, the composting process is usually divided into four phases: mesophilic phase (25–40 °C), thermophilic phase (35–65 °C), mesophilic phase, and maturation stage. In composting technologies, however, differentiation in only two phases is more common: active composting and curing.

In the *active composting phase* easily degradable components are decomposed quickly, causing high level of biological activity. The activity of these microorganisms generates a high demand of oxygen as well as a rise in temperature within the input material. At optimal moisture, oxygen and nutrient conditions, the temperature can reach to 55–65 °C degrees within a period of only 2 h. The high temperatures produced are important as they sanitize the compost. This process of reduction of human and plants pathogens, as well as elimination of weed seeds and insect larvae is called hygienization. Despite the importance of hygienization, temperatures have to be properly controlled and managed, as they can also result in the dropping of the moisture content below the optimal range (55–65%), impeding microorganisms, and slowing down the composting process. At moisture content lower than 40% the materials dry out. During this phase monitoring is essential to reach hygienization, avoid drying of the material and maintain the running of the process, and reduce odor generation and vectors attraction. The active composting time is dependent upon many factors, such as type of feedstock, the degree of feedstock preparation, the type of composting technology applied, climatic conditions, and the level of operator control and management.

During the *curing phase* slower biological processes take place (conversion of carbon into carbon dioxide and humus, and nitrogen into nitrates). As the more readily degradable materials in the feedstock are consumed, the types of microorganisms in the feedstock change and the overall populations become smaller. For this reason a lower oxygen demand and lower temperatures can be observed, which characterizes the curing step. If the curing takes place outdoors, climatic

conditions, mainly ambient temperatures, affect the level of biological activity. At cold, winter temperatures the microorganisms become dormant, slowing down the curing step. When the compost product is mature and stable, the curing is considered complete. *Stability* measures the biological activity in the compost material. Compost is stable when it is no longer undergoing rapid decomposition, meaning it shows no or only a low level of biological activity. At this stage the compost material can be used as soil conditioner. However, cured compost, as well as "fresh compost" after hygienization can be utilized as soil conditioner. This compost is also rich in nutrients and fresh organic but it has to be taken into account, that fresh compost smells bad and should not be plough in the soil after application. Maturity indicates the degree of phytotoxicity of the compost, which is usually caused by higher levels of ammonia or organic acids.

Factors Affecting the Composting Process There is a wide variety of chemical and physical factors affecting the growth of microorganisms, which are therefore relevant for the planning, operation, and monitoring of composting technologies.

Kind of substrate: Many properties of the compost process and the quality of the compost are determined by the input material used: C:N ratio, moisture content, pH level, interstices volume, and the particle size.

C:N ratio: The supply of carbon (C) relative to nitrogen (N) is an important quality of compost input material, as it is closely related to the speed of the degeneration process. At a good C:N ratio, microbes will use all the N for their own metabolic needs. As a general rule, the C:N ratio should be greater than 20 : 1. At C:N ratio lower than 20 : 1, the microorganisms have surplus N and it can be lost to the atmosphere as ammonia gas, leading to odor problems. Optimum C:N ratio is between 30 : 1 and 40 : 1. To achieve this, waste with narrow C:N-ratio (mostly wastes with low structure) might be mixed with waste with a wide C:N ratio (structure rich material).

Moisture and oxygen/aeration: Both moisture and oxygen supply are needed to keep the microorganisms alive, so both need to be provided in sufficient amounts during the composting process. Initial moisture contents should be 50–65% and water might need to be added during the process. To keep the process aerobic, the oxygen amount in the compost material should be greater than 12%. Moisture and aeration are closely related to each other, which has to be considered when managing the composting process, as aeration reduces the water content in the feedstock, drying up the material. Moisture and aeration are also closely related to the structure of the input material.

pH – level: The pH level of the input material also influences the activity of the microorganisms. For a good biological activity the pH should be between 5.5 and 8.

Structure: The structure of the input material determines its porosity, which is crucial when considering the aeration. For a good aeration and a fast composting process, the particle size should be between 25 and 100 mm.

Temperature: As already mentioned in Section 16.1.2 active composting phase temperature is important for the hygienization of the compost and it has to be

controlled together with the water content, as high temperatures might also dry up the material.

Mixing/agitation: The turning/mixing of the feedstock is important to achieve a better sanitation of the compost. It also breaks up clumps of particles re-establishing porosity and reintroduces air into the feedstock. The optimal agitation frequency is related, however, to temperature as agitation lowers the temperature.

Organic, Biodegradable or Bio-waste, Is It All the Same? From [1]

Although the three terms are often used as synonyms, there are legal differences between them in Europe. While *organic waste* has no legal definition, bio- and biodegradable waste are defined in the European legislation.

The European Waste Framework Directive (2008/98) defines *bio-waste* as "*biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises, and comparable waste from food processing plants.*" It, therefore, does not include forestry or agricultural residues, manure sewage sludge, or other biodegradable waste (natural textiles, paper, or processed wood).

The concept of *biodegradable waste* has a wider meaning. According to the European Landfill Directive (1999/31) biodegradable waste is waste that is capable of undergoing anaerobic or aerobic decomposition, such as food and garden waste and paper and cardboard. Particularly in landfills, biodegradable waste can have negative impacts when improperly managed.

As it can be seen, according to definitions many different organic materials can be counted to bio- and biodegradable waste. In MSW, the two main organic components are food waste from all kinds of origin and green waste. For simplicity reasons we will focus on these two fractions, although the principles and systems from the described technologies may also apply to other organic fractions.

16.1.1.2 Composting Technologies

The treatment capacity of composting plants[1] can vary from less than 1000 to more than 100 000 Mg per annum [4–9]. Depending on the capacity, input material, local conditions, and requirements the layout of the plant will differ significantly. Figure 16.1 shows a simplified layout of a composting plant illustrating the different possible treatment steps. Usually, different types of mechanical treatment (separation, size reduction, turning, mixing, etc.) are applied before, during, or/and after the composting process.

Although all composting technologies make use of the degradation processes described before Section 16.1.1.1, the technologies available differ considerably and range from simpler to more sophisticated systems. With regard to the rotting process the compost technologies concentrate on the active rotting step.

1 Small scale home composting methods are not considered here.

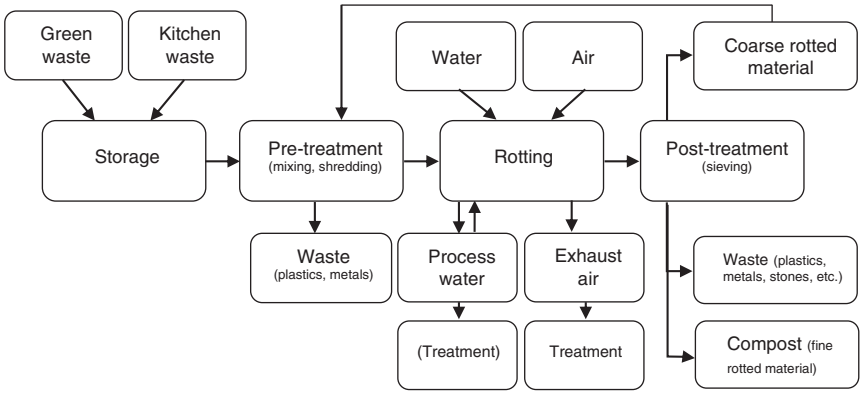

Figure 16.1 Simplified layout of a composting facility.

Common approaches to classify the facilities consider whether the active rotting system is as follows:

- Merely for "green" waste or for bio-waste from segregated collection
- Open, enclosed, or in-vessel
- Passively or actively aerated
- Static, semi-dynamic, or dynamic (mechanical agitation).

The differentiation in open, closed, and in-vessel facilities considers whether the rotting process takes place outdoors, in enclosed buildings or in reactors. The consideration of this aspect is of importance mainly when analyzing emissions (odor, water/leachate, exhaust gases) from the facility. Compared to the open system, the enclosed and in-vessel system achieve shorter retention times (5–10 days), less area requirements, better process and emissions control, as well as high quality and a consistent end product. On the other hand, their costs of construction and operation are higher. The material flow in in-vessel technologies can be as batch or continuous input.

Both the aeration and mechanical agitation of the compost material enhance the air supply in the compost influencing therefore the rotting time. The mechanical agitation describes the movement frequency of the compost material. In static composting systems the compost material is moved discontinuously (once a week or less). In semi-dynamic processes the movement of the material takes place regularly but discontinuously and is more than once per week. In dynamic processes the material is continuously moved, either several times a day or constantly (rotating).

In passive aeration systems, air is supplied to the compost material through natural air movement (gaseous diffusion, convection, and chimney effect). In these systems the porosity of the compost material determines the air exchange rate. To avoid anaerobic zones, mechanical agitation is used in some cases to re-establish the air content within the compost material. Actively aerated systems force air through the compost material. A positive aeration system pushes air from the

bottom to the surface of the pile, where it comes out. In a negative aeration system, air is pulled down from the surface to the base of the pile, where it is captured and treated in a bio-filter to minimize odor emissions. A bidirectional aeration system (combined pressure and suction aeration) combines the positive and negative systems switching between them. Any of the three systems work continuously or intermittently. Continuous operation work with lower air flow rates but it can lower the temperature of the pile so that pathogen destruction is not achieved. For this reason, intermittent operation is more common.

In the following text, the most common active rotting technologies[2] will be briefly described. It should be accounted that after active composting is completed mostly the curing of the compost takes place in open windrow composting.

Windrow Composting Windrow composting implies forming long rows of triangular or trapezoid cross-section, whose width is about twice the height. Usual windrow dimensions are a maximum of 3 m height and 6 m width, depending on the windrow turning machine. The dimensions are important as they should allow the generation of sufficient heat and the diffusion of air to the center of the windrow.

Windrows technology can range from simple open windrow (outdoor) to enclosed systems in a building. A further option is to cover the windrow with a membrane textile; this option is explained in detail in Section "Encapsulation with Semipermeable Membrane Cover." One disadvantage of open systems is the influence of the weather (rain, sun, wind). To minimize runoff and leachate problems from rain water, windrows can be placed under a roof or in a building, which will increase the facility costs. Closed systems can further retain odor arising from the process; however, due to the dimensions of the buildings the exhaust air volumes to be treated are significant. For a proper treatment with biofilters low exhaust air rates should be minimized. Further, windrow systems can be passively or actively aerated. Enclosed systems are usually combined with under floor ventilation. A problem from closed systems and especially of its actively aerated systems is corrosion of the building structure caused by water condensation.

In passively aerated systems, the windrows are turned regularly to introduce air in the pile and increase porosity so that the passive aeration is efficient at all times. At the same time, the turning also mixes the material so that material from the surface moves to the center of the windrow, where microorganisms will convert it into compost. The windrow size and spacing are determined by the equipment used for turning. Typical turning machines are front-end loaders, towered windrow turners, or self-propelled turners (see Figure 16.2). Some turners are also equipped with a watering system. To allow the turning and delivery equipment to work the working surface for the windrows has to be firm.

In Germany, windrows systems used for the active composting phase with a facility capacity higher than 6.500 Mg per annum are usually enclosed systems, so that odor emissions are avoided and exhaust gases are treated in a bio-filter.

2 Names for composting technologies differ slightly in literature, so denominations other than the one proposed here can also be found.

(a) (b)

Figure 16.2 (a) BACHKUS windrow turner [10], (b) BACKHUS Lane turner [10]. (With permission from BACKHUS EcoEngineers.)

Some windrows systems also use active aeration and occasional turning. In English literature, this windrow type is referred as *aerated static pile* and is considered an independent technology system.

Windrow composting is suitable for a wide range of feedstock and facility capacities with low infrastructure requirements. The disadvantages are the relatively long composting times and large area requirements. Further, in open systems the high odor potential is a problem.

Another special type of windrow composting with passive aeration and periodical agitation (semi-dynamic) is the so-called *matts* [4]. To form the matt-windrows, different layers of waste are alternatively put on top of each other, starting at the bottom with bulking materials to provide porosity. To avoid poor air distribution, the selection of the waste materials and the forming of the layers is essential. The bulking materials shall maintain the porosity throughout the entire composting process. Therefore, the system is mainly used for green waste. A system for leachate collection is needed. The composting time ranges from 3 to 4 months for the active phase and for a further 2–3 months for the curing phase. The curing usually takes place in common open windrows with 2–3 turnings.

Extended Beds Extended bed composting systems can be seen as a special form of windrow composting, where the organic material formed to a wide "extended windrow" instead of many narrow ones. In this way, the area requirements can be significantly reduced. Extended beds systems are, therefore, suitable for larger feedstock amounts and are mainly enclosed although open systems are possible. Usually, in enclosed systems, in-floor forced aeration (positive or negative) and agitation are combined. Agitation is performed by special remote controlled turning machines, which usually also water the compost material. Some of these turners can compensate the mass loss (due to the rotting of the material), so that after the turning the windrow reaches its initial height. The composting time in extended beds, range from 45 to 90 days.

Encapsulation with Semipermeable Membrane Cover This technology was developed as an improvement of the open windrow system to enhance operation and reduce odor emissions. Nowadays, the encapsulation with semi-permeable membrane cover is not limited to enclosed windrow composting systems but is also applied in silo designs, as well as in structural designs, such as box and tunnel systems, for composting, stabilization, and biodrying operations. Emission and process control during the biological treatment is accomplished by the semi-permeable behavior of the membrane and the sealing of the cover to the interfaces of the installation in combination with forced aeration. Due to the cover and its sealing function the encapsulated system is considered an enclosed technology.

The membrane is semi-permeable to gases but larger odor-causing molecules (ammonia, hydrogen sulfide, volatile fatty acids) cannot diffuse through the small pores. On the inner side of the membrane, condensation water accumulates, absorbs the odorous compounds, and precipitates back to the windrow. Back in the windrow odorous compounds are further decomposed.

The cover offers other advantages compared to open systems. It prevents rain water from infiltrating the windrow, reducing run-off and leachate problems. In addition, water loss from evaporation is reduced as the cover retains condensation water. Further, the cover reduces vector attraction. Compared to enclosed composting systems in buildings, membrane covers could be economically more favorable, since a building as enclosure is more cost-intensive in the construction, maintenance, and repairs. The life-time of membranes is, however, significantly shorter. Service life of 2–9 years depending on the robustness of the cover as well as on the handling practice have been reported (Personal Communication, W.L. GORE & ASSOCIATES GMBH.).

It is possible to modify open windrow systems into systems covered with membranes. For the aerated windrow composting with membrane cover, common open windrows are formed usually on a concrete pad with ground aeration trenches. Besides structural designs, in which the cover is mounted to a frame, the membrane cover is usually pulled over the windrow by an automatic mechanical winder. At the base of the windrows the membrane cover is sealed to the ground with weight to prevent air from escaping. Positive aeration allows the optimal functioning of the system as no exhaust air collection is then required, since encapsulation with a semi-permeable membrane cover acts as an integrated system combining biological treatment and emission control in one. Aeration is controlled by oxygen and temperature sensors connected to a control computer. The active composting time is 3–8 weeks. The curing can be completed in open windrows.

Channel and Tunnel Composting These enclosed technologies combine forced aeration and agitation. In channel systems, the waste material is piled between walls, which form a rectangular "channel" or lane open to the roof. The walls allow the feedstock to be piled vertically to the sides and ends, so that the space requirements are lower than that for simple windrow composting. The special turning machine moves on rails on top of the channel and works automatically

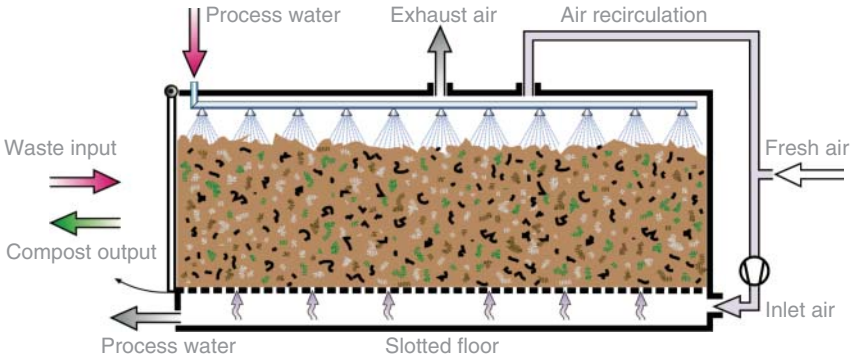

Figure 16.3 Schema of tunnel composting from STRABAG [11]. (With permission from STRABAG Umwelttechnik GMBH.)

(see Figure 16.2). Similarly to windrow composting the agitation of the material breaks up clumps of particles and maintains porosity. Each channel is separately aerated, watered, and periodically turned. Since the channels are placed in a building, corrosion and exhaust air treatment problems are the same as mentioned in closed windrow composting systems.

In tunnel systems (see Figure 16.3), the channels are closed to the top thereby minimizing the exhaust air volume. A further advantage compared to channels is that building damage due to corrosion is reduced, since the processed air is contained within the tunnel. The exhaust air is conducted to and treated in a biofilter. Similarly to channels, aeration, watering, and turning can be regulated separately for each tunnel. Some tunnel systems have only aeration but no agitation components. The composting period depends on the length of the tunnel, turning frequency, and desired compost quality at the end of the process. Common composting periods of commercial systems can range from 2 to 12 weeks. For both systems, channels and tunnels, the produced compost needs further curing.

Box- and Container Composting Box- and container composting are in-vessel, static technologies. The difference between them is the size of the containment where the active composting step takes place. The volume of rotting boxes is 50–60 m^3 while the volume of rotting containers is 20 m^3 and can be transported by a truck. Both systems are actively aerated. They both also have a leachate collection system and treat exhaust gases with a bio-filter. The technologies are modular so that it is easy to expand the capacity by adding a new box or container. However, due to the low capacity of each box/container their suitability for large-scale operations is limited. The curing of the compost after the active phase usually takes place in open windrow composting.

"Brikollare" – Composting Process The "brikollare" composting process is not widely spread. As of 2009, only five plants in Germany were operating with this technology [9]. In the process the shredded waste material is formed to briquettes

(~30 kg each) and stockpiled on palettes inside a closed building, where the static and passively aerated rotting process takes place. The water content of the briquettes varies from 50% to a maximum of 62%. The composting time varies from 3 to 6 weeks depending on the desired compost quality. After the rotting process the briquettes have a water content of approximately 20% and need further treatment and curing [4, 8].

Silo Composting In-vessel vertical silo is a continuous feed, static technology. It consists of a steel vessel, where every day waste material is loaded at the top and composted material is removed from the bottom of the silo. The material takes usually 14 days to move from the top to the bottom. Air is forced in the opposite direction: from the bottom of the silo through the composting mass. Water from the incoming air condenses at the top of the vessel and waters the incoming waste. The compost extracted from the silo after 14 days is cured, often, in a second aerated silo. Since the material is piled vertically, this technology has low area requirements but present compaction, temperature control, and air flow challenges.

Rotary Drum Composting In this in-vessel composting technology, the waste material is continuously agitated (dynamic system) and actively aerated. The rotating drum is slightly inclined so that the waste material tumbles from the upper (infeed) end to the lower (discharge) end. Air is added through the discharge end. As the waste material tumbles, waste and air get mixed. The time the waste spends in the rotating drum is determined by the speed of the rotation and the inclination of the drum. Generally, rotating drums are planned with 1–7 days composting time.

During the composting process in the drum the highly degradable materials are decomposed quickly, but the short composting time is not sufficient for the decomposition of more persistent materials, like lignin. For this reason, a second stage composting is needed for further decomposition.

16.1.1.3 Compost Use and Quality

Compost improves the physical, biological, and chemical properties of soils [12–14]. Therefore, it can be used as a soil conditioner in agriculture and horticulture, as topsoil for landscaping and land restoration or as component of growing media. The regulations and standards for compost use differ significantly across countries. In Europe, some countries have a complex network of regulations controlling the compost use, while others allow the use of compost without any legal directions. Usually, the regulations for compost use include dosage restrictions concerning the permissible quantity of compost (megagram dry matter) at a maximum heavy metal content (compost class) in a certain period of time, and the application as fertilizer considering the maximum nutrient supply to keep soils in a proper condition. These restrictions are mainly intended for application in agriculture as in most other uses, compost is applied infrequently and larger amounts are necessary [12].

In Germany, following amounts of compost are allowed for agricultural use: Class I 20 Mg dry matter in 3 years and compost Class II 30 Mg dry matter in

3 years (Biowaste Ordinance 1998). For nonagricultural use, the amounts of compost range from 10 to 65 Mg dry matter, depending on the use (Soil Protection Ordinance 1999). For fertilizing purposes the application of compost should occur according to good agricultural practice (Fertilizer Ordinance 2003). An overview of compost use regulations in other European countries is given in [12] or in the country reports of the European Compost Network (ECN) [13].

Further, the German Quality Assurance Organization of Compost (Bundergütegemeinschaft Kompost e.V. – BGK) [14] sets the quality parameters for compost. Important parameters include: hygienization, limit content for heavy metals (Pb, Hg, Cd, Ni, Cr,) and copper and zinc, nutrient content (N, P_2O_5, K_2O, MgO, CaO), and impurities contents (glass, plastics, stones).

16.1.1.4 Status of Composting in Europe and Germany

The ECN offers a good overview of the composting activities for most European countries [13]. Based on their own survey, the ECN counted ~30 million ton of source segregated biodegradable waste collected in Europe, half of which are garden waste. These are treated in over 2000 composting sites [15]. Furthermore, 800 small agricultural co-composting plants were identified mainly in Germany and Austria. The latest sites offer a large potential for rural areas in the eastern Member States [12].

The last analysis of the status of the composting technology in Germany [16] shows that the main waste fractions processed are bio-waste and separately collected green waste. The share of green waste decreases from 60% in plants with less than 10 000 ton per annum to 26% in plants with more than 30 000 ton per annum. While composting plants with lower capacities (10 000 ton per annum) are mainly open systems, plants with higher capacities (30 000 ton per annum) use mainly enclosed systems. For the segment between 10 000 and 30 000 ton per annum, the share of open and enclosed systems is equal. The technologies mostly used are windrow, extended windrow, covered windrow, tunnels, and box/container composting.

16.1.2 Anaerobic Digestion

AD uses the anaerobic microbial decomposition to break down separately collected organic waste, resulting in biogas and digestate as main products. AD gained importance in the last decades, mainly because the generated biogas can be used for energy purposes and is, therefore, considered a renewable energy source.

16.1.2.1 Process of Anaerobic Digestion

The process of AD can be used to treat separately collected, solid, organic waste as in composting [4–6, 17–19]. The range of possible input materials is, however, wider compared to the aerobic process, as AD can be also used to handle liquid wastes and sludge. The mixing of solid and liquid materials is also a possibility, which can offer environmental and economic benefits. In contrast to composting,

AD has one main limitation, namely that the AD process is not able to degrade lignin. A further difference is that AD needs thermal energy to achieve the necessary temperatures. A general equation for the AD process looks as follows:

$$\text{Organic matter} \Rightarrow \text{Biogas } (CO_2 + CH_4 + [H_2S] + [NH_3] + \text{trace gases}) + \text{digestate}$$

In AD microbes break down carbon compounds from the organic matter into biogas and digestate (for detailed information on microbes' species see [18]). In a typical AD system, about 50% of the organic matter is broken down. Compared to the aerobic decomposition (composting), AD has (since without oxygen) a lower airflow and lower heat production. To reach and maintain optimal temperatures additional heat is necessary. However, since the produced biogas has more energy than required, the process produces more energy than it consumes. For organic municipal waste, the common biogas production is approximately 100 Nm^3 biogas per Mg of input material. The main components of biogas are methane (CH_4) and carbon dioxide (CO_2), but it can also include hydrogen sulfide (H_2S) and ammonia (NH_3) as well as minor amounts of trace gases, such as siloxanes and different volatile organic compounds (VOCs). The typical composition of biogas varies from 55% to 70% methane, 30% to 45% carbon dioxide, and 200 to 4000 ppm hydrogen sulfide. The share of carbon dioxide to methane depends mainly on the input waste material. To maximize methane production, it is important to have a balanced feed of input materials. Most of the energy from the input material is shifted to the produced biogas as methane. Therefore, due to the methane content, biogas can be used to produce energy. The possible energy uses vary from production of electricity and heat in a combined heat and power unit, to feeding into the gas distribution system after conditioning of the biogas or as alternative fuel for vehicles. Thanks to these energy uses of the biogas, the energy balance of AD technologies is positive compared to composting.

Digestate is the solid or semi-solid material, which remains after the AD process. If the material after the AD process is liquid, it is then called effluent. Digestate is usually composted and used as fertilizer.

Microorganisms and Phases of the Anaerobic Digestion Process The process of AD consists of four successive steps due to the different microbial groups involved. In each step microbes convert the organic material into successive products, which will be used by the following microbes group and result in the production of biogas. The stages of the AD process are listed below (for a more detailed explanation see [4, 19]):

1) *Hydrolysis*: In this first stage, a specific set of microorganisms releases enzymes, which break down the large and complex organic molecules into smaller compounds. This stage works best at lower pH-levels (pH-level below 5) and is usually the limiting step as it is the slower step.
2) *Acidogenesis*: Fermentative microorganisms break down the hydrolyzed compounds into organic alcohols and acids, lowering the pH level in the digester.

3) *Acetogenesis*: In this stage, a new group of acid-tolerant microorganisms ferment further the organic acids and alcohols into shorter molecules (volatile fatty acids and hydrogen).
4) *Methanogenesis*: Strictly anaerobic methanogens microorganisms convert the products of the acetogenesis into biogas. The optimal pH-level for methanogens range from 6.5 to 7.2 (die at pH below 5).

The different microbes involved in each step require a different pH optimum level, making it difficult to design a system with optimal conditions for all microbes.

Factors Affecting the AD Process The main factors and operational conditions, which affect the AD process, are described in the following:

Moisture: Since AD systems have been developed to work at a certain moisture level, moisture is usually one of the most important factors to affect the process. If moisture is not at the proper range for the relevant type of AD system, the process will not work.

pH level: Since the microbes involved have a different pH optimum, one challenge in the AD process is to sustain the pH level within a proper range, so that all microbes can develop. Low pH levels can result when methanogens are inhibited and organic acids accumulate. The methanogen microbes can be even killed, if the pH lowers further.

Temperature/microbes: The technical AD process operates at mesophilic and thermophilic ranges. Depending on the temperatures different microorganisms will be present at the four process steps.

Solids retention time (SRT): SRT is important mainly for continuous systems and can be described as the average time that the solid material remains in the digester. At short SRF the degradation process cannot be completed, so that the full biogas potential from the input material cannot be captured. At too long SRT, the digester volume is not efficiently used.

C : N ratio and ammonia toxicity: Although nitrogen is an important nutrient for microbes in the AD process, too much nitrogen can cause the accumulation of ammonia in the digester, leading to ammonia toxicity and disturbing the AD process. The microbes most sensible to ammonia are methanogens; therefore, ammonia toxicity can disturb the quantity and quality of the biogas produced. To control the nitrogen concentrations, the C:N ratio of the input organic waste should be approximately 30.

Sulfide concentration: High concentrations of sulfide, as H_2S, hinder the AD process, leading to lower methane production. In organic MSW, proteins are typical sources of sulfur compounds.

16.1.2.2 AD Technologies

AD technologies comprise a mechanical pre-treatment stage before the digestion, the digestion itself and a post-treatment of the digestate, as well as the energy use of the biogas, as shown in Figure 16.4 [4, 5, 7, 20, 21]. Generally, the pre-treatment consists in the removal of metals and oversized materials. To enhance

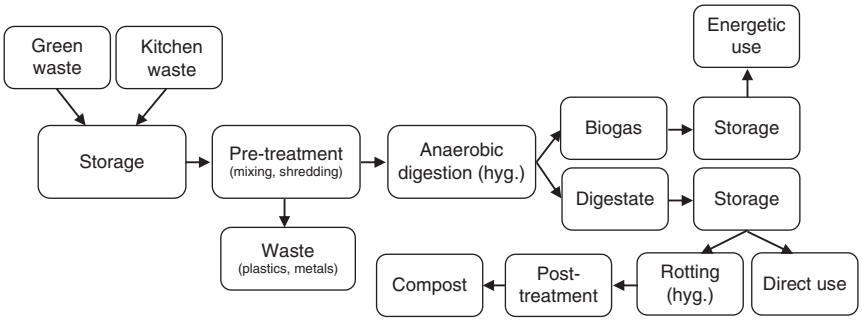

Figure 16.4 Simplified layout of an anaerobic digestion facility.

the digestion process, a more homogenous input material can be achieved by reducing the size of the feedstock (pulping, cutting, drumming, etc.).

There is a wide variety of engineered systems for AD, designed to optimize the biological process in order to produce biogas rich in methane for energy recovery. All technologies are enclosed and have specially designed vertical or horizontal reactors, where the feedstock breach down takes place. In the following text different criteria used to classify AD technologies are presented.

Wet/Dry Digestion The classification between wet and dry AD considers the dry solids content of the substrate. While the dry solids content in wet digestion ranges between 3% and 15%, it can vary from 15% to 45% in dry systems. Although "dry" digestion might suggest that there is no moisture involved in the process, this is not the case. Moreover, in dry digestion moisture is essential for the process.

Since wet digestion deals with materials suspended or dissolved in water, the feedstock material is pumped and the process is carried out in stirred, sealed digester (tanks). The mixing is essential for different reasons. One is to ensure sufficient contact between the microbes and the substrate since the liquid substrate mixture is prone to get split into two or more layers (light materials on top and dense materials on the bottom) leading to lower gas production. Another reason is to make sure that the optimal average solid retention time is maintained. For the mixing, different components can be applied: hydraulic, pneumatic (biogas recirculation), or mechanical stirring devices. Input material such as food waste, which can be easily converted to liquid, is best suitable for wet systems. To achieve the required low dry solid contents usually liquid manure is added.

The dry solid content of the feedstock for dry systems can differ significantly, showing different "structures." The feedstock for dry systems can consequently include materials such as garden waste and crops straw. Compared to wet systems, dry systems are less susceptible to physical contaminants (sands, fibers, etc.). Due to the input material's composition, the feedstock is usually piled in place.

Dry digestion systems can be further divided into different technologies. The two main systems applied for the treatment of source segregated MSW are percolation and plug flow systems. *Percolation technology* (Figure 16.5) uses usually a container or garage digester with a hydraulic gate. The digester is fed either directly

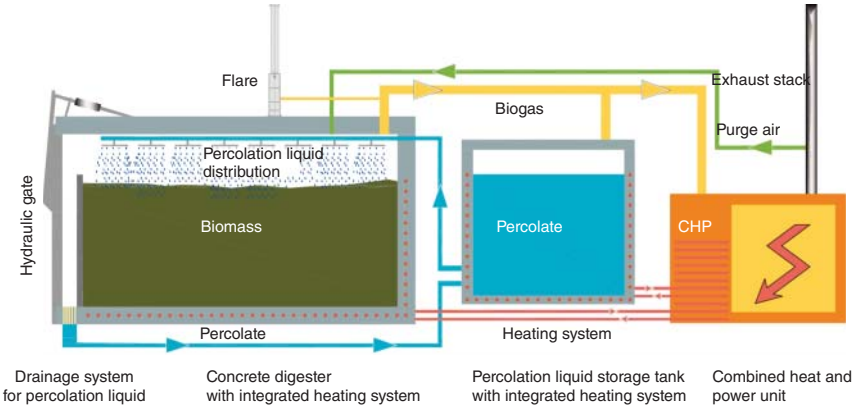

Figure 16.5 Schema of a percolation digester from BEKON [22]. (With permission from BEKON Energy Technologies GmbH & Co. KG.)

with a wheel loader or the feedstock is placed on a wire cage, which is inserted into the digester with a hook lift. The digester is equipped with a heating system and a percolation drainage system. The percolate is stored in a separate heated tank.

To accelerate the biogas production new feedstock is inoculated with digestate from the previous digestion. In the digester, the feedstock is at first aerated to allow the self-heating of the material and achieve the temperatures needed for the AD. After this short aerobic starting phase, the feedstock is irrigated with percolate and the anaerobic phase follows, lasting for 4–8 weeks (retention time). The irrigation can take place periodically or continuously and is stopped a few days before the end of the process to drain the material. Usually, 3 to 4 digesters are operated phase-delayed to reach a uniform gas production.

The acidity and pH-level of the percolate depends on the digestion stage. Therefore, by interchanging the percolate of different digesters at different stages (feeding the acidic percolate of a newly started digester to an already well-running digester, and vice versa, the percolate with low acidity and rich in bacteria from a well-running digester to a newly started one), the acidity at the start of the digestion process can be controlled.

In percolation technologies, the structure of the feedstock is essential to allow the irrigation of the percolate throughout the whole digestion process. The height of the feedstock pile in the digester is, therefore, limited to 2–3 m to avoid the compaction at lower layers due to the static pressure.

In *plug flow technology* (Figure 16.6) a horizontal lying cylindrical or rectangular digester is equipped either with a horizontal agitator or with several transversely located agitators. The electrically driven agitators help mainly the degassing. The direction of the agitation is changed periodically, so that the feedstock movement through the digester is a result of the material fed to the digester and takes place as a plug-flow continuously pushed forward by the fresh material.

The feedstock is usually fed to the digester with an auger system. Continuous flow systems integrate an inoculum loop to achieve rapid distribution of

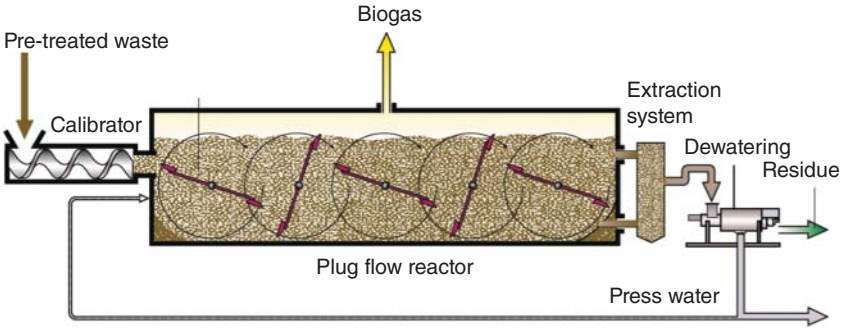

Figure 16.6 Schema of a plug flow digester from STABAG [11]. (With permission from STRABAG Umwelttechnik GMBH.)

microorganisms in the fresh material. The loop recycles a fraction of the digestate (from the end of the plug-flow) and mixes it with fresh feedstock.

At the back end of the digester the substrate is extracted and dewatered. The solid fraction and the excess liquid fraction are directly used as fertilizer or further treated. A part of the liquid fraction is used for the inoculation of the fresh feedstock.

Single- or Two-Stage Systems AD systems for source segregated organic waste can be also designed as single- or two-stage systems. The biological stages of the AD process need different optimal conditions, especially pH ranges. In a single-stage digestion system, all stages occur in the same vessel, at a pH between 6 and 7, which is not the optimal of the involved microbes. Two-stage systems attempt therefore, to optimize the pH for the microorganisms by operating in two different vessels with different conditions thereby dividing the AD stages. In the first vessel, the first two stages (Hydrolysis and Acidogenesis) occur, while in the second vessel conditions are optimized for the Acetogenesis and Methanogenesis. A disadvantage of this division, are higher costs for the construction and operation.

Mesophil or Thermophil Systems There are two possible operating temperature ranges that are favorable for the microbes in technical AD systems: the thermophilic range, with temperatures between 45 and 60 °C and the mesophilic range, with temperatures between 30 and 45 °C. Any AD system can be operated as a thermophilic or mesophilic system. To sanitize the material in mesophilic systems a pasteurization unit is used. At the beginning of technology development, systems were designed as mesophilic as the process was expected to be more stable and less energy was needed for heating. Nowadays, both systems are equally widely used for the treatment of source segregated organic waste.

Continuous or Batch Systems When considering the performance, technical AD can be operated as a continuous or as a batch process. In continuous systems, the substrate is regularly fed into the digester and the digestate is removed, where the

amounts of substrate and digestate are equal. The purpose of this performance is to achieve an approximately continuous production of biogas and a constant biogas quality. In batch systems, the digester is filled with the substrate and closed for the digestion period. Hence, the biogas amounts and quality vary with the time. To reach more continuous qualities and amounts of biogas, parallel batch systems can be used and operated consecutively.

16.1.2.3 Digestate Use and Quality

Due to its highly available N and P fractions, the main use of digestate is as fertilizer [12, 14]. The soil conditioner properties of digestate are lower than that of compost, except for the separated fiber fraction. In Europe, more than 95% of the produced digestate is applied directly in agriculture as liquid fertilizer. In Germany, liquid digestate is mainly used without further treatment as fertilizer in agriculture. From the plants handling organic waste, approximately 10% separate the liquid phase after digestion and produce compost out of the separated fiber. In Europe, most countries regulate the quality and use of digestate through waste or fertilizer legislation and are similar or the same than for compost [12].

As for compost, the German Quality Assurance Organization of Compost (Bundergütegemeinschaft Kompost e.V. – BGK) [14] sets the quality parameters for digestate. Important parameters are similar to that of compost and consider the hygienization of the digestate, limit contents for heavy metals (Pb, Hg, Cd, Ni, Cr) and copper and zinc, nutrient content (N, P_2O_5, K_2O, MgO, S), impurities contents (glass, plastics, stones) and a limit for organic acids content.

16.1.2.4 Status of Anaerobic Digestion in Europe and Germany

In 2010, 195 large AD sites with a capacity of 5 900 000 Mg organic waste were operational in Europe [12, 13, 16]. Additionally, 7500 agricultural digestion and co-digestion sites for agricultural residues, energy crops, and organic waste were operating in the same year. The produced digestate volume was ~56 million m^3 and the electricity production from biogas ~2.5 GW. [12].

In their country reports, the ECN offers a good overview of the number of AD plants in most European countries [13].

According to an analysis of the status of AD technology of 2014/2015, there are 75 AD sites in operation with at least 5000 ton per annum of biowaste as input. These sites have a total capacity of 38 million ton per annum for segregated organic waste [23].

16.2 Mechanical–Biological Treatment of Mixed Municipal Solid Waste

MBT of waste gained importance with the implementation of the European Landfill Directive (Council Directive 99/31/EC of 26 April 1999 on the landfill of waste) on 16 July 1999. The Directive aims to reduce the negative effects on the environment from the landfilling of waste. Among others, the Directive specifies that

biodegradable waste is no longer allowed to be landfilled and requires MSW to be pre-treated prior to its disposal [24]. At present, only waste incineration and MBT technologies are available as pre-treatment options for mixed MSW.

The treatment with MBT technologies implies effective material flow management applying mechanical and biological processes to convert and segregate the municipal waste into suitable outputs and marketable products [25]. Typical outputs are: a stabilized fraction for disposal, materials for recycling, refuse derived fuels (RDF)/solid recovered fuels (SRF) to be used as alternative fuel as well as a minor fraction of contaminated solid rejects and controlled water and gas emissions. Thus, MBT facilities achieve material and energy recovery of waste contributing to a sustainable resource management and reduction of greenhouse gases.

In addition to legal requirements, increasing energy prices are another reason for the growing importance of MBT plants. RDF/SRF has become a significant alternative fuel for coal-fired power plants, the cement industry and in mono-incineration plants (RDF plants) [26].

16.2.1 MBT Technologies

MBT comprises a wide range of technologies designed to handle MSW. The design and technical solution of MBT facilities is determined through the input material and the quality of the desired output. Considering that the waste allowed to be treated in MBT facilities can comprise up to 70 different waste codes,[3] it is clear that plants/operation concepts vary and are difficult to compare. Moreover, if the facility aims the production of alternative fuels, also the fuels quality characteristics are important for the plants layout and designed technical solution.

Despite these facts, MBT facilities can be classified into three main groups: MBT, Mechanical – Biological Stabilization (MBS), and Mechanical – Physical Stabilization (MPS). Their simplified layout is shown in Figure 16.7. The latter class is also denominated as mechanical treatment technology, as there is no biological step involved. However, they are counted as MBT if the facility produces RDF/SRF. As this paper concentrates on the biological treatment, the mechanical steps will not be described in detail (for further information on mechanical treatment possibilities see [25]).

16.2.1.1 MBT – Mechanical–Biological Treatment

MBT is the most common method for the material specific waste treatment and aims to produce a stabilized fraction for landfilling. In these MBT plants, the input waste is separated into different material flows: recycling materials,

3 The List of Waste (LoW) is a reference nomenclature providing a common terminology throughout Europe to improve the efficiency of waste management activities. Assignment of waste codes has a major impact on the transport, installation permits (usually granted for the processing of specific waste codes), decision about recyclability or as a basis for waste statistics. http://ec.europa.eu/environment/waste/framework/list.htm.

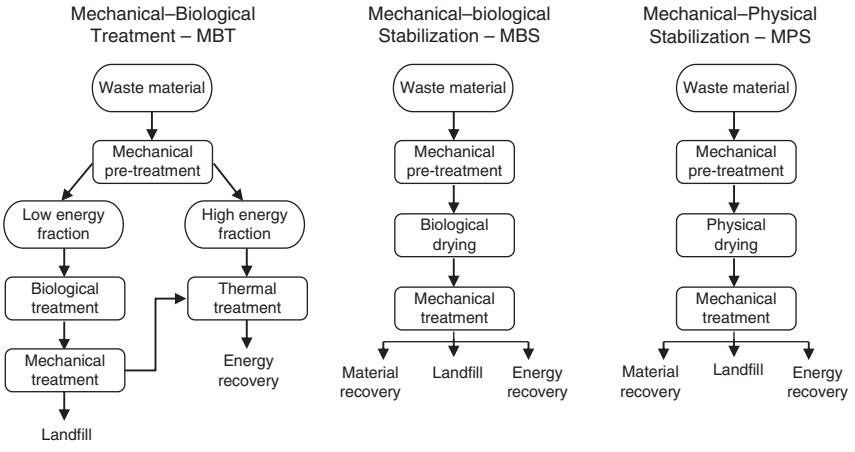

Figure 16.7 Simplified layout of the different MBT technologies.

energy recovery materials, and materials for further biological treatment. In the biological treatment the degradation of organic material takes place in a controlled environment within a matter of months. The same degradation process at a landfill would normally occur in a period of more than 50 years. The biological treatment at an MBT plant, therefore, reduces the potential of producing landfill gas by more than 95% [27].

The two possible processes for biological treatment are either the aerobic rotting process (in tunnels or windrows) or the AD process (dry or wet AD). Both processes, composting and AD, have been explained in detail in Section 16.1. In both cases the final product is a stabilized material, which can be safely disposed in a landfill.

16.2.1.2 MBS – Mechanical–Biological Stabilization

MBS plants are optimized for the production of RDF/SRF. In MBS, the waste is stabilized in a biological drying step prior to mechanical separation. *Biodrying* is a form of composting where the heat produced during the aerobic biological activity is used to dry the waste. Depending on the process concept either the whole waste amount or only the high calorific fraction is treated biologically.

After conditioning, the waste is fed to the biological drying, where it shall primarily loose moisture and achieve low degradation of the organic matter. In this way, most of the biomass content from the waste can be included into the RDF/SRF, which not only enlarges the RDF/SRF quantities, but also reduces the biodegradable material to be landfilled.

In the next mechanical treatment of ferrous and non-ferrous metals, inert materials and impurities are segregated and the high calorific fraction is divided into one or more RDF/SRF with different qualities.

16.2.1.3 MPS – Mechanical–Physical Stabilization

Since this plant concept has no biological step it will be described shortly. In MPS the high calorific fraction of the waste is separated by means of mechanical and physical processes and pre-treated to an RDF/SRF. The pre-treatment comprises the segregation of the low calorific fraction and metals together with a multistage crushing. If necessary, hazardous fractions are separated and the high calorific fraction dried using thermal energy (heat) [28].

16.2.1.4 Status for Germany and Europe

During the middle of 2005, the MBT plants had to establish themselves on the market by fulfilling the high requirements regarding emissions and quality of the products safely and continuously during the operation process. Therefore, over the past years plants with MBT technology have been and are continuously optimized. Starting problems are solved and process concepts have been adapted to the changing frame conditions in the waste market [29].

Between 2005 and 2011 the number of MBT plants in Europe increased by about 60% to more than 330 plants. The treatment capacities grew by almost 70% to 33 million annual Mg. It can be assumed that the number of MBT facilities will increase onto more than 450 plants and the capacity onto 46 million Mg within the next years [26].

From the total number of waste treatment plants with MBT technology the plants mainly aimed at producing a stabilized fraction for landfilling (with composting or digestion) make about 280 plants in Europe and are situated largely in Italy, Germany, Austria, France, and Spain [12].

Germany, Austria, and Italy have the longest tradition in the use of MBT technologies and have, therefore, higher treatment capacities than other European countries. Italy has the highest treatment capacity worldwide [27]. In Germany there are currently about 55 operating MBT plants [30], treating more between 5 and 6 million ton of unsegregated waste [31]. Nearly 30% of the incoming municipal waste is treated bio-mechanically.

MBT-Derived Fuels: Refused Derived Fuels (RDF)/Solid Recovered Fuels (SRF)

There are several terms describing the thermal recoverable waste fraction: solid waste fuels, secondary fuels, substitute fuels, alternative fuels, and so on. The two most established terms are RDF and SRF. While RDF is used for a longer time, it refers only to the high calorific fraction of the waste. SRF was introduced by the technical Committee of the European Committee for Standardization (CEN/TC 343) in 2011 and denotes an alternative fuel that is equipped to meet quality specifications. It is defined as "*solid fuel prepared from non-hazardous waste to be utilized from energy recovery in incineration or co-incineration plants, and meeting the classification and specifications requirements laid down in EN 15359.*"

16.3 Biological Treatment of Agricultural Waste

Agricultural waste is not included in MSW but since it is also biodegradable waste the different possibilities for handling source segregated MSW are also applicable for agricultural waste.

Agricultural waste includes mainly large amounts of crops residues and animal husbandry waste. Basically, both technologies, composting and AD, can be applied to treat these wastes. However, due to the different "structures" of the residues some systems might be more convenient than others. Large amounts of manure for instance are usually treated in wet AD systems. As mentioned in section 16.1.2.4, there were approximately 7500 agricultural digestion and co-digestion sites for agricultural residues, energy crops, and organic waste present in Europe in 2010 [12].

16.4 Conclusion

Biological waste treatment options: composting and ADs as well as mechanical biological treatment are well-established technologies in Germany, producing good quality products (respectively compost, digestate and biogas, RDF/SRF and/or a stabilized material for disposal). However, there are many European countries still landfilling most of their wastes, losing in this way, economic opportunities and causing unnecessary environmental constraints. By increasing the amounts of waste treated biologically in the EU, important amounts of greenhouse gas emissions could be avoided. Compost and digestate, could be applied in agriculture, reducing the amounts of chemical fertilizer or in the case of compost, and could even be used to address the problem of degrading soil quality. Further, biogas produced from organic waste could help increase the share of renewable energy and RDF/SRF could help reduce the use of fossil fuels.

The biological waste treatment technologies have achieved high levels of development in Germany and other European countries. Therefore, the focus is now on the *optimization* of the processes in each technology to improve the energy efficiency, the reduction of emissions, the recovery of materials, and so on. Further, the experiences have to be adapted for other countries, mainly for new EU countries, where waste management is still in the build-up process, and also for developing countries and emerging markets, where waste management is still rudimentary, causing environmental constraints and about 10% to 15% of climate relevant emissions.

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17
Energy Recovery from Organic Waste

Yutaka Nakashimada and Naomichi Nishio

17.1
Advantage of Methane Fermentation for Energy Recovery from Organic Matter

Various biological processes have been investigated to recover energy from organic wastes. A schematic drawing of such processes is shown in Figure 17.1. Organic matter is mainly composed of carbohydrates such as glucose, xylose, starch, and cellulose as well as lipids and proteins. Presently, carbohydrates are considered to be promising feedstock for energy production. Indeed, ethanol, the most common liquid fuel, is produced through alcohol fermentation by yeast directly from sugarcane or from cellulosic biomass via pretreatment. Acetone–butanol–ethanol fermentation has also been applied industrially before the petroleum industry was fully developed. Even hydrogen can be produced from carbohydrates since some strictly and facultative anaerobic bacteria produce hydrogen together with mainly volatile fatty acids (VFAs) such as acetate and butyrate.

Lipids, especially vegetable or animal fats, are expected to be useful as feedstock for biodiesel fuel, although some anaerobic microorganisms can ferment them to fatty acids, mainly acetate. Biodiesel fuels are defined as fatty acid methyl or ethyl esters produced from oils, and they are used as fuels in diesel engines and heating systems [1, 2]. Biodiesel fuels have various advantages such as they are an alternative to petroleum-based fuel, are renewable, have a favorable energy balance, produce lower harmful emissions, and are nontoxic; thus they have attracted much attention recently. Although biodiesel fuels are produced chemically and enzymatically, glycerol is essentially generated as a by-product [3, 4]. An increase in the production of biodiesel fuels would raise the problem of efficiently treating wastes containing glycerol. Some microbes ferment glycerol to produce biofuels and chemicals including hydrogen, ethanol, 1,3-propanediol, 2,3-butanediol, butanol, lactic acid, and succinic acid [5].

On the other hand, biological processes for energy recovery from proteins are restricted. Because they are composed of a variety of amino acids that have

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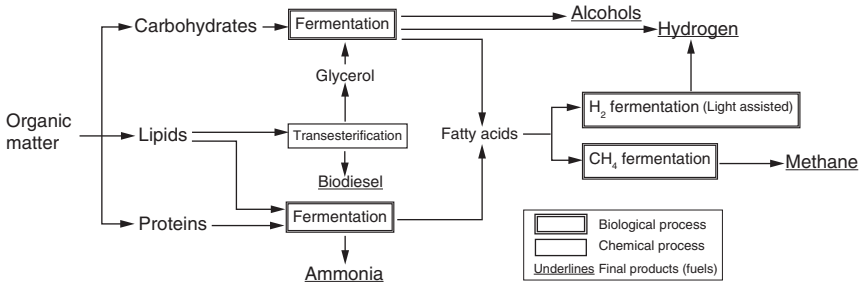


Figure 17.1 Production of various fuels by biological processes of methane fermentation.

different properties, production of the single chemical from them by a single microorganism is usually difficult. However, the use of microbial consortia would overcome this restriction. For most microorganisms, proteins are easily degradable organic matter. When anaerobic bacteria catabolize them as an energy source, they are converted to VFAs with the release of ammonia. Such fatty acids are feasible substrates for methane fermentation to produce methane as a single energy compound.

It has to be noted that carbohydrates, lipids, and even the alcohols produced from them can also be easily converted to fatty acids by many anaerobic bacteria. This implies that all of the main constituents in organic matter are converted to fatty acids, which are substrates for methane fermentation. This is why methane fermentation is a useful process in the wastewater treatment of various kinds of organic matter including food wastes, municipal wastes, and industrial wastes. Furthermore, the end products (methane and carbon dioxide) of methane fermentation are easily removed from wastewater and can be used for energy after the removal of carbon dioxide. Thus, methane fermentation is the most powerful way to recover energy from organic wastes.

17.2 Basic Knowledge of Methane Fermentation of Organic Wastes

The anaerobic microbial degradation of organic matter to methane, which is usually known as "anaerobic digestion" or "methane fermentation," has been used for the treatment of organic wastes and wastewater. In this process, methanogenic microbial consortia anaerobically degrade organic matter to produce methane and carbon dioxide. Methane fermentation is technologically simple, but its metabolism of organic matter to produce methane by microbial consortium is a relatively complex process. This complex microbial consortium leading to methane formation from organic substrates allows it to be applied to many types of organic matter. For example, the specific yields of biogas in relation to the kinds of substances degraded are given in Table 17.1 [6].

Table 17.1 Mean composition and specific yields of biogas in relation to the kind of substances degraded.

Substance	Gas yield (m^3 per kg-TS)	CH_4 content (Vol%)
Carbohydrates	0.79	50
Fats	1.27	68
Proteins	0.70	71
Municipal solid waste (MSW)	0.1–0.2	55–65
Biowaste	0.2–0.3	55–65
Sewage sludge	0.2–0.4	60–70
Manure	0.1–0.3	60–65

Source: Adapted from Ref. [6].

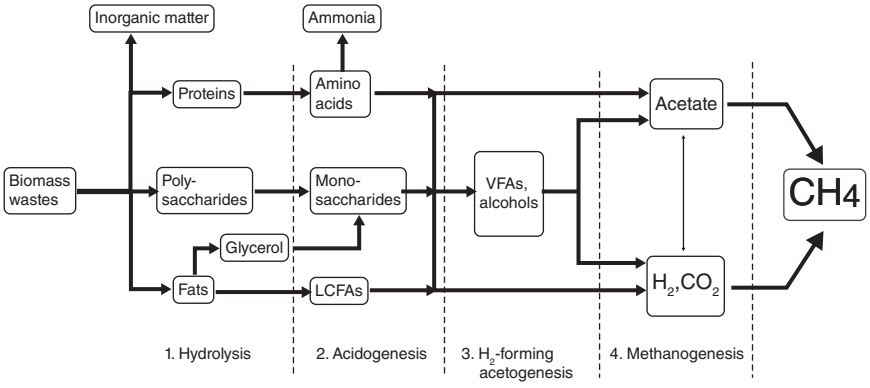


Figure 17.2 Methane production from biomass wastes by multistep reactions. LCFA, long-chain fatty acid; VFA, volatile fatty acid (such as propionate and butyrate).

In the methane fermentation process, a variety of microorganisms play different roles in the decomposition of organic material to produce methane [7]. The roles of the microorganisms in the methane fermentation process can be subdivided into the three phases of hydrolysis/acidogenesis, H_2-forming acetogenesis, and methanogenesis (Figure 17.2).

Hydrolysis is usually an extracellular biological process induced by hydrolytic enzymes (amylase, cellulase, lipase, protease, etc.). To efficiently hydrolyze complex particulate organic matter containing carbohydrates, lipids, and proteins, the matter should be disintegrated into small particulates by means of adequate biological or nonbiological pretreatments, because the extracellular enzymes secreted by hydrolyzing bacteria can attack the reaction sites on the particulates. The disintegration can be induced by nonbiological pretreatments such as physical shearing as well as heat and chemical treatments [8, 9]. Even without any such pretreatment, some degree of particulate organic matter is depolymerized into soluble substrates by extracellular hydrolytic enzymes. These enzymes hydrolyze

carbohydrates to soluble monosaccharides, proteins to amino acids, and lipids to long-chain fatty acids (LCFAs) and glycerol. Several fermentative bacterial groups can be involved in the hydrolysis process [10].

Hydrolyzed substrates are catabolically converted to a number of simpler molecules under anaerobic conditions. Monosaccharide is usually catabolized to reduced products such as lactate, propionate, and ethanol, or oxidized to end products such as acetate and butyrate to adjust the redox balance. H_2 and CO_2 are also produced to discharge excess electrons and carbons, respectively. The reaction is usually called *fermentation*, and it is a typical characteristic of anaerobic heterotrophic microorganisms to produce adenosine triphosphate (ATP), an energy molecule required for the growth and maintenance of cells. Thus, a wide variety of anaerobes participate in acidogenesis.

The degradation of mixed amino acids obtained by the hydrolysis of proteins occurs via two main pathways: one is a coupled reaction of oxidation/reduction with pairs of amino acids, which is called the Stickland reaction [11]; the other is an uncoupled reaction in which a single amino acid is oxidized with protons or carbon dioxide as the external electron acceptor [12]. The end products from the mixed amino acids are fatty acids, mainly acetate and ammonia.

Metabolites such as propionate, butyrate, and ethanol produced via the acidogenesis phase are further oxidized to acetate by H_2-producing acetogenesis. Acetogenesis plays an important role in methane fermentation because it supplies acetate, a direct substrate to acetoclastic methanogens, and avoids the accumulation of VFA that causes a significant drop of pH, resulting in the failure of methane fermentation. However, acetogenesis is a tricky reaction because the oxidation of fatty acids and alcohols is usually an endergonic reaction under standard biological conditions [13]. For example, when propionate is oxidized to H_2, acetate and carbon dioxide, the Gibbs free energy of the reaction ($\Delta G^{0\prime}$) is +76 kJ per reaction under the standard biological conditions (25 °C, 1 atm., pH 7). In the case where butyrate is oxidized, $\Delta G^{0\prime}$ is +48 kJ per reaction. Hence, the reactions do not proceed in the direction of oxidation under standard conditions. However, if the H_2 concentration is kept quite low, the Gibbs free energy becomes negative, and the oxidation of fatty acid proceeds with energy generation for the growth of acetogenic bacteria. The removal of H_2 by hydrogenotrophic methanogens enables the oxidation of VFAs by acetogenic bacteria. This implies an obligate dependence of acetogenic bacteria on methanogens [13, 14].

The substrates that methanogens utilize are very limited. In the industrial process of anaerobic digestion, the usual substrates for methane formation are H_2 and CO_2, or formate, by hydrogenotrophic methanogens, and acetate by acetoclastic methanogens.

All known methanogens are archaea and strict anaerobes. Several hydrogenotrophic methanogens such as *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, *Methanocellales*, and *Methanopyrales* have been isolated and characterized [7]. The removal of H_2 by hydrogenotrophic methanogens is indispensable for syntrophic acetogenesis of higher organic acids to proceed, as mentioned above. On the other hand, the diversity of acetoclastic methanogens,

which is important for the conversion of acetate to methane, is very limited. The acetoclastic methanogens reported so far are *Methanosarcinales*, which is the class including *Methanosarcina* and *Methanosaeta* [7]. *Methanosarcina* can consume a relatively high concentration of acetate and has the ability to use methyl compounds such as methanol. Although *Methanosaeta* can consume a lower concentration of acetate and plays a role in decreasing the chemical oxygen demand (COD) in the anaerobic digestion, since its growth rate is significantly low, the number of *Methanosaeta* cells significantly affects the performance of anaerobic digestion.

17.3 Conventional Methane Fermentation Process

Since anaerobic digestion has been a popular process for the treatment of organic wastes with energy recovery, a variety of processes have been developed. According to the International Energy Association (IEA), anaerobic digestion systems are classified according to operating temperature, mixing method, and percentage of total solids (TS) in the waste (Table 17.2) [15].

For anaerobic digestion, the continuously stirred tank reactor (CSTR) is commonly used with a wide range of TS contents and is applied to treat a variety of organic wastes such as agricultural wastes, food wastes, animal manure, and sewage sludge [16–18]. The contents of the reactor are continuously stirred for complete mixing, which is achieved with mechanical stirring, biogas recirculation, or their combination. Wastes are fed into the reactor continuously or

Table 17.2 Schematic overview of anaerobic digestion process classified by IEA.

TS content	Mixing	Temperature	Commercial plants
Low (~10%) wet system	CSTR[a)]	Thermo	Herning, Vegger
		Meso	Bellaria, DSD-CTA
	Plug flow	Thermo/meso	S-Uhde
	UASB[b)], EGSB[c)]	Thermo/meso	BTA, Paques
Medium (10–25%) semidry process	CSTR	Thermo/meso	WASSA
High (25–40%) dry process	Batch	Thermo	ANM, BioFerm
		Meso	Biocel
	CSTR	Thermo	Snamprogetti
	Intermittent mixing	Meso	Valorga
	Plug flow	Thermo	Dranco, Kompogas
		Meso	Funnell

Source: Adapted from Ref. [15].
a) Continuous stirred tank rector.
b) Upflow anaerobic sludge blanket.
c) Expanded granular sludge blanket.

intermittently, and the same amount of effluent is withdrawn from the reactor. The major issue regarding the anaerobic treatment of wastewater with a CSTR has typically been the extended retention times required to grow methanogens (>10 days) [7] and/or to disintegrate and hydrolyze persistent organic matter such as lignocellulosic biomass [19, 20] and sewage sludge [8]. Hence, the retention time in the reactor is typically in the range of 10–30 days, although it can be varied according to the characteristics of the organic wastes and the operating conditions, such as temperature.

To achieve rapid and effective anaerobic digestion, some processes have been developed, for example, the upflow anaerobic sludge blanket (UASB) [21, 22]. The UASB system is based on its capability to cause the self-granulation (flocculation) of anaerobic microbes associated with methane formation. The UASB system was first constructed by Lettinga *et al.* in the Netherlands [23]. In the system, the wastewater influent enters from the bottom of the reactor. The water flows upward through the blanket of methanogenic sludge, where the organic matter is anaerobically decomposed, and some of the methanogenic bacteria form granules by aggregating in the absence of any support matrix. The produced biogas is separated by a gas–solid–liquid separator and the clarified liquid is discharged as an effluent. The dense, compact granular sludge with high sedimentation properties naturally settles into the bottom, being adverse to the water flow. The upward flow of gas-containing granules through the blanket combined with the return downward flow of degassed granules creates continuous convection, enabling effective contact between the granules and wastewater. There is no energy-consuming mechanical or hydraulic agitation within the reactor. The concentration of granules involving methanogenic microbial consortia is very high compared to conventional continuous treatment processes, as it reaches 50–100 kg VS m^{-3} at the bottom and 5–40 kg VS m^{-3} at the upper part of the reactor [23]. A typical UASB reactor can operate at 10–20 kg COD m^{-3} per day [24]. UASB reactors are typically suited for use in wastewater streams with total suspension solids (TSS) <3% and a particle size <0.75 mm.

The expanded granular sludge blanket (EGSB) reactor is a variant of the UASB concept [25]. Internal mixing in the UASB is suboptimal, since such mixing produces dead space in the reactor that reduces the treatment efficiency. To overcome this problem, the upward flow velocity of the wastewater passing through the sludge bed is accelerated in the EGSB reactor. The increased superficial velocity is gained either by utilizing tall reactors or by incorporating an effluent recycle (or both). The increased superficial velocity (>4 m h^{-1}) causes partial expansion (fluidization) of the granular sludge bed, resulting in better wastewater–sludge contact. Accumulation of flocculent excess sludge between the sludge granules is also prevented. Compared to UASB reactors, EGSB systems can accommodate higher organic loading rates (up to 40 kg COD m^{-3} per day [26]). The EGSB reactor is suitable for low-strength soluble wastewaters (<1–2 g sCOD l^{-1}) or for wastewaters that contain inert or poorly biodegradable suspended particles, which should not be allowed to accumulate in the sludge bed.

Anaerobic fixed-bed reactors (AFBRs) have good potential for treating wastewaters. These reactors are filled with support carriers composed of organic or inorganic materials with high surface areas to immobilize the methanogenic microbial communities on their surfaces [27–29]. Since methanogenic communities not only attach to the surfaces of the media but are also present in the spaces of the media, a high-density microbial population can be retained in the reactor, resulting in low hydraulic retention times, which make the system economical [29] and resistant to organic and hydraulic shock loadings [30]. The selection of a support material is a critical issue in the successful development of the system because microbial adhesion is greatly influenced by the characteristics of the medium [31]. Different natural and artificial solid materials can be used as biofilm carriers. Commercial carrier elements are usually made of organic polymers such as polyethylene, polypropylene, or polyurethane, while natural carriers are essentially made of inorganic materials such as sand, gravel, pumice stone, porous glass beads, and zeolite [32].

17.4 Advanced Methane Fermentation Processes

To achieve rapid and effective anaerobic digestion, several processes have been developed, as mentioned previously. However, to enhance these processes, it is necessary to determine their applicability to recalcitrant organic wastes and wastewaters containing toxic compounds or high-solid organic materials. Although treating such organic wastes is unusual in methane fermentation, several advanced processes have been developed.

17.4.1 Methane Fermentation of Organic Wastes with High Salinity

Acid/alkali pretreated biomass, algal biomass cultured in a marine environment, or wastewaters discharged from the food processing industry may have high salt content, with especially high sodium concentrations. Indeed, it has been reported that the presence of sodium ions at low levels is beneficial for the growth of mesophilic anaerobes and is essential for the growth of methanogens (230–350 mg l^{-1}) because of their role in the oxidation of NADH and in the formation of ATP [33–35]. However, the methane fermentation of organic wastes with high salinity appears to be difficult because the salt tolerance in methane fermentation is usually low, that is, less than 3.0% NaCl (~12 000 mg l^{-1} as Na^+) in conventional systems [36] and less than 1.0% (3900 mg l^{-1} as Na^+) for *Methanosaeta* sp. [37]. McCarty [35] reported that a sodium concentration of 3500–5500 mg l^{-1} moderately inhibited methanogens, and this effect became strong above 8000 mg l^{-1}. Liu and Boone [38] reported an inhibitory effect of sodium on microorganisms participating in the anaerobic digestion of cellulose,

in which it was found that NaCl concentrations up to 0.25 M (~6000 mg l^{-1} as sodium ions) had little effect on the growth rate of propionate-, acetate-, or H_2-CO_2-degrading cultures, but the growth rate of lignocellulose-degrading populations was ~50% inhibited by 0.25 M NaCl. These reports suggested that a sodium concentration of 8000 mg l^{-1} may be critical to induce an inhibitory effect on methane fermentation, although the presence of other ions including potassium, calcium, and magnesium can play an antagonistic role with respect to potential sodium toxicity [39, 40].

Acclimation of the methanogenic sludge to high sodium concentrations over long periods was effective to improve tolerance and reduce the lag phase time [41, 42]. However, the sodium concentration should be stable because anaerobic microorganisms are sensitive to environmental changes [43]. An alternative strategy to overcoming sodium inhibition is to use marine microorganisms. Aspe *et al.* [44] compared marine sediments and fresh pig manure as anaerobic inocula to purify a saline effluent generated by the fishmeal industry during the hydraulic unloading of fish from ships, and found that the marine inoculum adapted better and faster at 37 °C, showing a specific methanogenic activity of 0.065 g CH_4-COD per g-VSS per day. Takeno *et al.* [45] reported methane production during the acidogenic fermentation of marine mud sediment contaminated with organic matter in artificial seawater. In the acidogenic fermentation of the mud sediment, almost all of the fatty acids produced were acetate, which is one of the most favorable substrates for methanogens. Hence, an efficient treatment of the mud sediment was carried out using a semicontinuous, two-stage reactor system, where the culture broth was circulated between acidogenic and methanogenic UASB reactors, yielding ~110 mmol methane from ~280 g wet wt. mud l^{-1} under stable operation at four-day intervals for one treatment period (Figure 17.3). The ability of the methanogenic activity of such marine sediments to treat brown algae was evaluated in detail, which showed higher specific rates of acidogenesis from brown algae, acetogenesis from propionate and butyrate, and hydrogenotrophic and acetoclastic methanogenesis than conventional mesophilic granular methanogenic sludge under the addition of 3% NaCl [46]. The marine sediments were successfully acclimated as methanogenic sludge to treat raw brown algae without dilution by pure water in processes in which ~90% water and 2–3% of salt were involved [47]. These acclimated sediments were successfully applied to the continuous culture for biogas production from nondiluted raw brown algae using a CSTR, in which stale biogas production at a hydraulic retention time of at least 45 days was achieved under saline conditions [48]. These findings imply that the use of marine microorganisms is an effective method of overcoming sodium inhibition.

17.4.2
Methane Fermentation of Nitrogen-Rich Organic Wastes with High Ammonia

Carbohydrates are favorable substrates for anaerobic digestion. On the other hand, proteins, nucleic acids, and urea are sometimes troublesome because the accumulation of ammonia released during their degradation is very toxic for

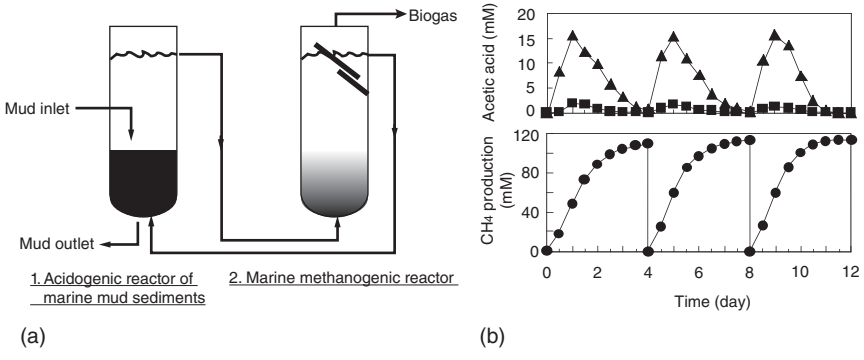


Figure 17.3 (a) Schematic diagram of the two-stage reactor system for methane fermentation of marine mud sediments. (b) Profiles of methane production and acetic acid in acidogenic reactor effluent (closed triangle) and in methanogenic reactor effluent (closed square) during batch treatment of mud sediment in a two-stage UASB reactor system. (Adapted from [45]; with permission ©2001, Springer-Verlag.)

methane production. The toxicity of ammonia varies according to its ionic form. Free ammonia (NH_3) has higher toxicity than the ammonium ion (NH_4^+) [33, 39]. It is speculated that this occurs because NH_3 is passively transported across the cell membrane and subsequently dissociates, leading to a change of intracellular pH to disrupt the cell homeostasis [49]. The inhibition is pH- and temperature-dependent because the relative ratio of NH_3 for total ammonia ($=NH_3 + NH_4^-$) depends on both pH and temperature. At neutral pH, the inhibition of methane production by ammonia is often a significant problem when the concentration of ammonia exceeds a critical level (usually 5000 mg-N l^{-1} in mesophilic and 3000 mg-N l^{-1} in thermophilic conditions) [50–52].

In the case of a wet process, ammonia is diluted to the level at which inhibition does not occur. On the other hand, the dry process is more sensitive to inhibitors, especially ammonia, because a high content of organic materials causes the terrible accumulation of ammonia [53]. Therefore, several techniques have been developed to avoid ammonia inhibition. The acclimation of methanogenic bacteria to high ammonia concentration is effective for maintaining the stable production of methane from an organic solution containing a high concentration of ammonia [51, 54–57]. Co-digestion of organic wastes that contain a high amount of nitrogen compounds and other wastes such as garbage and paper that have a relatively low nitrogen content has been applied to decrease the ammonia concentration to less than the threshold level [58, 59]. The stripping of ammonia from organic wastes may be an efficient method because it is sometimes difficult to collect a sufficient amount of low-nitrogen wastes to maintain the ammonia at less than the threshold level, and because concentrated ammonia can be used in pure ammonia fertilizers [60–62].

The process involves two reactors and an apparatus for ammonia stripping (Figure 17.4a). In the process, high-nitrogen organic wastes are anaerobically digested in the first reactor to release ammonia from nitrogen-containing

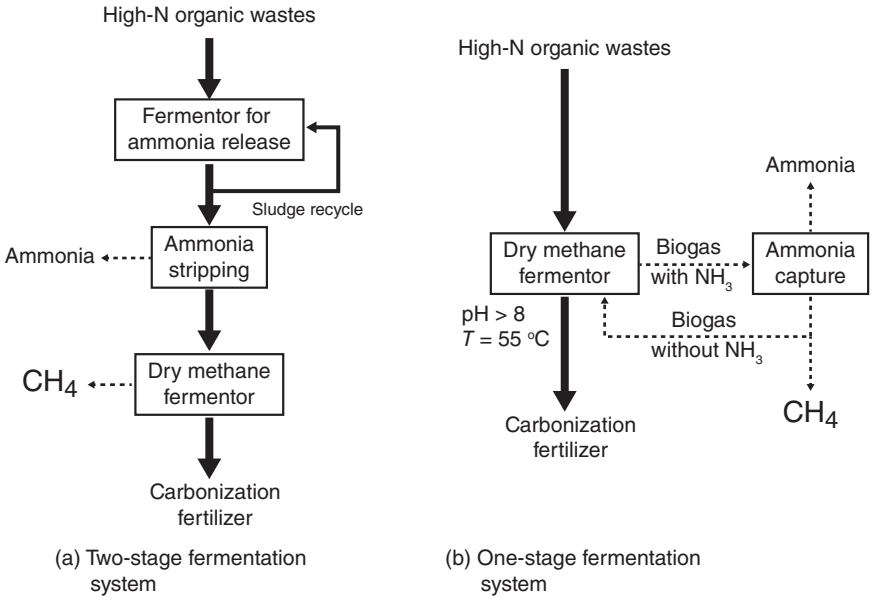


Figure 17.4 Ammonia–methane fermentation process for anaerobic digestion of nitrogen-rich organic wastes.

compounds. The ammonia is removed from the digested sludge by means of ammonia-stripping under alkaline pH and at a temperature higher than 70 °C, and then dry methane fermentation is performed. By using this process, when the raw or ammonia-stripped dehydrated waste activated sludge (DWAS) was fermented to methane under dry conditions (water content = 80%) in the repeated batch mode, only the treated DWAS was fermented to methane sustainably while the treatment of raw DWAS increased the ammonia production up to 7600 mg-N kg^{-1} wet sludge and stopped the methane production [60]. The ammonia–methane two-stage anaerobic digestion demonstrated successful methane production at a sludge retention time (SRT) of 20 days (6.5 g-VS kg^{-1} per day) in semicontinuous operation using a laboratory-scale reactor system, yielding biogas production of 0.58 Nm^3 per kg-VS with an average composition of 64% CH_4. The system can also be applied to model garbage waste (water content = 78%) containing ~6000 mg-N kg^{-1} wet weight of total nitrogen [62] and chicken manure (water content = 75%) containing ~22 000 mg-N kg^{-1} wet weight of total nitrogen [63]. Furthermore, to reduce the cost and the time consumed by the multistep process during dry fermentation, a reactor system equipped with an ammonia-capturing unit for methane production from chicken manure was developed (Figure 17.4b) [61]. At an initial pH of 8 and at 55 °C, 195 and 157 ml per g-VS of methane were successfully produced from the once-ammonia-stripped chicken manure and the mixture of once-ammonia-stripped chicken manure and raw chicken manure in the ratio of 1 : 1, respectively. In this method, the ammonia concentration was maintained at a level <2000 mg-N kg^{-1}

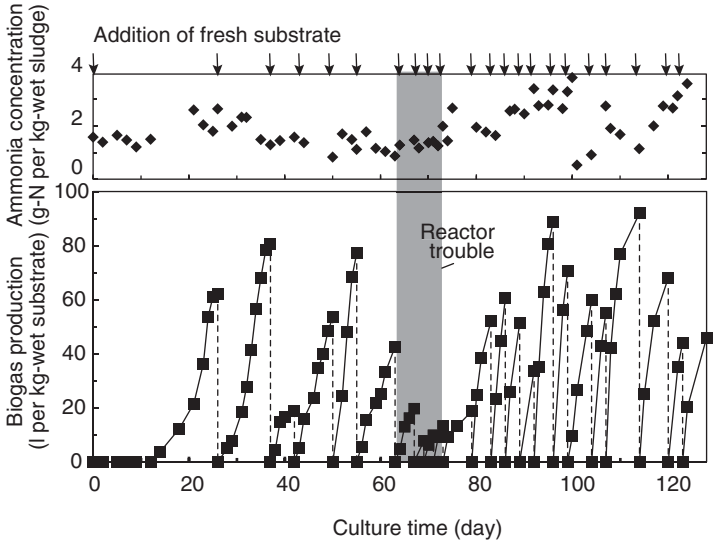


Figure 17.5 Methane fermentation of raw chicken manure by the one-stage reactor process with biogas recycle and ammonia capturing.

wet sludge in the reactor. Furthermore, even when 100% raw chicken manure was applied to the reactor, sustainable methane production was observed (Figure 17.5), resulting in a methane yield of 541 per kg-fresh chicken manure (unpublished data).

These findings demonstrate that ammonia is one of the most significant inhibitory factors in the methane fermentation of organic wastes. Adequate control of the ammonia concentration results in successful energy recovery from organic wastes that have been conventionally considered to be unfeasible for methane fermentation.

17.5
Hydrogen Production from Organic Wastes

17.5.1
Hydrogen Production Combining Methane Fermentation

Hydrogen gas (H_2) is expected to be a future clean energy source, and it can be biologically produced either by photosynthetic microorganisms or by fermentative anaerobes [64]. The enhancement of H_2 production by microorganisms for industrial use is being pursued through extensive research [65]. Hydrogen can be produced in the hydrolysis and acidogenesis stages in a methanogenic ecosystem (Figure 17.2). If high-rate and high-yield hydrogen production is achieved, it is possible for the hydrogen gas produced to be directly connected

to a fuel cell system without the need for any reforming. Furthermore, methane can be produced from fatty acids such as acetate, propionate, and butyrate that accumulate in the liquid broth after hydrogen fermentation. Hence the two-stage fermentation process combining hydrogen and methane production has been intensively researched and reviewed [65–67].

The two-stage process combining hydrogen and methane production exhibits several advantages compared with single-stage methane fermentation. First, the recovery of hydrogen prior to methane fermentation can achieve higher energy recovery for thermodynamic reasons. For example, when 1 mole glucose is applied to hydrogen fermentation and the maximum hydrogen yield is obtained by the complete conversion of substrate to acetate ($C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+$), 89.6 l per mol-glucose of hydrogen is produced, giving 1147 kJ mol^{-1}. Since 2 moles methane are produced from 2 moles acetate, 1779 kJ mol^{-1} of energy should be recovered as methane, where the energy yield is calculated based on the higher heating values of methane (39.7 kJ l^{-1}) and hydrogen (12.8 kJ l^{-1}). Hence, the two-stage process can theoretically yield 2926 kJ mol^{-1}. On the other hand, since 3 moles methane are produced from 1 mole glucose ($C_6H_{12}O_6 + 3H_2O \rightarrow 3CH_4 + 3HCO_3^- + 3H^+$), the energy yield of the single methane fermentation is 2668 kJ mol^{-1}. This simple calculation shows that the theoretical energy yield via the two-stage process is increased by 10% compared with single-stage methane fermentation. Furthermore, the two-stage process is more stable, and the process parameters such as pH, Organic loading rate, and Hydraulic retention time are more easily optimized via the separation of hydrogen and methane fermentation than via the single-stage process [17, 68]. A study by Mitani *et al*. [69] compared the two-stage hydrogen and methane process with the single-stage methanogenic process. In beer production facilities, high-rate methane fermentation processes such as those involving a UASB or EGSB reactor have been used to treat organic wastewater. However, it was difficult to treat the pressed filtrate from the spent malt in the lauter tun using a UASB reactor because it contains a high density of suspended matter, which is unfeasible for use in a UASB reactor. The two-stage process was applied to this pressed filtrate. When the continuous culture for hydrogen fermentation with an isolated *Clostridia* and the following UASB methane fermentation were carried out at 50 and 37 °C, respectively, the total amount of energy recovered as the sum of hydrogen and methane increased to 103 from 90 kJ l^{-1} of that by the single-stage methane fermentation. The result demonstrated effective wastewater treatment without the removal of suspended matter from the pressed filtrate and improvement of energy recovery by connecting the hydrogen fermenter before UASB methane fermentation.

17.5.2 Hydrogen Production by Various Anaerobic Bacteria

As mentioned previously, hydrogen can be generated by fermentative anaerobes in the hydrolysis and acidogenesis phases of anaerobic digestion [65]. Typical genera related to hydrogen fermentation are *Clostridium*, *Enterobacter*, *Escherichia*,

Thermotoga, and *Caldicellulosiruptor* [65, 70]. Among them, the mesophilic strictly anaerobic bacteria of *Clostridium* genus have received a great deal of attention for their production of either solvents (butanol and acetone) or acids (butyrate and acetate) as well as for their production of H_2 [71] because they can ferment hexoses and pentoses to H_2 with a theoretical maximum yield of 4 mol H_2 per mol hexose [72]. In this context, strictly anaerobic thermophilic bacteria such as those of *Thermotoga* and *Caldicellulosiruptor* genera are expected to be high-yielding hydrogen producers because higher temperatures (≥60 °C) are energetically more favorable for biological H_2 production [73], enabling thermophiles to achieve higher H_2 yields than mesophiles. Most of such thermophilic H_2 producers are able to ferment the hexoses and pentoses to H_2 with yields close to the theoretical maximum yield [70, 74].

There have also been multiple reports on H_2 production by facultative anaerobes such as *Enterobacter* [75–78], because they have a high growth rate and several advantageous properties similar to those of other members of the Enterobacteriaceae family. These properties include the ability to utilize a wide range of carbon sources, facultative anaerobicity, and the lack of an inhibitory effect of high H_2 pressure on H_2 generation [77]. However, the theoretical maximum H_2 yield of this bacterium from glucose is only 2 mol per mol hexose and is practically less than 1 mol per mol hexose, which is lower than that of *Clostridium* sp. [79]. *Enterobacter aerogenes* produces 2,3-butanediol (BD), ethanol, and organic acids (lactate, acetate, and formate) in addition to H_2 [80, 81]. Of these metabolites, ethanol, BD, and/or lactate are formed for the reoxidation of reducing equivalents such as NADH to maintain the intracellular redox balance (Figure 17.6). However, since H_2 is produced from formate, which is formed through the metabolism to acetyl-CoA from pyruvate via pyruvate-formate lyase, it is possible to theoretically produce a H_2 yield of 2 mol per mol glucose by stopping the production of BD and lactate. In this context, attempts were made to obtain mutants that had higher H_2 yields than the wild type [78, 82]. Recently, although the metabolic engineering of several microorganisms has been intensively researched for the purpose of enhanced hydrogen production [83, 84], the improvement of the H_2 yield that comes with conventional mutation is still useful because it is difficult to apply genetically engineered microorganisms to the treatment of organic wastes.

Mutants carrying defects in the structural genes for butanediol dehydrogenase (BDDH) and alcohol dehydrogenase (ADH) can be selected by the allyl alcohol (AA) method [85]. In this method, since AA is oxidized by ADH and/or BDDH to a toxic aldehyde (acrolein), mutants deficient in these enzymes can survive. A number of *Saccharomyces cerevisiae* X2180-1A and X2180-1B [86], *Escherichia coli* [87–89], and *Clostridium acetobutylicum* mutants [90] have been isolated by this method. To screen non- or low-acid producers, a proton suicide method [91, 92] is effective. The method is based on the lethal effects of bromine and bromite produced from a mixture of NaBr and $NaBrO_3$ during the production of acids such as lactate and acetate. Double mutation to *E. aerogenes* HU-101 by the AA and proton suicide methods to block the production of both alcoholic and acidic metabolites increased the H_2 yield to 1.17 mol per mol glucose, which is 2.1-fold

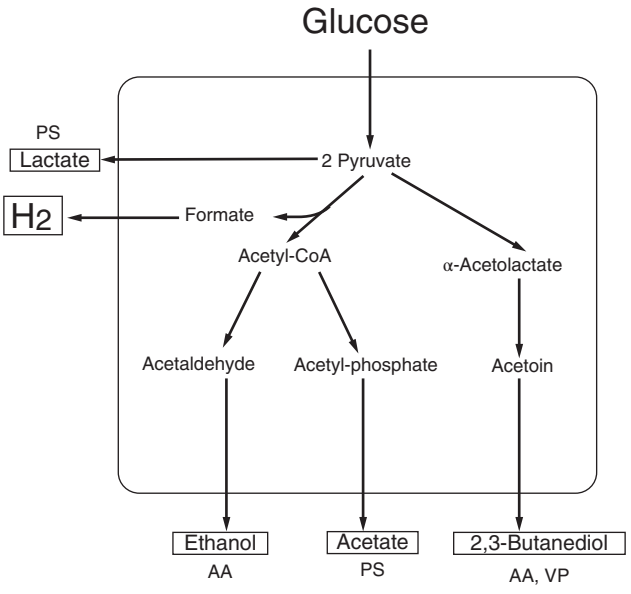


Figure 17.6 Anaerobic catabolism of *Enterobacter aerogenes* HU-101 and screening method applied for increasing hydrogen yield. AA, metabolite reduced by allyl-alcohol method; PS, metabolite reduced by proton-suicide method; VP, metabolite reduced by Voges–Proskauer (VP) test.

higher than that of the wild strain [78]. The mutant from *E. aerogenes* HU-101 with the higher H_2 yield could be screened using the Voges–Proskauer (VP) test [93]. 2,3-Butanediol is produced via α-acetolactate and acetoin through the three enzymatic steps of α-acetolactate synthase, α-acetolactate dehydrogenase, and acetoin reductase. Since acetoin can be detected by the VP test, a negative mutant of this test should be deficient in 2,3-butanediol production. The mutant VP-1 screened with the VP test successfully showed a high H_2 yield of 1.8 mol per mol glucose with decreased lactate and increased succinate yields [94]. Recently, Lu *et al*. [82] reported that the total hydrogen yield of the mutant obtained by atmospheric and room temperature plasma (ARTP) was increased by 26.4% (1.13 mol per mol glucose), mainly dependent on the increase of hydrogen production by the NADH pathway.

17.5.3 Feasible Substrates for Hydrogen Production

H_2 production is affected by various environmental factors such as substrates [95], culture pH [71], electron mediators [96], and the nature of the electron acceptor [97]. To optimize H_2 production, it is important to systematically determine the cellular responses to these environmental conditions. In this context, Nakashimada *et al*. [98] tested the effects of carbohydrates possessing

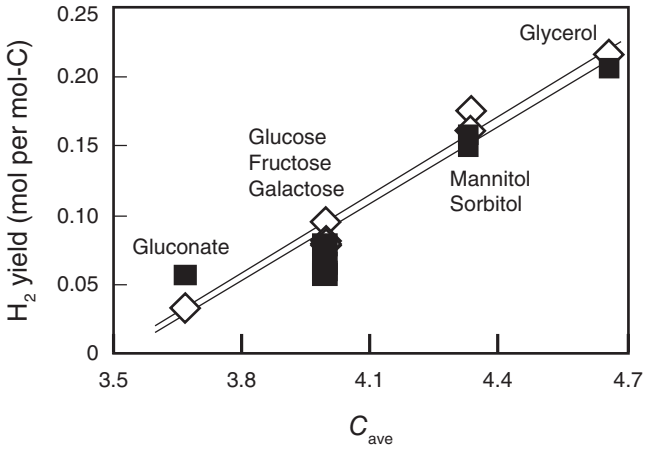


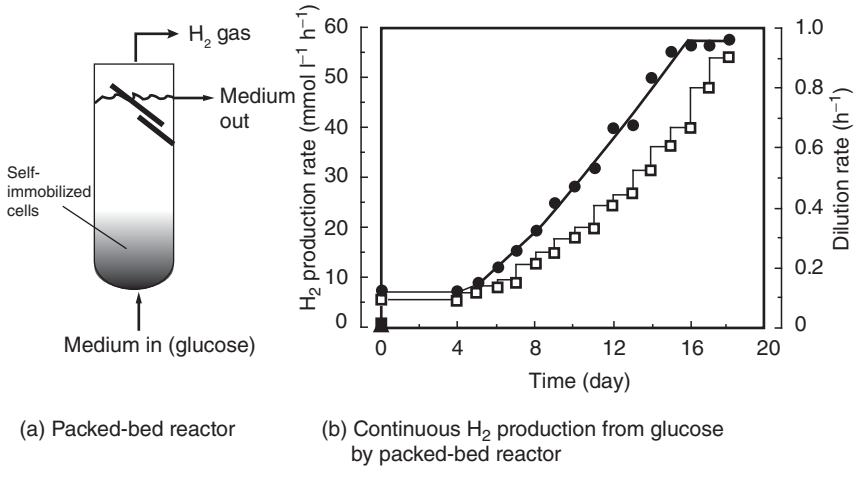
Figure 17.7 The relationship between H_2 yield and C_{ave} in *E. aerogenes* HU-101. Symbols: closed square, hydrogen; open diamond, ethanol. (Adapted from [98]; with permission ©2002 International Association for Hydrogen Energy. Published by Elsevier Ltd.)

various redox levels on the H_2 yield of *E. aerogenes* HU-101 and found a linear correlation between the H_2 yield and redox states of carbon sources, calculated as the available electrons per carbon for each carbon source (C_{ave}) as follows: C_{ave} = (degree of reduction in compound)/(number of carbons in one mole of compound), where the degree of reduction in the compound was calculated as C = 4, O = −2, H = 1. The relationship between H_2 yield and C_{ave} is illustrated in Figure 17.7, demonstrating that carbohydrate with higher C_{ave} obtains higher H_2 yields [98]. This clearly indicated that the redox state of the carbon sources directly affected the H_2 production by *E. aerogenes*, and carbon sources such as glycerol with higher redox states should be used to obtain higher hydrogen production.

Proteins have much lower hydrogen potential compared to carbohydrates. Xiao *et al*. [99] experimentally demonstrated that the maximum hydrogen yields of glucose and peptone as sole carbon sources were ~0.14 ml H_2 per mg glucose and 0.077 ml H_2 per mg protein, respectively, at a neutral initial pH in batch systems inoculated with thermally treated activated sludge.

17.5.4
Bioreactor for High-Rate Hydrogen Production

For industrial use, the rate of H_2 production is as important as the overall H_2 yield. A bioreactor with a high cell density is required, and a variety of reactor systems have been reported and reviewed [100]. For example, *E. aerogenes* HU-101 exhibited strong flocculation in a cylindrical glass column reactor during continuous culture [101]. A packed-bed reactor with immobilized cells has several advantages over a stirred-tank reactor because of its lower energy demands, high cell density



(a) Packed-bed reactor

(b) Continuous H_2 production from glucose by packed-bed reactor

Figure 17.8 Schematic drawing of (a) packed reactor system for self-immobilized cells of *E. aerogens* and (b) continuous H_2 production from glucose. (Adapted from [101]; with permission ©1998, Springer-Verlag, Berlin, Heidelberg.).

per reactor volume, and ease of scale-up due to the simple construction of the reactor. The use of flocculated cells increased the cell density in the reactor since the space utility of the reactor is maximized when no support materials are used. In a continuous culture using glucose as the substrate in the packed-bed reactor (Figure 17.8), cells from the high H_2-producing mutant AY-2 of E. aerogenes [78] began to flocculate and settle to the bottom of the reactor at 4 days after starting the continuous culture. Thereafter, the H_2 evolution rate increased linearly with a stepwise increase in the dilution rate until 0.67 h^{-1}. The flocculated cells continued to accumulate during the continuous culture. The cell density in the packed bed, estimated as mixed liquor volatile suspended solids (MLVSS), was 17 g l^{-1} and the H_2 evolution rate was 58 mmol l^{-1} h^{-1} (1.1 mol-H_2 per mol glucose) at 0.67 h^{-1}, which was nearly 2.0-fold higher than that of the wild strain HU-101.

Biodiesel fuels have attracted a great deal of attention recently because they are an alternative to petroleum-based fuel, are renewable and nontoxic, contribute to a favorable energy balance, and produce less harmful emissions than gasoline. Although biodiesel fuels are produced chemically and enzymatically, glycerol is essentially generated as the by-product [3, 4]. If there is an increase in the production of biodiesel fuels in the world, then the problem of efficiently treating wastes containing glycerol will need to be resolved. Since it was found that glycerol is the best substrate for H_2 production by *E. aerogenes* [98], H_2 production from glycerol-containing wastes discharged after the biodiesel manufacturing process was studied [102]. In continuous culture with a packed-bed reactor using self-immobilized cells, the maximum rate of H_2 production from pure glycerol was 80 mmol l^{-1} h^{-1}, yielding ethanol at 0.8 mol per mol-glycerol, while that from biodiesel wastes was only 30 mmol l^{-1} h^{-1}. However, using porous ceramics as a support material to fix cells in the reactor, the maximum H_2 production rate from

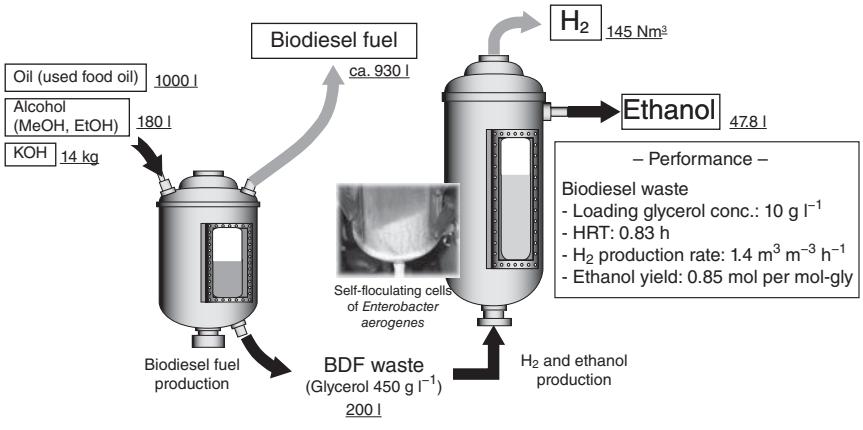


Figure 17.9 Process flow of biodiesel fuel production combined with H_2 and ethanol production from BDF waste containing glycerol. (Adapted from experimental data by Ito *et al*. [102].)

biodiesel wastes reached 63 mmol l^{-1} h^{-1}, obtaining an ethanol yield of 0.85 mol per mol glycerol. This indicates that we can produce biodiesel, H_2, and ethanol from vegetable oils and animal fats or their wastes (Figure 17.9).

17.6 Upgrading of Biogas from Organic Wastes Based on Biological Syngas Platform

There are several advantages to producing methane and H_2 from organic wastes as described above. However, it is seems that the recovery of energy from organic matter is most likely to occur via liquid fuels such as ethanol and butanol rather than gaseous fuels because liquid fuels have several advantages, including their high energy density, ease of transportation, and their high acceptability in our conventional petroleum-based society.

Although nonrenewable resources such as fossil oil, coal, and gas have been crucial to the development and maintenance of industrialized societies, the need for renewable fuels and industrial bulk substrates is escalating because fossil resources are finite and being depleted. Another motivation for the search for renewable energy compounds and technologies is the concern about global warming related to increased CO_2 concentrations in the atmosphere due to the burning of fossil resources. Although the use of biomass including organic wastes has been widely anticipated as a renewable and sustainable resource, the supply of biomass is not sufficient to satisfy the global demand for energy. Moreover, producing biomass does not necessarily decrease the potential for global warming because of the extensive use of fertilizers that can increase nitrous oxide emissions [103, 104]. Although solar, wind, and water power are also significant renewable energies, they are used mainly for generating electricity and not for

producing bulk substrates. New methods, therefore, should be developed for both the production of bulk chemicals and the production of energy from renewable resources that are not restricted to biomass.

One approach to solving these problems is the use of syngas, the mixture gas of H_2 and CO or of H_2 and CO_2, which is a simple and abundant energy source that will be available even after the complete depletion of fossil resources because it can be generated from various kinds of organic matter via thermal or biological decomposition [105, 106] and by the electrolysis of water [107]. If CO_2 can be used as a carbon source, it will contribute to global carbon recycling without the further emission of CO_2. In this context, syngas fermentation using acetogens, a group of anaerobic bacteria that can autotrophically grow not only on H_2 plus CO_2 but also on syngas, has received a great deal of attention [108, 109]. Syngas fermentation is a biological process for producing useful metabolites from the mixture gas of H_2, CO, and CO_2 generated by the gasification of various organic substances. The biological production of fuels and chemicals through syngas fermentation offers several advantages over conventional sugar fermentation technology, because in syngas fermentation, the whole biomass, including non-degradable components such as lignin, via gasification and renewable energies besides biomass such as solar, wind, and waterpower, can be used via their conversion to H_2 [110] (Figure 17.10).

17.6.1 Bioduel Production from Syngas by Acetogens

Typically, acetogens produce mainly acetate from H_2 plus CO_2 or CO via the reductive acetyl-CoA pathway, also referred to as the Wood–Ljungdahl pathway [111]. Briefly, the reductive acetyl-CoA pathway consists of two branches: the methyl branch and the carbonyl branch. These two branches provide reduced, single-carbon molecules that contribute to the formation of acetyl-CoA. In the case of autotrophic growth on CO_2 and H_2, on the methyl branch, a six-electron

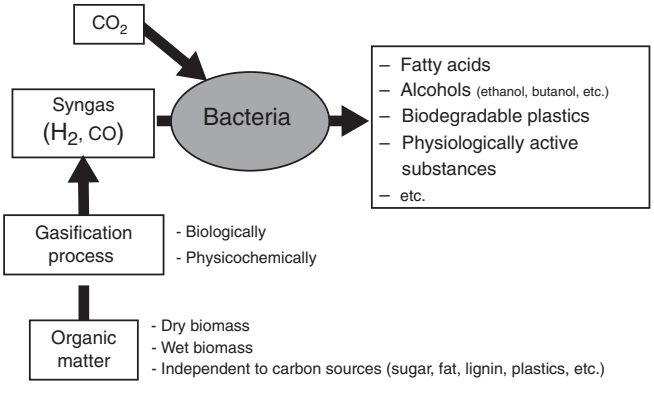


Figure 17.10 Production of biofuels and materials based on a syngas platform.

reduction of CO_2 yields a methyl moiety, while the carbonyl branch reduces CO_2 to CO which is bound to the carbon monoxide dehydrogenase/acetyl-CoA synthase complex. The complex unites the two branches by reacting these products with coenzyme A to yield acetyl-CoA [109]. Although the acetyl-CoA that is produced is mainly assimilated into the cellular biomass or converted to acetate, some of it can be converted to biofuels or chemicals.

A large number of mesophilic and thermophilic acetogens have been isolated so far [111, 112], and the production of biofuels and chemicals including ethanol [113, 114], *n*-propanol [115], *n*-butanol [115, 116], 2,3-butanediol [117], and hexanol [116] by mesophilic acetogens from syngas has been intensively investigated. On the other hand, the use of thermophilic acetogens in a microbial production based on a syngas platform has scarcely been considered, although the physiology has been well characterized [118] and the genome of *Moorella thermoacetica* has already been sequenced [119]. Sakai *et al*. first reported that thermophilic *Moorella* sp. HUC-221, a relative of *M. thermoacetica*, isolated from a mud sample collected from thermophilic groundwater in Japan, produced ethanol in addition to acetate from H_2 plus CO_2 [120]. HUC-221 produced 1.5 mM ethanol from 270 mM H_2 and 130 mM CO_2 in pH-uncontrolled batch cultures, while the total ethanol production reached 15.4 mmol l^{-1} in a cell-recycled repeated batch culture with pH-controlled at 5.8 [121]. If thermophilic acetogens can be applied for the production of alcohols such as ethanol or propanol from syngas, the recovery of the alcohols will be facilitated because their boiling temperature is close to that of the culture broth, thereby enabling the continuous distillation of alcohols. Furthermore, thermophilic bacteria have higher growth and metabolic rates than mesophilic bacteria, and the risk of microbial contamination is lower [122, 123]. In this context, thermophilic acetogens should be promising candidates for H_2 plus CO_2 or syngas fermentation compared to mesophilic bacteria.

17.6.2
Development of Genetic Engineering Tools of Acetogens

Genetic manipulation of the metabolic pathway by molecular biological techniques is a powerful method for the efficient production of a target product from syngas and H_2–CO_2. Recently, there has been remarkable progress in the development of genetic systems for acetogens. Protocols for transformation with plasmids constructed for *C. acetobutylicum* [124] and gene deletion via double crossover homologous recombination were developed for *Clostridium ljungdahlii* [125, 126]. A uracil-auxotrophic mutant (dpyrF) of thermophilic *M. thermoacetica* was constructed, allowing for both the positive and counter-selection of desired recombinants [127] by the disruption of *pyrF* for orotate monophosphate decarboxylase (PyrF), where it was effective in avoiding the decomposition of the introduced plasmids by the restriction–modification system according to the method reported by Yasui *et al.* [128]. For multiple gene manipulation and the direct transformation of an alternative strain that is desired for use as the host strain, a transformation system for *M. thermoacetica* ATCC39073 was developed

[129] using a thermostable kanamycin-resistant gene (*kanR*) derived from the plasmid pJH1 harbored by *Streptococcus faecalis* [130]. The tools can be applied to alternative strains of *Moorella* spp. The procedure of genetic manipulation could be used for two new thermophilic acetogen strains, Y72 and Y73, which were closely related to *M. thermoacetica* ATCC39073, isolated from a soil sample [131]. The transformation efficiency of strains Y72 and Y73 was 20-fold higher than that of strain ATCC39073. The reason for the higher transformation efficiency of Y72 compared to that of ATCC 39073 may be because Y72 possesses only two sets of genes considered to be involved in a restriction–modification system, which is half the number found in ATCC39073 [132].

The combination of metabolic engineering and synthetic biology approaches can be applied to accelerate the development of syngas fermentation processes by using such genetic tools and the recent advances in whole-genome sequencing [109]. For example, the deletion of genes for the putative bifunctional aldehyde/alcohol dehydrogenases from the *C. ljungdahlii* genome resulted in a significant decrease of ethanol production with the increase of acetate [126]. The introduction of plasmids bearing heterologous genes for the butanol synthesis pathway into *C. ljungdahlii* allowed transient butanol production [125]. The introduction of the functional lactate dehydrogenase gene (*T-ldh*) into *M. thermoacetica* dpyrF successfully transcribed *T-ldh* and exhibited higher LDH activity than ATCC39073 and dpyrF, yielding 6.8 mM of lactate from fructose. Berzin *et al*. [133] demonstrated that the complete elimination of acetate production by the deletion of the phosphotransacetylase gene and overexpression of synthetic alcohol dehydrogenase in *Clostridium* sp. MT653 resulted in a significant increase of the ethanol yield to 590 mM from a syngas blend mixture of 64% CO and 36% H_2 with no acetate production from 250 mM ethanol of the wild strain. The same group also reported the production of several biofuels and chemicals from syngas, including acetone [134], butanol [135], mevalonate [136], methanol, and formate [137], by a similar strategy, suggesting that the elimination of acetate production is effective in improving the production of the target end products. However, the elimination of acetate synthesis may prevent the concurrent synthesis of ATP via acetate kinase, and the impact on the growth energetics is not yet fully understood [126].

Significant growth in the research on and metabolic engineering of acetogens can be expected to improve the use of syngas or H_2/CO_2 obtained from biological and fossil organic wastes. Such research could also improve the production of both biofuels and commodity chemicals using technologies that are currently available.

17.7 Conclusions

In this chapter, we introduced the achievements that have been made thus far in the recovery of biological energy from organic wastes. The development of technologies for the recovery of energy from organic wastes is becoming an ever-more

important challenge due to the eventual depletion of fossil fuel resources and global warming. In the above context, biogas fermentation including methane is a timely technology because of its applicability to various types of organic waste, not simply wastes containing carbohydrates as in alcohol fermentation, and because of its role in the treatment of organic wastes. Furthermore, the present chapter described not only biogas production but also strategies for the upgrading of biogas to more useful biofuels and biomaterials by biological methods involving acetogens present in the ecosystem of methane fermentation. If the bioconversion of biogas is realized, a shift from a sugar-based to a syngas-based bioindustry for the production of desired fuels and chemicals from organic matter could occur. The studies reviewed in this chapter represent a few examples demonstrating that the ecosystem of methane fermentation is potentially extremely valuable as a biological resource. The physiology of anaerobic microorganisms is still unclear, but achieving a deep understanding of the physiology and metabolism of anaerobic bacteria will make a significant contribution to environmental protection and the welfare of humanity.

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18
Microbial Removal and Recovery of Metals from Wastewater

Michihiko Ike, Mitsuo Yamashita, and Masashi Kuroda

Our current high standard of living is supported by the extensive use of various metallic elements, not only typical metals such as iron (Fe) but also metalloids such as arsenic (As) and antimony (Sb) and nonmetals such as selenium (Se). In particular, rare metals and rare earth elements have recently become fundamental to the production of novel materials supplied to high-tech industries, and demand for them is growing. In this chapter, the term "metals" will be broadly defined as all the metallic elements mentioned earlier, including radionuclides. Mass consumption of metals increases the chance of their emission into the environment. In fact, based on PRTR (Pollutant Release and Transfer Registration, Japan) data [1], considerable amounts of various metals are moved into the water phase, including wastewater, in the course of their mining, refining, processing, use, and wastage, which may finally lead to their emission into the aquatic environment. For example, 745 tons (t) of manganese and 97 t of molybdenum were discharged into the aquatic environment in 2012 in Japan [1]. Emission of metals into the aquatic environment can cause severe environmental problems, for example, adverse effects of mercury (Hg) on human health and ecosystems, such as the Minamata tragedy [2]. Emission of metals into the environment is also recognized as a loss of valuable resources, leading to their depletion in future. Therefore, it is important to efficiently remove metals from wastewater and recover them as recyclable resources before they are discharged into the aquatic environment, for both environmental protection and sustainable use of metals. However, it is a tremendous challenge to remove/recover metals from wastewater, where they exist in extremely or relatively low concentrations and as mixtures of various compounds, when using existing physicochemical techniques such as adsorption, ion exchange, and chemical coagulation because of the high cost and huge energy and/or materials consumption. It should also be noted that strong acid and alkaline agents are often used in the physicochemical recovery processes, which are friendly neither to working environments nor to natural environments.

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To cope with the above-mentioned difficulties, the use of microbial reactions in metal removal/recovery has been proposed [3]. Such reactions include bioprecipitation, biomineralization, biovolatilization, biosorption, and bioleaching. Since microbial reactions are intrinsically material- and energy-efficient, microbial metal removal/recovery technologies can be cost effective and environmentally friendly. Microbial technologies provide definite advantages, including efficient targeting of metals, even at low concentrations or in the presence of other compounds, because of their high specificity derived from the nature of enzyme reactions. Further, the microbial reactions proceed under ambient temperatures and pressures and do not require strong acid or alkaline reagents, and are thus very safe and easy to use. Consequently, microbial metal removal/recovery shows great promise as a key technology for establishing healthy and sustainable human–metal relationships in the future. This chapter focuses on potential biotechnologies for removal/recovery of metals from wastewater using specific microbial reactions. Various microbial reactions available for metal removal/recovery will be reviewed, and an example of the research and development (R&D) of technologies for the recovery from wastewater will also be introduced [4–6].

18.1 Microbial Reactions Available for Metal Removal/Recovery

Certain types of microbes are known to catalyze metabolic reactions using a variety of metal elements, which considerably affect the geochemical cycles for metals [7]. The metabolic reactions include alteration of the redox state (oxidation/reduction), methylation/alkylation, and hydrogenation of the metals. Some microbes have evolved the ability to actively transport metals to the inside or outside of their cells and to efficiently concentrate them onto the cell surface, by which the microbes gain the advantage of utilizing low concentrations of metals as minor nutrients or the capability of tolerating metal toxicity. Further, microbes may generate various products that affect the behavior of metals inside the cells or in the environment, such as metal-binding proteins/peptides, acids, oxidants, and others. When these microbe–metal interactions lead to the transformation of metals in the water phase or soluble form into those in the solid or gaseous phase, they can be utilized to remove/recover metals from wastewater. When the microbe–metal interactions cause the mobilization of metals from the solid phase, they can be utilized to extract metals from wastes generated during wastewater treatment for recycling. Such microbial processes available for metal removal/recovery can be classified into bioprecipitation/biomineralization, biovolatilization, biosorption, and bioleaching, as conceptually illustrated in Figure 18.1.

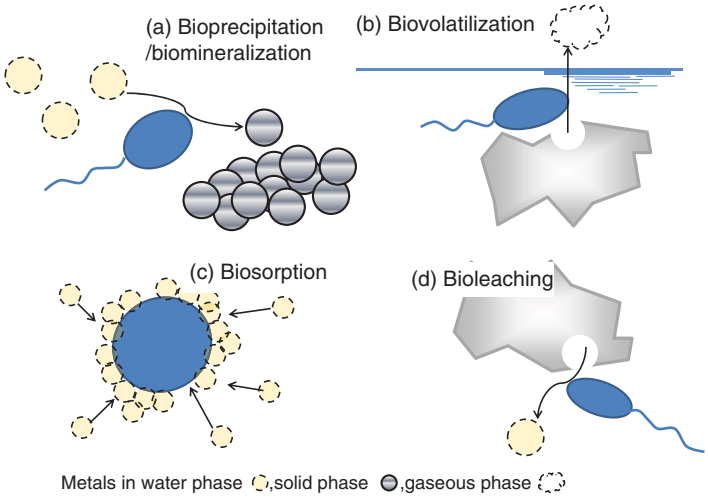

Figure 18.1 Microbial processes available for metal removal/recovery from wastewater: (a) bioprecipitation/biomineralization, (b) biovolatilization, (c) biosorption, and (d) bioleaching.

18.1.1 Bioprecipitation/Biomineralization

The microbial processes that lead to immobilization of soluble metals into the solid phase are called bioprecipitation or biomineralization [7–9]. Bioprecipitation enables removal/recovery of metals from the water phase by simple solid–liquid separation methods. Various kinds of microbial metabolic reactions can cause bioprecipitation of metals, and the known examples are summarized in Table 18.1.

Table 18.1 Examples of bioprecipitation.

Reduction of metals		
$Se(VI) \rightarrow Se(IV) \rightarrow Se(0)\downarrow$	$Te(VI) \rightarrow Te(IV) \rightarrow Te(0)\downarrow$	$Cr(VI) \rightarrow Cr(III)\downarrow$
$V(V) \rightarrow V(IV)\downarrow$	$Au(III) \rightarrow Au(0)\downarrow$	$Ag(I) \rightarrow Ag(0)\downarrow$
$Pd(II) \rightarrow Pd(0)\downarrow$	$U(VI) \rightarrow U(IV)\downarrow$	$Tc(VII) \rightarrow Tc(IV)\downarrow$
Oxidation of metals		
$Fe(II) \rightarrow Fe(III)\downarrow$	$Mn(II) \rightarrow Mn(III) \rightarrow Mn(IV)\downarrow$	
Sulfide precipitation		
$S(VI) \rightarrow S(III) \rightarrow S(II) \rightarrow S(0) \rightarrow S(-II)$: $S(-II) + M(II) \rightarrow MS\downarrow$		
Phosphate precipitation		
Poly-Pi → Pi: $2Pi + 3M(II) \rightarrow M_3Pi_2\downarrow$		

M, example of metal element; Pi, phosphate.

The most typical microbial metabolic reaction that can cause bioprecipitation is the reduction of metals to a lower redox state, lowering metal mobility. Through microbial reduction, some metals are transformed into insoluble elemental metals, and others into forms with lower solubility to precipitate with other minerals, and so on. A well-studied example of the reductive transformation of soluble metals into insoluble elemental metals is Se, where Se oxyanions (selenate and selenite), Se(VI) and Se(IV), are reduced to Se(0) particles [10]. The reductive microbial reactions have been utilized in the removal of Se from wastewater and contaminated water. For example, a pilot-scale Se removal bioreactor utilizing the Se(VI)-reducing bacterium *Thauera selanatis* has been successfully used for treating drainage water [11]. The microbial chromium (Cr) reduction process transforms highly soluble Cr(VI) into less soluble Cr(III), which can be easily precipitated as $Cr(OH)_3$ in slightly alkaline conditions, and this reaction has also been applied for wastewater treatment and water reclamation processes, though mostly at the lab scale [12, 13]. Other metals that can be reductively immobilized or precipitated include tellurium (Te), antimony (Sb), molybdenum (Mo), vanadium (V), gold (Au), silver (Ag), palladium (Pd), uranium (U), and technetium (Tc), and attempts have been made to establish the removal process for some of these [14, 15]. Metal reduction by microbes under anaerobic conditions is generally catalyzed by the corresponding metal reductases for anaerobic respiration: that is, the metals in oxidized forms can be reduced as electron acceptors, coupled with the oxidation of organic compounds or other electron donors. Therefore, the selection of suitable electron donors may be important to utilize such reductive metabolic reactions for developing metal removal/recovery processes. The importance of sulfur- and sulfate-reducing bacteria and iron-reducing bacteria has been recognized in the reductive bioprecipitation of various kinds of metals, such as Cr, Au, Ag, Pd, U, and Tc [7, 9], and understanding their physiological properties may aid in the design of metal removal/recovery processes that incorporate them. Microbial metal reduction can also proceed under aerobic conditions, and may be associated with metal detoxification mechanisms [16].

Microbial oxidation of certain metals can also lead to their immobilization. It has been well documented that ferrous iron [Fe(II)] in the water phase is not only abiotically but also microbiologically or enzymatically oxidized to precipitate as Fe(III) by iron-oxidizing bacteria. Similarly, dissolved manganese [Mn(II)] can be immobilized as oxidized Mn, such as Mn(III/IV), by manganese-oxidizing microbes. Fe-/Mn-oxidizing microbes can gain energy via oxidation processes and achieve autotrophic and/or heterotrophic growth. Microbiologically generated Fe/Mn precipitates may cause the concentration of other metals by coprecipitation or adsorption, possibly allowing for simultaneous removal of various metals from the water phase [17]. Fe/Mn bioprecipitation reactions have been applied to the removal of Fe/Mn and other metals from wastewater from mining industry as well as from groundwater [18, 19].

In addition to changes in the redox state of metals, other microbial metabolic reactions can cause the bioprecipitation of a wide range of metals even when they themselves cannot be reduced or oxidized. In such bioprecipitation processes, the

most important microbial reaction may be the generation of sulfide, which can form insoluble or hard-to-dissolve salts with a wide variety of metals. Sulfide can form precipitate with ions of copper (Cu), cadmium (Cd), bismuth (Bi), lead (Pb), Hg, Sb, and tin (Sn) under acidic conditions and with zinc (Zn), nickel (Ni), and cobalt (Co) under alkaline conditions; it is thus possible to remove these metal ions from the water phase [7]. Microbial sulfide formation is mediated by sulfate- or sulfur-reducing bacteria, which are anaerobic heterotrophs. They oxidize organic compounds using sulfate and elemental sulfur as terminal electron acceptors, eventually generating sulfide. Sulfide bioprecipitation may be applied to remove various metals from sulfur-containing wastewater, using sulfate-reducing bacteria under anaerobic conditions. Toxic metals in mining wastewater can be efficiently removed by lab-scale bioreactors using sulfate-reducing bacteria [20]. This concept has also been implemented in large-scale commercial bioreactors [21].

Similar to sulfide precipitation, microbial reactions that produce phosphates can also promote the precipitation of some metals as insoluble salts. Phosphate may be generated by polyphosphate hydrolysis by polyphosphate-accumulating microorganisms found in biological phosphorous removal processes. Microbial phosphate precipitation of zirconium (Zr), U, and lanthanum (La) and other heavy metals has been documented [22, 23].

18.1.2 Biovolatilization

Biovolatilization [7–9, 24] refers to microbial transformation of metals into their volatile forms, offering a way to convert the metals from the water phase to the gaseous phase. It may also be possible to remove metals from the solid phase by biovolatilization. Thus, we can apply this process to the removal of metals from wastewater and also from solid waste generated by wastewater treatment. If we can trap the volatilized metals that is off-gas from the wastewater treatment process (e.g., in a gas scrubber), they can be subsequently recovered. Biovolatilization of metals is generally associated with their methylation or alkylation by microbes, whereas volatilization of Hg may also be mediated by its reduction. Examples of metal biovolatilization are summarized in Table 18.2.

In contrast to metals that are immobilized into precipitates by their reduction, microbial reduction of Hg leads to its mobilization in the gaseous phase, because the end product, Hg(0), is volatile. Certain types of bacteria and fungi can reduce soluble Hg(II) to generate Hg(0) as a mechanism for resistance to or detoxification of Hg, and some Hg(II) reductases that catalyze this reaction have been cloned and characterized [25]. Use of bioreactors for the removal of Hg from contaminated water and sediment using biovolatilization has been documented [26].

Several types of metals are known to be microbially volatilized via methylation or alkylation, and those processes are considered to make significant contributions to geochemical cycling [27]. Among these, Se volatilization has received

Table 18.2 Examples of biovolatilization.

Reduction of metals	
$Hg(II) \rightarrow Hg(0) \uparrow$	
Methylation of metals	
$Hg \rightarrow (CH_3)Hg^+$, $(CH_3)_2Hg^+$	$Se \rightarrow (CH_3)_2Se$, $(CH_3)_2Se_2$
$Te \rightarrow (CH_3)_2Te$	$As \rightarrow (CH_3)AsH_2$, $(CH_3)_2AsH_1$, $(CH_3)_3As$
Hydrogenation of metals	
$Se \rightarrow H_2Se$	

the most attention for its potential bioremediation applications. Se in natural soil and water environments can be mobilized into the atmosphere by microbial volatilization [28]. In such processes, soluble or elemental Se is methylated to generate dimethyl selenide ($(CH_3)_2Se$; DMSe) and smaller amounts of dimethyl diselenide ($(CH_3)_2Se_2$; DMDSe). Se methylation is commonly observed and known to be mediated by various types of bacteria and fungi. Bioremediation of Se-contaminated water has been reported in the San Joaquin Valley and Kesterson reservoir using enhanced microbial methylation [29]. However, it should be noted that such applications of Se volatilization via methylation are limited to intrinsic or passive bioremediation systems, but engineered applications, such as wastewater treatment, have rarely been documented. Biovolatilization via methylation has also been relatively well studied in Hg, As, and Te. Hg(II) may be methylated to methyl mercury ($(CH_3)Hg^+$) and further into dimethyl mercury ($(CH_3)_2Hg^+$). Microbial methylation of As can produce mono-, di-, and tri-methyl arsines ($(CH_3)_nAsH_{3-n}$, $n = 1,2,3$). Dimethyl telluride ($(CH_3)_2Te$; DMTe) can be microbially produced as the main volatile compound of Te. Bioremediation applications of methylation of Hg and As are still in the R&D stage, similar to those of Se biovolatilization. In addition, some studies have reported on the methylation-mediated biovolatilization of other metals including Sn, Pb, Sb, and Bi [24, 30]. Although methylation of metals was believed to be a detoxification process for protecting microbes, it is considered questionable at present because methylation of metals often leads to an increase in toxicity. Biovolatilization may be caused not only by methylation/alkylation but also by hydrogenation for some metals [30]. The primary hurdle for application of biovolatilization to metal removal/recovery from wastewater may be the typically the slow reaction rate.

18.1.3 Biosorption

Biosorption [31–33] is defined as the removal of substances from the water phase or solution by sorption to biological materials, and is a physicochemical process rather than a biological process. Metals can be removed from the water

phase by biosorption with microbial biomass and/or by using microbial products as biosorbents, which are solid surfaces of bio-matrices capable of adsorbing or concentrating sorbates. Biosorption is recognized as an important process affecting the bioavailability of metals in natural environments, which may provide enhanced or inhibitory effects [7]. Sorption processes use chemically synthesized materials, including activated carbon and ion-exchange resins, rather than biomaterials, but have been applied to remove toxic heavy metals from industrial wastewater. Since biosorption is, in principle, identical to conventional sorption processes using chemically synthesized sorbents, it may also be applied to the removal/recovery of metals from wastewater. "Bioaccumulation" is another microbial metal removal process similar to biosorption. Although it is difficult to completely differentiate these two processes, they have slightly different definitions; bioaccumulation refers to the uptake of metals by living cells, whereas biosorption is the passive concentration of metals onto biomass, which may be inactive or even dead [32]. Thus, bioaccumulation can include the process of active transport of metals into cells and the consequent metabolic changes in the cells. In the application for metal removal/recovery, biosorption may have some advantages compared with bioaccumulation, such as the lack of a requirement for cell activity maintenance, a wider application range, a rapid removal/recovery rate, a high toxicity tolerance, and the potential for regeneration/reuse of the cells.

To date, a large number of studies have been performed to discover efficient biosorbents. These studies have suggested that almost all microbial biomass/biomaterials can bind certain types of metals. Although it is very difficult to choose a limited number of references to summarize the available knowledge on metal biosorbents, we have attempted to compile a list of some of the biosorbents reported for metal removal/recovery, as shown in Table 18.3. The possible

Table 18.3 Examples of reported biosorbents for metal removal/recovery.

Microbes	Metals removed	References
Bacteria		
Bacillus subtilis	Ag, Cd, Cu, La	[34]
Pseudomonas aeruginosa	Cd, Cu, Pb	[35]
Pseudomonas putida	Cd, Cu, Pb, Zn	[36]
Fungi		
Aspergillus niger	Cd, Cu, Ni, Pb	[37]
Penicillium canescens	As(III), Cd, Hg, Pb	[38]
Penicillium chrysogenum	Cd, Cr(VI), Cu, Ni, Pb, Th, U, Zn	[39–42]
Saccharomyces cerevisiae	Au, Ag, Cd, Co, Cr(VI), Cu, Fe, Hg, Ni, Pb, Pt, Th, U, Zn	[43–50]
Algae		
Chlorella vulgaris	Cd, Cr(VI), Cu, Fe(III), Ni, Pb, Zn	[51, 52]

microbial biosorbents include a wide range of taxonomic categories; bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa*, fungi (mainly yeasts and molds) such as *Aspergillus* and *Penicillium* species and *Saccharomyces cerevisiae*, and microalgae such as *Chlorella* species and their metabolic products, including biopolymers [31–33]. In addition, certain wastes or by-products containing microbial cells can be used as biosorbents [53, 54]. These include activated sludge from wastewater treatment processes, and fermentation by-products containing yeasts or mold mycelia, and so on. Utilization of such waste biomass as biosorbents is especially attractive from an economical viewpoint, and a wide variety of metals have been reported to be removed using the above-mentioned biosorbents. Toxic heavy metals are the major target for biosorption studies, including Cd, Co, Cr(VI), Cu, Hg, Ni, Pb, Sn, and Zn. Radionuclides such as americium (Am), cesium (Cs), europium (Eu), protactinium (Pa), strontium (Sr), thorium (Th), and U have also been reported to bind with certain biosorbents. Precious metals, including Ag, Au, Pd, and platinum (Pt), can also be recovered from the water phase by biosorption, though trials to remove rare metals and rare earth elements have been relatively limited (e.g., Ni, Mo, and neodymium (Nd)) [32, 55]. Thus, biosorption shows potential for application to a variety of areas, such as toxic metal wastewater treatment, decontamination of radionuclide-contaminated water, and recovery of valuable metals from industrial waste streams.

The mechanisms of metal biosorption have not been fully elucidated, owing to the complexity of the phenomena. They may be associated with combinations of ion exchange, complexation/chelation, adsorption by physical forces, electrostatic interactions, and micro-precipitation [31–33]. The mechanisms depend on the chemical components and spatial structure of the surface of the microbial cells, affecting capability or performance. In addition, the surface structure of microbial cells may determine the specificity or selectivity of the biosorbents. In particular, the chemical functional groups of the cell wall play important roles in metal biosorption, including carboxyl, carbonyl, amine, amide, thiol, imine, imidazole, sulfonate, phosphonate, and phosphodiester groups. For example, carboxyl groups bind metal cations because of their negative charge, whereas amines can attract both metal cations and oxy-anions, depending on pH. The physiological state of the microbial cells, dead or living, cultivated under nutrient-rich or nutrient-poor conditions, and so on, can also considerably change the properties of the biosorbents, because the chemical and physical structure of the microbial cell surface will be altered. Based on such principles, cell wall modification of microbial cells has been applied to improve or alter the metal-binding capability and specificity using physical treatments such as freeze–thaw and lyophilization, chemical treatments such as alkali/acid treatment, washing with detergents, and cross-linking with organic solvents [56]. In fact, it has been reported that alkali treatment of a fungal cell significantly enhanced its metal uptake capability [57]. Another promising approach to modifying the surface properties of microbial sorbents is genetic manipulation. Genes coding known metal-binding peptides, such as metallothionein and phytochelatin, can be utilized to create recombinant

microbes possessing high metal-binding capabilities [58]. The recombinant yeast *S. cerevisiae*, which displays the metal-binding histidine hexapeptide, was created to provide efficient Cu-binding capability [59].

For practical applications of metal biosorption, the selected microbial cells to be used as biosorbents should be immobilized onto/into a solid matrix to allow easy solid–liquid separation, regeneration/reuse, and maintenance at high density in bioreactors, because they are essentially small particles to be suspended in the water phase [32]. Immobilization of microbial cells with polymeric materials is an established technique, and sodium alginate, polysulfone, polyacrylamide, and polyurethane have all been used. An example of the limited number of commercialized biosorbents, AMT-BIOCLAIM™ (Visa Tech Ltd.) uses *B. subtilis* cells pretreated with a strong caustic solution, immobilized as porous balls onto polyethyleneimine and glutaraldehyde [60]. AlgaSORB™ (Bio-recovery Systems Inc.) uses *Chlorella* in silica or polyacrylamide gels [61]. These immobilized biosorbents can be successfully applied to metal removal/recovery from wastewater in traditional reactor configurations, such as packed-bed reactors and fluidized-bed reactors. When the microbial cells as the biosorbent can be taken up into naturally occurring biofilms/biomats, or grow in aggregate forms in bioreactors, they have the same advantages as the above-mentioned synthetically immobilized biosorbents [62].

18.1.4 Bioleaching

Certain microbial activities can dissolve or extract metals from the solid phase, and such processes are termed bioleaching [7, 8, 63, 64]. It may be used to extract metals from solid waste generated by wastewater treatment to yield concentrated metal solution/precipitate for efficient recycling. Such applications may also add significant value by reducing the risk related to solid waste disposal by removal of toxic heavy metals. When bioleaching is applied to the extraction and recovery of metals from mineral ores, it is also called *biomining* [65]. Microbes mediate bioleaching primarily through the formation of inorganic or organic acids, oxidation and reduction reactions of metals, and generation of complexing or chelating agents. The major categories of microbes that play key roles in bioleaching from solid waste are acidophilic chemolithoautotrophic bacteria, heterotrophic bacteria and fungi capable of generating organic acids, and other specific bacteria catalyzing bio-cyanidation (Table 18.4).

Bioleaching/biomining of mineral ores, especially low-grade ores, using acidophilic autotrophic bacteria has already become established in the bioindustry for extracting Cu, Au, U, and other metals [63], and is expected to have application for treating various types of solid waste, including sewage sludge and incineration ash, as well as other metal-rich wastes. Several studies have confirmed the significant potential of bioleaching for the recovery of metals from solid waste. The most commonly utilized bacteria in chemolithotrophic leaching belong to the genus *Acidithiobacillus*, represented by *Acidithiobacillus*

Table 18.4 Examples of reported microbes for bioleaching.

Microbes	Metals leached	Application	References
Acidophilic chemolithoautotrophs			
Acidithiobacillus ferrooxidans	Cd, Cr, Cu, Ni, Pb, Zn	Digested sewage sludge	[66, 67]
Acidithiobacillus thiooxidans	As, Cd, Cr, Cu, Ni, Zn	Fly ash (municipal waste)	[68]
Acidithiobacillus sp.	Cd, Co, LI, Ni	Spent battery	[69–71]
Acid-producing heterotrophs			
Aspergillus niger	Al, Mo, V	Petroleum refinery waste	[72, 73]
Penicillium sp.	Co, Cu, Ni, Zn	Mineral ores	[74]
Cyanide-forming bacteria			
Chromobacterium violaceum	Au	Waste electric device	[75, 76]
C. violaceum, Bacillus megaterium, Pseudomonas fluorescens	Au, Ag, Pt	Jewellery waste, automobile catalyst	[77]

ferrooxidans and *Acidithiobacillus thiooxidans*, which autotrophically oxidize sulfur (S), Fe(II), or both (sulfur- and iron-oxidizing bacteria). Thermophilic or mesophilic archaeal bacteria such as *Acidianus*, *Sulfolobus*, *Sulfococcus,* and *Ferroplasma* are known to catalyze the same oxidation reactions. They can oxidize metal sulfides directly to obtain electrons, generating relatively soluble metal sulfates and sulfuric acid (direct mechanism). In addition, the oxidation of reduced metals in the solid phase can be chemically catalyzed by Fe(III) generated from the bacterial oxidation of Fe(II), leading to metal solubilization (indirect mechanism). Sulfuric acid generated during these reactions can also contribute to metal extraction. The only important factors required for chemolithotrophic leaching are the supply of S, possibly Fe(II), and oxygen, and acidic conditions. Practical application of this process for metal recovery from wastewater-derived solid waste may be possible using established biomining processes such as dump mining, heap mining, and tank leaching. Lab-scale studies on bioleaching have been performed to remove toxic metals from sewage sludge samples [78, 79].

Heterotrophic microbes, characterized by the capacity for organic acid production, can also be utilized for bioleaching, termed *chemoorganotrophic leaching*. *Aspergillus* and *Penicillium* species have often been utilized in studies of metal removal/recovery from various types of solid waste [73, 80]. They produce low-molecular organic acids, including lactic, oxalic, citric, gluconic, and succinic acids, which contribute to metal solubilization. In addition, they may excrete extracellular metal-complexing/chelating agents, improving the extraction of metals. For chemoorganotrophic leaching, proper carbon sources should be supplied, though keeping the pH in the acidic range is not necessary.

A two-step process has been proposed to achieve efficient bioleaching with these microbes [74]. In this process, microbes are cultivated in a first reactor to generate metal-solubilizing agents, and a cell-free solution is subsequently placed in contact with the solid waste for metal extraction, avoiding the inhibition of the microbes by the toxic materials contained in the waste.

The well-known cyanogenic heterotrophic bacteria *Chromobacterium violaceum, Pseudomonas fluorescens*, and *Bacillus megaterium* can isolate Ag, Au, and Pt from electronic scraps (e-waste) and jewelry waste [75–77]. With appropriate carbon and nitrogen sources, they produce cyanide in the aqueous phase, and metal–cyanide complexes are formed to be solubilized. It may be possible to utilize this mechanism to recover precious metals from solid waste derived from industrial wastewater.

18.2
Selenium Recovery by *Pseudomonas stutzeri* NT-I

Microbial metal metabolic reactions and microbe–metal relations are considered to possess high potential for removing and recovering metals from wastewater. However, practical industrial applications have remained limited, in spite of the huge numbers of related studies. In particular, microbial recovery of metal as a resource has rarely been implemented at industrial or full scale, though some wastewater treatment bioreactors aimed at metal removal or detoxification have been implemented. In this section, our R&D for Se recovery utilizing bioprecipitation and biovolatilization catalyzed by a versatile selenium-metabolizing bacterial strain is introduced.

18.2.1
Pseudomonas stutzeri NT-I as a Versatile Tool for Selenium Recovery

Selenium, an element in the 16th group of the periodic table, is an important minor metal used as a semiconducting material for photovoltaic cells [81], quantum dots [82], and so on, and which, from another viewpoint, is known as a contaminant of aquatic environments [83]. Selenium species typically found in the environment are shown in Figure 18.2. Highly soluble and toxic selenate (SeO_4^{2-}; Se(VI)) and selenite (SeO_3^{2-}; Se(IV)) are the ions responsible for water contamination. Elemental selenium (Se^0) and metal selenides (Se(−II)) are insoluble minerals found in the pedosphere. Selenium is also known as an essential trace element for organisms, found as seleno-amino acids (Se(−II)) in the biosphere. Methyl selenides and hydrogen selenide (Se(−I) or Se(−II)) have volatile properties and is found in the atmosphere. Certain microbes can catalyze redox reactions of the selenium compounds to alter their chemical properties, contributing to cross-boundary transfers within different terrestrial phases on earth. Biological removal and recovery of selenium from the water phase is based on exploitation of microbial reactions. Successful studies on the biological removal of selenium have primarily been

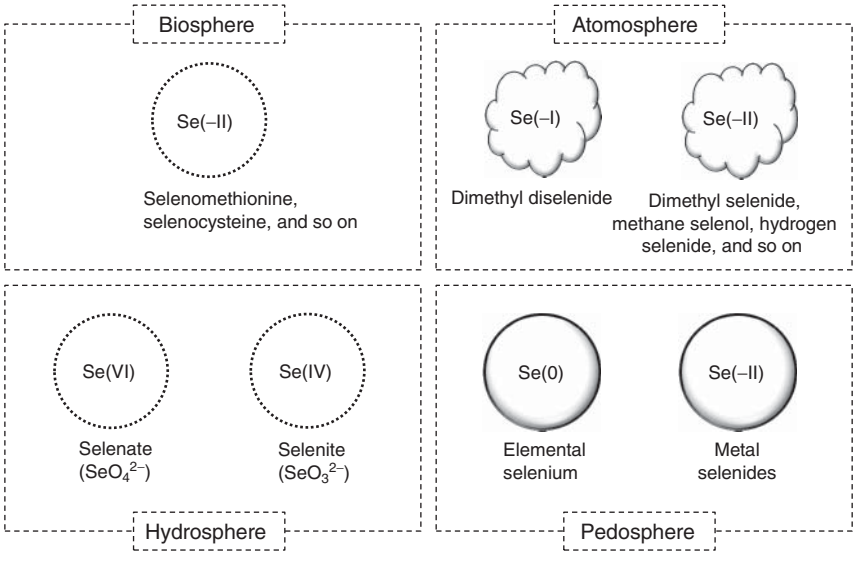

Figure 18.2 Selenium species in the environment. Roman numerals in parentheses indicate oxidation numbers.

reported in agricultural or mine wastewater, to date [84]. However, industrial wastewater containing higher concentrations of selenium should be targeted for economical recovery of selenium.

Therefore, we isolated *Pseudomonas stutzeri* NT-I from the drainage of a refinery producing selenium for industrial use [4]. Typical selenium metabolic reactions in aerobic and anaerobic conditions are shown in Figure 18.3. Strain NT-I

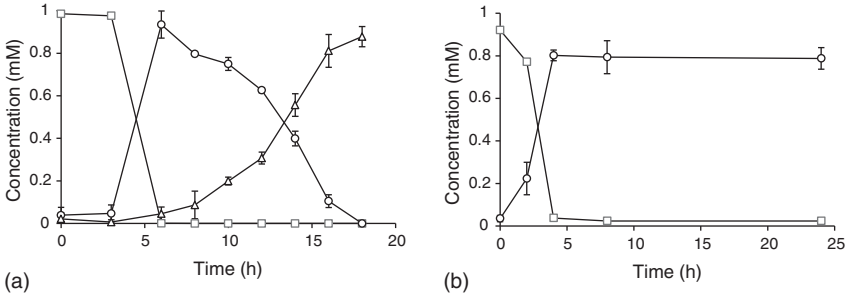

Figure 18.3 Typical time courses of selenate reduction by the strain NT-I [4]. (a) Reduction under aerobic conditions, and (b) reduction under anaerobic conditions. Symbols: open squares, selenate; open circles, selenite; open triangles, elemental selenium. Vertical bars represent the standard deviation of three independent experiments. (With permission ©2011 The Society for Biotechnology, Japan. Published by Elsevier B.V.)

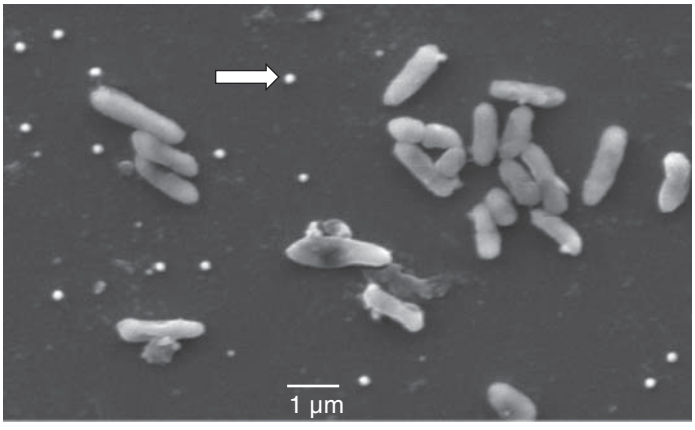

Figure 18.4 Scanning electron microscopy image of strain NT-I [4]. The arrow indicates a particle of elemental selenium. (With permission ©2011 The Society for Biotechnology, Japan. Published by Elsevier B.V.)

reduces selenate to selenite in both aerobic and anaerobic conditions, and then selenite to elemental selenium in aerobic conditions. Because the resulting elemental selenium forms small spherical particles with a diameter of 20 nm outside the cells (Figure 18.4), selenium could readily be separated from the cells by ultrafiltration as well as by sedimentation or centrifugation followed by incineration of the organic matter. Moreover, strain NT-I can reduce high concentrations of selenate (up to 10 mM) and selenite (up to 9 mM), which would enable application to wide range of industrial wastewater types.

During prolonged cultivation of strain NT-I in aerobic conditions, we serendipitously observed the disappearance of the dense red color derived from elemental selenium in the culture medium; we later determined that this marked the volatilization of selenium by the strain NT-I. Detailed analysis clarified that selenium was volatilized as methyl selenides with methyl sulfides by the strain NT-I [6]. Most of the microbial volatilizations of selenium previously reported were subtle metabolism [85–88]. In contrast, strain NT-I volatilized most of the selenium added to the medium, revealing a trait that shows great promise for industrial application in the future. Accordingly, strain NT-I, which catalyzes both bioprecipitation and biovolatilization by reductive reaction of selenium, could be a versatile tool for selenium recovery.

18.2.2
Selenium Recovery by Bioprecipitation

In the selenium refinery factory where strain NT-I was isolated, wastewater containing selenium (up to 124 mg l^{-1}) with high salinity (6–7%) is produced. The current process of wastewater treatment is based on physicochemical technologies,

employing the electric reduction of selenate to selenite, followed by sedimentation using a ferric flocculant. The concentration of selenium in the discharged inorganic sludge is too low (1%) to recycle economically. The triple costs of energy consumption for the electric reduction, excess flocculant, and sludge disposal place a financial burden on the factory.

We attempted to develop a wastewater treatment process using selenium bioprecipitation by strain NT-I [5]. Strain NT-I was inoculated at a pilot-scale swimbed reactor equipped with an interior acrylic biomass carrier, and the reactor was operated in micro-aerobic conditions for sequential batch processes. The wastewater was neutralized, diluted to reduce salinity, and supplemented with a carbon source (ethanol) and nutrient salts before treatment. Selenate or selenite was augmented when necessary. Neutralization and dilution were also included in the current physicochemical processes.

The first and second batches successfully reduced the selenium concentration to the Japanese effluent standard (0.1 mg $Se\,l^{-1}$) within 5 days from wastewater initially containing 60–70mgl^{-1} selenite. The third batch removed 30 mg l^{-1} selenate, which demonstrated that strain NT-I can remove selenate and selenite from actual wastewater, with appropriate pretreatment. Microbial community analysis revealed that bacteria other than strain NT-I coexisted in the reactor after 25 days of operation. Because selenate-reducing bacteria are uncommon [89], it appeared that the selenate reduction in the reactor was primarily performed by the strain NT-I, although coexisting bacteria could substantially contribute. Selenium was removed as elemental selenium in the biomass, which would be ready for refining by incineration because selenium concentration in the biomass was sufficiently high (calculated to be 30%). Selenium recovery might produce a profit over the treatment cost, depending on the price of selenium. Furthermore, we achieved selenium recovery by bioprecipitation from extracts from waste material in cement production and fly ash from sludge incineration, indicating a broad range of potential applications [90, 91].

18.2.3 Selenium Recovery by Biovolatilization

Separation of selenium and biomass, an essential step for selenium recovery by bioprecipitation, adds additional cost. Selenium recovery through biovolatilization can be advantageous because volatilized selenium is separated from the water phase automatically; therefore, we attempted to demonstrate the concept of utilizing vigorous selenium volatilization by strain NT-I [6]. Strain NT-I was cultured in a medium containing selenate using a simple bioreactor equipped with an apparatus containing concentrated HNO_3 for selenium trapping from the exhaust gas. As a result, ~80% of the selenium initially added was removed from the water phase, and ~80% of the volatilized selenium was recovered in concentrated HNO_3, with thrice the the amount of sulfur and a small amount of silicon, during 48 h of operation (Figure 18.5). Selenium refinement from a solution of such simple

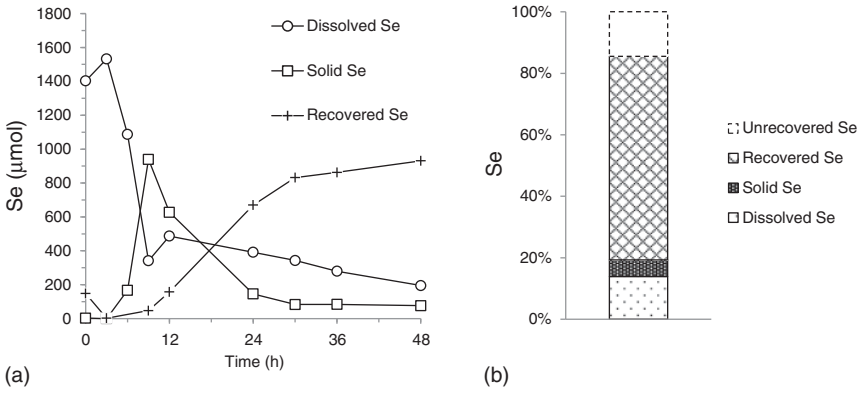

Figure 18.5 Recovery of Se through biovolatilization by strain NT-I [6]. (a) Time course of Se during cultivation. The vertical axis indicates the amount of Se in the culture and trapping solution. (b) Material balance of Se at 48 h. The ratio of Se in its respective phases to total Se in the jar fermenter at 0 h is indicated as a percentage. (With permission ©2012 Elsevier Ltd.)

composition should be easier than refining from the mixture with organic matter obtained by bioprecipitation.

However, two challenges remain: establishment of practical operating conditions to decrease the selenium concentration in treated water and to maximize the recovery rate, and increasing the purity of the recovered selenium by eliminating sulfur. To solve these problems, discovery of the molecular mechanism of selenium metabolism and additional biochemical engineering will be necessary. It has been suggested that anaerobic respiration similar to that of *Thauera selenatis* is responsible for selenate reduction in strain NT-I [92]. However, the limitation of selenite reduction and selenium volatilization to aerobic conditions implies that sufficient energy obtained by aerobic respiration is essential for them. Similarly, the simultaneous volatilization of selenium and sulfur implies that selenium metabolism is related to sulfur metabolism in strain NT-I. Therefore, it appears that an "omics" approach, such as genomics, transcriptomics, or metabolomics, should be employed to reveal the relationship between selenate metabolism and the global metabolic pathways.

18.3
Future Prospects

18.3.1
Toward Environmental Conservation and Solutions to Resource Depletion

As discussed thus far, various microbial mechanisms are being proposed for a wide range of applications in environmental conservation technologies, including

bioremediation of metal-contaminated wastewater, waste material, and soil. However, much of this effort has been limited to laboratory- and pilot-scale experiments. Microbial technologies for metal removal/recovery clearly show a strong potential for application in environmental and resource conservation owing to their favorable economic and environmental characteristics. R&D of environmentally adaptable microbial technologies for metal removal/recovery may also be conducted in the metal industry where biotechnology has not been applied to date, including metal mining, metal processing, new materials development, anti-degradation protection for metal structures, and metals analysis and measurement. To date, these microbial technologies for metal removal/recovery have primarily been applied in environmental conservation, such as treatment and detoxification of metal-contaminated wastewater and waste material, and remediation of contaminated environments such as soil. In addition to common toxic metals such as As, Cd, Hg, and Pb, rare metals such as Sb, Se, and Mo are also being subjected to regulation, raising a requirement for treatment technologies that can be applied to a wider range of metals than do previous technologies [69, 93, 94]. There is a distinct market for treatment technologies for decontamination of environmentally regulated metals, and an opportunity for development and application exists in taking advantage of the economic efficiency of microbial technologies for metal removal/recovery. However, these technologies have yet to overcome their limitations of slower treatment speed and higher uncertainty, compared to physicochemical methods. Biotechnological methods are currently suitable for low-cost, low-environmental-impact treatment of contaminated idle land, or for treatment of farmlands that results in minimal loss of soil texture. A stable market for these technologies is expected to emerge as their environmental conservation value becomes firmly established.

Precious and rare metals are usually discussed in terms of depletion of resources rather than environmental treatment. Providing a stable supply of scarce elements for the future poses a difficult problem. Recovery and recycling of precious metals such as Au and Ag, platinum group of metals such as Pd and Pt, and rare metals such as Mo, Ni, and Se from wastewater and waste treatment facilities pose an important challenge for resource conservation, but recent progress has been made, and this technology should become available in the near future [93, 95, 96]. When low-cost technologies, such as purification and treatment, are converted to dynamic technologies that provide benefits, such as resource recovery and development, these low-cost technologies can serve as catalysts for creating a new industry as novel technologies by evolving from static technologies to construct a recycling-based society. Practical application of microbial technologies for metal removal/recovery in the environmental conservation field may open the door for novel biological catalysts, biomaterials, and utilization in industrial production technologies with high market value. Refining and processing of metals generally employs strong acids or high temperature, and takes place under severe conditions with high energy expenditure. If these processes could be replaced by environmentally friendly bioprocesses, or if concentration of specific

metals – which currently depends on chemically synthesized resins – could be replaced by biomaterials, then microbial technologies for metal removal/recovery could make an even greater contribution toward the development of a sustainable society. Biotechnology is a rapidly evolving field where new biological reactions, enzymes, DNA, microbes, and plants are discovered every day, and which is revealing new metabolic reactions involving metals. If these biological reactions are utilized to their full potential, they may lead to development of new technologies in the field of resource supply. Development and application of microbial technologies for metal removal/recovery may help to solve some of the largest problems of the century, such as environmental degradation and resource depletion.

18.3.2 Development of Removal and Recovery Strategies for Other Elements

Microbial technologies for metals removal/recovery in wastewater and waste material could be laterally extended to other elements if microbes that effectively catalyze the same reactions can be obtained. Acquisition of microbes with the capability to metabolize metals is difficult to achieve through normal screening processes, but it has to be attempted first. In fact, microbes have already been identified that perform various functions, including oxidation and reduction of As, oxidation of Mn, reduction of Cr, reduction of Pd and Pt, adsorption and concentration of radioactive elements and rare earth elements, vaporization of mercury ions, elution of metals from incinerated ash, and biomineralization of Te [3, 15]. The use of rapidly developing techniques for genomic analysis and bioinformatics to study global microbe and DNA databases may help in identifying potentially useful biological resources that have so far remained undiscovered.

18.3.3 Potential for Practical Application

Several problems remain to be solved before microbial technologies for metal removal/recovery in wastewater and waste material can be put into practice. The major problems are the slow reaction speed compared to physicochemical reactions and the instability of microbial reactions due to the inhibition of their growth and metabolism by other toxic substances, including metals, that are found in wastewater and waste materials. Although these issues pose problems for the industrial use of microbial processes, they are also inevitable so long as organisms are involved, and they cannot be eliminated. However, these problems may be overcome to a substantial extent if microbes that can tolerate toxic metals and other substances can be isolated and cultured with practical applications in mind, and by improving preprocessing steps for microbial treatment of wastewater and solid wastes.

18.4 Conclusions

Various microbial metabolic reactions of metals and microbe–metal interactions have been reported, and their application to metal removal/recovery from wastewater have attracted a great deal of attention in recent years. Microbial metal removal/recovery technologies are considered to possess several advantages over existing physicochemical technologies, because they typically consume less materials and energy, are more cost effective, do not generate hazardous by-products, and are thus in greater harmony with the natural environment; using this technology can help avoid harsh working conditions such as high pressure and temperature and use of hazardous reagents, and this technology is efficient for targeting metals even in low concentrations and in the co-presence of other compounds because of its high specificity. In spite of the recognition of the great potential of microbial metal removal/recovery, practical industrial applications have been limited to date. In particular, applications of microbial recovery of metal as resources, which can contribute to both environmental conservation and sustainable resource utilization, have yet to be realized at large scales. That means microbial metal recovery technologies are still in the FS (feasibility study) or R&D stages. To establish practical microbial metal recovery technologies, further R&D may be required for screening of promising microbial agents, for detailed characterization of these agents, especially at the genomic level, for understanding the underlying mechanisms, optimization of design and operation of bioreactors/processes, verification of the designed processes using an actual wastewater matrix, field development, downstream processing of recovered metals, and so on. It should also be emphasized that a multidisciplinary approach may be necessary to fully develop metal recovery technologies, with participation of scientists and engineers not only from biotechnology and environmental engineering but also from microbiology, microbial ecology, chemical engineering, hydraulic engineering, materials science, and so on, and even from social sciences such as economics and politics.

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19
Sustainable Use of Phosphorus Through Bio-Based Recycling

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19.1
Introduction

Phosphorus (P) is one of the most critical elements in biological building blocks and plays a crucial role in the cellular energy metabolism of all living organisms [1]. Since no other element can substitute for P in biochemical processes, humans ultimately rely on P availability. Modern farming is reliant on phosphate (P_i)-derived fertilizers to enhance agricultural production in soils [2]. Therefore, P availability is critical to our present and future food security [3]. Moreover, P is widely used as an important raw material in a variety of industrial products, including beverages, food, and pharmaceuticals [4].

Today, P is mostly obtained from mined P_i rock, which is nonrenewable on a human timescale [5]. However, natural reserves of high-grade P_i rock (27 – 37 wt% P_2O_5) are limited and rapidly running out on a global scale. P_i rock is becoming more and more a valuable and strategic material, because P scarcity is linked to our food security [3]. On the other hand, increased input of P_i into lakes, bays, and other surface waters causes nuisance phytoplankton growth known as *eutrophication* [6]. Eutrophication is a complex problem with serious effects on the state and health of aquatic ecosystems, imposing large economic and ecological costs [7]. Algal blooms degrade water quality by producing an offensive odor and taste. The nuisance growth of algae renders boating and fishing difficult and discourages swimming. Excessive growth of algae consumes dissolved oxygen, when they are decomposed by aerobic bacteria. Algal toxin production is also a serious problem in drinking water supplies [8]. Hence, it should be noted that the inefficient use of P not only accelerates the depletion of high-grade P_i rock but also causes environmental damage to aquatic systems by turning this essential resource into a pollutant.

Despite the potential shortage of P_i rock, a substantial amount of P in eroded soil particles and also in urban waste flows into the ocean from where P recovery is practically infeasible [9]. To prevent the P loss associated with human activities, increased attention has been paid to closing the loop on the anthropogenic P

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cycle [10]. Two main pathways toward the sustainable use of P are (i) minimizing P losses in the supply and demand chain, and (ii) recycling P from potential secondary resources [3]. Improving P_i rock mining, beneficiation technologies, and agricultural practices can contribute to preventing P losses in the supply and demand chain [3, 10]. Biotechnological and physicochemical methods have the potential to contribute to the recovery and recycling P from secondary resources [11, 12].

Agriculture is a major user of P, accounting for 80–85% of the world's P consumption [9]. The majority of P consumed from food and feed ends up in human wastes and animal manures, which are large sources of recyclable P. Since human wastes are discharged of in municipal wastewater, it is one of the major P sinks in urban areas [13]. Aiming to control the eutrophication problem, various technologies have already been developed for removing P from wastewater [11, 13]. However, not all the P removal technologies can be applied to P recovery for recycling because the quality of the recovered product is critical to P recycling. For instance, chemical precipitation with Al^{3+} or Fe^{3+}, which is a commonly used technology for P removal, makes P recycling more difficult compared to biological P removal. These cations form insoluble P precipitates, which are poorly utilized by plants when applied to agricultural land as fertilizer. If P is removed by precipitation with Al^{3+} or Fe^{3+}, it needs to be converted to a more plant-available form using wet chemical or thermochemical technologies [13]. Biological P removal is more amenable to P recycling, because P is accumulated by sludge microorganisms in the form of polyphosphate (polyP), which can be further recovered as soluble P_i for recycling [12].

19.2 Microbiological Basis

19.2.1 P_i Acquisition in Bacteria

Bacteria use P_i as the preferred P source (Figure 19.1). When P_i is available in excess, P_i is taken up by the P_i inorganic transport (Pit) system that is expressed constitutively [14]. Under these conditions, bacteria can store P_i in excess of their requirement for growth in the form of polyP [14]. On the other hand, since bacteria are often subjected to P_i deficiency in nature, they have evolved complex systems to survive under P_i starvation conditions. P_i starvation induces the P_i-specific transport (Pst) system, which serves as a major scavenger of P_i residues [15]. The Pst system of *Escherichia coli* comprises four distinct subunits encoded by the *pstS*, *pstA*, *pstB*, and *pstC* genes [16]. These genes, together with the *phoU* gene, form the *pst* operon and are involved in the regulation of the P_i (*pho*) regulon [17]. The *phoU* gene encodes a negative regulator of the *pho* regulon.

Biologically synthesized polyP is a linear polymer of P_i with a chain length of up to 1000 residues or more [17]. The enzyme responsible for polyP biosynthesis

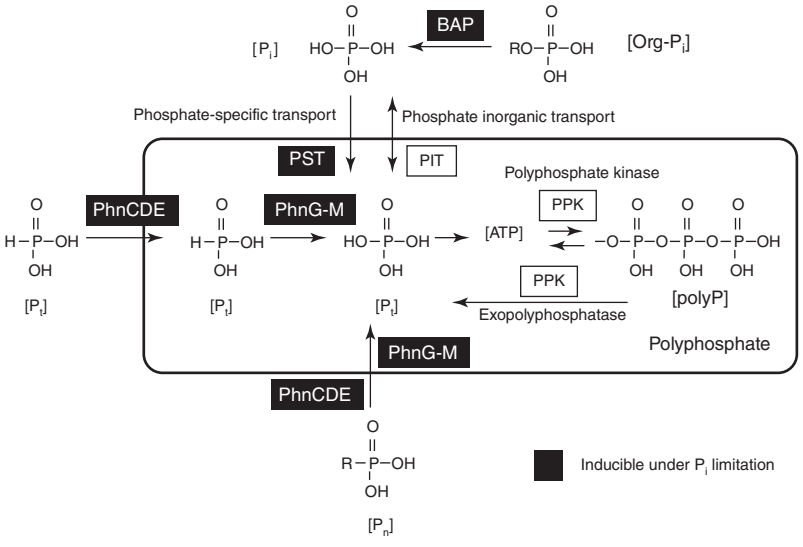

Figure 19.1 P acquisition in bacteria [12]. (With permission ©2009 The Society for Biotechnology, Japan. Published by Elsevier B.V.)

is polyP kinase (PPK), which polymerizes the terminal P_i of ATP into polyP in a freely reversible reaction [18]. The utilization and degradation of polyP is catalyzed by polyPases, including an exopolyPase (PPX), and several polyP-specific kinases, including polyP-glucokinase and polyP-fructokinase [18].

19.2.2
Bacterial polyP Accumulation

Bacteria exhibit two distinct patterns of polyP accumulation, known as P_i luxury uptake and polyP overplus [12]. P_i luxury uptake occurs when bacterial cells are subjected to nutritional imbalance unfavorable for growth. For example, *E. coli* can accumulate polyP when it is subjected to amino acid (nitrogen) starvation [19]. The molecular mechanism of polyP accumulation under nitrogen starvation has been described by Kuroda *et al*. [20]. Briefly, guanosine pentaphosphate (pppGpp), which is the major regulatory signal for amino acid starvation, inhibits PPX activity without affecting PPK. This leads to polyP accumulation in *E. coli* in response to the nitrogen starvation. The resulting polyP forms a complex with Lon protease, enabling the activation of protein degradation to supply free amino acids. On the other hand, many bacteria exhibit polyP overplus when P_i is added to cells previously subjected to P_i starvation. The molecular mechanism of polyP overplus has been determined with *Klebsiella aerogenes* [21]. Induced expression of the *ppk* gene encoding PPK and the *pst* genes under P_i starvation is responsible for polyP overplus in *K. aerogenes*.

Bacterial polyP accumulation can be enhanced by genetic modification [22]. A genetically modified strain of *E. coli* could accumulate approximately 16% of

its dry weight as P (73% as P_2O_5). This P content of the *E. coli* recombinant far exceeded that of high-grade P_i rock. Genetically modified microorganisms have no practical use in an open system such as activated sludge process. However, spontaneous mutations of the *phoU* gene enable bacteria to accumulate high levels of polyP even under P_i excess. Such mutations can be caused by the *N*-methyl-*N'*-vitro-*N*-nitrosoguanidine (NTG) mutagenesis [23]. Since the *phoU* mutants express alkaline phosphatase constitutively, they can be easily screened as blue-colored colonies on P_i-excess agar plates containing 5-bromo-4-chloro-3-indolyl-phosphate (X-P_i). This simple technology allows us to enhance the ability of sludge bacteria to accumulate polyP without using the recombinant DNA technology [24].

19.3 Bio-Based P Recycling

Basically, bio-based P recycling from wastewater consists of (i) biological P removal from wastewater, (ii) P_i release in a more condensed form from P-rich sludge biomass or P_i leaching from sludge incineration ash, and (iii) P_i precipitation from solution with inorganic cations such as Ca^{2+} or Mg^{2+}. Through this process, P is recovered in the form of either calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HAP] or struvite ($MgNH_4PO_4{\cdot}6H_2O$, magnesium ammonium phosphate) [13, 25, 26]. The recovered products can be used as an effective slow-release fertilizer in agriculture depending on the pH [25]. From the industrial viewpoint, HAP recovery is more promising because it has versatile applications in the manufacturing industry [26]. There are many technological options to remove, release, and precipitate P_i.

19.3.1 Biological P Removal

Although many technologies have been proposed to remove P from wastewater, biological P removal is a relatively inexpensive and environmentally sustainable option [13, 27]. Biological P removal can avoid consuming a lot of chemicals and correspondingly reduce the cost of sludge disposal [11]. In addition, it is achievable in existing activated sludge processes with a minimum modification on the operating regimes [11]. Activated sludge processes, which are commonly used for treating wastewater, can remove 50–70% of the P_i normally found in municipal wastewater [13]. Moreover, when activated sludge is subjected to alternate anaerobic and aerobic cycles, sludge microorganisms can accumulate high levels of cellular P_i in the form of polyP [12, 28]. This phenomenon is known as enhanced biological phosphorus removal (EBPR).

EBPR has already become a well-established process applied in many full-scale wastewater treatment plants (WWTPs). There is no doubt that EBPR primarily relies on the ability of sludge microorganisms to accumulate polyP. However,

despite extensive works on EBPR microbiology and biochemistry, the detailed mechanism still remains unclear [29]. This is attributable to the complex nature of activated sludge. The complexity and variability of the microbial population hinder taking molecular and genetic approaches except fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and metagenomic techniques. It seems difficult, if not impossible, to clarify the detailed mechanism of EBPR at a molecular level.

EBPR processes can remove typically 80–90% of influent P in WWTPs. The alternate anaerobic–aerobic cycle can be installed by introducing an anaerobic zone ahead of an aerobic stage [11]. In a conventional WWTP, this can be readily realized by shutting off aeration at the upstream part of an aeration tank and returning the sludge to this region from a secondary settler. EBPR sludge contains considerable amounts of P (typically 3–5 dry wt%), together with organic matter and other plant nutrients such as nitrogen, sulfur, and potassium. However, it also contains residues formed from the addition of precipitating agents in the wastewater treatment, organic contaminants, and potentially toxic trace elements such as chromium, mercury, and cadmium [30]. These potential pollutants may become enriched in the soil in the long run if the sludge is directly applied to agricultural land. To avoid these potential risks, it is recommended to process EBPR sludge into fertilizers that are safer to store, handle, and apply [31].

19.3.2
P_i Release from polyP-Rich Sludge

P_i is released from EBPR sludge in the more concentrated form by various technologies, including heat treatment [32], anaerobiosis [33], anaerobic digestion [34], and incineration followed by chemical leaching [30]. PolyP can be released from sludge by heating at 70 °C for ~1 h [32]. The released polyP is degraded to P_i in solution during heat treatment. Since sludge settleability is unchanged before and after heat treatment, the P-rich solution can be readily separated from sludge solids by sedimentation [35]. An obvious disadvantage of this technology is the additional cost for heating the sludge at 70 °C. Heat exchangers may help minimize heat energy loss, thereby enabling cost reduction [12].

PolyP-rich activated sludge can release P_i, but not polyP, back into solution when it is subjected to anaerobiosis [33]. Although the detailed mechanism is unclear, this is considered as a simple and the most inexpensive option to release P_i from sludge biomass in a side stream [33]. For instance, this phenomenon has been applied to releasing P_i from sludge biomass in a small anaerobic P_i stripper tank [33]. The addition of a readily decomposable organic acid such as acetate or propionate can significantly enhance the rate of anaerobic P_i release. However, the rate and extent of P_i release by anaerobiosis are less prominent than those achieved by heating at 70 °C.

Anaerobic sludge digestion is a well-established process to stabilize waste sludge from the EBPR process [34]. It is one of the most technically mature and cost-effective processes to convert sludge to methane-rich bioenergy (biogas), thereby

reducing volume, odor, and pathogens [34]. During anaerobic sludge digestion, P_i is released to the sludge liquor in a more condensed form. However, P_i derived from polyP-rich sludge often causes uncontrolled precipitation of struvite on pipe walls and equipment surfaces of anaerobic digestion processes [25]. This leads to operational problems and significantly increases maintenance costs in waste sludge management. The possibility of struvite formation increases with a rise in the P content of waste sludge. Hence, P_i recovery prior to anaerobic digestion may reduce the potential of struvite deposition. One technical option for P_i removal prior to anaerobic digestion is the above-mentioned heat treatment [32]. Many studies have shown that heat treatment prior to anaerobic digestion can improve digestibility and methane productivity [36]. Therefore, the heat treatment prior to anaerobic digestion may be beneficial not only for controlling the struvite deposition problem but also for improving methane productivity [35].

Sludge incineration followed by chemical P_i leaching is another option for P_i release from polyP-rich sludge [37]. When EBPR sludge is incinerated in a mono-incineration plant, P is concentrated in the ash and can be released by acid or alkaline leaching. One issue of the chemical P_i leaching is how to separate the valuable P from problematic heavy metals. Various separation technologies, including solvent extraction [38] and ion exchange [39], are available to remove heavy metals. Solvent extraction involves the transfer of metal compounds from an aqueous solution to an organic solvent using extracting chemical agents [38]. Ion exchange is achieved by ion exchangers with specific affinity to definite metal ions or groups of metals. This enables replacing heavy metal ions by nontoxic ones such as Na^+ and K^+ [39]. However, it is unavoidable that the P recovery cost increases with increasing consumption of chemicals. The main alternative for chemical P_i leaching from sludge incineration ash is via thermochemical methods [31]. For instance, problematic heavy metals can be removed from sludge incineration ash by heating at 900–1000 °C in the presence of KCl or $MgCl_2$. P loss during the thermochemical treatment can be minimized by granulating sludge incineration ash into pellets as a pretreatment. The thermochemically treated sludge ash can be used as NPK fertilizer after being supplemented with NH_4NO_3 and K_2SO_4. Since fertilizer is a relatively inexpensive commodity, the potential of the thermochemical method as a P recycling option depends on the fuel cost for heating sludge incineration ash [40].

19.3.3 P_i Recovery from Aqueous Solution

P_i can be recovered from an aqueous solution in the form of either HAP or struvite. HAP has a chemical formula similar to that of bone mineral and is commonly used as a bioceramic owing to its biological properties of biocompatibility [41]. HAP is formed from unstable calcium phosphates such as dicalcium phosphate dehydrate ($CaHPO_4 \cdot 2H_2O$, brushite) and amorphous calcium phosphate ($Ca_3(PO_4)_2 \cdot nH_2O$, ACP) during and after P_i precipitation with Ca^{2+} [42]. P_i precipitation with Ca^{2+} is influenced by many factors such as the Ca/P molar ratio, pH, ionic strength,

temperature, and coexisting ions [43]. Extensive studies have been carried out on P_i precipitation with Ca^{2+}, enabling this process to become a well-established P_i recovery option. P_i recovered in the form of HAP can be used as a raw material for the manufacturing of commercial fertilizers.

As mentioned earlier, struvite often forms in anaerobic sludge digestion processes where high concentrations of P_i and ammonium are present. The blockage of pipes by struvite precipitation causes significant operational problems which lead to an increase in the maintenance costs of WWTPs. However, if struvite precipitation is minimized in the digester, high struvite production from P_i-rich stream can be achieved in the downstream crystallization process [25]. P_i recovery by controlled struvite crystallization has been one of the most widely recommended technologies for treating sludge digestion liquors [25]. A high-quality struvite pellet product has been marketed as a slow-release fertilizer [43].

P_i can also be recovered from aqueous solutions using crystalline calcium silicate hydrates (CSHs) [44]. CSHs occur naturally and produced technically during the production of gas concrete [13]. CSHs release Ca^{2+} into P_i-bearing water, thereby triggering the Ca–P crystallization and its deposition onto the surface of CSHs. This technology has the merit of reducing the consumption of chemicals and correspondingly decreasing the cost of P recovery [44]. To avoid struvite scaling problems, it is effective to directly add CSHs to an anaerobic digester, thereby decreasing the P_i concentration in the digested sludge [45].

The use of amorphous calcium silicate hydrates (A-CSHs) is a new cost-effective option to recover P_i from aqueous solution [42]. A-CSHs can be synthesized using unlimitedly available materials such as siliceous shale and $Ca(OH)_2$ at low costs. Since the reaction of P_i with A-CSHs occurs at pH 7–9, it is not required to adjust the solution pH to a high alkaline pH. The high settleability, filterability, and dewaterability of the recovered P are the advantages of A-CSHs over conventional $CaCl_2$ and $Ca(OH)_2$ (Figure 19.2). No chemical coagulants are required for P_i recovery by A-CSHs. Moreover, unlike $Ca(OH)_2$, no significant carbonate inhibition occurs with P_i recovery with A-CSHs. Although the detailed mechanism for P_i recovery by A-CSHs remains unclear, it seems likely that P_i is exchanged with soluble silicate polymers in A-CSHs, enabling P_i to bind to them. Importantly, the P recovered by A-CSHs can be directly used as by-product P_i fertilizer. *In situ* experiments at a WWTP have shown that heavy metals such as As, Cd, Cr, Cu. Hg, Pb, and Zn are not enriched in the recovered products [46]. A-CSHs have great potential as a beneficial material for P recycling.

Biological P removal has been implemented in many WWTPs, aiming to control eutrophication in aquatic systems. However, only a minor portion of removed P is currently recycled for the manufacture of fertilizers. Despite its precious resource potential, it is mostly disposed of as landfill or construction materials without being recycled. This is because essentially no regulation requires P reuse and recycling. Lack of policy measures allows the wastewater treatment sector to neglect P recycling, which may be considered as an extra service. Emerging issues for the implementation of P recycling include the high capital cost for plant construction and the difficulty in establishing stable channels for the distribution and

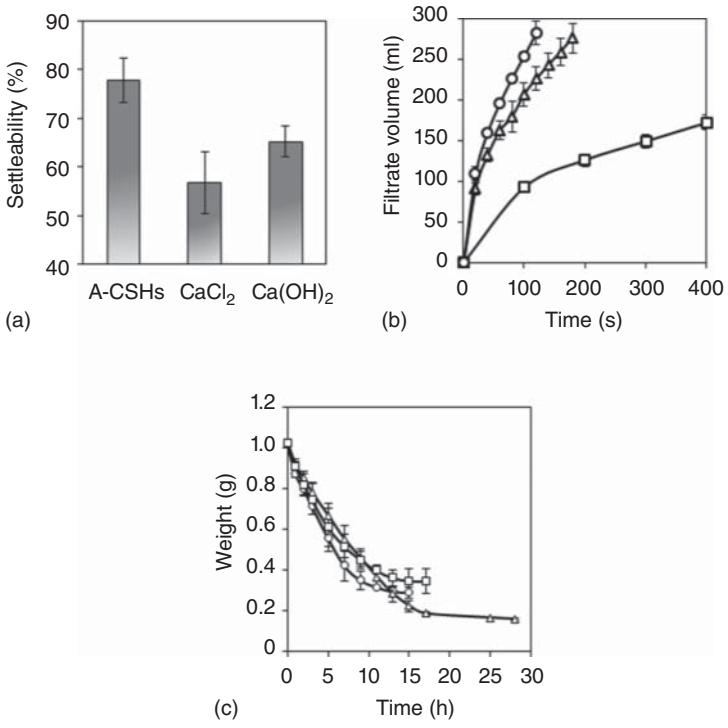

Figure 19.2 Settleability, filterability, and dewaterability of P recovered by A-CSHs, $CaCl_2$, and $Ca(OH)_2$ [42]. The settleability (a), filterability (b), and dewaterability (c) of recovered P were assessed by the method described previously [42]. Symbols are A-CSHs (circles), $Ca(OH)_2$ (squares), and $CaCl_2$ (triangles). (With permission ©2013 Elsevier Ltd.)

sale of recovered products. To overcome these challenges, it is critical that the wastewater treatment sector works in close collaboration with government agencies and the fertilizer industry.

19.4 Other Options for P Recycling

19.4.1 Land Application of Biosolids

The application of biosolids (treated sewage sludges) to agricultural land is the simplest option to recycle P from wastewater. The nutrient-rich organic solids are considered as a low-grade fertilizer and soil amendment to improve the chemical and physical properties of soil [47]. However, a number of factors make the land application of biosolids increasingly difficult [40]. As mentioned earlier,

the residual sludge from wastewater treatment contains a variety of organic contaminants, heavy metals, and pathogens. Although most risk assessments demonstrate that the majority of the contaminants do not place human health at risk, continued vigilance in assessing the significance and implications of potential pollutants is necessary to ensure the long-term security of the land application of biosolids [48].

Urine diversion is another option for the application of human waste to agriculture [49]. Urine diversion sanitation technology, which can divert urine away from human excreta via a specially designed toilet, has been developed and applied in mainly developing countries [50]. The source separation of urine can improve effluent quality and save energy utilization and investment costs of the receiving wastewater treatment. Recycling urine as a liquid fertilizer has the potential to provide approximately 15% P required to fertilize cereal crops on a global basis. However, human urine also contains ingested pharmaceuticals and hormones. If urine sources are sterile and lack chemical contaminants, they may be used as liquid fertilizer after a precautionary waiting period [50].

19.4.2 Animal Manure Management

Animal manure has often been considered as a waste product and disposed of by application to land within a narrow region of where it is produced [51]. However, animal manure is one of major secondary P resources that have the potential to reduce reliance on mineral P_i fertilizer. Animal manure is a valuable source of plant nutrients and organic matter if it is adequately managed and applied. Reuse of animal manure is currently hindered by its bulky nature, the risk of transmitting pathogens, the contamination of heavy metals, undesirable odor, and the geographical separation of livestock farming from crop production. Additionally, where animal manure has been applied as organic fertilizer over a long term, P_i overaccumulation in soil causes increased transfer of P_i to surrounding water bodies, leading to eutrophication [51].

To sustainably use animal manure as fertilizer, manure treatment technologies have been developed and are still being developed [52]. Among them is anaerobic digestion of animal manure, which can offer substantial benefits, including onsite energy generation, production of stable liquid fertilizers and high-quality soil amendment, reduction in odors, and reduction in ground and surface water contamination [53]. The unavoidable issue is the associated investment costs for large-size reactors and the handling, dewatering, and disposal of the digested residues. Like biosolids, animal manure contains potential organic pollutants such as veterinary medicines, antibiotics, and biocides. To minimize the health and environmental risks, P needs to be recovered from animal waste and transformed into a form of marketable product, thereby easing the storage, movement, and application of manure nutrients. Incineration ash of animal manure, particularly chicken manure, contains a considerable amount of P and can be easily processed into fertilizers.

19.4.3 Biosolubilization of Immobilized P_i

P is present in inorganic and organic forms in agricultural soil. Although chemical fertilizers contain large amounts of soluble inorganic P_i, it is easily and rapidly immobilized by forming complexes with Al^{3+}, Fe^{3+}, Ca^{2+}, and Mn^{2+}, depending on the soil type [54]. Many microorganisms are able to solubilize and mineralize P pools in soil. They produce organic acids such as butyrate, citrate, and gluconate, which can convert unavailable P to soluble P_i at decreased pH [55, 56]. P-solubilizing microorganisms, predominantly bacteria, can contribute to the P efficiency of plants by increasing the P cycling in agricultural soil. P-solubilizing bacteria can also be economically and environmentally useful for bioleaching P_i from high-P iron ore and low-grade P_i rock [57]. Molecular breeding of plants that can use P_i efficiently may be a helpful strategy for agriculture. However, taking into account public acceptance issues on genetically modified plants, investments may be more effective if aimed at the use of P-solubilizing microorganisms.

19.4.4 Industrial P Recycling

Approximately 15% of the global P demand comes from the manufacturing industry [3, 58]. For instance, high-grade phosphoric acid is used as an iron-coating material in the automotive industry, etching agents for aluminum line pattern substrates of computer chips and liquid crystal panels, food additives, chemical catalysts, and flame retardants. P is also one of the crucial raw materials for the production of rechargeable batteries such as lithium ion batteries. In the industrial manufacturing sector, considerable attention has been paid to P_i removal from wastewater, because P_i emission is strictly regulated by national or local governments to control eutrophication [59, 60].

Recently, P recycling has been considered as an economically beneficial option, because it may lead to the reduction of waste disposal costs by turning P-rich waste into a resource [46]. For instance, considerable attention has been paid to P recovery in wastewater treatment processes of the fermentation and food industries [61]. Nucleic acid fermentation processes use P_i as an essential raw material, thereby generating P_i-rich wastewater. P_i is removed from the wastewater by biological P removal and precipitation with $Ca(OH)_2$ [61]. P recovery has also been implemented in an edible oil refining process, which uses high-grade phosphoric acid to remove impurities from crude vegetable oil [46]. Since no harmful substance is used in the edible oil refinery process, the quality of the recovered product makes it suitable for use in fertilizers for agricultural purposes. The P recovered in the form of HAP is sold as a raw material to local fertilizer companies [46].

In the manufacture of liquid crystal glass substrates, P_i is commonly used as an aluminum etching agent [62]. Although the P_i-rich wastewater can be chemically treated with $FeCl_3$ or $Ca(OH)_2$, this generates large amounts of precipitates,

which must be disposed of as industrial wastes. A membrane system has been developed for recovering P from the spent etching solution of a liquid crystal substrate manufacturing process [46]. P_i is separated from spent etching solutions using a two-step reverse osmosis membrane system. This technology uses essentially no chemicals to recover P_i from spent etching solutions. The resulting P_i solution contains no impurities such as heavy metals.

In terms of quantity, P recycling in the steel-making industry is particularly important [63]. For instance, the amount of P emitted into steel-making slag is estimated to be ~2 times larger than that ending up in sewage sludge in Japan [64]. Coal and iron ore, which are essential raw materials for the manufacture of iron and steel, contain small amounts of P (typically <0.03 mass% of P_2O_5). Since P has detrimental effects on the mechanical properties of steel, it is removed into steel-making slag at concentrations as high as 2–10 mass% of P_2O_5 [64]. Hence, dephosphorization slag from the steel-making industry is considered as a quantitatively important secondary P resource. Laboratory experiments have demonstrated that P is potentially recovered from dephosphorization slag using wet magnetic separation [64] and carbonate flux treatment [65]. Dephosphorization slag can also be reduced in an iron bath furnace to simultaneously extract iron, manganese, and P [66]. P-enriched slag can be separated from the hot metal yield and used as P fertilizer. The global production of iron ore is ~2000 million tons per year (mt/y) [67]. Assuming that iron ore contains 0.05% of P on average, ~1.0 mt/y of P could be potentially recovered from dephosphorization slag. This is equivalent to one-third of industrial P demand worldwide (2.8 mt/y).

19.5 Conclusions

The cheap supply of P is crucial to our food security [3]. Sustainable P use must ensure that all the world's farmers have sufficient access to P in the long run to produce enough food to support humanity while minimizing adverse environmental and social impacts [3]. However, because of the limited supply and increasing demand of P worldwide, cheap P fertilizer is becoming a thing of the past.

Much P is lost along the way when passing from mine to field to fork [3]. The losses are significant in arable land and livestock production because of overuse of P fertilizer and improper management of animal manure. Improved agricultural efficiency can contribute much to the achievement of P sustainability [68]. In addition to cutting usage, recycling P is crucial to the sustainable use of P. Biotechnology is involved in the demand and consumption chain of P, ranging from crop production, livestock farming, and food processing to waste treatment. Hence, biotechnology has the potential to improve the efficiency of anthropogenic P use if adequate and full consideration is given to its role in the demand and consumption chain. To fully realize P recycling, it is necessary to establish P_i refinery technology that can recover P from a wide variety of secondary P resources and use it for recycling (Figure 19.3). P-solubilizing microorganisms play fundamental

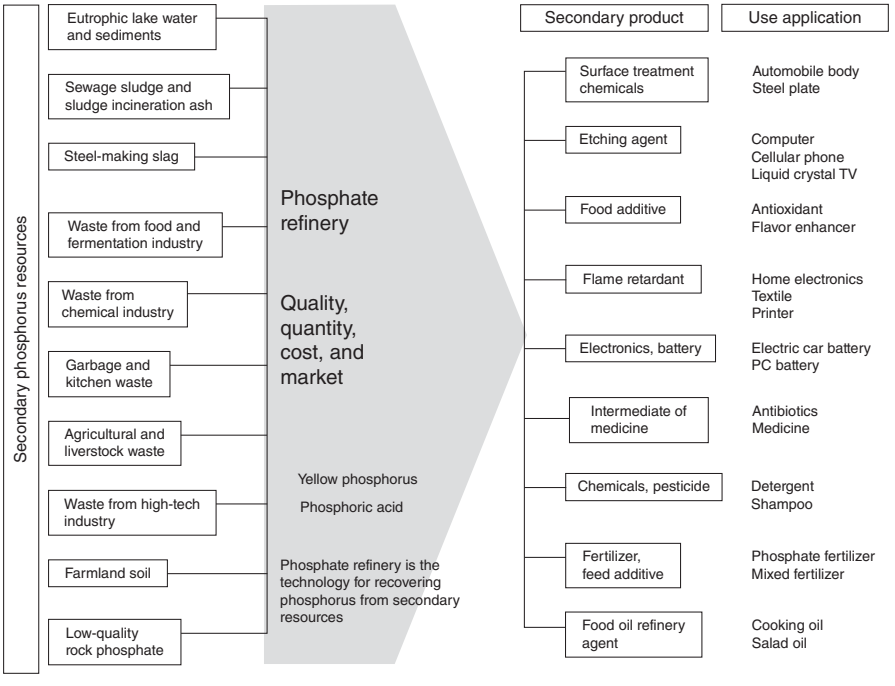

Figure 19.3 P_i refinery technology.

roles in biogeochemical P cycling in natural and agricultural ecosystems [69]. A biotechnological option for P_i refinery may be given by the use of P-solubilizing microorganims. Microbial P_i refinery technology is just starting and needs to fully exploit the potential of microbes to valorize P-containing wastes.

Despite the recent development of P recovery technology, implementation of P recycling is hindered by a variety of socioeconomic reasons such as a lack of public awareness, immature markets for recovered P, and insufficient support from governments. As a result, only a limited amount of potential secondary P resources has been used for recycling. When compared with P removal from wastewater, P recycling is more difficult to implement, because it needs to create channels for the distribution and sale of the recovered products. The implementation of P recycling requires the integration of technology innovation, corporate strategies, and public policies. It is particularly important to realize synergies with pollution control, energy/materials recovery, and reducing wastes. A sustainable and safe strategy for P recycling is expected to benefit our present and future society.

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