HANDBOOK OF FOOD BIOENGINEERING
VOLUME 14

# ADVANCES IN BIOTECHNOLOGY FOR FOOD INDUSTRY


Edited by
Alina Maria Holban
Alexandru Mihai Grumezescu

# *Advances in Biotechnology for Food Industry*

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## Handbook of Food Bioengineering, Volume 14

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ACADEMIC PRESS
An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1800, San Diego, CA 92101-4495, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom



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**Library of Congress Cataloging-in-Publication Data**
A catalog record for this book is available from the Library of Congress

**British Library Cataloguing-in-Publication Data**
A catalogue record for this book is available from the British Library

ISBN: 978-0-12-811443-8



*Publisher:* Andre G. Wolff
*Acquisition Editor:* Nina Rosa de Araujo Bandeira
*Editorial Project Manager:* Jaclyn A. Truesdell
*Production Project Manager:* Mohanapriyan Rajendran
*Designer:* Matthew Limbert

Typeset by Thomson Digital

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# Foreword

In the last 50 years an increasing number of modified and alternative foods have been developed using various tools of science, engineering, and biotechnology. The result is that today most of the available commercial food is somehow modified and improved, and made to look better, taste different, and be commercially attractive. These food products have entered in the domestic first and then the international markets, currently representing a great industry in most countries. Sometimes these products are considered as life-supporting alternatives, neither good nor bad, and sometimes they are just seen as luxury foods. In the context of a permanently growing population, changing climate, and strong anthropological influence, food resources became limited in large parts of the Earth. Obtaining a better and more resistant crop quickly and with improved nutritional value would represent the Holy Grail for the food industry. However, such a crop could pose negative effects on the environment and consumer health, as most of the current approaches involve the use of powerful and broad-spectrum pesticides, genetic engineered plants and animals, or bioelements with unknown and difficult-to-predict effects. Numerous questions have emerged with the introduction of engineered foods, many of them pertaining to their safe use for human consumption and ecosystems, long-term expectations, benefits, challenges associated with their use, and most important, their economic impact.

The progress made in the food industry by the development of applicative engineering and biotechnologies is impressive and many of the advances are oriented to solve the world food crisis in a constantly increasing population: from genetic engineering to improved preservatives and advanced materials for innovative food quality control and packaging. In the present era, innovative technologies and state-of-the-art research progress has allowed the development of a new and rapidly changing food industry, able to bottom-up all known and accepted facts in the traditional food management. The huge amount of available information, many times is difficult to validate, and the variety of approaches, which could seem overwhelming and lead to misunderstandings, is yet a valuable resource of manipulation for the population as a whole.

The series entitled *Handbook of Food Bioengineering* brings together a comprehensive collection of volumes to reveal the most current progress and perspectives in the field of food engineering. The editors have selected the most interesting and intriguing topics, and have dissected them in 20 thematic volumes, allowing readers to find the description of

basic processes and also the up-to-date innovations in the field. Although the series is mainly dedicated to the engineering, research, and biotechnological sectors, a wide audience could benefit from this impressive and updated information on the food industry. This is because of the overall style of the book, outstanding authors of the chapters, numerous illustrations, images, and well-structured chapters, which are easy to understand. Nonetheless, the most novel approaches and technologies could be of a great relevance for researchers and engineers working in the field of bioengineering.

Current approaches, regulations, safety issues, and the perspective of innovative applications are highlighted and thoroughly dissected in this series. This work comes as a useful tool to understand where we are and where we are heading to in the food industry, while being amazed by the great variety of approaches and innovations, which constantly changes the idea of the "food of the future."

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# Series Preface

The food sector represents one of the most important industries in terms of extent, investment, and diversity. In a permanently changing society, dietary needs and preferences are widely variable. Along with offering a great technological support for innovative and appreciated products, the current food industry should also cover the basic needs of an ever-increasing population. In this context, engineering, research, and technology have been combined to offer sustainable solutions in the food industry for a healthy and satisfied population.

Massive progress is constantly being made in this dynamic field, but most of the recent information remains poorly revealed to the large population. This series emerged out of our need, and that of many others, to bring together the most relevant and innovative available approaches in the intriguing field of food bioengineering. In this work we present relevant aspects in a pertinent and easy-to-understand sequence, beginning with the basic aspects of food production and concluding with the most novel technologies and approaches for processing, preservation, and packaging. Hot topics, such as genetically modified foods, food additives, and foodborne diseases, are thoroughly dissected in dedicated volumes, which reveal the newest trends, current products, and applicable regulations.

While health and well-being are key drivers of the food industry, market forces strive for innovation throughout the complete food chain, including raw material/ingredient sourcing, food processing, quality control of finished products, and packaging. Scientists and industry stakeholders have already identified potential uses of new and highly investigated concepts, such as nanotechnology, in virtually every segment of the food industry, from agriculture (i.e., pesticide production and processing, fertilizer or vaccine delivery, animal and plant pathogen detection, and targeted genetic engineering) to food production and processing (i.e., encapsulation of flavor or odor enhancers, food textural or quality improvement, and new gelation- or viscosity-enhancing agents), food packaging (i.e., pathogen, physicochemical, and mechanical agents sensors; anticounterfeiting devices; UV protection; and the design of stronger, more impermeable polymer films), and nutrient supplements (i.e., nutraceuticals, higher stability and bioavailability of food bioactives, etc.).

The series entitled *Handbook of Food Bioengineering* comprises 20 thematic volumes; each volume presenting focused information on a particular topic discussed in 15 chapters each. The volumes and approached topics of this multivolume series are:

Volume 1: Food Biosynthesis

Volume 2: Food Bioconversion

Volume 3: Soft Chemistry and Food Fermentation

Volume 4: Ingredients Extraction by Physicochemical Methods in Food

Volume 5: Microbial Production of Food Ingredients and Additives

Volume 6: Genetically Engineered Foods

Volume 7: Natural and Artificial Flavoring Agents and Food Dyes

Volume 8: Therapeutic Foods

Volume 9: Food Packaging and Preservation

Volume 10: Microbial Contamination and Food Degradation

Volume 11: Diet, Microbiome and Health

Volume 12: Impact of Nanoscience in the Food Industry

Volume 13: Food Quality: Balancing Health and Disease

Volume 14: Advances in Biotechnology for Food Industry

Volume 15: Foodborne Diseases

Volume 16: Food Control and Biosecurity

Volume 17: Alternative and Replacement Foods

Volume 18: Food Processing for Increased Quality and Consumption

Volume 19: Role of Materials Science in Food Bioengineering

Volume 20: Biopolymers for Food Design

The series begins with a volume on *Food Biosynthesis*, which reveals the concept of food production through biological processes and also the main bioelements that could be involved in food production and processing. The second volume, *Food Bioconversion*, highlights aspects related to food modification in a biological manner. A key aspect of this volume is represented by waste bioconversion as a supportive approach in the current waste crisis and massive pollution of the planet Earth. In the third volume, *Soft Chemistry and Food Fermentation*, we

aim to discuss several aspects regarding not only to the varieties and impacts of fermentative processes, but also the range of chemical processes that mimic some biological processes in the context of the current and future biofood industry. Volume 4, *Ingredients Extraction by Physicochemical Methods in Food*, brings the readers into the world of ingredients and the methods that can be applied for their extraction and purification. Both traditional and most of the modern techniques can be found in dedicated chapters of this volume. On the other hand, in volume 5, *Microbial Production of Food Ingredients and Additives*, biological methods of ingredient production, emphasizing microbial processes, are revealed and discussed. In volume 6, *Genetically Engineered Foods*, the delicate subject of genetically engineered plants and animals to develop modified foods is thoroughly dissected. Further, in volume 7, *Natural and Artificial Flavoring Agents and Food Dyes*, another hot topic in food industry—flavoring and dyes—is scientifically commented and valuable examples of natural and artificial compounds are generously offered. Volume 8, *Therapeutic Foods*, reveals the most utilized and investigated foods with therapeutic values. Moreover, basic and future approaches for traditional and alternative medicine, utilizing medicinal foods, are presented here. In volume 9, *Food Packaging and Preservation*, the most recent, innovative, and interesting technologies and advances in food packaging, novel preservatives, and preservation methods are presented. On the other hand, important aspects in the field of *Microbial Contamination and Food Degradation* are shown in volume 10. Highly debated topics in modern society: *Diet*, *Microbiome and Health* are significantly discussed in volume 11. Volume 12 highlights the *Impact of Nanoscience in the Food Industry*, presenting the most recent advances in the field of applicative nanotechnology with great impacts on the food industry. Additionally, volume 13 entitled *Food Quality*: *Balancing Health and Disease* reveals the current knowledge and concerns regarding the influence of food quality on the overall health of population and potential food-related diseases. In volume 14, *Advances in Biotechnology for Food Industry*, up-to-date information regarding the progress of biotechnology in the construction of the future food industry is revealed. Improved technologies, new concepts, and perspectives are highlighted in this work. The topic of *Foodborne Diseases* is also well documented within this series in volume 15. Moreover, *Food Control and Biosecurity* aspects, as well as current regulations and food safety concerns are discussed in the volume 16. In volume 17, *Alternative and Replacement Foods*, another broad-interest concept is reviewed. The use and research of traditional food alternatives currently gain increasing terrain and this quick emerging trend has a significant impact on the food industry. Another related hot topic, *Food Processing for Increased Quality and Consumption*, is considered in volume 18. The final two volumes rely on the massive progress made in material science and the great applicative impacts of this progress on the food industry. Volume 19, *Role of Materials Science in Food Bioengineering*, offers a perspective and a scientific introduction in the science of engineered materials, with important applications in food research and technology. Finally, in volume 20, *Biopolymers for Food Design*, we discuss the advantages and challenges related to the development of improved and smart biopolymers for the food industry.

All 20 volumes of this comprehensive collection were carefully composed not only to offer basic knowledge for facilitating understanding of nonspecialist readers, but also to offer valuable information regarding the newest trends and advances in food engineering, which is useful for researchers and specialized readers. Each volume could be treated individually as a useful source of knowledge for a particular topic in the extensive field of food engineering or as a dedicated and explicit part of the whole series.

This series is primarily dedicated to scientists, academicians, engineers, industrial representatives, innovative technology representatives, medical doctors, and also to any nonspecialist reader willing to learn about the recent innovations and future perspectives in the dynamic field of food bioengineering.

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# *Preface for Volume 14: Advances in Biotechnology for Food Industry*

The progress of biotechnology has greatly impacted the modern food industry. Microbial production of food ingredients was modulated and utilized by food industry to obtain specific and valuable food products, which are widely obtained and commercialized worldwide. Quality, quantity, and diversity of food products are increased by the contribution of biotechnology to fulfill very wide nutritional requirements and food preferences. Novel techniques and approaches in food production and processing allowed for a real food revolution, pushing the limits of traditional food concept. Although easier to obtain, numerous modern food products fail to provide necessary nutritive components. In this context, biotechnology was utilized to design and obtain valuable high quality food products and supplements with a great nutritional value. Moreover, the faster and cheaper production of basic food products was also possible by the progress of food biotechnology.

Since the technological progress on this field is rapidly increasing to ensure an efficient emergence of the very diverse food industry, it is very difficult to acknowledge all the major achievements. The purpose of this book is to reveal and discuss the main scientific progress made in the field of food biotechnology, while providing a strong technical base to understand future progress. Recent advances made on basic technologies to obtain wide-interest food products, as well as innovative novel approaches are highlighted.

The volume contains 15 chapters prepared by outstanding authors from Bulgaria, Germany, the United States, Brazil, Portugal, India, Poland, Turkey, Mexico, China, and Spain.

Selected manuscripts are clearly illustrated and contain accessible information for a wide audience, especially food scientists, materials science researchers, biotechnologists, biochemists, engineers; and also for any reader interested in learning about the most interesting and recent advances regarding biotechnological progress in the food industry.

Chapter 1, *Role of Biotechnology in the Agrofood Industry*, written by Trejo-Perea et al. discusses about the progress of various scientific techniques to modify, improve, and increase the value of various foods of animal, plant, and microorganism origin of economic interest.

In Chapter 2, *Biotechnology in Food Processing and Preservation: An Overview*, Ghoshal aims to highlight the applications of biotechnology in food processing and preservation. Biotechnology is discussed as a factor to improve the edibility, consistency, and shelf life of food by preventing growth of unwanted microorganisms naturally present in foods, and also to promote the production of valuable food components.

In Chapter 3, entitled *Enzymes and Food Industry: A Consolidated Marriage*, Gomes et al. provide an overview of enzymatic applications in the food industry, as well as the newly progresses in this area. In the last century, the development of enzyme-based technologies greatly impacted food industry, as these biomolecules catalyze biochemical reactions with high specificity and mild physicochemical conditions.

Chapter 4, *Lactic Acid Bacteria—From Nature through Food to Health*, prepared by Teneva-Angelova et al., presents recent achievements in environmental diversity of lactic acid bacteria (LAB) (traditional and alternative sources), studies on biological activities of LAB, as well as their role in the functional food formulations.

In Chapter 5, *Development of Controlled Cocultivations for Reproducible Results in Fermentation Processes in Food Biotechnology*, Johannes et al. give valuable examples of the simultaneous utilization of different microorganisms in industrial processes and the development of controlled and reproducible coculture fermentation enabling the combination of traditional fermented food with industrial production processes.

In Chapter 6, *Enumeration and Identification of Probiotic Bacteria in Food Matrices*, Zielińska et al. provide methods of enumeration and identification of probiotic bacteria in food according to culture-dependent and culture-independent techniques, such as phenotyping, biochemical, physical, immunological, and molecular biology methods. In particular, the direct identification potential of molecular tools, such as DNA, RNA, and peptide analysis is highlighted.

In Chapter 7, *Improvement of Ripened Cheese Quality and Safety With* Thymus mastichina *L. Bioactive Extracts*, Carvalho et al. present a case study of the efficiency of *T. mastichina* L. bioactive extracts in the improvement of safety and quality of ripened cheese during production and processing.

Chapter 8, *Potential of High Hydrostatic Pressure to Improve the Production of Plants Used as Food*, was prepared by Altuner. High hydrostatic pressure (HHP) is used in food processing for several purposes, such as sterilization, coagulation, and gelation; however, there are some new potential uses of HHP in different areas besides food processing, such as conducting this method on plants used as food, in order to change some physiological and biochemical properties, which can be accepted as an improvement, when it is compared to the unpressurized plant samples.

Chapter 9, *Corrosion in Electronic Sensors Used in Manufacturing Processes Decrease the Quality in the Seafood Industry*, written by Badilla and Gaynor, offers a statistical analysis to evaluate the operation yielding of the electronic sensors used in the industrial systems, industrial equipment, and industrial machinery. Also, this chapter reveals an evaluation of the cost originated by the presence of corrosion in the metallic surfaces of the electronic sensors and the determination of the principal pollution agents that damage the metallic surfaces of electronic sensors.

In Chapter 10, *Biotechnology of Ice Wine Production*, Jing et al. discuss about the management of extreme osmophilia in the must of ice wine, which is one of the critical goals in the biotechnology of ice wines. The control of sensory quality and longevity of these sweet wines that make them something really special is also discussed here.

Chapter 11, *Metagenomics of Traditional Beverages*, written by Morales et al., includes the applications of culture-independent methods, such as molecular techniques: DGGE, ARDRA, and second-generation sequencing as pyrosequencing and illumina technology, to identify those microorganisms involved in fermentation of traditional beverages and microorganisms isolated from these beverages. The impact of molecular technologies on food industry is highlighted in this chapter.

In Chapter 12, *Process Engineering Applying Supercritical Technology for Obtaining Functional and Therapeutic Products*, Silva et al. present a detailed assessment of such technology and the range of bioactive compounds produced, aiming to present a protocol of experimental activities in the field of food processing for increased consumption.

Chapter 13, *Sugar Beet Pulp as a Source of Valuable Biotechnological Products*, written by Joanna et al., describes the composition of sugar beet pulp, the chemical pretreatment methods that can be used to obtain suitable media for microbial cultivation, the microorganisms used in various biotechnological processes, and new strategies for the production of valuable compounds, including lactic acid, propylene glycol, furfural, furfuryl alcohol, and tetrahydrofurfuryl alcohol. The solutions presented here have the potential to generate additional revenue for businesses, from the sale of new products, such as food, animal feed, and green chemicals.

In Chapter 14, *Biofilms in Food Industry: Mitigation Using Bacteriophage*, Laxmi and Bhat describe the capability of a newly isolated bacteriophage of industrial impact, designed as ΦPAP-1 to resist under hostile environmental conditions and manifests broad antibiofilm activity against a large host range. This bacteriophage could be considered a biocontrol agent with great impact especially on food industry.

Chapter 15, *Bioactive Properties and Biotechnological Production of Human Milk Oligosaccharides*, written by Yebra et al., discusses the effects of human milk

oligosaccharides on human health, being involved in the protection against gastrointestinal infections, prebiotic effects, and antiadhesive effect against enteropathogens. The main sources with high content of milk oligosaccharides, such as various milk formulations are presented in this chapter.

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CHAPTER 1

# Role of Biotechnology in the Agrofood Industry

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## 1 Brief Summary of Agrofood Industry Role Worldwide

The agroindustrial sector is known as the manufacturing sector that processes raw materials and intermediate products derived from agriculture, fisheries, and forestry. This sector is taken to include manufacturers of food, beverages and tobacco, textiles and clothing, wood products and furniture, paper, paper products and printing, and rubber and rubber products, as in Food and Agriculture Organization of the United Nations (FAO, 2013). Agroindustry is part of the agribusiness that includes suppliers to the agricultural, fisheries, and forestry sectors and distributors of food and nonfood outputs from agroindustry (Henson and Cranfield, 2009).

The demand for food and agricultural products is changing in unprecedented ways according to recent notices from FAO. Products, such as fish, meat, and dairy, as well as grains, oil crops, and higher nutraceutical value animal or vegetal products are increased, basically trying to provide options to overcome the higher problems provoked by several nontransmisible chronic diseases as obesity, diabetes, and so on (Guevara-Gonzalez and Torres-Pacheco, 2014; García-Mier et al., 2013; Jimenez-Garcia et al., 2013). The changing structure of agrofood demand offers high opportunities for diversification and add value in agriculture. In this sense, changing consumer demand during the last decade of the 20th century witnessed a diversification of production in developing countries into nontraditional fruits and vegetables (FAO, 2007).

In recent years, the relationship between fruit and vegetable consumption and health has been the main target of a great deal of scientific research involving the identification of specific plant components that promote health benefits. Bioactive compounds are classified into three major compounds classes of secondary metabolites found in plant-derived foods that may convey health benefits as shown in Table 1.1.

The accelerated growth of agroindustries in developing countries also risks in equity, sustainability, and inclusiveness. Moreover, the transformation of agroprocessing from

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00001-3

**Table 1.1: Potential health benefits ascribe to three main classes of phytochemicals (García-Mier et al., 2013).**

| References | Active Compounds | Findings of Potential Health Benefits |
|---|---|---|
| Cardador-Martínez et al. (2002), Feregrino-Pérez et al. (2011), Fresco et al. (2006), González-Molina et al. (2010), Hanhineva et al. (2010), Jiao et al. (2010), Manach et al. (2004), Silva et al. (2012), Sur et al. (2008) | Polyphenols | Antiproliferative, antimutagenic, antioxidant, estrogenic, antimicrobial, antiinflammatory, anticarcinogenic, cardioprotective, antiitch, hypocholesterolemic, antidiabetic activity. |
| Krinsky and Johnson (2005), Lage et al. (2010), Mathabe et al. (2008), Patil et al. (2011), Yahia and Ornelas-Paz (2010) | Terpenes | Antioxidant activity, cancer prevention, cardioprotective activity, protection against eye diseases (cataracts, macular degeneration), antimicrobial, antidiabetic activity. |
| Herraiz and Galisteo (2003), Kabashima et al. (2010), Monteiro et al. (2012), Wang et al. (2007), Yang et al. (2007) | Alkaloids | Antioxidant, antitumor, anticancer, antiinflammatory activity, rheumatoid arthritis, hypertension. |

the informal to the formal sector has great implications through the supply chain to the final consumer. Agroindustrialization involves valuable opportunities and benefits for developing countries, especially for overall processes of industrialization and economic development, export performance, food safety, and quality. Meanwhile, there are several potential adverse effects on those engaged in informal sector agroprocessing enterprises, because of that processes of agroindustrialization must be attuned with overall processes of economic restructuring. Hereinafter, agroindustries will change on a global scale, with new opportunities and challenges for developing countries, and suggesting that the future of agroindustrialization will be somewhat different than in the past.

The growth in demand for value-added food and agricultural products draws an attention to agroindustries to develop in the context of economic growth, food security, and to cope with poverty strategies (Da Silva et al., 2009). The World Bank reported in 2008 that 800 million people are considered poor, subsisting with less than US $1 per day. In addition, 75% of poor people come from rural areas. This situation locates agrofood industries as an important element trying to change this condition worldwide. Importantly, it must be considered that agrofood industries must be sustainable only if they consider the competitiveness in term of costs, prices, operational efficiencies, product offers, and other associated parameters that can benefit the farmers (Da Silva et al., 2009; Jimenez-Garcia et al., 2013). Several frontier knowledge in biotechnological and mechatronic fields regarding the food production, concept called as biosystems engineering (Guevara-Gonzalez and Torres-Pacheco, 2014), must be necessary to be included in the functioning of agrofood industries of competitiveness required for success as aforementioned. Subsequent sections of this chapter will try to describe

some possibilities of application of these strategies to support present and future agrofood industries in generating competitiveness.

## 2 Genetically Modified Food

### 2.1 Main Concepts

Transgenic organisms are a type of genetically modified organism (GMO) that has genetic material from another species that provides a useful trait. Specifically, transgenic organisms are those generated using recombinant DNA technology (Key et al., 2008). There are other types of GMOs, such as cisgenic and transplantomic ones, the former produced by modifying chloroplasts and the latter using genetic material from the same species (crossable-sexually compatible) (Ruf et al., 2007; Schouten et al., 2006). Thus, depending on the type of GMO it would be the regulation and environmental concerns to be considered if produced for industrial purposes.

### 2.2 Concerns With GMO for Agrofood Industries

Based on the previous concepts, genetically modified (GM) foods are those produced from organisms whose genetic material has been modified in such a way that it does not occur naturally. GM foods available nowadays come mostly from plants, but it is expected that in future, foods based on GM microorganisms or animals will be commonly found in the markets. GM plants have mainly been developed to improve yield by several strategies. In the future, it is expected genetic modification should be aimed at altering the nutrient content of food, reducing allergenic activity, and/or improving the efficiency of food production systems. Obviously, all GM foods must be tested before they reach the market. FAO/WHO Codex guidelines exist for risk analysis of the GM food. It is known that several concerns regarding GM food is nowadays discussed worldwide, with several pros and cons in this sense for the application of this type of organisms in agrofood production. However, some recent studies found that for consumers, quality of food is the most important factor in determining their purchase intentions toward GM food (Ismail et al., 2012). Other studies, warrant a regional perception about the possibility of GM food perception, and information on controversial issues predominantly come from newspapers (Botelho and Kurtz, 2008).

### 2.3 Current and Future Possibilities for GM Food

In current years, the state of California has experienced a huge problem with drought. California is in this situation so the government has signed two measures to fund $1 billion to drought-tolerance projects. The drought problem has just started and it is expected that the situation will further increase during the 21st century. Based on the aforementioned scenario, these problems will cause huge losses in crop production unless the research in

agricultural systems finds strategies of production using less water. Monsanto has carried out plant breeding for drought-resistance among others. Monsanto's Drought Gard corn is currently the only commercially available GM engineered and released to cope with drought conditions. Now, the unraveling of the fundamentals of efficient water use in plants' genomes is a current task in several laboratories worldwide. The objective in this case is to maintain or improve productivity with less water. It has been found elsewhere that the overexpression of epidermal patterning factor 2 (*epf2*) gene, produce *Arabidopsis thaliana* plants with increased tolerance to drought environments (Franks et al., 2015). This gene encodes for a small peptide, which participate in regulating the number of stomata. Genetic manipulation of the *epf2* expression was carried out to develop a plant with fewer stomata per leaf, expecting to have a more efficient water use. This latter generated variety was able to use less water without sacrificing the plant performance, especially in a high carbon dioxide atmosphere, both aspects are very important in the climate change current scenario. The critics toward GMOs are based on the currently available varieties, as RoundUp Ready (herbicide-resistant) and Bt (pesticide-producing) varieties. Another example of plant with more environmentally friendly products is a novel antipest potato plant, reported in Germany (Zhang et al., 2015). This GMO is able to defend the plant against the Colorado potato beetle, a beast referred to as an "International Superpest" because it has evolved resistance to all major insecticide classes in the past century. This plant copes with the pest through the RNA interference. An enzyme called Dicer into small pieces cuts using a double-stranded RNA in cells about 21 nucleotides long, named as short interfering RNAs.

These RNAs bind to a complementary strand RNA, then placing a target on the RNA molecule. Agricultural sciences are studying how to get plants to produce dsRNAs that are harmless to them but cope to pests that are provoking the problems in agriculture. Transgenic potato plants that produce dsRNAs were designed to target β-actin and shrub, two genes in the beetle that are absolutely essential for survival. This latter example is not the first one in this sense to use RNAi to control the pest; however, it was the first example to show that the insecticidal RNAs are produced within the chloroplasts instead of the main cells, thus avoiding the plant's own RNA Dicer. With this strategy, that is, inserting genes to the chloroplasts (transplantomic strategy); the plants were able to kill potato beetles with high efficiency. This gene silencing approach provided plant protection in absence of chemicals and without the synthesis of foreign proteins in the plant, thus no provoking allergies. This situation is especially important given that public support of GMOs is currently difficult task to solve, as many harbor fears that genetic manipulation will provoke several problems. These studies displayed various possibilities that genetic engineers try to solve using biotechnology. In addition to drought and pests, crops are being designed to cope with nutritional deficiencies and vaccines production. In the current scenario of climate change, agriculture will need to change or evolve with it, to maintain the required adjustments within the plants with an unpredictable environment. The future of agriculture will depend on the creative

strategies of geneticists and the GMOs produced. These new varieties are expected to be able to carry out several important features that current ones cannot (Zhang et al., 2015).

## 3 "Omics" Approaches in Agrofood Industry

### 3.1 Generalities

The current advances in genomics have been very remarkable in the recent years. The publication of the human genome in 2001 was a milestone, followed by the first crop (rice) in 2002 and the first farm animal (chicken) in 2004. The FAO Biotechnology Forum 2012 evaluated the impacts that genomics and other related omics technologies (proteomic, transcriptomic, metabolomics, etc.) have had on food and agriculture. Sequencing the genome of an organism and the use of bioinformatics has provided important tools for the discovery of biological insights. Thus, a lot of information can be obtained and organized based on the sequential biological processes in the cell, and then used in several metabolic pathways. The aforementioned study of these "omes" is called genomics, transcriptomics, proteomics and metabolomics regarding to genes, transcripts, proteins, or metabolites, respectively. In addition to these four main "omics," several other subdisciplines have emerged, such as the epigenomics which studies modifications, like DNA methylation and histone modification, that influence which genes are turned on or off in different cells at different times without changing the DNA sequence. The genome is quite stable but the levels of mRNA, proteins, and metabolites can change considerably depending on the kind of cells/tissues/organs that are sampled, on time and on a wide range of environments. The first plant genome to be sequenced in the year 2000 was *A. thaliana,* highly studied as plant model. In 2002, the first crop sequenced and published was, the rice genome. Since 2011, the sequenced plant genomes have included those of the cacao, cassava, cucumber, and others (Morrell et al., 2012). In recent years, several plants have been included in the list of sequenced plants. Recently, chickpea (*Cicer arietinum*) was sequenced, an important legume for food security in many developing countries (Varshney et al., 2013). They produced a draft genome sequence of a specific chickpea variety (called CDC Frontier) that was obtained using the whole genomic shotgun strategy, a method in which the entire genome is randomly fragmented into small pieces that are sequenced, and then these sequences are subsequently assembled using computational methods to produce a consensus sequence (Feuillet et al., 2011). From their sequence, it was estimated that the chickpea genome is 738-Mb long and contains 28,269 genes (averaging 3,055 bp in length). Moreover, Varshney et al. (2013) used whole genome resequencing to study the genetic diversity among 29 elite chickpea varieties from different countries. With this approach, a reference genome is available for a given species and now making possible to identify nearly 4 million polymorphisms, including single nucleotide polymorphisms (SNPs) and short insertions and deletions that can be used for future genetic breeding programs or to identify genes involved in traits of interest (Bentley, 2006).

The first livestock genome sequenced was the chicken (*Gallus gallus*) in 2004. Subsequently, those of the cow, horse, pig, rabbit, sheep, turkey, and goat have also been sequenced and published (Dong et al., 2013; Fan et al., 2010). In the case of aquatic animals the genome of the pufferfish, *Rugu rubripes*, was published in 2002 and showed a genome size of about 365 Mb. Other fishes, such as the medaka and zebrafish (model species in developmental biology), and the stickleback (a model used for studies of adaptation and speciation), and those of the Atlantic cod and Nile tilapia (both important food fish) have been sequenced (Munguia-Fragozo et al., 2015). Bernardi et al. (2012) reported that nearly 60 fish species are currently being sequenced and 100 more species have been identified and considered for sequencing in close future. Recently, a transcriptomic study in tilapia was reported regarding effects of photoperiod in gene expression of this species (Fuentes-Silva et al., 2015). Finally, microorganisms are also an important group to be considered in agrofood industry because some of them can be considered as food or as an accessory to produce the food (e.g., biofertlizers). Microorganisms constitute the majority of organisms whose genomes have been sequenced, especially those related to disease. Small genome size and role in food and agriculture implicate that sequencing fungi, bacteria, or viruses and knowing their sequences are important to design better strategies to improve their uses.

The list of microorganisms whose genomes have been sequenced is presented elsewhere (Nelson and Garrity, 2012). For bacteria, the list includes pathogenic strains (*Brucella abortus*, *Cronobacter sakazakii*, *Dickeya zeae*, *Enterobacter cloacae*, *Geobacillus thermoglucosidans*, *Lactococcus garvieae*, and a new member from the *Treponema* genus, found in the cow rumen). The viruses included in the list are the avian influenza virus in China; two types of bluetongue viruses, which infect ruminant animals in China; two strains of sacbrood viruses, which infect the honeybee in Korea; an isolate of soybean Putnam virus, which infects soybeans; and an isolate of Peste des petits ruminants virus from wild bharal sheep. For fungus *Aspergillus oryzae,* Strain 3.042 is highly used for the production of soy sauce and other fermented foods in China. Finally, in the case of metagenomics, that is, the genomic analysis of entire microbial communities has become more important in the last decade and it is expected to be more in close future (Relman, 2011). Several types of environmental samples are currently used to study the genomes of the microorganisms they contain, even in the worldwide microbiome project headed by Dr. Craig Venter.

## 3.2 Applications of "Omics" in Food and Agriculture

### 3.2.1 Genetic improvement of populations

Genetic improvement of crops, livestock, aquatic animal, or microorganisms for specific purposes is one of the main possibilities for the use of genomics and the other "omics" in food and agriculture, among other purposes. Via these strategies, researchers and breeders might obtain direct access to insights about the functioning of the genes in an individual. In this sense, the genome sequencing strategies leaded to the identification

of numbers of molecular markers (e.g., SNPs) scattered throughout the entire genome (Varshney et al., 2013). SNPs association with traits of interest can be reached for genetic improvement in an approach called marker-assisted selection (Gonzalez-Chavira et al., 2006). Commercially available "SNP chips" have been developed that allow individuals to be genotyped for tens of thousands of SNP markers distributed across the genome. A number of strategies are available to use the large number of markers for genetic improvement. One strategy is the use of association mapping or genome-wide association studies, the genome-wide marker alleles associated with the trait of interest are first identified and then markers with significant associations with the trait are used to predict breeding values (Hayes and Goddard, 2010). Genomic selection is another strategy used to predict breeding values. Both aforementioned strategies have been used in animal and plant breeding (Hayes and Goddard, 2010; Varshney et al., 2012). Omics tools are used to identify individual genes affecting important traits and thus to understand how they function. Thus, all this knowledge can be used for genetic breeding programs within the population (Hayes et al., 2013; Varshney et al., 2012), or transferring the gene to another species for development of a GMO.

### *3.2.2 Characterization and management of genetic resources for food and agriculture*

Besides genomics, other "omics" strategies have provided a wide range of new tools to study the current genetic resources for food and agriculture. For example, a study comparing the genomes of wild and domestic pigs from Europe and Asia has been reported. The results indicated that the Asian and European pigs were separated from each other roughly 1 million years ago and there was a very clear distinction between European and Asian breeds (Groenen et al., 2012).

These genomic methodologies have also been important in the characterization, study, and preservation of wild populations. These tools have been used to estimate the effective population size of the North Sea houting (*Coregonus oxyrhynchus*) and to study genetic interactions between stocked hatchery strain brown trout (*Salmo trutta*) and wild brown trout populations (FAO, 2013). On the other hand, the metagenomic approach has been used to characterize and to study the diversity of several complex microbial ecosystems that are relevant to food and agriculture; that is, to study the microbial community in the rumen (McSweeney and Mackie, 2012).

### *3.2.3 Food and agricultural product authentication*

Genomic molecular markers have also been used in studies to confirm the authenticity of commercially available agrofood products. Wilkinson et al. (2012) described the use of a high density SNP genotyping assay to authenticate pork products from specific British pig breeds.

### *3.2.4 Pathogen detection*

Genomics, transcriptomics, and proteomics are transforming the approaches to detect, prevent, and treat food-borne pathogens. Genome sequencing efforts will improve outbreak detection

and source tracking, helping in creating large amounts of food-borne pathogen genome sequence data, which will be available for data mining efforts that could facilitate and provide new insights into food-borne pathogen biology and transmission, as source attributions. Omics tools are starting to yield practical food safety solutions (Bergholz et al., 2014). Transcriptomics and proteomics are being used for a rational development of new control strategies for food-borne pathogens. A recent study identified that fluoro-phenyl-styrene-sulfonamide specifically inhibits activation of the general stress response sigma factor, SigB, in *Listeria monocytogenes* (Palmer et al., 2011). When this SigB factor is induced by *L. monocytogenes* in foods, compounds, such as FPSS may be useful as an additional control measure to inhibit the general stress response, and reduce survival of the pathogen. Transcriptomics data from food-borne pathogens submitted to different environmental stresses have identified biomarkers related to specific resistance characteristics of several pathogens (Den Besten et al., 2010). Mathematical modeling of these data is important to predict the microbial behavior and have also the potential to improve control measurements (Abee et al., 2011).

### 3.2.5 Vaccine development

Genomics is also used to develop vaccines to prevent some diseases, both in livestock and fish. The identification of potential antigen epitopes is one of the key steps in vaccine development, thus resulting in more effective usage. The field of omics technologies has revolutionized the way in which new microbial antigens are identified. The study of the genome in an organism will help in the development of new vaccines. For instance, the release of the Atlantic cod genome provided new knowledge about the gene control of its immune system, then, with this knowledge, new findings helped to develop more targeted vaccines for this species (Star et al., 2011).

## 4 Automation Role in Agrofood Industry

Today's world is subject to important changes from the environmental point of view. Based on this, it is valid to assume that the way we produce foods must necessarily evolve to the generation of systems that consider the aspect of "sustainability." Thus, for future production systems, food production with these features (biosystems) should be the most suitable option to support the worldwide growth of the population in an environmentally friendly way. Biosystems, to be a real alternative in food production, should consider basic studies of various disciplines with the production of plants, animals, and microorganisms, among others. These efforts must be related to aspects of production control engineering as well as recycling systems and waste inputs. Some important research fields regarding food production are new production strategies of plants using the development of electronic instrumentation for monitoring production at different levels, recycling agroindustry residues, environmentally friendly approaches for food production, among others (Castañeda-Miranda et al., 2014; Guevara-Gonzalez and Torres-Pacheco, 2014).

At present, several biotic and abiotic factors have affected the food production drastically that day by day diminish the quantity and quality of the worldwide food; this menaces the food security, principally in less developed countries. According to FAO in 2013, there are 868 millions of persons suffering from malnutrition around the world and millions of them are found in Asian and African countries; nonetheless, the highest percentage is concentrated in Africa (FAO, 2013), where there are countries that have 65% with nutrition problems. To battle these factors that decrease the quality and quantity of food around the world, the agriculture has incorporated technology that goes from employing simple sensors, such as temperature sensors to sophisticated instruments, such as phytomonitor (Contreras-Medina et al., 2012; Millan-Almaraz et al., 2010). In addition to biotechnology, the use of automation and instrumentation technology in agriculture is commonly called precision agriculture and its goal is, by monitoring variables affecting the final production and quality of the plants, to increase the quantity and quality of the food production, and by consequence decrease the affection provoked by the abiotic and biotic factors. The process going from sowing to having a product ready to be sold, has several stages that must be carefully monitored to ensure a product of high quality and ready to be consumed; to reach this, the plant need to be monitored during the sow-to-ripening and postharvesting processes; to do this, several kinds of technology and techniques has been used.

### *4.1 Image Processing in Food Industry*

In the last decade, the use of technology in food industry has been increased greatly, principally due to the high demanding market that day by day requires and demand products of high quality. Also, the market restriction in the last few years has pursued the use of technology in food production (Acosta-Navarrete et al., 2014). The systems of visual inspections normally consist of a light source, a camera, commonly a couple charge device for capturing the image and a computational system for extracting features of images. Commonly, these kinds of systems are used in production lines, where human activity is repetitive and the products need to be manufactured very rapidly, so that decision making must be based on fast and accurate assessments during the overall process. The advantage of having these systems is that they offers repeatability and accuracy by eliminating subjectivity, tiredness, slowness, and the absorbance of the cost related to human inspection (Acosta-Navarrete et al., 2014). The system captures the image by using a camera, scanner, videos, and so on. Subsequently, it converts the image into digital format and after this, a preprocessing stage is generally required to highlight the region of interest and to remove noise that could interfere at the time of extracting important features; the principal objective of a preprocessing stage is to segment the region of interest to finally apply a processing stage that is in charge of recognizing and interpreting the image, always seeking to make sense to the object of interest of the images. In addition, having optimal conditions to acquire a good quality image, permit having a less complex preprocessing algorithm.

### 4.2 Photosynthesis Monitoring in Food Production

Photosynthetic process is the most important biochemical reaction in the world because it allows plants to transform sunlight energy into chemical energy (Millan-Almaraz et al., 2013). Because of this, plants fix carbon dioxide ($CO_2$) and release oxygen ($O_2$). The high importance of this process produces 90% of the planet biomass (Acosta-Navarrete et al., 2014). Thus, more accurate photosynthesis measurements are necessary to establish comparisons and understand plant productivity or biomass accumulation at the leaf, plant, canopy, or community levels as well as their interaction and response to environmental, chemical, or biological factors that generate stress conditions (Acosta-Navarrete et al., 2014). Here, it can be inferred that it is very important to perform the photosynthesis measurement because it reflects the whole crop biomass production, which is very important to maintain high yields at the new generation plant factories.

There are many methods to perform photosynthesis measurements. However, the gas exchange method is currently the most commonly utilized technique to achieve this by measuring carbon dioxide exchange. Because of this, the method is widely utilized in commercial equipment and experimental setups to measure individual leaves, whole plants, plant canopy, and even forests (Millan-Almaraz et al., 2010). This methodology is based on isolating the specimen or sample under analysis in a closed chamber and to measure the initial gas concentration when the chamber is closed. After a few minutes, the chamber has been closed; changes in the proportions of gases from the air inside the chamber produced by the plant are recorded. Consequently, it is possible to measure the gas exchange by analyzing $O_2$ or $CO_2$ concentration (Acosta-Navarrete et al., 2014). There are two types of gas exchange designs: closed chambers where the sample is completely enclosed to measure the difference in gas without contact with the outside air and the open chambers where air can freely enter and leave the chamber flowing through the sample (Millan-Almaraz et al., 2010).

### 4.3 Sensing Sap Flow in Plants

In plants, there are two main vascular tissues at the stem, which are responsible for water and nutrition transport in the sap. Xylem is the first one and is responsible for water transport at the inner radius of a plant stem. In contrast, phloem is located in the outer radius of a plant stem and is responsible for transporting photosynthesis products and other nutrients, such as sugars (Acosta-Navarrete et al., 2014). Sap flow measurement is an instrumentation technique in which sensors are introduced in the plant xylem at stem or trunk to measure temperature differences that indicates sap flow information, such as direction and density (Acosta-Navarrete et al., 2014).

Sap flow measurement has been utilized for many years to analyze different kinds of plant and its relations to water, soil, and atmosphere conditions. In plants, sap flow has a specific

trend to increase at middle day hours and starts to decrease at evening to finally reach its minimum value during night. Transpiration is another useful variable that allows detecting water-related stress phenomena in plants (Millan-Almaraz et al., 2010). However, it results as complex and costly when it is compared to sap flow technique that only requires temperature sensors and a heating element, which are cheap and easy to be implemented (Davis et al., 2012). To perform sap flow measurements, there are three main methodologies: heat ratio method, heat balance, and thermal dissipation method (Millan-Almaraz et al., 2010).

### 4.4 Plant Morphological Sensors

As aforementioned, plants have a sap flow system where the xylem transport water and phloem transport sugars and other nutrients, and transpiration is a water loss mechanism which occurs during $CO_2$ exchange process during photosynthesis. As a consequence of water and nutrient flow across the plant, stems, and trunks produce diameter variations, which are completely related to sap flow. Therefore, a steam diameter decrease occurs during the day, while it increases during nighttime (Korpela et al., 2010). Consequently, this stem diameter information is very useful for plant physiology research purposes and plant production systems where drought and other water-related stress conditions need to be monitored with accuracy (Millan-Almaraz et al., 2010). There are two main types of sensing elements which are utilized for morphological sensors: strain gauges and linear-variable-displacement-transducers. The design of morphological sensors varies and depends on plant characteristics, such as size, shape, or location. For example, there are fruit growth sensors, which are designed to measure microvariations on apples, tomato, or other fruits size. Also, there are stem diameter sensors for different types of plants stems or trunks (Korpela et al., 2010).

## 5 Climate Control for Food Production

Food security is one of main global challenges in the current century. Word population will increase up, at least by the middle of the 21st century, and demand of food will rise. Moreover, problems, such as climate change and pests have a profound impact on food production. Then, it is easy to notice that traditional agricultural schemes cannot deal with these problems and an alternative solution has to be implemented (García-Mier et al., 2013). Greenhouses are building structures that allow the creation of an indoor microclimate for crop development, protecting plants from severe external climate conditions or pest invasion. The greenhouse microclimate can be manipulated by control actions, such as heating, ventilation, $CO_2$ enrichment to name a few; to provide appropriate environmental conditions. These modifications imply additional use of energy in the production process. Furthermore, it requires a control system that minimizes the energy consumption while keeping the state

variables as close as possible to the optimum crop physiological reference. Horticulture in greenhouse conditions is a rapidly expanding interest and is consequently increasing in its economic and social importance (Kolokotsa et al., 2010; Trejo-Perea et al., 2009).

The greenhouse climate control problem consists in the creation of a favorable environment for crop development, looking for high yield, quality, and low operative costs. Along the time, researches have shown that this is a very difficult task due to the complexity of the greenhouse environment. Even small changes in the structure or location could drastically redefine the problem. For example, microclimate variables, such as temperature and relative humidity (RH) are highly nonlinear and strongly coupled, and the greenhouse is largely perturbed by the outside weather. Also the aforementioned objectives (yield, quality, and low cost) present conflict of interest because generally, high control precision agriculture requires extra energy consumption (Acosta-Navarrete et al., 2014; Haigen et al., 2010).

## 5.1 Greenhouse

The greenhouse-crop system can be considered as a solar collector involving sensible and latent heat exchanges. Different research has been conducted regarding climate control for protected agriculture applications. The main objective of these investigations is to find an accurate model that represents the greenhouse environmental dynamics and an efficient and a flexible controller that adjusts the microclimate variables of interest. This problem has been the focus of many researchers worldwide who have analyzed, experimented, and proposed many climate control systems to manipulate variables, such as temperature, RH, $CO_2$ enrichment, radiation, and many others that are necessary to generate the fundamental conditions for successful protected agriculture (Van Straten et al., 2010). The simplest way to represent the greenhouse system, including external perturbations, microclimate variables, and even possible control inputs are presented in Fig. 1.1.

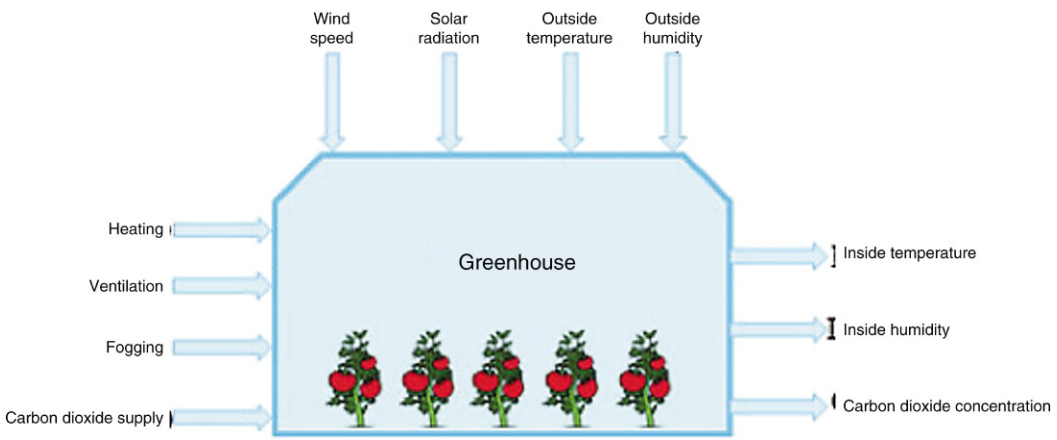


**Figure 1.1: A Typical Greenhouse Climate Model (Van Straten et al., 2010).**

The importance of aforementioned greenhouse climate model lies in most control theories, which require the mathematical model of the system for tuning and simulating the proposed algorithms. Different mathematical greenhouse models have been developed based on this scheme. A simple model of the temperature changes in a greenhouse can be described by the differential equation [Eq. (1.1)].

$$\frac{dT_G}{dt} = \frac{1}{C}\left[K_{out,air}(T_0 - T_G) + q_h\right] \tag{1.1}$$

where the $T_G$ is the greenhouse internal air temperature, $C$ the greenhouse thermal capacity, $K_{out,air}$ is the heat loss coefficient from greenhouse air to outside air. To be the external air temperature and $q_h$ is the heating power. Despite easiness of the model, is widely accepted and provides a quick, inexpensive, flexible, and repeatable way to compare how the greenhouse temperature responses to certain control methodology.

Duarte-Galvan et al. (2012) show classification for the control techniques for greenhouse climate, which are divided into two main fields; the first one is usually called conventional control consisting of climate controllers, which try to control the greenhouse microclimate just by reducing the deviation between set point of the interest variables and measured values to zero. Examples of conventional controllers are ON/OFF, proportional integral derivative controller (PID), other classical controllers, and also artificial intelligence (AI) paradigms, such as artificial neural networks (ANNs), fuzzy logic systems (FLS), genetic algorithms (GAs), among others. The other field is optimal control, in which factors, such as greenhouse dynamic behavior, actuator capabilities, water and energy consumption, and meanly the crop response are taken into account. Expert systems and model predictive control (MPC) are widely accepted for optimal control purposes (Fig. 1.2).

However, aforementioned AI-based techniques can be also considered like optimal production controllers when they reach objectives, such us optimal crop growth, reduction of the associate costs, reduction of residues, improvement of energy, and water use efficiency (Ramírez-Arias et al., 2012).

Conventional controllers were widely used since computational tools were introduced in protected agriculture until the end of 20th century. Nevertheless, the increase in power computational capabilities with cost reductions in the next decade allowed the application of more complex algorithms, which deals with the optimal control scheme. In the rest of this chapter, the review of protected agriculture techniques will be focused on optimal control taking into consideration both modeling and controllers.

## 5.2 Optimal Control and Modeling

The objective of modern greenhouse industry is a sustainable crop production system by reducing water and energy consumption and biocide use while maintaining a high crop

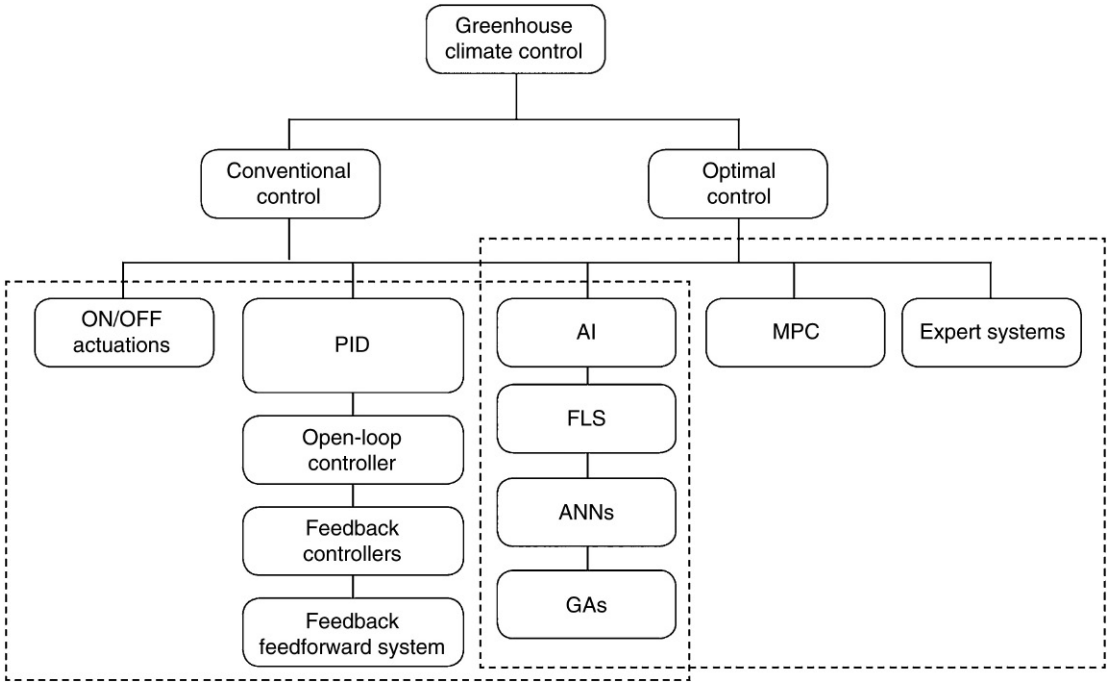


**Figure 1.2: Greenhouse Control Theories Classification (Duarte-Galvan et al., 2012).**
*AI*, Artificial intelligence; *ANNs*, artificial neural networks; *FLS*, fuzzy logic systems; *GAs*, genetic algorithms; *MPC*, model predictive control; *PID*, proportional integral derivatives.

quality and yield. In addition to new materials and advance building methodologies, this can be achieved by modifying the microclimate control strategy. However, the optimal control scheme is slowly accepted in practice due to the lack of reliable crop development models for the wide variety of crops. Also, experimental probes that show advantages and clear assessment of the risks involved and the theoretical limitation is required. To effectively validate the performance of proposed optimal control algorithms, greenhouse models, which include parameters related with crop response are required. Dynamic mathematical modeling is an essential issue to be solved to achieve a good performance of the controller. Thus, two different strategies for computing greenhouse models are found in the literature. The first one is based in physical laws, and the second in an approximation of the behavior without a priori information, that is: polynomial fitting, ANNs, and so on. Modeling by physics laws is complicated because to achieve a reliable model, lots of parameters have to be included in the model. But, it has proved that physics-laws models had a better goodness-of-fit than black-box models (Blasco et al., 2007). However, adjusting these parameters is complicated. An alternative was purposed by Guzmán-Cruz et al. (2009), which used and compared different evolutionary algorithms (EA), such as GAs, evolutionary strategies, and evolutionary programming to calibrate the parameters that define the greenhouse inside temperature and

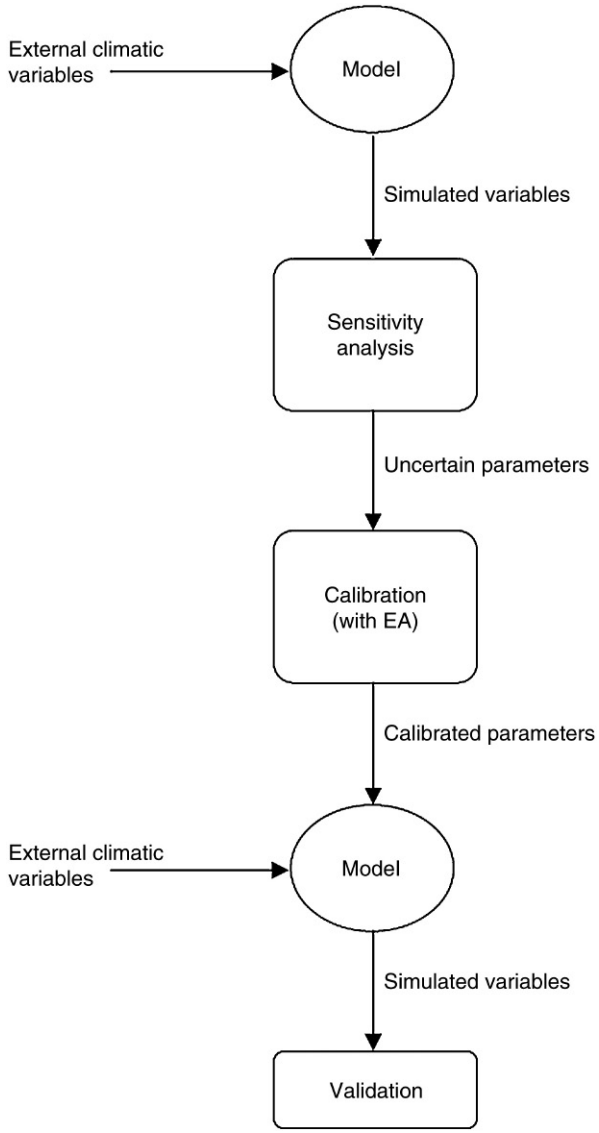


**Figure 1.3: Calibration Diagram of Greenhouse Model by Evolutionary Algorithms (Guzmán-Cruz et al., 2009).**
*EA*, evolutionary algorithms.

RH within a greenhouse with tomato crop. The calibration consisted in an optimization problem which works altering model parameters until getting a better fit between estimated and measured data (Fig. 1.3). Results show a better performance of evolutionary programming to predict the air temperature and RH behavior; however, least squares and sequential quadratic programming methods slightly improve the estimation of temperature, but present lot of inaccuracy for RH.

Despite the favorable results obtained by the aforementioned models, many authors argue that a more detailed knowledge about phenomena is required to increase the performance of controller and thus to meet objectives of modern agriculture. Several works deal with the problem of model plant behavior and influence on the greenhouse microclimate. These behaviors could be related with plant growth, transpiration, or photosynthesis (Acosta-Navarrete et al., 2014).

Other works focus to understand deventilation phenomena inside the greenhouse by models considering the heterogeneity of the system and how the different segments of the atmosphere interact with plan canopy or external environment. Computational fluid dynamics also serves to study ventilation, its effects on inside temperature and humidity, and how it is affected by external wind direction (De la Torre-Gea et al., 2014). Finally, based on these studies, different methodologies were purposed to design the greenhouse geometry, the correct orientation, and even the required equipment to achieve a desirable yield and quality (Vanthoor et al., 2011). Also, software which use all the aforementioned models were developed to provide a tool that could predict the greenhouse behavior under certain weather characteristics prior to its installation, equipment available, and greenhouse materials could also be included in this web-based application (Fitz-Rodríguez et al., 2010).

### 5.3 Controllers

The advantages of using optimal instead of conventional greenhouse climate control relies in that an optimal control approach to greenhouse climate control fully exploits scientific quantitative knowledge concerning the greenhouse atmosphere, the soil, the equipment, the crop, and their interactions. All these aspects must be captured in a mathematical dynamic model that deals with the problem of maximizing the profit, achieving welfare of the crop through practices that minimizes production costs (Van Straten et al., 2010). Robust controllers were applied in protected agriculture because of its ability to deal with uncertain parameters, disturbances, or modeling mistakes. Horticultural research has indicated that for the majority of plants, crop growth responds to long-term average temperatures rather than specific day and night temperature profiles. This principle is used in the well-known temperature integration technique (TI) where it is possible to adjust the set point temperature in a flexible way to obtain a desired average temperature instead at fixed value over the time. Therefore, energy savings could be obtained by decreasing flexibility to the heating set points, when conditions are favorable and lowering it, when they are not. This knowledge has been applied in the design of a tool available to exploit the interaction between photosynthesis and growth according to empiric knowledge. The method is based on varying heating set points using previously recorded information to achieve the desired average for any user-defined period. Meanwhile, a decoupled process with fast temperature response (e.g., photosynthesis or stress) are processes of slow response in time. The objective was to improve the temperature integration concept by introducing dynamic temperature constrains; these flexible

boundaries depend on the underlying crop process while increasing the potential for energy saving in greenhouses. Despite the promising energy savings obtained with temperature integration, the potential of this technique is limited by humidity if usual set points are maintained, because the high relation between those variables counteracts the TI. A promising solution is the use of a process-based humidity regimen. In this regimen, RH set points can freely move within a range, avoiding affect the TI objectives. However, as humidity changes could highly affect the crop quality and yield, the set points of its duration period were calculated to avoid plant-affecting processes, such as Ca-deficiency, plant water stress, crop growth, crop development, and air-borne fungal diseases (Körner and Challa, 2004).

As was previously mentioned, the grower intervention had not been completely avoided in the crop management. Then, decision support tools that assists the grower to choose the most appropriate climate regimen was proposed (Gupta et al., 2010). These regimens choose the most appropriate climate for plants according to its phonologic state to obtain the optimal gains of sustainability and plant quality. The greenhouse climate and crop model are studied separately and jointly considering the effects of six different regimes with increasing degrees of freedom for various climate variables, which include crop model, temperature integration, dynamic humidity control, and negative DIF regimes (DIF 1/4 the difference between average day temperature and average night temperature, and therefore reduces the use of chemical growth regulators) (Körner and Van Straten, 2008).

Phytocontrol is a new theory that proposes the use of the plant physiological responses as input signal to establish the set points in the climate controller (Ton et al., 2001). Also, this has not proved to be a stable and reliable method, because it is necessary to gather a lot of information to prove the reliability of this theory (Linker and Seginer, 2003). Nevertheless, different types of controllers have emerged demonstrating the advantages and disadvantages between them, better performance for some actions among other characteristics.

### *5.4 Some Specific Applications of Automation and Modeling in Agrofood*

Our research group has been working on several aspects related to applications of biotechnology and automation processes to help food production at different levels. In this section, some examples of these researches will be described briefly. First, phytomonitoring strategies of photosynthesis, transpiration, and crop damage by phytopathogens have been developed to improve control systems for crops production under greenhouse conditions (Contreras-Medina et al., 2012; Espinosa-Calderon et al., 2012; Millan-Almaraz et al., 2010). Plant transpiration constitutes the plants evolving adaptation to exchange moisture with a dry atmosphere. Thus, accurate measurement methods for plant transpiration are also required. Thus, a smart sensor combining five primary sensors was proposed measuring air temperature, leaf temperature, air RH, plant out RH, and ambient light (Millan-Almaraz et al., 2010). A field programmable gate array (FPGA) based unit was employed to develop

several signal-processing algorithms to the primary sensor readings to reduce the signal noise and improving its quality. Once filtered the primary sensor readings, the dynamic of transpiration, stomatal conductance, leaf-air-temperature-difference, and vapor pressure deficit are calculated in real-time. Finally, transpiration related stress conditions can be detected in real-time because of the use of online processing and embedded communications capabilities (Millan-Almaraz et al., 2010). On the other hand, plant responses to physiological function disorders are called symptoms and they are caused principally by pathogens and nutritional deficiencies. Plant symptoms are commonly used as indicators of the health and nutrition status of plants. Currently, the most popular method to quantify plant symptoms is based on visual estimations. However, this method is inaccurate and imprecise because of its subjectivity. Computational vision has been used in plant symptom quantification because of its accuracy and precision. There are methods to obtain information about the health and nutritional status of plants based on reflectance and chlorophyll fluorescence, but they use expensive equipment and are frequently destructive. Therefore, systems able to quantify plant symptoms overcoming the aforementioned disadvantages that can serve as indicators of health and nutrition in plants are desirable. An FPGA-based smart sensor is able to perform nondestructive, real-time, and in situ analysis of leaf images to quantify multiple symptoms presented by diseased and malnourished plants can serve as indicator of the health and nutrition in plants. The effectiveness of the proposed smart-sensor was successfully tested by analyzing diseased and malnourished plants (Contreras-Medina et al., 2012). To propose a simple and accurate strategy for photosynthesis estimation, a study in pepper plants were carried out by relating RUBISCO gene expression, and $CO_2$ exchange, as well as a relationship between leaf temperature and photosynthesis. This relationship found was strong as it can be used to design new devices for estimating photosynthesis in a non-invasive way (Espinosa-Calderon et al., 2012).

Mathematical modeling using ANN has been used to estimate carotenoid content during ripening in tomato fruits (Vazquez-Cruz et al., 2013). Commonly carotenoid determinations in tomato are performed in full ripening tomatoes. In this work six tomato-ripening stages were established. The relationship among color parameters (L*, a*, b*, and hue), maturity stages, and leaf area with the lycopene and β-carotene concentration was analyzed with different regression models. The $R^2$ values were low, showing that lycopene and β-carotene content was not well correlated with color during the ripening stages. The objective of this work was to provide an ANN model including leaf area index (LAI) and color readings as inputs to solve this lack of fit of the regression models for carotenoid estimations in tomatoes. Two multilayer perceptrons (MLPs) were trained and validated, with six input variables and one output variable, to estimate the concentration of both carotenoids in tomato samples at different ripening stages. Comparing the results of the MLPs with those obtained by regression models, it was concluded that when the MLPs were used within the range studied, they were able to estimate lycopene and β-carotene concentrations of tomato

with accuracy and reliability, solving the lack of fit by regression models (Vazquez-Cruz et al., 2013).

Moreover, nitrogen (N) is an important macronutrient to reach adequate crop yields. A problem with the use of nitrogen fertilizers is that farmers apply excessive amounts of this fertilizer to crop in the fields, causing high environmental pollution. Methods for monitoring nitrogen with crops have been developed to improve N fertilizer management. The majority of these methods are based on leaf or canopy optical-property measurements. Electrical impedance (bioimpedance) has been applied to determine the physiological and nutritional status of plant tissue, but no studies related to plant-N contents are reported (Muñoz-Huerta et al., 2013). A research aiming to analyze how plants electrical impedance response is affected by their N status was successfully carried out. The advantages and disadvantages of plant-N sensing techniques are shown in Table 1.2 (Muñoz-Huerta et al., 2013).

Four sets of lettuce (*Lactuca sativa* L.) with different N-source concentrations per set were used. Total nitrogen and electrical impedance spectra (in a 1–100 kHz frequency range) were measured 5 times per set, 3 times every other day. Minimum phase angles of impedance spectra were detected and analyzed, together with the frequency value in which they occurred, and their magnitude at that frequency. High and positive correlation was observed between plant N content and frequency values at minimum phase angle with no significant variations detected between days of measurement. These results suggest that electrical impedance can be sensitive to plant N status. Thus, as aforementioned in the research studied described, there are several possibilities of apply biotechnological and automation knowledge to develop new devices for crop quality monitoring in harvest and postharvest levels. Additionally, these devices could be employed mainly in greenhouse or even in open field.

## 6 Concluding Remarks

In recent times, concerns about the impact of the food that people consume on their own health, as well as the social and environmental consequences that it entails, have led to major changes in all steps of the agrofood chain including all the agents from the producer to the retailer (García-Mier et al., 2013). These phenomena comprise complex technologic, social, economic, and environmental linkages that require integrated research approaches, that is, the changes in consumer's demand and their consequences need to be considered from all these different points of view. While agrofood is a basic requirement for human survival and well being, the ability to ensure food security, that is, access to sufficient nutritious food is determined by multiple social and political factors. At the same time, food production as the foundation of food security also provides the potential for building a new politics to work toward sustainability. Currently, the agricultural challenge is to get crop yield and quality at the same time. Since ancient times, agricultural practices seek to confer comfort to plants through stress reduction. For this reason, the use of greenhouse has emerged in

**Table 1.2: Advantages and disadvantages of plant N-sensing techniques (Muñoz-Huerta et al., 2013).**

| Techniques | | | | Advantages | Disadvantages |
|---|---|---|---|---|---|
| Tissue analysis | | | Kjeldahl digestion | Reference method to estimate total N contents (protein, amino acids, nucleic acids, etc.). | Invasive and destructive; time-consuming; toxic reagents used; sample preprocessing requirements. |
| | | | Dumas combustion | No nitrate and nitrite reduction | Destructive; nitrogen loss due to incomplete combustion; sample preprocessing requirements. |
| Optical meters | Leaf level | Transmittance | SPAD | Noninvasive due to the high correlation between N status and leaf chlorophyll contents; Portability. | Unable to detect over fertilized crops due to the chlorophyll saturation; low sensitivity for detecting N stress at early stages. |
| | | Fluorescence | Dualex/ multiplex | Can eliminate erroneous signals from bare soil; distinguish between different N treatments in shadow of full sunlight; portability; multiplex is able to detect N deficiency among other stresses (i.e., pathologies, water stress). | They are still not able to be used as ground-based remote sensor; in spite of this, fluorescence sensors could be used for monitoring larger crop areas in the near future. |
| | Canopy level | Ground-based | Passive sensors: FieldSpec CropScan LI 1800 | Can detect a greater crop field area than leaf level meters. | Calibration is required. Sunlight dependence. |
| | | | Digital cameras | Do not require sophisticated instruments. | Sunlight dependence; although, recent studies have reported the use of fuzzy logic controllers for reducing sunlight effects, more research focused on crop N status analyses is still required. |
| | | | Actrive sensors: GreenSeeker Yara N-Sensor CropCircle | No dependence on sunlight due to their own light sources; Yara captures more biomass per unit of soil surface, and measures and record a wide waveband; GreenSeeker can describe the variation in the crop canopy according to the crop's N status, even close to N saturation. | Expensive equipment; saturation due to the biomass increasing. GreenSeeker limitations because it is able to measure only two wavelengths; Yara performance could not be able to detect plant N status when they are close to N saturation; GreenSeeker reaches saturation earlier than CropScan when measuring crop N status during growth stage. |
| | | Satellite-mounted | QuickBird | Allows the entire field analysis | Expensive imagery; interferences by atmospheric conditions; slow turnaround; despite this, new satellite constellations are coming with higher spectral, spatial and temporal resolutions, improving their ability to determine crop N status. |

| | | | |
|---|---|---|---|
| Sap and electrical meters | Nitrate test strips | High correlation between N status and plant sap nitrate concentration; cheap and portable; quick measurements. | Nitrate variations caused by light exposure; plant sap dilution is required; destructive; no other N forms are considered, so it is not able to measure total N in plant tissue. |
| | Nitrate ISE | Due to the high correlation between N status and sap nitrate concentration, N content can be estimated by means of nitrate ions in plant sap; a wider operative range than nitrate strips. | Sensitivity to other ions, such as chloride, bicarbonate, and nitrite; nitrate concentration depends on other variables than N status (i.e., diurnal variation, sampling procedures); destructive; calibration is needed; sap dilutions are required; no other N forms are considered, so it is not able to measure total N in plant tissue. |
| | Electrical impedance spectroscopy | Direct measurement of plant tissue electrical properties. | Invasive; electrode polarization effects; there are few studies focused on plant nutrient sensing. |

the perspective of reducing the stress produced by inadequate weather parameters, such as temperature, radiation, and RH parameters as well as pest diseases. Consequently, they have allowed growing crops in an efficient way in relative safety from the outside world. However, this practice has brought a decrease in the production of phytochemicals with health implications, because these compounds are produced by the plant in response to stress conditions. Besides, because plants are sessile organisms, synthesis of phytochemicals represents a major strategy for counteracting unfavorable conditions.

Thus, the use of biotechnological strategies, either GM or not organisms, represents a viable strategy to achieve both crop yield and bioactives, that is, the challenges assumed by conventional and organic agriculture, respectively. Indeed, questions around the use of biotechnology must be solved in short coming years, to be included without problems in agrofood systems. The advances in automation strategies together with the improvement in greenhouse building and climate control will lead to conquer the goal of the 21st century: produce more and better food for an increasingly demanding population in a sustainable manner. For this reason, this chapter addressed a vision about the role of biotechnology in the agrofood production and characterization, considering the option of automation strategies that might be included in these systems to potentiate agrofood production in the present century.

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CHAPTER 2

# Biotechnology in Food Processing and Preservation: An Overview

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## 1 Introduction

To fabricate and develop commercial products and methods by employing molecular methods that utilize entire or fractions of living organisms is known as modern biotechnology. Modern biotechnology is relatively recent and quick growing division of the molecular biology that was introduced 30 years ago with the development of the first recombinant gene. Biotechnology is changing our way of living by affecting the foods, drinks, medicine, and cloths. The application of biotechnological methods in the food and agricultural industry has great repercussion on the society. Biotechnology has the maximum potential to resolve the pressing need of hunger today and thus help to avoid mass starvation in the coming future.

Through domestication and agricultural activities of breeding and selection, plants were developed into food crops that permit fabrication of more healthy, safer, tastier, and nutritious edible item. Various other aspects of biotechnology, such as medical biotechnology, also known as red biotechnology, help us in gene therapy, initial stage identification of various diseases, such as cancer, diabetes, Parkinson's, Alzheimer's, and atherosclerosis resulting in early stage treatment and eventually curing these diseases.

Palatable foods and potable beverages can be manufactured by converting relatively huge amount of perishable and nonedible food materials into more useful and shelf stable products by using various unit operations and technologies. Any kind of technology applied in the food processing must be safe and good quality and the final product must be free from any health hazard. Definition of safe food is the food, which is chemically, physically, and microbiologically free from any harmful material; or the level of contaminant present in the food will not cause any harm to public health. Nowadays, consumers are interested to pay the premium for quality food products that are safe and convenient.

A range of technologies is applied at different levels and scale of operation in food processing across the developing country. Low input and conventional technology includes drying,

**Advances in Biotechnology for Food Industry**
http://dx.doi.org/10.1016/B978-0-12-811443-8.00002-5

evaporation, canning, dehydration, freezing, vacuum packing, osmotic dehydration, sugar crystallization, etc.

Processing assures food security by minimizing waste production and reducing the food chain and increasing food availability and marketability. The purpose of food processing is to improve its quality and security. Food safety is a scientific discipline, which ensures that a particular food will not be the reason of any injury to the consumer when it is manufactured and eaten according to its deliberate use. Biotechnology plays a pivotal role to improve the taste, flavors, color, texture, aroma of foods, and its aesthetic and nutritional value; it is extensively used in many countries. Food undergoes fermentation by intentional inoculation or by natural fermentation and eventually these desirable changes appear due to fermentation by microorganisms and/or their enzymes, flavor, fragrance, food additives, and other value-added products. These high value products are used in food and nonfood use and also imported to other countries.

Food processing involves various unit operations and techniques to convert raw, perishable, and inedible products to consumable form with enhanced quality and shelf life. To produce a safe and high quality food, the process and manufacturing protocol used in the food processing must be of food grade, that is, free from health hazards. Safe food can be defined as the food that contains no harmful components that affects human health and nutrition. Biotechnology is also widely employed as a tool in diagnostics to monitor food safety, prevent, and diagnose food-borne illnesses and verify the origin of foods. Techniques applied in the assurance of food safety focus on the detection and monitoring of hazards whether biological, chemical, or physical. Fermentation is generally used to make desirable changes in food. Fermentation can be carried out naturally or by intentional inoculation. Fermentation is the process in which carbohydrates are converted into alcohol and carbon dioxide or organic acids when yeasts, bacteria, or a combination of them works on the food in the absence of air. Fermentation is used to produce wine, beer, cider, leavening of bread, and lactic acid.

Ghoshal (2012) studied the effect of xylanase from *Penicillium citrinum* on rheological properties of whole-wheat dough. Linear viscoelastic range was observed from 0.1% to 1%. The amplitude sweep test established that G′ and G″ were higher in xylanase containing dough as compared to control. The values of power law coefficient, $x$ and $y$ were higher in xylanase containing dough, which showed higher dependency on strain. In weak gel model parameters, higher A value revealed the stronger starch gluten network in xylanase containing dough, while lower value of $z$ represented the higher dependency of both the modulus (G′ and G″) on the strain. Creep compliance data revealed that control dough is stronger than xylanase containing dough. Creep test data of control and xylanase containing dough were fitted to Peleg, Kelvin, and Burger Models to check the adequacy of fitting of creep data in to different mathematical models. Peleg model, as well as six-element Kelvin model described well the creep behavior of control and xylanase containing dough samples. Large deformation of dough in terms of uniaxial extensibility and unfermented dough stickiness study revealed that

xylanase containing bread exhibited greater extensibility and less resistance to extension as compared to control samples. It is found that $R_m$ (maximum resistance) was higher in control than enzyme treated dough but extensibility ($E$) increased with enzyme supplementation. Therefore, it was concluded that xylanase addition makes the dough softer. SEM study revealed that addition of xylanase resulted in continuous and closed gluten network in which starch granules are embedded. Higher magnification revealed that large starch granules were more swollen and evenly dispersed within the protein matrix.

Ghoshal et al. (2013) studied the effect of xylanase on whole wheat bread. The quality of bread containing xylanase was improved with respect to specific volume and moisture loss, textural properties, color, thermal properties, and sensory properties. Firmness values and enthalpy values of stored samples were fitted in Avrami equation. Xylanase addition resulted in the reduced rate of staling in bread. A 20% reduction of limiting firmness value was observed in bread containing xylanase. Using Avrami equation, calculated values of firmness and enthalpy were determined and plotted. The calculated values were in agreement with the experimental values. During storage, bread-containing xylanase was softer as compared to control. From the analysis of various staling properties examined, it can be inferred that bread stales at both ambient (25°C) and cold (4°C) temperature, but the rate was lowest at cold (4°C) temperature in bread containing xylanase. From the aforementioned study, it was proved that partially purified xylanase could be used to improve the color, texture, and sensory properties of whole wheat bread.

Kaur and Ghoshal (2016) studied the biocolor production using selected fruits and vegetable peel and orange red color was extracted using the strain *Blakeslea trispora* (+) MTCC 884 by solid-state fermentation. Analytical determination of color using UV-spectrophotometer produced maximum absorbance at 449 nm which confirmed that extracted color was β-carotene and the peak of HPLC analysis curves at retention time of 12–14 min further confirmed the chromatograms of β-carotene. It has been observed that β-carotene production was influenced by the parameters, such as pH, temperature, and incubation time. It was also concluded that 96 h at 28°C and pH 6.2 were the most appropriate environmental parameters for the production of β-carotene. Mass spectroscopy of extracted color displayed the $m/z$ value at 537.608 agreeing to the presence of β-carotene. LCMS analysis of extracted color gave the eluted peaks of trans β-carotene (Rt 13.37) confirmed the presence of β-carotene.

Natural fermentation leads human history. The earliest evidence of fermentation dates back to 7000–6000 BC. It was an alcoholic beverage, made from fruits, rice, and honey in the Neolithic age in Chinese village of Jiahu. Winemaking was prevalent in 6000 BC in Georgia. There was a jar containing traces of 7000 years old wine displayed at the University of Pennsylvania, excavated from mountains in Iran. Also the traces provided the proofs regarding the production of fermented products in Babylon c.3000 BC, ancient Egypt c.3150 BC, pre-Hispanic Mexico c.2000 BC, and Sudan c.1500 BC.

Louis Pasteur, French chemist was first to connect yeast to fermentation in 1856. He defined fermentation as respiration without air. Fermentation, useful for conversion of sugars and other carbohydrates into preservatives and other organic acids, is the result of his research. Fermentation is generally used in food processing as it:

- modifies diet by enrichment of flavors, aromas, and food texture;
- preserves food by production of acids;
- enriches food with protein, essential amino acids, and vitamins;
- removes antinutritional factors; and
- decreases process time.

This review is regarding the recent advances in biotechnology and its applications in food processing and manufacturing of various foods from transgenic plants, animals, and microorganisms. Plants are the primary source of food for humans and feed for livestock. Through domestication and agricultural activities of breeding and selection, plants were developed into food crops that serve as the major source of dietary carbohydrates, lipids, proteins, vitamins, and minerals for humans and livestock. This part of the article discusses the occurrence of genetic engineering to improve the quality of milk in cattle, reduce the fat content in swine, increase the growth and productivity in poultry, and provide tolerance against freezing temperatures in fish. The fabrication of a variety of proteins by using mammary glands and eggs as bioreactors and modification of microorganisms by genetic engineering for improvement of food products has also been discussed. Various biotechnological techniques for the identification of transgenic substances and harmful pathogens are also described.

### 1.1 Methods to Improve the Quality of Microbial Strain

In traditional biotechnology, microbial cultures are improved for use in food processing application by improving the quality of microorganisms and the yield of metabolites using mutagenesis, conjugation, and hybridization (for yeast *Saccharomyces cerevisia* strain used in baking, brewing, and beverage production methods).

Recombinant genetic engineering is the best-known technique to alter the purified microbial strain related to food fermentations following the norms and regulations as per customer awareness. Genetically modified (GM) strains are applied in the manufacture of enzymes, vitamins, PUFA, amino acids and other fatty acids (Tables 2.1 and 2.2).

## 2 Genetically Modified Plants

### 2.1 Methods of Production of Genetically Modified Plant

GM modified plants are generated by the biolistic method (Particle gun method) or by *Agrobacterium tumefactions* mediated transformation method. In biolistic or gene gun

**Table 2.1: Applications of some food additives and processing aids derived from Genetically modified (GM) microorganisms.**

| Applications | Categories of Food Additives | Food Additives |
|---|---|---|
| Cheese making | Enzymes | Rennet |
| Manufacturing of high fructose corn syrup | | Isomerase, amylase |
| "Lite" beer | | Pullulanase |
| Meat tenderizer | | Proteases |
| Juice, beer clarification, and bread manufacturing | | Xylanase |
| Nutritional supplement | Amino acids | Methionine, lysine, and tryptophan |
| Ingredient in sweetener production | | Aspartic acid and phenylalanine |
| Acidulant | Organic acids | Citric acid, acetic acid, benzoic, and probionic acid |
| Flavoring and coloring agents | Flavors and pigments | Vanillin and monascin |
| Nonnutritive sweeteners | Low-calorie products | Aspartame, thaumatin, and monellin |
| Food additives, and cooking oil | | Modified fatty acids triglycerides |
| Animal and human food supplement | Single-cell protein | |
| Stabilizers, thickeners, and gelling agents | Microbial polysaccharides | Xanthan gum |

**Table 2.2: Enzymes from GM microorganisms.**

| Enzymes | Source Microorganisms |
|---|---|
| Chymosin | *Aspergillus niger* |
| Phytase | |
| Lipase | |
| Aspartic proteinase | *Aspergillus oryzae* |
| Esterase-lipase | |
| Glucose oxidase | |
| Laccase | |
| Lipase | |
| α-Amylase | *Bacillus licheniformis* |
| Pullulanase | |
| α-Acetolactate decarboxylase | *Bacillus subtilis* |
| α-Amylase | |
| Maltogenic amlyase | |
| Pullulanase | |
| Chymosin | *Escherichia coli* K-12 |
| Xylanase | *Fusarium venenatum* |
| Chymosin | *Kluyveromyces marxianus* var. *lactis* |
| α-Amylase | *Pseudomonas fluorescens* |
| Pectin lyase | *Trichoderma reesei* |

method, the gene is directly shot at the plant cell under high pressure. This method has been applied successfully for many crops, especially monocots, such as wheat or maize, for which transformation using *A. tumefaction's* has been less successful. This technique is clean and safe. The only disadvantage of this process is that serious damage can happen to the cellular tissue. In agrobacterium-mediated piece of DNA, which infects a plant is integrated into a plant chromosome, through a tumor inducing plasmid along with the genetically engineered (GE) strain.

Recent advancements in plant sciences and agricultural biotechnology offer new opportunities and possibilities to improve the yield, quality, and production economics of food crops. Few examples are given to generate vitamin, mineral, and essential nutrient-rich transgenic plants (Klein et al., 1987).

## 2.2 Vitamin-Rich Plants

Vitamins play a vital task on human health by varying metabolic circumstances and supporting the biochemical processes that liberate energy from foods during digestion, making of hormones, blood cells, nervous-system chemicals, and other genetic materials. Deficiency of any vitamin can cause serious health disorder. Transgenic plants can be manufactured using knowledge of biotechnology, with increased content of vitamins in certain crops.

### 2.2.1 Vitamin-A

Reduced form of vitamin A (retinal) is the source of rhodopsin. Rhodopsin is essential for vision and also employs to preserve epithelial and immune cells. The retinoic acid is essential during the embryonic development and for homeostasis in adult body and its scarcity develops the sign of night blindness to total blindness. Fruits and vegetables are principal source of β-carotene and are predecessors of vitamin-A (Fig. 2.1). In carotenoid pathway, the extent of β-carotene formed by plants can be enhanced by increasing the flux by raising the availability of carotenoid precursors, by communicating enzymes in the early part of the pathway between geranyl pyrophosphate and lycopene (Fig. 2.2).

For example, transgenic rice was developed by Swiss Federal Institute of Technology, Zurich, Switzerland in collaboration with University of Freiburg. In this transgenic rice, expressing genes for β-carotene was incorporated (Potrykus, 2001). Four steps are involved in β-carotene biosynthesis of rice grain (Bartley et al., 1994). This rice appeared yellow in color. β-Carotene (1.6 μg) is present in selected line per gram of rice endosperm, and was recognized as "golden rice." The first strategy is production of carotenoid over expression in tomato by using DXP synthase that enhances flux in the entire pathway and enhances the total carotenoid content (Enfissi et al., 2005).

Cho et al. (2016) studied the comparison of nutritional characteristics of transgenic rice containing CaMsrB2 gene and traditional variety. This study was conducted to compare

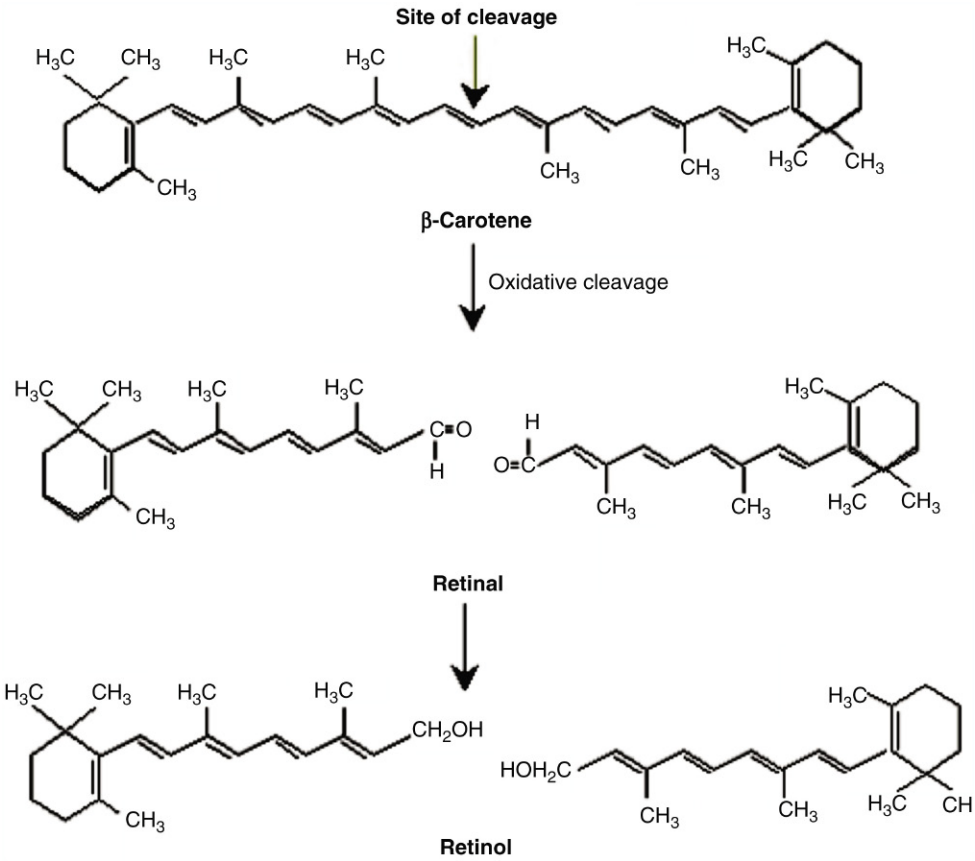


**Figure 2.1: Biosynthesis of Vitamin A.**

nutritional profiles of compositional analysis in terms of proximate components, lipid profiles, amino acid profile, and vitamin contents, and antinutrients between transgenic drought-tolerant Agb0103 rice harboring the pepper methionine sulfoxide reductase B2 gene CaMsrB2 and the parental rice cultivar, "Ilmi" as a nontransgenic control. And found that Agb0103 rice with improved resistance to drought is nutritionally equivalent to the parental rice cultivar.

### *2.2.2 Vitamin-C*

Ascorbic acid is an important antioxidant and cofactor for various enzymes. It improves immunity, boost cardiovascular functions, alleviate ailment relating to connective tissue (Davey et al., 2000), and it is essential for iron metabolism (Hallberg et al., 1989). Humans cannot synthesize ascorbic acid due to absence of L-gulono-1,4-lactone oxidoreductase, which is needed during biosynthesis of ascorbic acid. Vitamin-C-rich plants are the only dietary sources of vitamin-C for humans (Davey et al., 2000). In plants, biosynthesis of

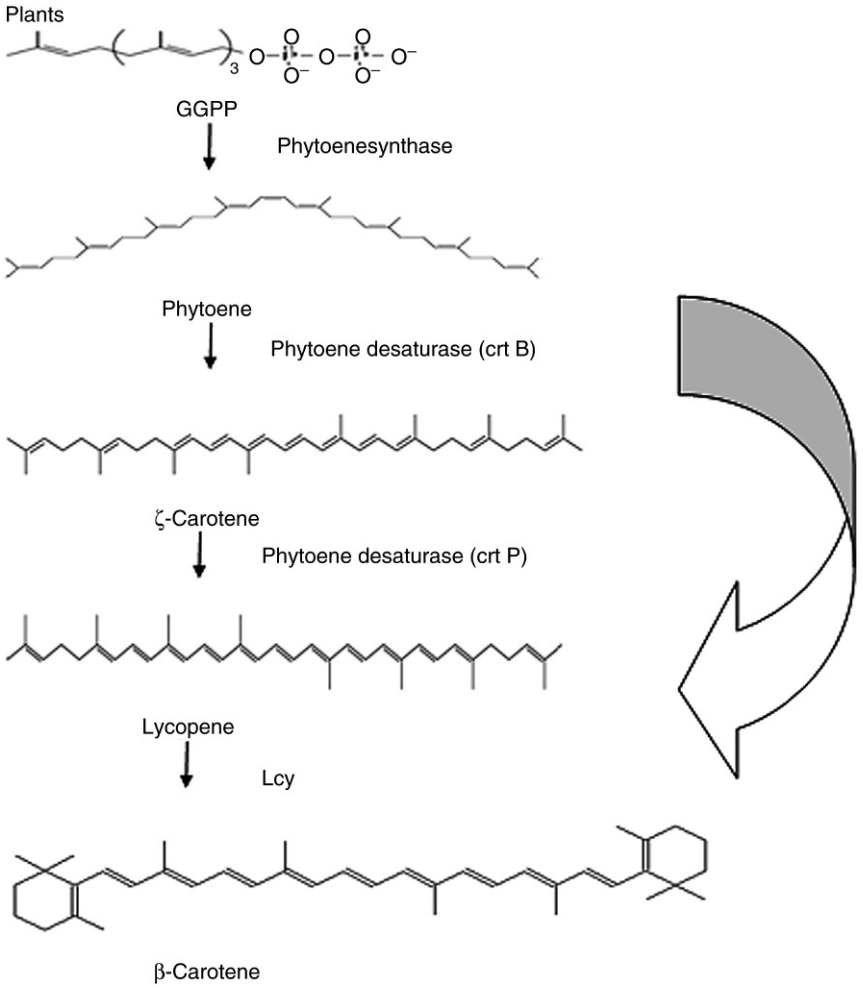


**Figure 2.2: Conversion of β-Carotene From Geranyl Geranyl Pyrophosphate (GGPP).**
*Lcy*, Lycopene cyclase.

vitamin-C takes place in two ways. First, with the conversion of D-galactouronic acid to L-galactouronic acid by D-galactouronic acid reductase enzyme followed by conversion of L-galactouronic acid to L-galactano-1,4-lactone, immediate predecessor of ascorbic acid. Agius et al. (2003) isolated D-galacturonic acid reductase enzyme encoding gene from strawberry (Fig. 2.3) (Smirnoff et al., 2001; Wheeler et al., 1998) and characterized as *galUR*.

In alternative method vitamin-C is synthesized by recycling (Smirnoff et al., 2001; Washko et al., 1992; Wheeler et al., 1998). Chen et al. (2003) hypothesized that by enhancing the expression of DHAR in plants, ascorbic acid synthesis also increases, and a proficient ascorbate recovery would be accomplished. Following the ascorbate recycling pathway,

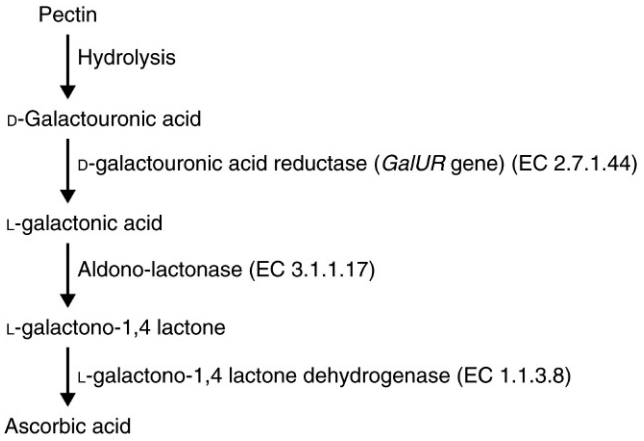


**Figure 2.3: Biosynthesis of Vitamin C.**

expressing the rice *dhar* gene in multivitamin maize, 6 times enhanced level of ascorbate compared to normal level had been observed (Fig. 2.4).

### *2.2.3 Vitamin-E*

Vitamin E belongs to tocotrienol and tocopherol families and is lipid soluble. Mainly in plants, vitamin is produced during photosynthesis (Hess, 1993). Vitamin-E is important because of its therapeutic properties. It is best known for its activity against cancer, degenerative disorders, and cholesterol (Theriault et al., 1999). Tocotrienol is more powerful antioxidant than tocopherol but not absorbed as readily. Researchers have taken initiative to grow vitamin-E-rich plants. Various methods are being used.

Vitamin-E is not a single vitamin; actually it describes eight fat-soluble antioxidants from the tocotrienol and tocopherol families that are synthesized by plants photosynthetic pathway

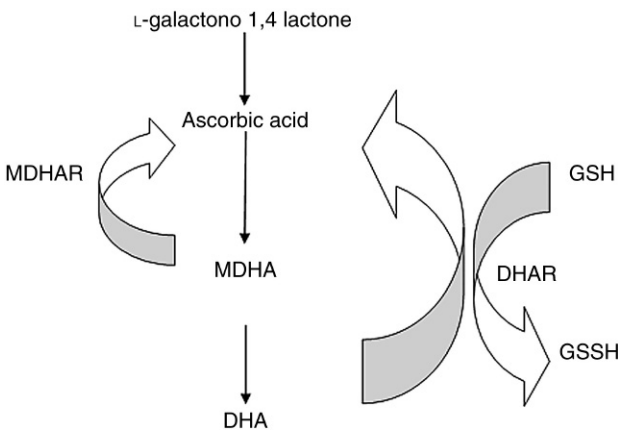


**Figure 2.4: Recycling of Ascorbic Acid.**

**Table 2.3: Essential minerals for human being.**

| Macronutrients | Micronutrients |
|---|---|
| Ca, P, Na, Mg, Cl, S, Si | Fe, F, Zn, Cu, Co, I, Se, Mn, Mb, Cr |

(Hess, 1993). During biosynthesis of both tocopherols and tocotrienols, homogentisic acid formation from *p*-hydroxyphenyl-pyruvate is the first step and it is catalyzed by the enzyme *p*-hydroxyphenyl-pyruvate dioxygenase (EC 1.13.11.27) (Grusack and DellaPenna, 1999). In another method, identification and isolation of a novel monocot gene that encodes HGGT; (specific enzyme for tocotrienol synthesis) is done for enhancement of vitamin E (Cahoon et al., 2003). A 10–15-fold increase in tocotrienol synthesis is noticed in bioengineered barley with HGGT.

The third way involves manipulation of final step in biosynthesis of vitamin-E to enhance the vitamin-E content. Hereby using the enzyme γ-tocopherol methyl transferase as catalyst the conversion of γ-tocotrienol and γ-tocopherol to α-tocotrienol and α-tocopherol is taken place (Shintani and DellaPenna, 1998).

## 2.3 Essential Minerals

Essential mineral content (micro and macronutrients) are listed in Table 2.3.

### 2.3.1 Iron

Iron insufficiency is the most common mineral malnutrition worldwide; more than 2 billion people suffer from iron deficiency along with primary clinical symptom of anemia about half of iron deficiency cases. To avoid this, several techniques, such as enrichment of food with iron and other functional ingredients are being exploited (Maberly et al., 1994), but the success is limited, especially in developing nations. So instead of supplementation, the new techniques of bioengineering are used to enhance the essential mineral content in staple food crops. Strategies implied are over expression of ferreting, store large quantity of bio available iron, and the expression of photoset, which degrade phytate, which inhibit many essential mineral, (Ravindran et al., 1995) and help to absorb easily stored iron in the human digestive system. Goto et al. (1999) introduced soybean ferritin cDNA into rice plants, which endorsed the accretion of iron in rice grain endosperm 3 times more than the untransformed plants. Lucca et al. (2002) inserted fungal (*Aspergillus niger*) phytase cDNA in rice and increased degradation of phytic acid was observed.

## 2.4 Essential Amino Acids

Human beings cannot synthesize essential amino acids on their own. Out of 20 amino acids, nine amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine,

tryptophan, and valine are essential amino acids. Animal proteins are the complete sources of amino acids. Plant sources, such as cereals are deficient in lysine and threonine, legumes are deficient in tryptophan, methionine, and/or cysteine. To make more nutritious plant sources, it is essential to incorporate amino acid-rich gene and to make GE plant. Amino acid deficiency can be tackled by applying two GE approaches: (1) introducing engineering plants to generate proteins including essential amino acids; and (2) incorporating engineering design in amino acid metabolism to enhance the accessibility of essential amino acids in the free amino acid basket. Lysine was chosen first in both the approaches. As lysine deficiency results in fatigue, lack of concentration, bad temper, pale eyes, delayed growth, hair loss, anemia, and reproductive problems. Zheng et al. (1995) had taken initiative to introduce β-phaseolin, a gene from the common bean (*Phaseolus vulgaris*) to fabricate transgenic rice with improved lysine content. To transmit gene protoplast, mediated alteration process was used. Transgenic maize seeds express the AH protein contained up to 32% more protein than wild-type seeds and contained higher levels of lysine, tryptophan, and isoleucine. To produce lysine, methionine, and tyrosine-rich transgenic potato, *Agrobacterrium* mediated transformation was done.

## 2.5 Essential Phytochemicals

Phytochemicals are precious for human nutrition. Indoles, isothiocyanates, and sulforaphane from vegetables, such as broccoli, alylic sulfides from onions and garlic and isoflavonoids from soybeans are known as plant phytochemicals. These are present in high concentration in raw foods but intensities are reduced during processing and handling (Wang and Murphy, 1996). Enhanced amount of phytochemicals in foods can resolve this difficulty. Two genes IFS1/IFS2, encoding for isoflavone synthase in soybean are revealed and expressed in *Arabidopsis thaliana*, to activate the synthesis of isoflavonoid genistin (Jung et al., 2000).

## 2.6 Isoflavonoids

Flavonoids are a group of phytochemicals responsible for the pigmentation in plant, feed deterrence, wood protection, protection from fungi and insects, and introduction of genes for root nodulation. Anthocyanins, condensed tannins, and isoflavonoids, are flavonoids, actually they are phytochemicals (Buchanan et al., 2001). The major resources of isoflavonoids can be achieved by consumption of soybean-rich products. Isoflavonoid levels can decrease by 50% during soya seed processing for traditional soy foods (Wang and Murphy, 1996). Escalating isoflavonoid quantity in soybean could solve this problem. Alternatively, development of other isoflavonoid-rich crops that can create this powerful compound therefore, widens their consumption. Jung et al. (2000) recognized *IFS1/IFS2*, two soybean genes encoding isoflavone synthase, and expressed these genes in *A. thaliana*, generating the synthesis of the isoflavonoid genistein. Approximately 2 ng/μg of fresh plant weight, genistein was produced (Jung et al., 2000).

**Table 2.4: Fermented foods of different countries.**

| S. No. | Fermented Foods | Countries of Origin |
|---|---|---|
| 1 | Amazake, atchara, bai-ming, belacan, burong mangga, com ruou, dalok, doenjang, douchi, jeruk, lambanog, kimchi, kombucha, leppet-so, narezushi, miang, miso, nata de coco, nata de pina, natto, sake, seokbakji, soju, soy sauce, stinky tofu, szechwan cabbage, tai-tan tsoi, chiraki, tape, tempeh, and totkal kimchi | East and Southeast Asia |
| 2 | Kumis (mare milk), kefir, and shubat (camel milk) | Central Asia |
| 3 | Achar, appam, dosa, dhokla, dahi (yogurt), idli, kaanji, mixed pickle, ngari, hawaichaar, jaand (rice beer), sinki, tongba, and paneer | India |
| 4 | Fermented millet porridge, garri, hibiscus seed, hot pepper sauce, injera, lamoun makbouss, laxoox, mauoloh, msir, mslalla, oilseed, ogi, ogili, ogiri, and iru | Africa |
| 5 | Sourdough bread, cultured milk, chicha, elderberry wine, kombucha, pickling (pickled vegetables), sauerkraut, lupin seed, oilseed, chocolate, vanilla, tabasco, tibicos, pulque, and mikyuk (fermented bowhead whale) | United States |
| 6 | Kushuk, lamoun makbouss, mekhalel, torshi, boza | Middle East |
| 7 | Rakfisk, sauerkraut, pickled cucumber, surströmming, mead, elderberry wine, salami, sucuk, prosciutto, cultured milk products, such as quark, kefir, filmjölk, crème fraîche, smetana, skyr, rakı, and tupí | Europe |
| 8 | Poi, kaanga pirau (rotten corn), and sago | Oceania |
| 9 | Idli, dosa, dhokla, jellabi, kefir, and kam | India |

## 2.7 Enzymes

Enzymes occur in all living organisms and catalyze biochemical reactions that are necessary to support life (Olempska-Beer, 2008). They are commonly used in food processing, preservation, and raw ingredient manufacturing. The use of recombinant DNA technology has made it possible to manufacture novel enzymes that are tailored to specific food processing conditions. Alpha amylases with increased heat stability have been engineered for use in the production of high-fructose corn syrups. These improvements were accomplished by introducing changes in the α-amylase amino acid sequences through DNA sequence modifications of the α-amylase genes (Olempska-Beer, 2008). Enzymes derived from recombinant microorganisms are listed in Table 2.2 and other application of enzymes in food processing is listed in Tables 2.4–2.6. Application of enzymes in food preservation and manufacturing has historically been considered nontoxic.

## 2.8 Flavors, Amino Acids, and Sweeteners

Volatile organic chemicals, such as flavors and aromas are the sensory principles of many consumer products and govern their acceptance and market success (Berger, 2009). Flavors produced using microorganisms currently compete with those from traditional agricultural sources. According to Berger (2009), more than 100 commercial aroma chemicals are derived

**Table 2.5: Categorization of different fermented foods on the basis of raw materials.**

| S. No. | Raw Materials Used | Names of Products |
|---|---|---|
| 1 | Cereal-based (with/without pulses) fermented foods | Amazake, beer, bread, choujiu, gamju, injera, kvass, makgeolli, murri, ogi, rejuvelac, sake, sikhye, sourdough, sowans, rice wine, malt whisky, grain whisky, idli, dosa, vodka, and boza |
| 2 | Milk-based fermented foods | Some kinds of cheese also, kefir, kumis (mare milk), shubat (camel milk), cultured milk products, such as quark, filmjölk, crème fraîche, smetana, skyr, and yogurt |
| 3 | Vegetable, BS, and unripe fruits-based fermented foods | Kimchi, mixed pickle, sauerkraut, Indian pickle, gundruk, and tursu |
| 4 | Pulse (legume)-based fermented foods | Cheonggukjang, doenjang, miso, natto, soy sauce, stinky tofu, tempeh, oncom, soybean paste, Beijing mung bean milk, kinama, and iru |
| 5 | Honey-based | Mead and metheglin |
| 6 | Tea-based | Pu-erh tea and kombucha |
| 7 | Fish-based | Bagoong, faseekh, fish sauce, Garum, Hákarl, jeotgal, rakfisk, shrimp paste, surströmming, and shidal |
| 8 | Meat-based fermented foods | Chorizo, salami, sucuk, pepperoni, nem chua, som moo, and saucisson |

BS, Bamboo shoot.

using biotechnology either through the screening for overproducers, the elucidation of metabolic pathways and precursors or through the application of conventional bioengineering. Recombinant DNA technologies have also enhanced efficiency in the production of nonnutritive sweeteners, such as aspartame and thaumatin. Market development has been particularly dynamic for the flavor enhancer glutamate (Leuchtenberger et al., 2005), which is produced by the fermentation of sugar sources, such as molasses, sucrose, or glucose using high-performance strains of *Corynebacterium glutamicum* and *Escherichia coli* (Table 2.7).

Balsamo et al. (2016) studied proteome comparison of grains from two maize genotypes, with colorless kernel pericarp, P1-ww and red kernel pericarp, P1-rr. Two-dimensional gel electrophoresis (2-DE) was performed from univariate analysis identified three soluble protein extracts of each maize genotype and 55 proteins spots. Multivariate analysis showed the separation of the two maize genotypes proteome profiles using 2-DE data.

Enzyme plays a vital role in oil extraction, purification, and modification. Microbial lipase is extensively used in oil extraction, purification, and oil modification. Commercial Cocoa Butter Equivalent may be produced by combination of different processing steps, including blending, interesterification, fractionation, and refining using lipase (Fig. 2.5).

## *2.9 DNA Vaccine*

GE DNA is introduced directly into the body to defend an animal against any disease where cell can produce an antigen, resulting in a protective immunological response. There has

**Table 2.6: Production of enzymes using different microorganisms and substrates.**

| Enzymes | Microorganisms | Substrates |
|---|---|---|
| Cellulase, β-glycosidase, CMCase, laccase, xylanase, polygalactouronase, ligninase | Strains of *Aspergillus* sp., *Trichoderma* sp., *Lentinula* sp., *Penicillium* sp., *Pleurotus* sp., *Sporotrichum* sp., *pulverulentum*, *Cerrena* sp., *Bortritis* sp., *Gliocladium* sp., *Phanerochaete* sp., etc. | Bagasse, coconut coir pith, rice husk, rice straw, wheat bran, wheat straw, tea waste, sweet sorghum, silage, sugar beet pulp, saw-dust, grape-wine cutting waste, palm oil mill waste, sago hampas, cassava waste, sweet sorghum, soy hull, paddy straw, etc. |
| Xylanases, β-xylosidase, α-arabinofuranosidase, acetoesterase, catechol-oxidase | Strains of *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., *Phlebia radiate*, *P. eryngii*, *Melanocarpus albomyces*, *P. sanguineous*, *Thermomyces lanuginose*, *Thermascus aurantiacus*, *Talaromyces emersonii*, *Thermomono spora* sp. | Rice straw, corn hull, corncobs, wheat bran, wheat straw, bagasse, rice straw, cotton stalks, soy hull, kraft pulp, sugar beet pulp, rice husk, apple pomace, corn cobs, coffee processing waste, barley straw, and oat straw |
| Laccase, Li-peroxidase, Mn-peroxidase, aryl alcohol oxidase, catalase, phenol oxidase | Strains of *Penicillium* sp., *Pleurotus* sp., *Phlebia radiate*, *Trametes versicolor*, *Flammulina velutipse*, *Polyporus* sp., *Panus tigrinus*, *Trichoderma versicolor* | Bagasse, wheat bran, wheat straw, sawdust, cotton stalk, kraft lignin, cellulose powder, and wood chips |
| Protease (acidic, neutral, and alkaline) | Strains of *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., *Bacillus* sp., *Trichoderma* sp. | Wheat bran, sunflower flour, coffee husk, soybean meal, rice bran, corn bran, rice hull, aspen wood, sweet potato residue, and waste hair |
| Lipase | Strain of candida sp., *Aspergillus* sp., *Rhizopus* sp., *Neurospora sitophila*, *P. candidam*, *Mucor* sp. | Wheat bran, peanut cake, and rice bran |
| α-Galactosidase, β-galactosidase | *A. niger*, *A. oryzae*, *Fanscaeus*, *Rhizomucor*, *Kluyveromyces lactis* | Wheat bran and soybean cake |
| α-Amylase, β-amylase, glucoamylase | Strains of *Aspergillus* sp., *Rhizopus* sp., *Mucor* sp., *Bacillus* sp., *Saccharomyces* sp. | Wheat bran, rice bran, rice husk, coconut cake, tea waste, cassava, bagasse, banana waste, corn flour, saw dust, soybean meal, sweet potato, potato, rice hull, and sugar beet pulp |
| Glutaminase | *Vibrio costicola* | Wheat bran, rice husk, saw dust, and coconut cake |
| Inulinases | *Staphylococcus* sp., *K. lactis* | Wheat bran and soybean cake |
| Phytases | *A. ficuum*, *A. carbonarius.* | Canola meal |
| Tannases | *Rhizopus oryzae* | Wheat bran + tannic acid |
| Feruloyl para-coumaroyl esterase | *Penicillium pinophilum* | Wheat straw |

been promising research using the vaccines for viral, bacterial, and parasitic diseases, several tumor types, etc., and eventually several DNA vaccines have been released for veterinary use. Among all only one DNA vaccine has been approved for human use, DNA vaccines may have a number of potential advantages over conventional vaccines, including the ability to induce a wider range of immune response type.

**Table 2.7: Production of color, flavor, organic acid and other products using different microorganisms and substrates.**

| Products | Particular Constituents | Organisms Used | Substrates Used |
|---|---|---|---|
| Color | Orange pigment | *Monacus* sp. | Agroindustrial residue |
| | Pigments | *Monascus purfureus* | Sugarcane bagasse |
| | Carotenoids | *Penicillium* sp. | Corn meal |
| Flavor | 2,5-DMP (Fruity aroma) | *B. natto* | Soybeans |
| | Fruity aroma | *Ceratocystis fimbriata* | Agroindustrial waste, cassava waste, apple pomace, soybean |
| | Acetaldehyde and 3-methyl butanol | *R. oryzae* | Tropical agrowaste residue |
| | Tetramethyl pyrazine (nutty and roasty flavor) | *B. subtilis* | Soybean |
| | Acetaldehyde and 3-methyl butanol | *R. oryzae* | Tropical agrowaste residue |
| | Strong pine apple aroma | *C. fimbriata* | Coffee husk |
| | Monoterpene alcohol and isoamyl acetate (fruity flavor) | *K. marxianus* | Cassava baggasse, giant palm bran |
| Organic acid | Lactic acid | *R. oryzae* | Sugarcane bagasse |
| | Lactic acid | *Lactobacillus paracasei* | Sweet sorghum |
| | Lactic acid | *Lactobacillus amylophilus GV6* | Wheat bran |
| | Citric acid | *A. niger* | Agroindustrial residue |
| | L-Glutamic acid | *Brevibacterium* sp. | Sugarcane bagasse |
| Gum | Xanthan gum | *X. campestris* | Apple pomace, grape pomace, citrus peels, spent malts, etc. |
| Biofuel | Ethanol | *Saccharomyces cerevisia*, *Schwanniomyces castellii*, *Zymomonus mobilis*, *Candida utilis*, *Tarula utilis* | Apple pomace, sorghum carob pods, sugar beet, sweet sorghum, sweet potato, wheat flour, rice starch |
| Vitamins | Vitamin B12, B6, riboflavin, thiamin, nicotinic acid, nicotinamide | *Citrobactewrfreundii*, *Klebsiella pneumoniae*, *Rhizopus oligosporus*, *R. arrhizus*, *R. stolonifer* | Soybean tempeh |
| Surfactant | Biosurfactants | *Bacillus subtillis* | Agroindustrial residues, molasses |
| Biocontrol agent | Biopesticides/ bioherbicide | *Entomopathogenic and Mycoparasitic fungi* | Sweet sorghum, rice flour, perlite-cornmeal agar |
| Antibiotics | Penicillin, cyclosporin, cephamycin, tetracyclin | *P. notatum*, *P. crysogenum*, *Tolypocladium inflatum*, *Fusarium solani*, *Neocosmospora varinfecta*, *Nocardia lactumdurans*, *Streptomices catteya*, *S. clauverigerus*, *Streptomyces viridefaciens*, *etc.* | Agroindustrial residue |
| Others | Gibberellic acid | *Gibberella fujikuroi* | Agroindustrial residue |

2,5-DMP, 2,5-Dimethyl pyrazine.

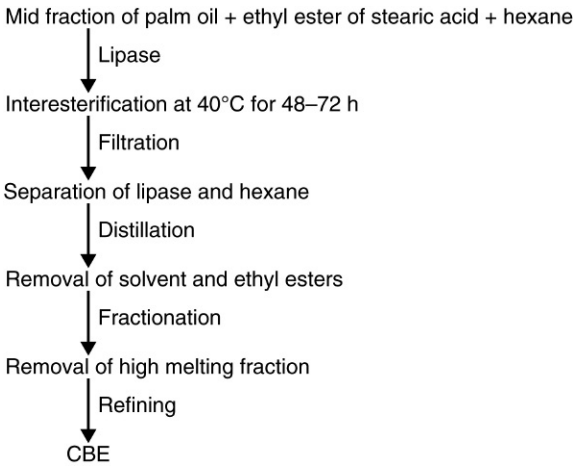


**Figure 2.5: Manufacturing of Cocoa Butter Equivalent (CBE) Using Lipase.**

## 3 Bioengineered Animals

Genetic engineering or bioengineering in animals can be defined as the deliberate changes in animal genome using the techniques of modern biotechnology. It provides various tools for improving animal welfare and health. Techniques, such as artificial insemination, embryo transfer, in vitro fertilization, cloning, etc. are used to improve the genetic makeup of animals. It provides various benefits, such as healthier offspring, healthier and safer food production from animals, consistent quality, disease resistance, etc. Nowadays, four techniques are being used to fabricate bioengineered animals, such as:

1. Transfer of nuclear material
2. Microinjection
3. Viral vector infection
4. Transfer of embryonic stem cell

### 3.1 Transgenic Dairy Cattle for Modified Milk

Bovine milk is well known as ideal food human as it contains balanced amount of vitamins and minerals, such as calcium and also good source of essential amino acids (Karatzas and Turner, 1997). An adult person can fulfill the calcium requirements by consuming two glasses of milk and milk products (Rinzler et al., 1999). Casein share majority, for example, 80% of total milk protein and is very nutritious (Brophy et al., 2003). αS1, αS2, β, κ casein determine the physicochemical properties of milk and any alteration in protein composition can affect milk's functional characteristics. Amount of protein content in milk significantly influence the cheese-making process, its yield, and nutritional

feature (McMahon and Brown, 1984). κ and β casein are the most important milk protein. Enhanced κ-casein content can decrease the size of the micelles, which provide improved heat stability while β-casein is more phosphorylated and connected to calcium phosphate, which affects calcium levels of milk (Dalgleish et al., 1989; Jimenez Flores and Richardson, 1998). The modification in milk composition is not limited to proteins only but can be extended to manipulation to the lactose, metabolic enzymes, milk fat and minerals in milk. Milk modification in transgenic animals have different applications; making it suitable for infants. Lacto-ferrin is an iron-binding protein has antimicrobial properties and may also mediate some effects of inflammation and have a role in regulating various components of the immune system. Its level in human milk is about 1 g/L (in human colostrum about 7 g/L). As the levels of lacto-ferrin in cow's milk is only about one-tenth that in human milk, this has caught the attention of those involved in designing human milk replacement formulas. Experiments are currently underway to add other naturally occurring human milk proteins—also having antimicrobial properties—and genes to alter the fatty-acid composition of milk and to make more healthier enriched mix suitable for heart patients.

### *3.2 Increased Muscle Growth in Cattle*

Myostatin (MSTN) is a well-known growth differentiation factor 8 (GDF-8), a member of transforming growth factor β (TGF-β) family, releases myocytes, which inhibit myogenesis so decreased the muscle growth and differentiation. These are the families of animals that possess mutations with this gene, which display an enhanced muscling phenotype, a desirable agricultural trait. The myostatin gene is highly conserved in other species of animals, such as human, bovine, rat, murine, zebrafish, chicken, and turkey. The myostatin knockout mice have been developed with increased lean muscles mass, which enlarged the hip and shoulder of transgenic mice. The homozygous of animals of such knockout animals have achieved 2–3 times more muscle weight as compared to the normal ones. Myostatin may bind to $NH_2$-terminal or prodomain portion of the protein noncovalently with mature myostatin, eventually inhibition occur (Thies et al., 2001). Mature myostatin interfere due to over expression of prodomain (Yang et al. 2001).

### *3.3 Transgenic Swine With Reduced Fat Content*

Bovine somatotropin, a growth hormone, is formed in the pituitary glands of young cattle and is well known for accelerating the milk production in lactating cows (Leury et al., 2003), though it is safe for human consumption. It gets inactivated during digestion in bovine gut making it biologically inactive for humans (Etherton, 1991).

Pursel et al. (1989) reported that recombinant bovine growth hormone (rBGH) caused decreased fat content in transgenic pigs. To introduce rBGH into pig genome microinjection

of pronuclear technique was adopted by controlling with mouse metallothionein-I (MT) promoter. First transgenic pigs were produced by artificial insemination with the sperms of rBGH transgenic males in nontransgenic females. The decrease in fatty acid content was noticed in transgenic pigs in comparison to the controls but there was no significant difference among the cholesterol levels. Thus, the consumers can get benefits from pork products with lower fatty acids, if the BGH secretion levels are controlled accurately (Solomon et al., 1994).

### 3.4 Transgenic Poultry: Egg as Bioreactors

Exogenous proteins are introduced to satisfy the consumer demand of various proteins used in biopharmaceuticals and it is produced with the help of egg, which was used as bioreactor in egg white (Gilbert, 1984). As the egg white is sterile in nature and has long shelf life and it is controlled by a single ovalbumin gene (Harvey et al., 2002; Tranter and Board, 1982). Harvey et al. (2002) introduced a bacterial gene β-lactamase from *E. coli* in egg white of transgenic chicken, expressed, and secreted. Expression of the gene was done using replication-defective retroviral vector introduced from avian leucosis' virus. Omnipresent cytomegalovirus promoter was used to exaggerate the expression. The protein β-lactamase was biologically active and the expression levels linger constant till fourth generation of transgenic hens. Though the levels of expression are far below those required for commercialization and an intense effort is underway to develop promoters driving much higher levels of expression but the results demonstrated the possibility of to use chicken's egg as a bioreactor.

### 3.5 Bioengineered Fish

Fish, an important protein source for the majority of people on the planet, is still primarily gathered from the wild, with serious consequences. Heavy investments into fishing fleets and technology, and ever-increasing yields, put the ocean's fisheries under increasing stress. Many fishing grounds are already overfished to the point that their future viability is threatened. GM fish have considerable potential to further increase the yield of fish farms but have prompted serious concerns both in Europe and the USA about the possible environmental impact on wild species. To overcome these concerns and address public resistance to biotechnology, it is therefore important to develop a sound, reliable and widely accepted method of estimating the potential for harm caused by GM fish escaping into the wild. For example, few companies have come forward for the commercialization of Atlantic salmon carrying growth hormone gene from Chinook salmon (Zbikowska, 2003). The main difficulty is the risk involved with the introduction of transgenic fish in the wild (Muir and Howard, 1999). According to Rasak et al. (1999) though there is no method till now for 100% sterilization, aforementioned obstacles could be avoided by sterilization of transgenic fish.

### 3.6 Improving Fish Growth Rate

To improve fish growth rates fish can be cloned and recognized from various fishes, by introducing fish growth hormones (Devlin et al., 1994; Du et al., 1992). Rahman et al. (1998) reported that at University of Southampton in the United Kingdom, a transgenic fish (*Orechromis niloticus*) has been generated and cloned. It is transformed with genes from several salmons. After research, the best results are found from the Chinook salmon growth hormone, gene that was microinjected to the fertilized fish egg. A successful integration and the transfer of transgenes to second generations have been reported. The growth rate of transgenic tilapia was found to be 33% higher than the wild type of tilapia, reducing farmer's production cost.

### 3.7 Increasing Antifreeze Property in Fish

Antifreeze proteins (AFPs) and antifreeze glycoprotein's (AFGs) can be introduced in the plasma of many fish species to incorporate antifreeze property that protects from freezing at subzero temperature in the cold water (Davies and Hew, 1990; Davies and Sykes, 1997). These proteins inhibit the formation of ice crystals of fish serum by lowering its freezing temperature (DeVries, 1984). These are important from aquaculture point of view because most of the fish, such as salmon and tilapia, cannot produce them naturally and thus cannot survive under low temperature environment in the northern Atlantic coast, which is very common in creating it a main obstacle for offshore aquaculture (Hew et al., 1995). The freeze tolerant salmon has been developed to cut down the cost of manufacture. There are two types of AFPs one-skin type small polypeptides and other liver type small polypeptides (Gong et al., 1996; Hew et al., 1986). For freeze tolerance capacity of Atlantic salmon, liver type AFP was used, the genes were injected and inserted in chromosomes of fertilized eggs.

The identical level of liver-specific expression and seasonal variation similar to those in winter flounder and protein activity were reported up to three succeeding generations of transgenic salmon. The expression levels and protein activity were observed up to three generations of transgenic salmon. Hew et al. (1999) reported that AFP levels in the blood of transgenic fish till three generation were lower (250 μg/mL) winter flounder (10–20 mg/mL) and therefore, inadequate to provide freeze resistance to the salmon. Research has been focusing on to discuss better antifreeze properties by altering gene structure.

## 4 Bioengineered Microorganisms

More than 5000 years, human beings have, consciously and innocently, made use of natural fermentation of a range of food items, which comprise bread, dairy products, alcoholic beverages, vegetable products, and meat products. But it was more in recent times, just in the last century, that researchers comprehend that the method of fermentation was done by the exploitation of microorganisms and that each microorganism accountable for a particular

food processing could be isolated and identified. Now, with superior bioengineering practice, it is possible to characterize the important food strains with high precision, isolation, and improvement of genes involved in the process of fermentation, and transfer desirable traits between strains or even between different organisms.

### 4.1 Elimination of Carcinogenic Compounds

One of the most important, commonly used, and very well known microorganisms is *S. cerevisiae*, known as Brewer's yeast in the food industry. In bread manufacturing, yeast is generally used as a leavening agent and during alcoholic beverage manufacturing, yeast is used as starter culture and grain residues or molasses are used as raw material for fermentation. During fermentation, occasionally undesirable products are produced. Recombinant DNA technology is the only solution. With the help of recombinant DNA technology, undesirable by-products can be removed by incorporating properties in the GM yeast strain. Ethylcarbamate or urethane is the unwanted by-product of foods and beverages during yeast fermentation. Ethylcarbamate or urethane is the probable carcinogenic material (Aldhous, 1990; Ough, 1976). Reduction of ethylcarbamate from alcoholic beverage is the main plunge of the alcoholic beverage industry; a large amount of investment has been done for the research (Dequin, 2001). The spontaneous reaction between ethanol and urea produces ethylcarbamate, which is the degradation product of grape arginine. In the presence of yeasts, which produces arginase and that catalyzes arginine degradation. If arginase is blocked, the reaction of arginine to urea can be prevented; therefore, ethanol to ethylcarbamate conversion can be stopped. A transgenic yeast strain was developed by inactivating *CAR1 gene*, which encodes for the enzyme arginase (EC 3.5.3.1) (Dequin, 2001; Kitamoto et al., 1991) to reduce the formation of urea in sake.

By incorporating an unproductive *CAR1* gene in the area of the arginase gene, the scientist developed the mutant yeast strain, flanked by DNA sequence homologous to the region of the arginase gene. The useless gene was incorporated into the active *CAR1 gene* through homologous recombination, in the yeast chromosome, and disturbed its function. As a result, urea was detached and ethylcarbamate was not produce during sake fermentation. The analogous tactic can be applied to remove ethylcarbamate from other alcoholic beverages including wine (Kitamoto et al., 1991).

### 4.2 Inhibition of Pathogenic Bacteria

During manufacturing of fermented food to enhance the security, hygiene, and proficiency in the food industry, the major thrust is to maintain the originality of the inoculating bacterial culture and also particular protective cultures (Gardner et al., 2001). Generally, inoculating or starter culture is used to instigate an industrial fermentation. Inoculating or starter culture is a mixture of particular microbial strain. The inoculating cultures offer the food a unique odor and consistency and protective culture helps to maintain the originality of the food by keeping unaltered properties, and also stop growing objectionable pathogenic organisms (Geisen and

Holzapfel, 1996). Though every time it is not possible but for convenience during fermented food manufacturing, the same microorganism should be used as starter and protective cultures. With the help of genetic engineering, better strains can be developed, which can be used as inoculating and protective cultures, therefore novel characteristics can be amplified and undesirable properties can be suppressed (Hansen, 2002).

The genetic engineering research increases microbial strains efficiency, improves process stability, and improves product safety during optimization of inoculating cultures (Geisen and Holzapfel, 1996). During the development of selected fermented foods, for example, mold-ripened cheese, degradation of lactic acid by fungal organism results increase of pH level (alkaline). *Listeria monocytogenes*, a food born pathogen, get suitable environment to proliferate in the alkaline *pH*. This type of circumstances can be prevented by using single starter culture, which has enhanced properties to inhibit the growth of such hazardous pathogens. The lysozyme (EC 3.2.1.17) can be used efficiently to prevent *Listeria* in food. Van de Guchte et al. (1992) incorporated the gene responsible for lysozyme formation in *Lactococcus lactis*, the bacterium strain. This bacterial strain expressed and secreted lysozyme at high levels after the genetic transformation. Lysozyme-encoding genes from *E. coli* bacteriophages T4 and lambda was cloned, in wide-host-range vectors and expressed in *L. lactis*. Biologically active lysozyme were produced and secreted by the transgenic *L. lactis* strains, indicates that transgenic *L. lactis* strains can be applied both as a starter and protective culture (Van de Guchte et al. 1992).

### *4.3 Natural Sweetener Produced by Microorganisms*

Microorganisms can be extensively used in manufacturing and improvement of food product along with flavor enhancer in fermented food manufacturing practices. Nowadays, many of the methods use synthetic chemical additives for flavoring of food products (Vanderhaegen et al., 2003). Nowadays, consumers are very health conscious and aware of health hazards caused by harmful synthetic flavoring of chemicals; so they prefer foods with natural food grade flavors (Armstrong and Yamazaki, 1986; Cheetham, 1993). Among thousands of natural volatile and synthetic fragrances, only a few hundred are regularly applied to food manufacturing in industrial scale (Somogyi, 1996). Bioflavors can be extracted from (1) plant sources (2) specific bioengineered microorganisms by biosynthesis. Bioflavors production by fermentation provides certain beneficial effect, such as the process is cheaper when produced and applied in large-scale without depending on natural resources and plant material (Krings and Berger, 1998). The wood sugar xylitol, a white crystalline material, taste is similar to sugar, is made from abundantly available xylose, the plant sugar (Nigam and Singh, 1995). Insulin is not required for metabolism; therefore, it is suitable for diabetic patients (Pepper and Olinger, 1988). Xylitol can prevent tooth decomposition in children and adults as it provides prevention from *Streptococcus mutans*, the bacteria responsible for cavity formation in human. Sweetening index of xylitol and sucrose are 60 and 100, respectively and xylitol provides 40% less calories than sucrose. Metabolism of xylitol is very slow

and human body can utilize partially. Xylitol is FDA approved sweeteners. Xylitol is used as sweeteners in foods since 1960s in United States for special dietary purposes (Emodi, 1978). Due to its extensive use in fermentation industry yeast (*S. cerevisiae*) can convert xylose to xylitol. Therefore, it is used for the commercial production of xylitol. Govinden et al. (2001) introduced *XYL1* gene into *S. cerevisiae* isolated from *Candida shehatae*, novel xylose reductase (EC 1.1.1.21). The *XYL1* gene from *Candida* was cloned behind the *PGK1* promoter and the construct was introduced and transformed into yeast by electroporation into the yeast expression vector pJC1. Effect of cosubstrate was evaluated by using glucose, galactose, and maltose on xylitol production from xylose. When glucose was used as cosubstrate from 50 g/L of xylose, highest xylitol yield of 15 g/L was obtained.

### 4.4 Production of Carotenoid in Microorganisms

Carotenoid is a group of structurally different coloring body present in plants, animals, and microorganisms. These pigments have multiple numbers of biological functions; for example, it provides color, produce hormone, harvest light, and have photo protection properties (Campbell and Reece, 2002). Carotenoids are principally used as natural food colorants, vitamin supplements in animal feed, and nutraceuticals in the pharmaceutical industry. Current studies have suggested numerous nutritional health benefits from the consumption of carotenoids. Carotenoids (astaxanthin, β-carotene, and lycopene) are very popular due to high antioxidant properties, which prevent harmful diseases, such as cancers, cardiovascular, inflammation from arthritis, boost immune system, and provide relief from pain (Giovannucci et al., 1995; Jyocouchi et al., 1991; Miki, 1991). Pharmaceutical industry uses extract of carotenoids from plant sources. As the cell wall of Microalgae, such as *Haematococcus pluvialis* is thicker, extraction of carotenoid astaxanthin is not easy though it generates high amounts of β-carotene. Using GE tools, effort has been taken to generate carotenoid-rich organisms. *Candida utilis*, the edible yeast is "generally recognized as safe," is a potential organism for industrial carotenoid and large-scale production of glutathione peptides and has already being successfully accomplished in *C. utilis* (Boze et al., 1992).

Carotenoids are manufactured from farnesyl pyrophosphate, the precursor of carotene in microorganisms and plants. Carotenoids, lycopene, β-carotene, and astaxanthin were synthesized by recombinant gene and cloned. Miura et al. (1998) developed a de novo biosynthesis process in *C. utilis* where bacterial genes encode for enzymes in the biosynthetic pathway. Four *Erwinia uredovora* genes (*crtE*, *crtB*, *crtI*, *crtY*) and two (*crtZ*, *crtW*) genes from *Agrobacterium aurantiacum* were used for manufacture of four different plasmids. According to report, following designs were recommended:

For lycopene production, plasmid pCLR1EBI-3 containing *crtE*, *crtI*, and *crtB*; β-carotene plasmid pCRAL10EBIY-3 containing the genes *crtY*, *crtI*, *crtE*, and *crtB*; astaxanthin synthesis in a dual plasmid system for pCLEIZ1 containing *crtE*, *crtI*, and *crtZ for* pCLBWY1 containing *crtW*, *crtY*, and *crtB*.

By electroporation technique, these genes were incorporated into the yeast chromosome. Then the plasmids were linearized by restriction digest and transformed into *C. utilis*. It was reported that the resultant transgenic yeast produced considerable amounts of lycopene (1.1 mg/g dry weight), β-carotene (0.4 mg/g dry weight), and astaxanthin (0.4 mg/g dry weight).

Those were the equivalent quantity found in microorganisms, which produces these carotenoids naturally (Miura et al., 1998). It indicates that *C. utilis* has a great potential for use in large-scale production of commercially important carotenoids.

## 5 Detection Methods

To maintain the food safety, various methods have been developed to ensure the traceability of genetic material. The detection methods are used to achieve the consumer faith by ensuring their safety.

### 5.1 Transgene Detection Method

Transgenic food products can contaminate the nontransgenic material; therefore, protection is needed. One very popular example is the contamination of corn from bioengineered corns. Though such events can have negative impact on the consumer acceptance for GM foods, so to increase their potential achievement and approval, most influential, handy, and cost proficient method is real-time quantitative PCR (Higuchi et al., 1992). Principal rudiments used today for detection of GMOs is antibiotic resistance markers and promoters, but they are not always perfect since the same signature sequences can be found in multiple number of GMOs, and are also injurious for humans and atmosphere. European Union in 2004 has already been banned on the usage of such markers. Compulsory tagging of GMO foods through a 1% threshold level for the existence of transgenic material was established. R&D scientists from the German company, Icon Genetics, proposed a novel idea for worldwide recognition of GMOs (Marillonet et al., 2003). They proposed the standardized coding procedure for accumulating the nontranscribed DNA based technical information to transgene before its insertion to the genome. Nucleotide based triplet is the basis of coding, which consists of any of the 26 letters of latin alphabet, an Arabic numeral from 0 to 9, and then a space character, giving a total of 37 characters (Marillonet et al., 2003). The researchers could introduce biologically unbiased, nongenetic coding series that interpret into unique information, for example, the name of the company, production date, and place of production, product model, and serial number. PCR performance and sequencing of the fragment is needed to read the DNA-encoded information.

Another method to detect GMO involves distinctive genomic progression flanking the transgene. Thermal asymmetric interlaced-PCR approach is rapid and is a proficient method to magnify the indefinite sequences adjacent to the known incorporation sites. Supplementation specific primers are used along with the arbitrary degenerate primers, which are intended to fluctuate at their annealing temperatures.

Amplification of marker gene followed by real-time PCR performance with quantitative tool is the two consecutive steps for accurate DNA quantification. The quantitative tools are:

1. DNA binding dyes (Morrison et al., 1998),
2. fluorescent oligonucleotides (Whitcombe et al., 1999),
3. molecular beacons (Tyagi and Kramer, 1996),
4. fluorescence resonance energy transfer probes (Wittwer et al., 1997),
5. TaqMan probes (Heid et al., 1996).

The application of three oligonucleotides in PCR reaction turns out to be highly unambiguous. The detection process comprises amplification by primers and then detection by annealing. TaqMan probe provides quantifiable fluorescence light. The intensity of light confirms the quantity of target gene present in the food sample.

### 5.2 Food Pathogen Detection

Food poisoning occurs due to the presence of toxin producing bacteria, such as *Salmonella*, *Vibrio*, *Listeria*, and *E. coli*. O157:H7 is the most harmful strain of *E. coli*, which produces Shiga toxins (*stx1* and *stx2)*. These Shiga toxins injure the lining of large intestine causing severe diarrhea and dehydration eventually, which have an effect on other organs also and finally if absorbed in blood stream can be fatal (Riley et al., 1985). Mead et al. (1999) reported about 73,480 illnesses and 61 deaths in USA rooted from ground beef, unpasteurized milk, and roasted beef. These products if not preserved properly can contaminate and can be responsible for the transmission of food borne illness. PCR was used to detect the pathogen, as PCR is fast, sensitive, and reliable. Traditionally, PCR follows gel electrophoresis process and analyses very small number of samples during one run. PCR-ELISA approach, which is 100-fold rapid and more accurate screening for the detection of *E. coli* O157:H7 and other STEC were reported. With the help of robots, the process can be automated in near future and also permitted rapid, susceptible, accurate, and large-scale screening of microorganisms that produce the Shiga toxins. Using explicit biotin-labeled PCR primers, this method can also be applied to detect any other food pathogens.

## 6 Conclusions

Fabrication of herbicide, insects, and pathogen-resistant transgenic plants of many food crops have led to the enhanced production at low production cost. Three times faster growing transgenic salmon can be manufactured by introducing foreign genes in salmon. Development of engineered foods with better characteristics in terms of nutrition, taste, quality, and safety are the principle of the next generation modern biotechnology. Fabrication of new vegetable solid fat with lower amount of saturated fatty acids, production of more nutritious healthier meat, bioextraction of plant bioactive nutraceuticals, which provides excellent health benefits

but it is present in very low quantity in plant. Biotechnologically modified foods not only help to produce novel compounds, but also make improvements in existing ones, as well as provide food safety and security.

As a whole, biotechnology offers numerous innovative tools like new sensors to identify microorganisms and their toxin using number of biosensors, as well as DNA probes, PMCA, ELISA tests. PCR has been developed to detect the existence of infectious pathogenic agents, such as bacteria, virus, fungi, etc. It is proved that using biotechnology in modern food, variety of more nutritious, healthier, tastier, secure, durable, safe, and convenient food products can be delivered to the world at more affordable prices.

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CHAPTER 3

# Enzymes and Food Industry: A Consolidated Marriage

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## 1 Introduction

The use of enzymes has greatly contributed to the development of mankind, especially for their application in food processing. Enzymes had been already used for food processing since ancient times, long before the discovery of enzymes as biological catalysts in fact. The use of ficin for milk clotting is such an old habit that it is described in Homer's Iliad, an epic poem written around the 8th century BC. Another example relates to the use of enzymes as meat tenderizers. It is estimated that natives of some Pacific islands have used papaya juice as meat tenderizer for centuries. With the advent of British colonization of these islands in the 18th century, a number of applications of papaya, including as meat tenderizer and for wound healing, were introduced in Europe (Copeland, 2000).

With the scientific development observed in the 19th and 20th centuries, knowledge about the properties of enzymes consolidated their use for industrial and commercial purposes. The first companies dedicated to the trading enzymes formulations were founded in the late 19th century, although at that time, the definition of enzymes as biocatalysts was not yet well established. In 1874, Christian Hansen started a company to trade in rennet; an extract of pepsin and chymosin obtained from the stomach of young calves, and used it to coagulate the milk. Later, in 1890, the Japanese entrepreneur Takamine founded his company in the USA for the production and commercialization of takadiastase, a mixture of proteases and amylases obtained through the cultivation of *Aspergillus oryzae* in wheat bran. Throughout the 20th century, the properties and structure of several enzymes were revealed, and thus the development of enzymatic processes for the industrial purposes was boosted (Poulsen and Buchholz, 2003; Vasic-Racki, 2006). The establishment of biotechnology has made possible to improve the biocatalysts properties to meet the most varied industrial processes (Fernandes, 2010).

The enzyme market has expanded in recent years and it is expected that in the coming years, the demand for enzymes will continue to increase. Global enzyme market is expected to be

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00003-7

worth US $7 billions in 2017, and the market for food enzymes represents a considerable share of this market (The Freedonia Group, 2014). The market for food enzymes was expected to reach US $2.94 billions in 2021. Moreover, this estimate highlights a potential growth of baking and dairy industries. The use of enzymes in food processing and the increased consumer awareness on the benefits of enzyme applications in food industries are the main reasons for their growth in the food market (Markets and Markets, 2016).

Enzymes are considered natural food ingredients or additives. Enzymes from microbial origin are safe, since there are no concerns about the presence of viruses or toxins, which exists in enzymes from animal origin. Furthermore, some groups with the restrictions on the food composition and preparation, as vegetarians, can readily accept foods processed by enzymes (Beermann and Hartung, 2012). In addition, enzymes show high specificity, which make them ideal components for promoting desirable modifications in food without changing its properties.

This chapter brings an overview on the use of enzymatic treatments for improving meat, milk, and bread quality. The importance of enzymatic treatments and the impact of endogenous enzymatic systems are highlighted. In addition, some advances in the area are mentioned, such as the use of immobilized enzymes and the search for novel enzymes in new sources of genetic diversity, such as metagenomic libraries.

## 2 Enzymes and Meat Quality

The worldwide meat consumption has increased in the last decades, and this trend is expected to continue, especially because of population growth and economic improvement in developing countries. With the improvement in income, consumers consider mainly quality attributes rather than price when choosing products (Henchion et al., 2014). Therefore, the quality traits of food products will be of greater importance to meet increasingly demanding consumers.

The meat quality traits exert considerable influence on consumer preference. In an increasingly competitive market, it is very important for the food industry to understand and identify these traits to attend customer expectations. At the moment, the most frequent quality attributes of meat consumption include tenderness, juiciness, and flavor (Troy and Kerry, 2010).

The texture is a complex characteristic derived from sensory perception of food. This perception is determined by the mechanical properties of the food, which is, in turn, a characteristic resulting from its micro- and macrostructures. Once the texture is dependent on the sensory characteristics, a wide variation in perception between individuals can be found. The texture is a complex attribute, determined by several parameters. Some of the food texture parameters include hardness, cohesiveness, viscosity, elasticity, and

adhesiveness. Tenderness, the most appreciable meat trait by consumers, is a texture property related to cohesiveness (chewiness). Thus, tenderness is a quality trait associated with the food, which exhibits little resistance to fragmentation during mastication (Bourne, 2002; Szczesniak, 2002).

The meat texture is a property determined essentially by myofibrils and connective tissues associated with skeletal muscle (Nishimura, 2015). Therefore, the structure of these two components of skeletal muscle and the modifications undergone by them in the postmortem period are crucial to the development of meat quality traits.

## 3 Muscle Structure and Postmortem Biochemistry

The sarcomere (Fig. 3.1) is the basic unit of muscle fiber, responsible for its contractile properties. It consists of a set of protein complexes organized in a fine manner, constituting the basic structure of the myofibril. Two of these structures are directly involved in muscle contraction: the thin and thick filaments. Thin filaments consist of assemblies of actin (the most abundant protein in the muscle), which are anchored to the borders of the sarcomere, a region named Z-disk, and extend to the center region. Actin filaments are associated to nebulin and chains of troponin and tropomyosin, work mainly as stabilizers of their structure and modulators of their interaction. The Z-disk is composed mainly by the dimeric protein α-actinin, which is responsible for anchoring thin filaments of neighbor sarcomeres. Thick filaments are composed of myosin (the second most abundant protein in muscle fiber) and originate from the center of the sarcomere, expanding to the borders and intersecting with thin filaments. In the central region, thick filaments are anchored to and originate from the M-line. Myosin moieties are composed of tail and head regions: the tails regions are anchored to the thick filaments, while the head regions present ATPase activity and interact with actin filament during muscle contraction. Another important component of the sarcomere is titin,

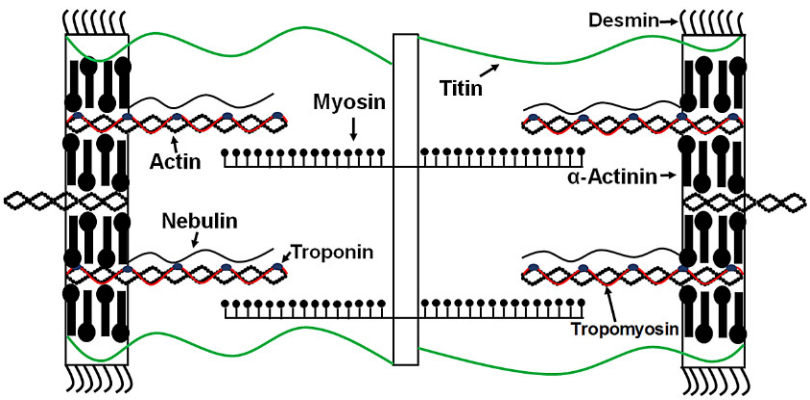


**Figure 3.1: Molecular Structure of a Sarcomere.**

the third most abundant protein in muscle fiber, and the largest protein known so far. Titin is anchored to Z-line, and expand throughout the sarcomere, being anchored again at the M-line. Titin is a component of considerable importance for maintenance of sarcomere structural integrity and stiffness, as well as for the correct spatial orientation of sarcomere components, such as myosin (Clark et al., 2002).

The connective tissue is composed primarily of collagen and proteoglycans, being responsible for maintaining the integrity of skeletal muscle. The connective tissue is organized in three different levels: the endomysium, a layer of connective tissue surrounding each muscle fiber; the perimysium, a layer of connective tissue delimiting bundles (sets) of muscle fibers; and the epimysium, a sheet of connective tissue involving the whole muscle. The stiffness conferred to muscle is a property originated specially by interactions between collagen fibers (Nishimura, 2015; Purslow, 2014).

After slaughter, various biochemical changes occur in skeletal muscle, and these changes are crucial for the transformation of muscle into meat and the development of quality meat characteristics. Muscle remains metabolically active after slaughter. The depletion of oxygen and shortage of available energy favor the intensification of anaerobic metabolism. Consequently, the muscle pH drops, due to the accumulation of lactic acid. The limited energy availability causes an increase in ionic strength, since the active pumps cannot remove the surplus of ions, such as calcium ($Ca^{2+}$) and potassium ($K^{+}$). The shortage of ATP also favors an irreversible association of actin and myosin, keeping the muscle in contracted form (Lomiwes et al., 2014; Lonergan et al., 2010; Paredi et al., 2012). The postmortem process of muscle transformation to meat presents three phases. Immediately after slaughter, energy reserves still allow the maintenance of muscle elasticity, and no texture changes are observed, defining the short, initial phase called prerigor. With the shortage of energy, and the metabolic changes previously discussed, the restriction imposed to actin/myosin association culminates to increase muscle toughness, and these events characterize the rigor phase (since the irreversible association between actin and myosin generates the so-called rigor bonds). The last step, postrigor phase, is characterized by the events involved in the meat tenderization. This last step is highly influenced by intrinsic (e.g., breed, age, and genetic variants) and extrinsic factors (e.g., temperature and handling conditions) (Lonergan et al., 2010; Sentandreu et al., 2002). Biochemical alterations that take place in muscle after slaughter favor the action of endogenous proteases.

## 4 Enzymatic Tenderization—Endogenous Enzymes

Calpains are the most studied endogenous proteases involved in the meat tenderization process, and are also considered the main agent responsible for the modifications in structural proteins that reflect changes in the meat texture. Calpains are cysteine-proteases belonging to the papain superfamily. These enzymes are $Ca^{2+}$-dependents and characterized by a

processing action, where there is no deep degradation of their substrate. Calpains are usually organized in dimers—one subunit containing the catalytic site, and another subunit related to activity regulation—and are ubiquitously distributed in tissues, where they play important role in several metabolic steps, such as removal of damaged proteins. In muscle, calpain activity is regulated, among other ways, by a specific inhibitor named calpastatin. This protein contains four inhibitory subunits, and its role, as inhibitor is also $Ca^{2+}$-dependent. Calpastatin may be subjected to degradation by calpains, but even the products of this degradation possess some inhibitory capacity (Ono and Sorimachi, 2012).

μ-Calpain and m-calpain are the most well characterized proteoforms of calpains. The denominations of these two proteoforms (μ-calpain and m-calpain) have origin in the levels of required $Ca^{2+}$ for half-maximum activity, with requirements in micromolar and millimolar ranges, respectively. Calpains show the highest activity at pH range of 7.2–8.2. Both proteoforms autolyze in the presence of $Ca^{2+}$. This is an interesting feature, since the autoproteolysis renders more activated forms and the calcium requirements for reaching half-activity decrease considerably. μ-Calpain and m-calpain have the molecular weight reduced to 76 and 78 kDa, respectively. The regulatory subunit, common to both proteoforms, has its size reduced from 28 to 18 kDa. The in vitro evaluation of calpains for substrate preferences has shown a great number of target proteins. Regarding the myofibrillar proteins, calpains can readily degrade desmin, nebulin, filamin, titin, tropomyosin, and troponin, while proteolysis of native actin and myosin is performed at a very slow pace (Goll et al., 2003; Lonergan et al., 2010).

The calpain activity is essential to the development of meat tenderization. For instance, Uytterhaegen et al. (1994) applied several inhibitors to evaluate the role of endogenous proteases in the texture changes observed during meat ageing. Results showed that the activity of cysteine-proteases was found to be crucial, since the application of a specific inhibitor to this class of protease completely affected tenderization. Besides that, the inhibitors of aspartate and serine proteases did not compromise tenderness development, reinforcing the important role played by cysteine-proteases. However, the endogenous cysteine-proteases from muscle include calpains and some types of cathepsins, such as cathepsin D, B, L, H, and I. In order to investigate which of these enzymes would be involved in the degradation of key structural proteins of the muscle fiber, inhibitors specific to each enzyme were applied. Results showed that calpain is the protease that effectively promotes degradation of myofibrillar proteins, such as desmin and titin, which leads to the weakening of myofibril structure, and ultimately, to the development of meat tenderness, while the cathepsins-inhibitors used did not influenced the development of meat tenderness significantly.

Nevertheless, since there are two proteoforms of calpains (μ and m) in muscles during postmortem period, which one of them is most responsible for the structural modifications of myofibrillar proteins observed during meat maturation is still an unclear issue. Geesink and Koohmaraie (1999) evaluated the behavior of μ-calpain under conditions that resembled those

of postmortem muscle. Within this context, they found out a significant autolysis of μ-calpain after 24 h of incubation, myofibrillar protein degradation up to 7 days, even in the presence of variable ratios of calpastatin. After 7 days of incubation, the proteolytic activity of μ-calpain was significantly reduced. This result was due to the instability of the autolyzed enzyme under high ionic strength, which was generated by the ion leakage in organelles. Thus, the authors attributed μ-calpain for the major proteolytic changes that are responsible for the meat tenderization.

Boehm and colleagues evaluated the dynamics of calpains (μ and m), and proposed a different model for the role played by these proteoforms during the development of meat tenderness. These authors also worked under similar conditions to the postmortem muscle, and they found that activity levels of m-calpain slightly decreased during 7 days of postmortem, while μ-calpain and calpastatin levels presented a high declination rate during the same period. In a similar trend to the previous work, the autolysis of μ-calpain was almost completed on the first day, while no autolysis was observed for m-calpain during the evaluated period. After 7 days of incubation, μ-calpain activity decreased significantly. It is worthy to mention that the autolysis of calpains was directly related to the availability of $Ca^{2+}$. Interestingly, it was found that μ-calpain became increasingly linked to myofibrillar proteins during this period, with the bound enzyme fraction representing about 50%–60% after 7 days of incubation. The authors pointed out that m-calpains could play an important role in the meat tenderization after a period of 7 days. At this stage, several changes in muscle conditions could favor the activity of these enzymes, including the increase in $Ca^{2+}$ levels and the reduced levels of calpastatin, which certainly would not be high enough to inhibit m-calpain activity (Boehm et al., 1998).

The function of other muscle proteases (cathepsins, caspases, and proteasomes) in the process of meat tenderization has also been evaluated. As described by Huang et al. (2011), caspase-3 and caspase-6 can degrade titin, desmin, nebulin, and troponin. However, the action of these proteolytic systems during the transformation of muscle to meat is still not a consensus. The action of other proteases in meat tenderization seems to be more evident in conditions not favorable to the activity of calpains. For instance, Wang et al. (2013) evaluated the texture development of duck meat during several temperatures of cooking (30–90°C). The best texture was achieved at 70°C, but at this temperature, calpain activity was too low. In fact, calpain total activity decreased from 30°C onward. Besides calpain, three other types of cathepsins were monitored (B, D, and L), with cathepsin D being the most thermostable, and directly associated to the development of the best texture at 70°C.

The development of meat tenderness is a complex process, influenced by both endogenous and exogenous factors. Calpain and calpastatin levels were found to be highly variable between different muscle fiber types, depending on the genetic diversity and regulation of gene expression. Different genotypes found in calpain-encoding gene of Korean

cattle (Hanwoo) were related to different levels of meat tenderness (Chung et al., 2014). According to the muscle tissue evaluated, the expression of genes encoding for calpains and calpastatin were different, with the most accentuated differences in the expression of regulatory subunits of calpains and calpastatins (Chung et al., 2014). The differences in calpain:calpastatin ratio was clearly related to the meat tenderness, since higher ratios were found in muscles known to be more easily tenderizable (Muroya et al., 2012). Preslaughter conditions may also influence the development of meat tenderness. The behavior of steers and heifers was evaluated before slaughter by Gruber et al. (2010), aiming at correlating stress condition with final tenderness of meat. The authors found a correlation between preslaughter stress, increased levels for plasmatic epinephrine and lactate, and meat toughness.

## 5 Enzymatic Tenderization—Exogenous Enzymes

Given the complexity and heterogeneity of meat tenderization, it is expected that some meat pieces remain tough. Since meat tenderness is an appreciated trait by consumers, tough pieces present low values. Several efforts have been intensely carried out in order to favor the correct development of meat tenderness, and ultimately, increase the economic value of tough meat pieces. Electrical stimulation is usually applied immediately after slaughter and its main effect consist in accelerating the metabolic changes in muscle (e.g., glycolysis), leading to a faster pH decline, and consequently, to accelerated tenderness development (Simmons et al., 2008). Also, the application of salt solutions, such as calcium chloride, stimulates endogenous activity of proteases, especially the calcium-dependent calpains (Kong et al., 2006; Whipple and Koohmaraie, 1993).

Another important strategy largely used is the application of exogenous enzymes to improve meat tenderness (Table 3.1). Plant proteases are the most commonly used enzymes in meat processing. These enzymes work in a broad range of temperature and pH, with activity against a high diversity of substrates. Papain, bromelain, and ficin are plant proteases

**Table 3.1: The main enzymes involved in the process of meat tenderization.**

| Enzyme | Origin | Substrates |
|---|---|---|
| Calpain | Endogenous | Myofibrillar proteins, mainly desmin, titin, troponin, and nebulin. Minor action on actin and myosin |
| Papain | *C. papaya* latex | Action on both myofibrillar proteins and collagen (Sullivan and Calkins, 2010) |
| Bromelain | *A. comosus* extract (stem or fruit) | |
| Ficin | *F. glabrata* latex | |
| Zingibain | Ginger (*Z. officinale*) extract | |
| Protease formulation | *B. subtillis* | |
| Protease formulation | *A. niger* | |

recognized as safe agents (GRAS status) for application in foods, as certified by the US Food and Drug Administration (FDA, 2016), and these are also authorized for application in the meat and poultry carcasses (USDA, 1999).

Papain (E.C 3.4.22.2) is extracted from the latex of *Carica papaya*. This latex contains a mixture of several proteases, including caricain, chymopapain, glycyl endopeptidase, and papain, which is the minor component (accounting for 5%–8% of total latex proteases). Bromelain refers to the aqueous extract of the stem and immature fruits of pineapple (*Ananas comosus*). The extract consists of a complex mixture of several enzymes that can be obtained from different plant tissues, including the stem bromelain (E.C 3.4.22.32) and fruit bromelain (E.C 3.4.22.33). Ficin (E.C 3.4.22.3) is found in the latex of the species of *Ficus*. It is isolated mainly from the latex of *Ficus glabrata* (Feijoo-Siota and Villa, 2011). These proteases are classified as cysteine-proteases, or thiol-proteases, due to the presence of cysteine as one of the catalytic amino acid residues present in the active site (Feijoo-Siota and Villa, 2011).

The production of plant proteases usually involves the enzyme extraction from plant tissues. Several purification techniques have been applied to obtain high-grade purity plant proteases. These purification techniques include liquid–liquid extraction (Hebbar et al., 2012), ethanol precipitation (Soares et al., 2012), and chromatography (Devakate et al., 2009). On the other hand, alternative strategies have emerged to facilitate the production and purification steps. Amid et al. (2011) evaluated the heterologous expression of a gene encoding for a stem bromelain in *Escherichia coli*. It was possible to obtain an enzyme with similar characteristics when compared to a commercial sample, and with much higher purity obtained in only one chromatographic step. The authors also pointed out the economic impact of using recombinant enzymes, since it is possible to obtain large amounts of purified enzyme in lesser time.

Other plant proteases have been also evaluated regarding their potential for meat tenderization. Ginger (*Zingiber officinale*) extract, denominated zingibain, was first described as a source of proteases with potential as meat tenderizers in the 1970s (Thompson et al., 1973). Two major proteases are found in ginger rhizome and both are classified as cysteine-proteases (Choi and Laursen, 2000). In addition, actinidin, a cysteine-protease from kiwifruit juice, first described by Arcus (1959), has also been evaluated for tenderization in meat.

Microbial proteases have appeared as potential enzymes for application as meat tenderizers. A major advantage for the evaluation of alternative enzymes is the over-tenderization effect normally associated to some plant proteases, which can lead to undesirable texture traits. The high thermal stability of plant proteases may also represent a problem for cooked meat, since enzymatic tenderization could persist during cooking and lead to the same overtenderization problems (Ha et al., 2013). While plant proteases production depends on crop and climatic conditions that can lead to price fluctuations, microbial proteases, or proteases expressed in

microorganisms can be produced throughout the year (Bekhit et al., 2014). Some microbial enzymes are recognized safe for use in food processing, such as proteolytic extracts from *Bacillus subtillis* and *Bacillus amyloliquefaciens*, containing subtilisin (E.C. 3.4.21.62) and neutral protease (E.C. 3.4.24.28) (FDA, 2016). Proteolytic enzymes of *A. oryzae* are allowed to use as tenderizers agents in meat and poultry (USDA, 1999).

Several works have reported the potential of both microbial and plant proteases for use in meat tenderization. Sullivan and Calkins evaluated the effects of bromelain, ficin, papain, fresh ginger extract, and proteases produced by *B. subtillis* and *A. oryzae* over meat samples with high and low connective tissue content. All the enzyme preparations tested were able to reduce shear force of the samples, and degrade myofibrillar proteins, and collagen. Proteases from microbial origin tended to act more over myofibrillar proteins than in connective tissue, while an inverse trend was observed in plant proteases, except for ficin, which showed a more balanced action against the two muscular components. Papain presented the best action at improving meat texture, whereas in sensory evaluation papain treated-samples were the best scored. The potential of *A. oryzae* protease was also highlighted, since the samples treated with this formulation presented similar quality traits with those treated with bromelain (Sullivan and Calkins, 2010).

Ha and coworkers evaluated four commercial plant protease formulations, composed of papain, bromelain, actinidin, and zingibain, in order to determine the substrate specificity of these enzymes. All enzymes were able to degrade both connective tissue and myofibrillar proteins, but with different speficities. Actinidin formulation showed activity against a wide range of myofibrillar proteins, with the detected hydrolysis of actin, myosin, nebulin, titin, filamin, actinin, and desmin. On the other hand, the formulations based on papain, bromelain, and zingibain showed restricted activity against actin, myosin, titin, and nebulin. Differences in collagen degradation were also observed, with zingibain exhibitting the highest collagenolytic activity (Ha et al., 2012). Ketnawa and Rawdkuen (2011) evaluated the tenderization effect of a bromelain extract from pineapple peels over beef, chicken, and squid meat samples. The extract catalyzed the degradation of both myofibrillar (actin and myosin), as well as connective tissue proteins, evidencing its potential for meat tenderization.

## 6 Enzymes for Dairy

The habit of consuming milk dates back to the prehistoric times. About 11,000 years ago, animal domestication and farming become to replace hunting and gathering livelihood of ancient communities in the Middle East, more specifically in the Crescent Fertile region. This event profoundly influenced social organization in human communities, as well as allowed the transition from seminomadism to sedentary lifestyle, and marked the beginning of the Neolithic period. Domestication allowed the use of animals to obtain a variable range of products other than meat, such as milk and wool, and for the generation of tensile strength for

many different activities, including transport and farming practices (Curry, 2013; Leonardi et al., 2012).

The first evidences of the addition of milk to the Neolithic man diet dates back from the 7th to the 5th millennium BC, as evidenced by the analysis of fat profiles in pottery sherds found in European archeological sites. These evidences demonstrated the use of perforated ceramic pots probably intended for separation of curd and whey, indicating that, in the early Neolithic, milk was used in a processed form, as cheese. Processing of milk allowed the generation of stable products, available for longer periods, since natural milk is more easily perishable. Besides that, milk processing resulted in products with reduced lactose content and suitable for consumption by adults (Evershed et al., 2008; Salque et al., 2013).

## 7 Milk Structure and Composition

Milk represents an important source of energy and nutrients for the world population. It could not be different, since milk is prepared to meet newborns nutrition requirements as their sole food at the first months of life. Cow milk represents globally the most produced and consumed milk (83% of total production), and is responsible for supplying considerable proportion of the dietetic requirements of proteins, fats, calcium, phosphorous, vitamins, and amino acids. The main carbohydrate found in milk is lactose, which account for approximately 4% (w/w) of total milk (Wijesinha-Bettoni and Burlingame, 2013).

Milk corresponds to a complex aqueous solution, mainly composed of water, proteins, carbohydrates, and fat. Lactose, a disaccharide of glucose and galactose linked by a β (1→4) glycosidic bond, presents concentration ranging between 4% and 6%, depending on the species evaluated. Lactose is responsible for some milk properties, such as osmotic pressure and, together with fats, represents the main energy source for the newborn. Besides lactose, lower amounts of other oligosaccharides are also present in milk, most of them derived from lactose through the action of transferases. Milk fats comprehend mainly triglycerides and are dispersed in milk serum (whey) and organized as fat globules. Phospholipids and some surface proteins are responsible for the stabilization of these globules. Milk is a valuable source of lipids, because a considerable diversity of saturated and unsaturated fatty acids is in the composition of milk fat. Moreover, milk fat globules are important structures for adsorbing and transporting of hydrophobic nutrients, such as vitamins, through a hydrophilic medium. Milk also contains significant amounts of minerals, among them calcium, potassium, and phosphor, as well as immunoglobulins and vitamins, namely vitamin A (retinol), vitamin C, vitamin E, biotin (vitamin B7), cobalamin (vitamin B12), and riboflavin (vitamin B2) (Fox, 2009; Jenness, 1999).

The protein content of milk is 3%–6%, depending on the species considered (Wijesinha-Bettoni and Burlingame, 2013). Protein structure and interactions directly affect milk

properties and so, knowledge and manipulation of milk proteins properties is responsible for the generation of a great variety of dairy products, such as cheese and yogurt. Caseins are the majority of proteins in milk, representing between 70% and 80% of total milk proteins (Bobe et al., 1998; Bovenhuis et al., 2013; Jensen et al., 2012).

Four different subtypes of caseins are found in milk: $\alpha_{s1}$-, $\alpha_{s2}$-, β-, and κ-casein, which accounts for about 38, 10, 35, and 12% of total casein, respectively. An understanding of the different structural characteristic of these casein subtypes is important to a better comprehension of the milk properties. In the composition of caseins, a considerable proportion of proline residues is present, rendering them a rigid structure characterized by defined secondary and tertiary structures. As a consequence of this rigid organization, caseins exhibit thermal stability, a crucial property as some dairy products may be subjected to heat treatments without severe changes in milk properties. Moreover, structural organization results in the exposition of hydrophobic residues, favoring the association between casein molecules and forming aggregates, an important characteristic for the supramolecular organization of caseins in milk. Casein subtypes, β, $\alpha_{s1}$, and $\alpha_{s2}$, contain phosphorylated serine residues in their structure, which confer to them the ability to bind to positively charged ions, especially calcium ions. As discussed posteriorly, this feature also contributes to structural organization and stability of caseins in milk. In addition to caseins, other proteins are also present in milk. The second most abundant proteins are β-lactoglobulin and α-lactalbumin, which present different characteristics in comparison with caseins. These proteins present defined secondary and tertiary structures, do not organize in aggregates, and are less phosphorylated. β-Lactoglobulin is an important allergenic for newborns (Fox, 2009).

Given the trend of milk caseins to self-associate, these proteins are found organized in supramolecular assemblies, called micelles (Fig. 3.2). Casein micelles influence several properties of milk, and so, knowledge about their structure is important to comprehend the modifications undergone by milk during processing steps in order to produce dairy products.

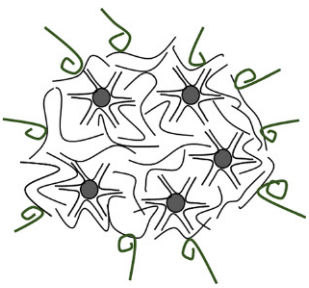

**Figure 3.2: Structure of a Casein Micelle.**
α- and β-Caseins are represented as black traces, forming a network with calcium phosphate nanoclusters (*gray spheres*). κ-Casein is represented as green traces on the surface of the micelle, indicating that CMP extends outside the micelle.

The organization of casein micelles is essentially governed by the affinity of different casein proteoforms to calcium. This characteristic is determined by the level of phosphorylation of the proteins, that is, the most phosphorylated a casein molecule is, the higher its affinity to calcium. In casein micelles, calcium exists as nanometric aggregates of calcium phosphate, named nanoclusters. Since α- and β-caseins present more affinity to calcium (given their high content of phosphate groups), these proteoforms interact to nanoclusters, and stabilize these mineral aggregates. This is an important feature regarding nutritional aspects. Calcium ions do not present good solubility, and the stabilization promoted by caseins allows the presence of considerable amounts of this divalent cation in the composition of milk. Besides interacting with nanoclusters, caseins also interact with other casein molecules, leading to the formation of an organized casein-nanocluster net, that compose the essential structure of casein micelle. However, given that milk is liquid in its natural state, the net formed by proteins cannot be continued. The limitation of micelle size is determined by κ-casein, since this proteoform presents only a few phosphorylated sites and does not show high affinity for calcium. κ-Casein locates at the borders of micelles, delimitating their volume. It is worthy to emphasize that micelle inner part is not solid. On the contrary, its reticulated structure allows the transit of water, and the access of several molecules, such as enzymes (Dalgleish and Corredig, 2012; Shukla et al., 2009). Moreover, κ-casein also plays an important role in avoiding agglomeration of micelles. Its C-terminal end, more specifically 64 terminal amino acids, presents differential properties, such as net charge, posttranslational modifications (glycosylations and some phosporylations), resulting in more hydrophilic behavior. This portion of κ-casein, named casein macropeptide (CMP), is extended outside to the casein, making a superficial layer that avoid micelles fusion (Hernández-Hernández et al., 2011; Kreuß et al., 2009; Martinez et al., 2011).

## 8 Milk Clotting Enzymes

The production of most dairy products, such as cheese and yogurt, relies on the generation of milk gels. These gels appear as a consequence of aggregation of casein proteins, originating two distinct phases: a jellified solid phase (curd), containing caseins, and a liquid phase (whey), containing milk soluble components, including β-lactoglobulin and α-lactalbumin, lactose, and salts. Essentially, casein aggregation happens due to the loss of repulsion between micelles. Since this repulsion is conferred by CMP localized at the surfaces of micelles, the formation of milk gels is related to the alterations in this portion of κ-casein.

Milk gel formation can be induced by enzymatic (rennet) digestion, or through acidification of milk. This acidification can be reached by the use of chemicals or with the inoculation of milk associated with a bacterial culture. In the acidification process, pH decrease leads to a neutralization of net charge in CMP, which, in turn, favors the micelle aggregation. The aggregation reaches its maximum at the isoelectric point of caseins, that is, approximately

at pH interval of 4.6–4.8. During milk acidification, most of the calcium phosphate from nanoclusters of casein micelles is solubilized (Lucey and Singh, 1998).

Chymosin (E.C 3.4.23.4), the most used enzyme in milk clotting, presents its physiological function of coagulating milk in neonates' stomach, retaining it for a longer time and allowing the action of other digestive enzymes, such as pepsin. It is present in lactating mammals, and in young ruminants, it is located in the fourth stomach (abomasum). The mechanism of peptide bond hydrolysis catalyzed by chymosin, and also by pepsin, involves the participation of two aspartic acids as catalytic residues, which classify both enzymes as aspartate proteases. The reaction involves the nucleophilic attack against carbonyl on the peptide bond through a water molecule. Enzymes belonging to these groups show optimal activity at acidic pH range (Yegin and Dekker, 2013).

Bovine chymosin catalyzes the cleavage of peptide bond between phenylalanine (Phe105) and methionine (Met106) residues in the CMP, promoting the release of CMP to the serum (whey), and the aggregation of casein micelles. The action of chymosin is driven by the binding to the amino acid sequence (97–112 residues) of κ-casein. This binding is important for the appropriate orientation of catalytic residues, as well as for the structural remodeling of active site (Palmer et al., 2010; Sørensen et al., 2013).

The traditional mode of production of chymosin involves direct enzyme extraction from abomasa of calves. The resulting extract is called rennet. The commercial rennet production began in Europe in the 19th century. The traditional rennet production at the industrial scale consists of washing the stomachs with saline solution, with subsequent defatting. Then, the material is drought, and milled, when it is ready for the extraction step. In this step, the milled material is extracted with a saline solution with pH around 6.0, and acidified for the activation of the prorenin (zymogen) to renin. The extract is equilibrated with pH value of approximately 5.7, and submitted to filtration procedures. It can be commercialized in either liquid or powder forms (Placek et al., 1960). Rennet comprises two major stomachic proteases: chymosin and pepsin. Chymosin presents high specificity for casein micelles. This characteristic is of paramount importance, since it reflects in a high clotting activity. On the other hand, pepsin does not possess such specificity, and its indiscriminate attack on casein compromise proper milk clotting. Therefore, the proportion between the activities of chymosin and pepsin directly impacts the rennet quality (Jacob et al., 2011). Diet can influence the production and proportion of chymosin. Moschopoulou et al. (2009) reported that abomasa from lambs fed with milk yielded higher amounts of chymosin and in a higher proportion than those fed with grass.

With the advent of biotechnology, heterologous expression techniques allowed the production of proteins of interest in large scale, and with more efficiency. In the early 1990s, FDA approved the use of recombinant chymosin for milk processing. Since then, it is estimated that recombinant chymosins account for 80% of global milk clotting enzymes market

(Johnson and Lucey, 2006). Recombinant chymosin has 100% chymosin activity, and very low nonspecific proteolytic activity. This is useful, since conventional rennet contains about 20% pepsin activity, which accounts for undesirable degradation of casein (Kumar et al., 2010). The characteristics of recombinant chymosins from other species have been evaluated. Vallejo et al. (2012) evaluated the performance of bovine, camel, goat, and buffalo chymosins regarding the effect of temperature, pH, and inhibitors, and kinetic parameters, and found that goat recombinant chymosin showed a better performance, evidencing the potential of this enzyme for cheese making processes. Interestingly, camel chymosin has been indicated as a potential substitute for bovine chymosin. The first enzyme presents 70% higher activity against CMP, and a specific clotting activity 7 times higher than bovine chymosin. The strength of the curd formed by camel chymosin action was similar when compared to the curd formed by the bovine one, evidencing the potential of the former enzyme for cheese production (Kappeler et al., 2006). Curiously, bovine chymosin present low clotting activity on camel milk, probably due to differences in the camel κ-casein amino acid sequence (Sørensen et al., 2011).

Several studies have been carried out in order to investigate the effect of milk characteristics on the properties of gels forms upon the action of chymosin. For instance, Glantz et al. (2010) evaluated several milk samples regarding their properties and the influence on milk gelation. Gel formation was favored when casein micelles tended to be small. In turn, formation of smaller micelles was related to lower pH, and smaller amounts of potassium and magnesium. In addition, the strength of the gel formed correlated with milk pH, and concentration of proteins and lactose, with more strength gels formed at lower pH, lower protein concentration, and higher lactose content. Gustavsson et al. (2014) also detected significant correlation between micelle size and milk gelation properties, and it was found that total calcium concentration, as well as ionic (soluble) calcium content also affected gelation properties. Analysis of noncoagulating milk samples revealed lower content of total calcium, and surprisingly, the content of β-lactalbumin, a whey protein, was lower in the samples with defective coagulation.

Some traditional cheesemakers use rennet from other animal sources, mainly some small ruminants, such as kids and lambs. This is a common characteristic associated to the traditional cheeses produced in the Mediterranean region. Instead of using liquid rennet, the abomasa of young unweaned animals are used to produce a paste. The production of rennet paste has some similarities to the liquid form production. Basically, the abomasum undergoes a defatting step, and subsequently is salted, dried, and finally grounded and blended to a format of paste. The production of rennet paste is totally artisanal. Additionally, the rennet paste presents a different composition in comparison with the commercial liquid rennet. In the first formulation, there is a higher level of lipases and the presence of a typical microbiota, which includes lactobacilli. These two features contribute to the development of unique sensorial traits during the ripening of traditional cheeses (Moschopoulou, 2011).

For cheese production, bovine chymosin (or rennet extract) is considered a standard, especially due to its high specificity toward the hydrolysis of CMP. However, the production of chymosin is expensive, and raises questions about the risks of disease transmission. Furthermore, cheese production and consumption has increased in the last years, and the availability of chymosin may not be enough to attend the increasing worldwide demand (Yegin and Dekker, 2013). In this context, several alternative enzymes have been evaluated regarding their potential as substitutes for chymosin. Candidates must have properties as similar as those of chymosin, namely specificity toward CMP (which reflect in high-clotting activity and curd yield), activity at the same conditions of cheesemaking (temperature and pH), and thermolability, since it is not desirable for the persistence of considerable amounts of residual activity during maturation, because of undesirable sensorial characteristics of the final product (Jacob et al., 2011).

The potential of plant proteases as substitutes for chymosin has been reported. Mazorra-Manzano et al. (2013) evaluated the potential of three plant protease extracts obtained from kiwi fruit (*Actinidia deliciosa* L.), melon (*Cucumis melo* L.), and ginger (*Z. officinale*), regarding their potential for milk clotting. Despite showing a clotting activity 67 times smaller than chymosin, kiwifruit extract-treated samples presented curd yield and texture properties similar to those of chymosin-treated samples. The potential of kiwi fruit extracts was also confirmed by Grozdanovic et al. (2013). With the use of these extracts, it was possible to obtain curd with similar rheological properties as that obtained with the use of chymosin. The authors emphasize the potential source of kiwi fruit wastes for low-cost extraction of proteases. Additionally, the clotting properties of religiosin B, isolated from *Ficus religiosa* latex have been described (Kumari et al., 2012). The enzymes show high specific clotting activity, when compared to other plant proteases, presenting the potential for use in cheesemaking processes.

However, the application of plant proteases is sometimes limited by the high nonspecific activities demonstrated by these enzymes. The unspecific degradation of caseins results in the loss of proteins to the whey fraction, and consequently, reduces curd yield and physical properties of milk gel formed. Additionally, degradation of casein usually leads to the release of peptides that impact sensory traits, such as hydrophobic peptides associated to bitterness (Roseiro et al., 2003; Shah et al., 2014).

Nevertheless, plant extracts are used in the production of some traditional cheeses in the Mediterranean region. A well-known example is the use of cardoon (*Cynara cardunculus*) extract in the production of several traditional cheeses in Spain and Portugal. These cheeses are produced with sheep and goat milk, and the use of a plant protease confers their typical characteristics, such as a soft creamy texture and a specific slightly bitter flavor. The clotting extract is obtained from cardoon flowers, which are air-dried, and then soaked in water. The fresh extract is used for milk clotting. Two main aspartic proteases compose the extract: cynarase and cardosin, which are present in many proteoforms (Roseiro et al., 2003).

Recently, the proteoform cardosin B has been successfully expressed in *Kluyveromyces lactis*. Through genetic engineering, it was possible to obtain the mature form of the enzyme without the need of an activation step. The expressed product showed similar characteristics to the original cardosin, representing an important result for the large-scale production of this protease (Almeida et al., 2014).

## 9 Enzymes in Cheese Ripening

After coagulation treatment the formed curd, which presents soft and weak sensorial characteristics, is maintained at rest, in a step known as ripening. During this step, the strong, typical sensorial traits of the cheese are developed, mainly due to microbial metabolic activity, especially over lipids, lactose, and also the casein network. The release of metabolic compounds, such as free amino acids and free fatty acids, confers the typical aroma of the cheese. The conditions of storage of curds, the curd-associated microbiota, and the chemical structure of curds make the ripening process to be subjected to high variability, which in turn, contribute to the generation of a great variety of cheese types (McSweeney and Sousa, 2000).

Enzymatic treatments have been employed for the reduction of the ripening time, as well as to enhance sensorial characteristics. Kilcawley et al. (2012) evaluated the use of three commercial proteolytic preparations during cheddar cheese ripening. The formulations were applied before clotting. The treated cheese samples presented distinct and enhanced flavor and aroma when compared to the control, reinforcing the importance of enzymatic treatment in the development of sensorial traits of cheddar cheese. The differences in these traits come from the proteolytic activity of the formulations, which made available peptides and amino acids for assimilation by starter bacteria, and consequently leading to an increased diversity of metabolites released.

Encapsulated enzymes have also been evaluated as cheese ripening accelerators. Lipase encapsulated in three different polymers—κ-carragenan, gellan, and sodium alginate—was evaluated in the acceleration of ripening of Kashar, a common cheese found in Turkey, Mediterranean region, and in the Balkan Peninsula. Cheeses treated with immobilized enzymes presented considerably higher amounts of free fatty acids in relation to the control, evidencing the positive effect of the addition of enzymes for the enhancement of sensorial traits. However, when cheese samples were subjected to sensorial evaluation, the positive effects conferred by the immobilized lipases were time-dependent, with sensorial traits becoming undesirable at the later stages of ripening. This was associated with excessive lipolysis, leading to the increase in rancidity. These results are positive in the sense that positive sensorial traits appeared early due to the presence of enzymes. However, it also shows that the release of enzymes must be more controlled, in order to avoid excessive degradation of cheese components, which lead to negative sensorial characteristics (Akin et al., 2012). In an attempt to control overripening in cheese samples made with raw

ewe milk, Calzada et al. (2013) evaluated the use of high pressure for some weeks after cheese preparation, and monitored the characteristics throughout the ripening period, and during the refrigerated storage of the cheese. Results showed a retarded effect over protein degradation, and conservation of the sensory attributes produced during ripening when pressurized samples were compared to the controls. These results reveal that the use of high pressure processing of cheese as a technological tool to control the action of enzymes at the postripening period.

## 10 Lactose-Free Milk

In ancient periods, milk was consumed mainly in processed forms, such as cheese, butter, and yogurt. In this way, milk nutrients and energy would be available for a larger period without spoiling, and also the lower contents of lactose allowed the consumption of dairy products by adults.

Lactose is hydrolyzed by lactase (β-D-galactosidase, E.C. 3.2.1.23). In humans, lactase is highly expressed in newborns, given that lactose is an important energy source during early life stage. After weaning period, lactase expression levels gradually decrease, being low, or even absent, in adults. In absence of lactase, lactose cannot be absorbed in gut trait, and its accumulation causes intestinal disturbs, with undesirable symptoms, such as flatulence and diarrhea. However, about 35% of world population keeps lactase expression in sufficient levels even in adulthood, allowing milk consumption without collateral effects. This characteristic increased as a consequence of a single nucleotide mutation in lactase-encoding genes, and was favored in regions where milking practices were established. It is estimated that the mutation appeared in Europe 6000–8000 years ago, and also in Africa and in the Arabian Peninsula, at different times and as a result of independent events (Ingram et al., 2009; Itan et al., 2010; Leonardi et al., 2012). The capacity of lactose digesting in adulthood, allowed the incorporation of milk in human diet, conferring a great advantage given the nutritional value of milk, regarding not only energy sources, but also vitamins and minerals. Milk consumption remarkably contributed to economic and social development of societies (Cook, 2014).

However, 65% of world population tends to express, in several degrees, some lactose intolerance. Given the importance of milk to human diet, it is of paramount importance that lactose-intolerant individuals could consume milk and dairy products. Lactose can be degraded in milk essentially by two ways: acid or enzymatic hydrolysis. When the acid hydrolysis is executed, it renders milk with undesirable sensorial characteristics (e.g., a brown color), requires further neutralization, and can damage industrial installations. In this sense, enzymatic hydrolysis has several advantages, given the specific action of the enzymes, without promoting considerable changes in sensorial attributes of the products. Lactase can be obtained from several sources, but lactases destined for industrial uses are from microbial

origin. The first use proposed of lactase in milk and dairy was made in the 1950s. The technological development of lactase-free milk and dairy goods started from the 1970s, with the release of the first commercial lactases (Harju et al., 2012).

Mahoney and Adamchuk (1980) evaluated the influence of milk components on the activity of a recombinant lactase produced by *Kluyveromyces fragilis*. Enzymatic activity was affected mainly by ions, with potassium, magnesium, and manganese working as activators, and sodium and calcium as inhibitors. Milk and whey proteins also activated lactase activity, but this does not seem a specific interaction between these proteins and lactase, since a similar effect was also observed from the incubation of the enzyme with other proteins, such as ovalbumin.

## 11 Enzyme in the Production of Galactooligosaccharides

Apart its use at the production of lactose-free milk and dairy products, lactase is also important in the synthesis of nondigestible oligosaccharides derived from lactose. The amount of nondigestible oligosaccharides production is estimated in 50,000–80,000 tons per year, with lactose-derived oligosaccharides accounting for 40% of this production. These compounds are largely used as sweeteners, in substitutions of sucrose, given their low cariogenicity and low caloric content. Additionally, this nondigestible oligosaccharides show prebiotic effect, that is, they stimulate the growth of beneficial gut bacteria, such as bifidobacteria and lactobacilli (Gänzle, 2012).

The performance of three commercial β-galactosidases from *K. fragilis, Bacillus Circulans*, and *A. oryzae* on the production of galactooligosaccharides from bovine skim milk was evaluated by Rodriguez-Colinas et al. (2014). Enzymes were evaluated at 4°C and 40°C. Enzymes presented different performances for the production of galactooligosaccharides and for the degradation of lactose. At 40°C, lactase from *B. circulans* yielded the highest concentration of galactooligomers, representing 16% of total carbohydrates. Lactase produced by *K. fragilis* produced oligomers that represented 15% of the total carbohydrates, but an interesting feature of this enzyme is that maximal production of oligomers was reached with degradation of 95% of total lactose. The maximal production of oligomers by *B. circulans* lactase was achieved with consumption of 50% of the total lactose, with the further decrease in lactose content not reflecting in an increase of oligomers concentration due to their degradation. Lactase from *A. oryzae* produced the lowest amount of lactooligomers, with maximal achieved at degradation of 43% of the total lactose. Enzyme origin also influenced the linkages present in the oligosaccharides evaluated. *B. circulans* lactase tended to form oligomers with β (1→4) bonds, while *K. fragilis* lactase formed oligomers with predominant β (1→6) bonds. This is another positive feature of this latter enzyme, since β (1→6) are more resistant to hydrolysis. At 4°C, lactase produced from *B. circulans* showed similar performance, while the enzyme produced from *K. fragilis* produced lower amounts

of oligomers, but at high lactose degradation levels. Thus, through the enzymatic treatment of milk, it is possible to obtain a product with low lactose content and, at the same time, with valuable concentrations of prebiotic galactooligomers.

The major drawbacks for enzymatic production of galactooligosaccharides are related to product inhibition, as well as thermal stability of the enzymes. In the course of transferase activity of lactase, inhibitory products (glucose or galactose) increasingly accumulate in the reaction media, affecting the rate of oligomers synthesis. In this sense, enzymes with more resistance to product inhibition are preferred. Another strategy for reducing inhibition is the immobilization of enzymes, which is effective when enzymes are subject to noncompetitive inhibition. Additionally, enzyme immobilization can lower production costs, since the enzyme can be recycled, and also allows a more controllable process when immobilized enzymes are held in reactors. The production of oligomers is favored at high lactose concentrations. However, since lactose present poor solubility at environmental temperatures, concentrated solutions can be achieved only at high temperatures, and thus, the use of thermostable enzymes could permit the synthesis of oligomers at such conditions. In this sense, an intense search for thermostable enzymes has been carried out, focusing specially in thermophilic microorganisms (Park and Oh, 2010).

Recently, some β-galactosidases with suitable properties for application in the dairy industry have been isolated from metagenomic libraries. Gupta and coworkers reported the isolation of a thermostable and alkalophilic β-galactosidase from geothermal springs. The enzyme presented optimal activity at 65°C, low lactase activity, but high efficiency of transglycosylation, with a wide diversity of possible acceptors, evidencing the potential of the enzyme for the efficient synthesis of a great variety of galactooligosaccharides (Gupta et al., 2012). A thermostable β-galactosidase was also reported by Zhang et al. (2013), and this enzyme presented higher tolerance to glucose and galactose inhibition than the commercial equivalents.

## 12 Enzymes Applications in Baking

### 12.1 Wheat Kernel Structure and Composition

Wheat grain structure consists, essentially, of three fractions (Fig. 3.3). Considering the nutritional value of wheat grain, the most important fraction is the endosperm, which is specialized in the storage of nutrients necessary for germination and early development of seed. Further, the endosperm is composed of two distinct tissues: aleurone, a layer of cells existent in the periphery of the endosperm, and the starchy endosperm that, as suggested by its name, is the tissue involved in the storage of the nutrients. The embryo (germ) is associated with the endosperm, and is located at the basal portion of the seed. Surrounding and involving the endosperm and the embryo, there are several layers of protective tissues. An epidermal layer (nucellar epidermis) involves the entire endosperm and embryo. Above

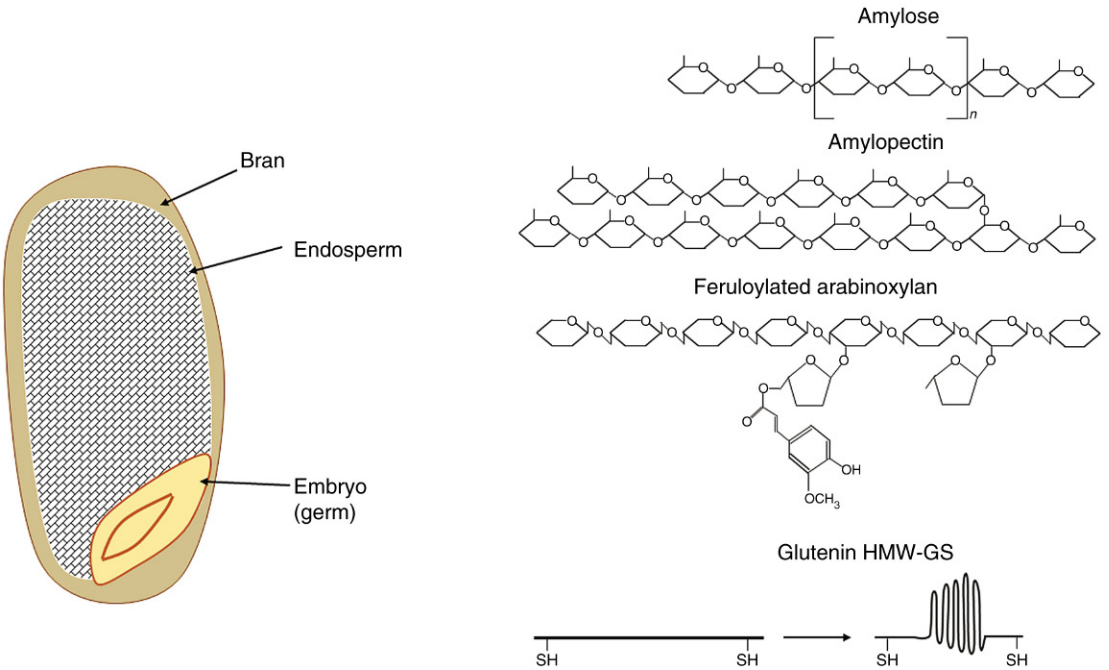


**Figure 3.3: Schematic Representations of the Components of Wheat Kernel and Wheat Flour Affecting Dough Quality.**

Starch (amylose and amylopectin), feruloylated arabinoxylans, and high-molecular weight glutenin subunit (HMW-GS). HMG-GS is showed in its native state with its secondary structure in a hydrated state.

the nucleus, it is found a seed resistant coating named testa. Ultimately, the pericarp involves the entire seed. It is a tissue derived from ovary, and organized in three sublayers: endocarp, mesocarp, and exocarp. During milling, most of the aleurone and the external layers are removed from the endosperm. This residue is named wheat bran. Posteriorly, the germ is also removed, during the refining of flour (Evers and Millar, 2002; Xiong et al., 2013).

Starch is the major compound in wheat endosperm, accounting for 80% of the dry weight. It is a branched homopolymer constituted of backbones of glucose units linked by $\alpha(1\rightarrow4)$ bonds, and branches, also with $\alpha(1\rightarrow4)$-linked glucose linear chains associated to backbones through $\alpha(1\rightarrow6)$ bonds. The frequency of branching differentiates two starch structures: the poorly-branched amylose, and the highly branched amylopectin (Wang et al., 2014). Starch is organized in granules, forming a semicrystalline structure. Parallel glucose chains in amylopectin associate with each other, forming double helices, which constitute the crystalline and compact structure in starch. The points corresponding to branches characterize the amorphous region of starch. Amylose, in turn, presents a single helix structure, and it is associated with the amorphous fraction of starch. The macromolecular structure of amylopectin and amylose confers a typical organization for starch granules, consisting of

an internal structure composed of concentric rings (Zeeman et al., 2010). Wheat endosperm starch presents 75% of amylopectin and 25% of amylose (Shewry et al., 2013).

Proteins, the second most stored polymer in endosperm, correspond to globulins and gluten proteins. Storage proteins are organized in globules from different origins: some proteins are transported via Golgi complex to the vacuole, while others remain retained at the endoplasmic reticulum. The distribution of total storage proteins follows a gradient, with a higher concentration at the edge of endosperm (subaleurone layers) and decreasing toward the center region. In mature endosperm, protein bodies merge, and constitute a continuous matrix that involves starch granules (Shewry and Halford, 2002; Tosi et al., 2011).

Gluten proteins represent the majority of the storage proteins in wheat endosperm. Gluten proteins are also known as prolamins due to the high content of proline and glutamine residues in their composition. Based on their solubility in alcoholic solutions, gluten proteins are divided in two groups: gliadins and glutenins. Gliadins are monomeric proteins, classified into three groups considering their amino acid composition: α/β-, γ-, and ω-gliadins. α/β- and γ-gliadins present molecular weight at the range of 28–35 kDa. The N-terminal domain of these proteins is characterized by the presence of repetitive motifs of glutamine, proline, phenylalanine, and tyrosine, with different length of the repetitive sequences in α/β- and γ-gliadins. The C-terminal domains of both types of gliadins are homologous and without repetitive sequences. Additionally, both types of gliadins contain cysteine residues, related with the formation of intramolecular disulfide bonds. ω-gliadins, in turn, are characterized by higher contents of glutamine, proline, and phenylalanine, and usually lack of cysteine residues. In wheat gluten, α/β- and γ-gliadins are more common than ω-gliadins (Shewry and Halford, 2002; Wieser, 2007).

Unlike gliadins, glutenins consist of polymeric proteins, with some assemblies reaching high molecular weight aggregates. It is of great importance to stress out the role of disulfide bonds between glutenin subunits in the formation of its macromolecular structure. Glutenin presents two types of subunits, classified in function of their molecular masses: the low molecular weight glutenin subunits (LMW-GS) and the high molecular weight corresponding subunit (HMW-GS). LMW-GS presents similarities with α/β- and γ-gliadins regarding molecular mass and amino acid composition. As in gliadins, the N-terminal domain of LMW-GS contains motifs of repeated sequences of amino acids, rich in proline and glutamine. These subunits also contain cysteine groups involved in intermolecular crosslinks. HMW-GS and LMW-GS display molecular weight ranges of 60–80 and 32–35 kDa, respectively. The structure of these glutenin subunits is organized in three different domains: at the N- and C-end of the molecule, the structure is determined by nonrepetitive amino acids sequences. Most of the cysteine residues are found in these two terminal domains. The domain defined by the inner region between C and N termini is characterized by the presence of repetitive peptides composed by proline, glutamine, glycine, and tyrosine. In all the gluten proteins, the presence of repetitive amino acids motifs is related to secondary structures

of great importance for their properties, especially β-turns (Shewry and Halford, 2002; Wieser, 2007).

Although not so abundant as starch and proteins, nonstarch polysaccharides are also present in wheat endosperm, corresponding to 2%–3% of total weight (Shewry et al., 2013). These components derive mainly from endosperm cell walls, and are nutritionally valuable, since they constitute the dietary fibers. The inclusion of fiber in the diet has several health benefits. Wheat bran fibers are associated to high antioxidant activity and immunomodulatory effects (Hromádková et al., 2013), and also, stimulate the proliferation of beneficial gut microbial populations, such as bifidobacteria, and contribute to the reduction of cholesterol levels (François et al., 2014; Maki et al., 2012; Zhang et al., 2011).

Arabinoxylan, a major component of the hemicellulosic fraction of the cell wall, is the most common nonstarch polysaccharide found in the endosperm. It accounts for about 70% of the total nonstarch polysaccharides, which also include β-glucan (20%) and cellulose (2%). In wheat flower, arabinoxylan content is about 1.9% of the mass (Shewry et al., 2013). Arabinoxylan is a heteropolymer consisting of backbone of D-xylose units linked by β (1→4) glycosidic bonds. Arabinosyl residues can be found laterally attached to the xylose residues, at the O-2 and/or O-3. Additionally, some moieties of ferulic acid can be esterified at the O-5 of some of the arabinosyl residues (Scheller and Ulvskov, 2010).

## 12.2 Wheat Processing—Dough Formation

Wheat flour is consumed in several processed forms. In some of them, viscoelastic properties of flour are determinant for the final product. This is clear in breadmaking, since the properties of the dough define the final volume and bread texture. Dough is defined as a semisolid mass with viscoelastical behavior and resistance to mixing, obtained after the mixture of flour and water (and other ingredients usually used in baking, such as oil and salt). The rheological properties of dough are attributed essentially to gluten proteins, although the interaction between the different components of flour exerts influence on these properties. The process of kneading is of paramount importance in dough preparation, since the mechanical energy applied to the mixture is responsible for the release of glutenins and gliadins from protein bodies, with the consequent hydration and organization of gluten network (Cauvain, 2015).

Gluten network development is macroscopically evidenced by an increase in dough consistency, a fundamental step to the development of optimal rheological properties that allows the production of dough with potential to generate high quality grade bread, that is, bread with good volume, and well textured (fine and soft) crumb. Bread quality is directly influenced by dough rheological characteristics, namely elasticity and viscosity. In this sense, when dough reaches its best viscoelastic properties, it is characterized as a mass with textural

stability, and also organized in such a way that allows gas retention during fermentation (Cauvain, 2015).

Gluten proteins determine viscoelastic properties of wheat, which are directly related to the secondary structure exhibited by these proteins during dough development. Tatham et al. (1985) determined the secondary structures of gliadin and glutenin subunits. It was reported that ω-gliadin presents a conformation containing β-turns regularly distributed throughout its structure. In addition, α/β- and γ-gliadins and LMW-GS present similar structures, containing both α-helices and β-turns. The structure of HMW-GS was characterized by α-helices at the C- and N-termini, assuming a globular-shaped structure, and by several β-turns in the central sequence between these regions. It is important to note that the β-turns are formed by the repetitive motifs previously described (Tatham et al., 1985).

Glutenin subunits constitute the backbone of gluten network in dough. These subunits are associated to each other through disulfide crosslinks, considering that the cysteine residues are located at the C- and N- terminal domains. During dough formation, the backbone of gluten network is organized in the direction of mixing, and the establishment of disulfide crosslinks between glutenin subunits is essential to ensure the integrity and cohesiveness of the network. In fact, during the step of mixing, disulfide bonds are constantly broken and rebuilt. Besides the disulfide bonds, hydrogen bonds and hydrophobic interactions play important role in the interaction between chains, and also between the components of the gluten network. Gliadin proteins associate to glutenin chains through these intermolecular interactions. Given this pattern of interaction between the components of the gluten network, it can be assumed that the glutenin portion contributes mainly to the elastic properties of gluten, while the gliadin subunit influences on gluten viscosity (Shewry et al., 2001, 2002).

The repetitive amino acid motifs located in the central sequence of HMW-GS are organized into β-turns, as previously mentioned. Further, these several β-turns in sequence form a helical structure, the β-spiral, that works as a type of molecular spring being the structure responsible for gluten elastic properties (Tatham et al., 1985). Water plays a crucial role in the gluten network development, especially in the secondary structure of HMG-GS. Prior to the addition of water to flour, HMW-GS are associated through hydrogen bonds in a very compact structure. As water is added, the hydrogen interactions between amino acid residues are increasingly substituted by amino acid—water hydrogen bonds. This hydration of gluten leads to the formation of two distinct regions: some portions of the molecule that are hydrated, named loops, and the remaining portions of the molecule where the hydrogen bonds between amino acids residues are still present, named trains. Typical secondary structures arise from HMG-GS hydration: the organization of β-sheet in the trains, where amino acids interact through hydrogen bonds; and the organization of β-turns (and, as a consequence, of β-spirals) in the loop, where the establishment of hydrogen bonds between amino acids

(especially the glutamine residues present in the repetitive motifs) and water is essential to the appearing of these structures. The further hydration favors a higher proportion of β-turns (Belton, 1999).

The β-spiral domain at HMG-GS subunits under appropriated hydration is mainly responsible for the elastic properties of gluten. During the stretching of the gluten network, the first deformed portions are the loops, and if the stretching increases, the β-sheet trains undergo a slippage movement. Once the stretching movement has ceased, the stretched structures tend to restore their original and more stable conformation (Belton, 1999; Shewry et al., 2001). Gluten extensibility is dependent of the ratio between trains and loops, as a higher energy required to deform the first is expected. In addition, the extension can lead to the interconversion of the two structural forms. During extension of gluten, Wellner et al. (2005) observed the formation of β-sheet structures and a decrease in the proportion of β-turn structures. With the reversal, the original proportions between these two structures were restored.

## 12.3 Enzymatic Improvement of Dough

Several enzymes can be used to improve dough characteristics by promoting modifications on flour components. The main enzymes applied to dough improvement are summarized in Table 3.2.

**Table 3.2: The main enzymes involved in the process of dough improvement.**

| Enzyme | Class | Substrate | Effects on Dough |
|---|---|---|---|
| α-Amylase | Hydrolase | Starch | • Release of sugars, which favors higher bread volume.<br>• Release of glucose contributes to enhance the development of crust brown color.<br>• Release of dextrins with high-polymerization degree, which present antistaling effect.<br>• Degradation of damaged starch globules, which favors correct water distribution. |
| Pentosanase (xylanase) | Hydrolase | Pentosans (arabinoxylans) | • Degradation of pentosans, which promotes correct water redistribution, with consequent correct development of gluten network. |
| Transglutaminase | Transferase | Wheat proteins | • Formation of crosslinks between glutamine and lysine residues, contributing to enhance dough strength. |
| Glucose/Pyranose oxidase | Oxidase | Monosaccharides | • Oxidation of SH groups by hydrogen peroxide, favoring gluten proteins linking, and so, contributing to the formation of a stronger dough.<br>• The oxidative activity of hydrogen peroxide also favors the crosslink between arabinoxylans (via ferulic acid moieties) and gluten proteins, also exerting strengthening effect on dough. |

## 12.4 α-Amylases

Amylases have been largely used in dough improvement. The amylolytic activity during dough formation contributes to increase the concentration of fermentable oligomers, leading to final bread with higher volume (Bae et al., 2014). In addition, the products released by amylases, especially monomeric glucose, can enhance the development of a typical brown color in bread crust, as result of caramelization reactions that occur during baking (Ahmad et al., 2012).

During the process of milling, part of starch granules underwent mechanical damage. When this damage is very extensive, the resulting dough presents different viscoelastic properties, which compromises baking performance. These modifications are caused by the higher water absorption of damaged starch, which affects gluten formation. Also, dough made from highly damaged starch flour presents a decrease in its consistency, leading to a reduced volume after fermentation (Barrera et al., 2007). Barrera et al. (2016) evaluated the effect of adding amylase and glucoamylase on flours with high levels of damage starch, and concluded that these two enzymes, especially when used together, could reverse most of the changes observed in dough mechanical and viscoelastic properties.

Once the process of breadmaking is finished, it is of great interest to the industry, the maintenance of product characteristics during shelf life as close as possible to the characteristics of the fresh product. The quality traits of the product during its shelf life influences considerably consumers' perception and the deep modification of these attributes may lead to significant waste.

Texture changes are common during bread shelf life, with staling as an intensely researched process. During bread baking, starch granules undergo structural modifications because of heating. In this case, granules become deformed and molecular interactions between starch molecules change in function of the high temperature and water interaction. These changes characterize the process of starch gelatinization. As the baked bread cools, starch molecules start to reassociate, but in a different way than in the original globule, with a different crystallinity. This process of starch molecules agglomeration after heating is called retrogradation. The different polymers that make up the starch behave differently in retrogradation process. Upon heating, amylose rapidly leaves the starch globule and accumulates in the outer space, and with cooling, undergoes rapid retrogradation. Amylopectin, in turn, recrystallizes more slowly. During starch retrogradation, there is mobility in the water available in the media, and the newly formed starch structures confine water inside their structure. Since water is a fundamental plasticizer of bread, the flow of water to the retrograded starch structures affects texture properties of bread. The mainly affected texture is crumb texture, which becomes firmer. This process is known as bread staling. Since retrogradation of amylopectin is slower, the changes in this portion of starch considerably contribute to crumb texture modifications during bread shelf life (Fadda et al., 2014; Gray and Bemiller, 2003; Wang et al., 2015).

The potential of enzymes, especially α-amylases, as antistaling agents has been studied. The antistaling effect of α-amylases is probably due to dextrins with high polymerization degree liberated by the action of these enzymes that can interfere with starch retrogradation (Gray and Bemiller, 2003). Giannone et al. (2016) evaluated the effects of amylase commercial formulations on moisture and textural characteristics during the shelf life (90 days) of a durum wheat bread. In all the enzyme-treated samples, moisture loss was slower than the control. Amylases also had a positive effect on maintaining crumb softness. Interestingly, in this work a formulation composed of amylase and lipase was used, and the combined use of the enzymes had a better effect on the texture improvement and conservation throughout the shelf life than the other formulations evaluated, which reinforce the importance of using combined enzymes in order to explore their cooperative effect. The positive effect of lipases was attributed to the enhanced inhibition of retrogradation exerted by monoacylglycerols in the presence of dextrins. The potential of xylanase for antistaling use was reported by Ahmad et al. (2012). Hydrolysis products resulting from the action of xylanase over arabinoxylan could increase moisture retention, favoring crumb softness.

### 12.5 Pentosanases (Xylanases)

As mentioned previously, the suitable development of the gluten network is a crucial event for ensuring the quality of bread. However, some flour components can impair this process, leading to a final defective gluten network. The main agents responsible to this interference are nonstarch polysaccharides, in which major one is pentosan (arabinoxylan). Doring et al. (2015) verified a negative impact over gluten network development exerted by excessive amounts of arabinoxylans. High concentrations of these carbohydrates significantly compromised the formation of the gluten network.

The effects of pentosans on wheat characteristics have been intensely studied, and some mechanisms of the interaction between pentosans and gluten proteins have been proposed. One mechanism relies on the fact that pentosans can interact with water and, consequently, reduce the level of available water. As mentioned previously, water availability is a crucial parameter for the correct assemble of glutenin subunits and, consequently, to the development of a gluten network with its rheological characteristics favorable for yielding good quality bread. Another possible mechanism has to do with the interactions of gluten proteins with ferulic acid moieties presented in arabinoxylan. These interactions, which occur via weak Van der Waals bonds, and interfere at the process of gluten network formation (Wang et al., 2003, 2004a,b).

Given the effect exerted by pentosans in bread quality, the use of pentosanases (xylanases) has been intensely studied in order to evaluate the potential of these enzymes for dough improvement. A xylanase produced by *Aspergillus niger* was evaluated for dough and bread improvement in two different steps of processing (Ahmad et al., 2012). First one, the enzyme

was added to wheat flour at mixing stage, or to the kernels during tempering (tempering is a step in which kernels are wetted in order to facilitate the removal of bran during milling). The enzyme was able to positively impact all characteristics tested for dough (dryness, stiffness, elasticity, extensibility, and coherency) and bread (volume and density, moisture retention, and sensory evaluation). An interesting result was that the application of lower xylanase loads during tempering yielded more satisfactory results than the use of higher enzyme loads during mixing. The authors pointed out that the utilization of dough improving enzymes during tempering may be a viable approach, since the enzymes would have more time to act over wheat components.

## 12.6 Enzymatic Dough Strengthening: Transglutaminase and Glucose/Pyranose Oxidase

As previously discussed, the correct development of gluten network is crucial for the good quality of final bread. However, in some situations, gluten-free products must be available in order to attend some specific consumers. This is the case, for instance, of people with coeliac disease, which consists of an immune reaction to gluten. Therefore, several strategies have been applied in order to develop bread and related products without gluten, but resembling the characteristics of conventional gluten products as much as possible. One of these strategies includes the enrichment of starch with nonwheat proteins, such as those from milk, egg, and legumes. Since these proteins do not have the same viscoelastic properties as gluten, their crosslinking would allow the development of a network that could favor the formation of stable dough structure (Matos and Rosell, 2014). In this sense, the use of enzymes to enhance crosslinking between proteins has been studied not only to the development of gluten-free products, but also to assist in the improvement of wheat dough.

Transglutaminase (E.C 2.3.2.13) is a transferase that catalyzes the formation of bonds between the group carboxamide of glutamine and amine groups of an acceptor molecule. In protein systems, transglutaminase promotes the formation of bonds between glutamine (donor) and lysine (acceptor) residues, constituting crosslinks between protein chains (Kieliszek and Misiewicz, 2014).

The use of transglutaminase for dough improvement has been reported. Moore et al. (2006) evaluated the effects of the addition of transglutaminase to gluten-free bread made with three different sources of protein: skim milk, egg, and soy. It was concluded that transglutaminase addition improved bread volume and crumb texture when bread was made with skim milk and egg. Transglutaminase was also used for wheat dough improvement. Seravalli et al. (2011) evaluated the effect of transglutaminase addition during a process of breadmaking, and found an increase in the polymerization degree of glutenin, more specifically of HMW-GS. This result evidenced the positive effects of the enzyme to promote protein crosslinking and favoring the development of stronger gluten network that ultimately result in bread with improved volume and crumb texture.

Oxidative enzymes have also demonstrated positive effects when applied to dough, especially by improving the strength of gluten network. The most representative and studied oxidative enzyme is glucose-oxidase (β-D-glucose:oxygen 1-oxidoreductase—EC 1.1.3.4). This enzyme is a flavoprotein that catalyzes the oxidation of D-glucose to D-glucono-δ-lactone, which is spontaneously converted into gluconic acid. The electrons taken during glucose oxidation are transferred to the FAD domain, and are further used in the reduction of $O_2$, with the production of hydrogen peroxide ($H_2O_2$) as second product (Bankar et al., 2009).

Glucose oxidase is the most known and used oxidative enzyme for dough improvement. The $H_2O_2$ produced by the enzyme is involved in the oxidation of wheat flour components, leading to the intensification of crosslinks, and ultimately, generating a stronger dough. The effects of oxidation on sulfhydryl (SH) groups of gluten proteins have been reported, resulting in enhanced crosslinks and ultimately in a stronger gluten network. The activity of glucose oxidase in dough has been associated to an increase in aggregation of gliadin particles, and in case of glutenins, HMW subunit seems to be more sensitive to the oxidative-induced changes (Bonet et al., 2006).

Recently, pyranose oxidase (EC 1.1.3.10) has drawn the attention for its use in dough improvement. This enzyme shows similar mechanism of action compared to glucose oxidase, except for the fact that the oxidized carbon by pyranose oxidase corresponds to C2 carbon, while glucose oxidase attacks carbon C1. Pyranose oxidase is produced by several lignocellulolytic fungi, including white rot fungi, and it is involved in lignin degradation, since the hydrogen peroxide produced by the action of this enzyme can be used by lignin peroxidases (Giffhorn, 2000). Pyranose oxidase is able to act over a wider range of substrates, including D-galactose, L-arabinose, and D-xylose. A pyranose oxidase of *Trametes versicolor* presented higher affinity to glucose and $O_2$ than glucose oxidase of *A. niger* (Km value for glucose oxidase was approximately 73-fold higher and enzymatic efficiency for pyranose oxidase was 12-times higher). This could favor the first enzyme, since in dough the levels of free glucose, as well as oxygen availability would be limited (Decamps et al., 2012b).

Application of glucose oxidase and pyranose oxidase significantly improved dough strength and resistance to mixing, with the latter enzyme showing better performance. The use of these enzymes also reduced the time needed for dough development, with their action promoting the formation of consistent dough, less prone to collapse, in a shorter time. So, the enzymes presented potential for industrial use (Decamps et al., 2012a). The effects of glucose and pyranose oxidase on dough strength and viscosity has been associated to the oxidation of SH groups caused by hydrogen peroxide released as product of the oxidation of carbohydrates by these two enzymes. Thus, the binding of gluten proteins is increased and the incorporation of other components to the gluten network, such as arabinoxylan, may be responsible for property changes observed in dough. This last hypothesis is sustained by the fact that arabinoxylan may be linked to gluten proteins, as evidenced by the reduction of

extractable content of this polysaccharide, as well as of ferulic acid when wheat extracts were treated with glucose and pyranose oxidase. This last fact is related to the oxidation of ferulic acid moieties, which leads to arabinoxylan association to gluten fraction, and ultimately, an increase in dough viscosity (Decamps et al., 2013). However, both works emphasize that the benefits of using oxidases in dough improvement is dose-dependent, that is, an excessive load of these enzymes could lead to over oxidation of dough components, causing an excessive increase in viscoelastic strength, with consequent undesirable sensorial traits in bread, such as low specific volume.

### 12.7 Synergism

The synergic effect of different enzymes on dough modification has also been evaluated. Investigation of cooperative action of different enzymes could lead to the development of cheaper formulations, since the synergistic effect allow the use of lower amounts of enzymes and, some time, allows better results than those obtained with the use of chemical compounds (Bueno et al., 2016). Steffolani et al. (2012) evaluated the potential of glucose-oxidase, xylanase, and α-amylase in the improvement of dough and bread properties. The action of glucose-oxidase promoted an increase in dough hardness, probably by increasing the crosslink between gluten proteins, while xylanase levels were positively correlated with dough stickiness. The synergic action of α-amylase and xylanases was observed in the specific volume of bread and crumb texture. The positive effects that result from the interaction between these enzymes were associated to the release of sugar by α-amylase and by the stabilization of gas cells by the products released when xylanase act on pentosans. Thus, a higher volume of gas was produced during fermentation, and this gas could be retained in the cells, leading to higher specific volume, creating a more porous and softer crumb.

## 13 Conclusions

Enzymes have been used for the improvement of food from ancient times. With the generation of knowledge about the properties of enzymes, and with the advent of biotechnology, these biological catalysts have gained considerable importance in food processing. The specific action of enzymes makes them useful additives for the improvement of food. In this chapter, we have shown the use of enzymatic treatment to improve the meat, milk, and wheat products. Proteases and carbohydrases are widely used in various processes, including meat tenderization, milk clotting for cheesemaking, enzymatic digestion of lactose, from milk galactooligosaccharides production, and improvement of dough properties. The prospects of enzymes market for the coming years reinforce the increased use of enzymes in the food industry. Additionally, the exploration of new sources of biodiversity, and advances in the field of protein engineering will allow the discovery and development of new catalysts, suitable for use in various industrial processes.

## Acknowledgments

The author acknowledges the receipt of financial support from the Brazilian National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES), and Foundation for Research Support of the Federal District (FAPDF).

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CHAPTER 4

# Lactic Acid Bacteria—From Nature Through Food to Health

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## 1 Introduction

For ancient times, lactic acid bacteria (LAB) have been used for fermentation of different foods and underlie one of the oldest methods employed for food preservation known to man. The use of LAB dates back to 6000 BC, with descriptions of fermentation in dairy foods and meat dating back to 1500 BC, and in plants to 300 BC. The typical LAB are Gram-positive, nonspore-forming, nonmotile, catalase-negative, devoid of cytochromes, anaerobic to aerotolerant cocci or rods, which are acid-resistant and produce lactic acid (LA) as the main end product of carbohydrate fermentation.

LAB are widespread microorganisms, which can be found in any ecosystem and product, such as plants, fermented foods, and the mucosal surfaces of the human body.

The plants are preferred source for isolation of LAB due to their specific flavor-forming and metabolic activities. The demand for new solutions to improve starter communities provoked the idea to exploit the biodiversity in unique natural systems. Each specified plant species provides a unique environment in terms of competitive microorganisms, natural vegetal antagonists, as well as accessibility, type, and concentration of the substrate in various physical factors. These conditions allow the growth of a typical epiphytic microflora by means of which a population emerges and a chain of fermentation processes commences after the plant material is prepared for fermentation. Plant-derived strains of lactobacteria have demonstrated tolerance to high pH values and salt concentrations, ability to ferment various types of carbohydrates, and a high level of stress resistance compared to those of dairy origin. Furthermore, no significant differences were noted in the fermentation characteristics and profiles of enzymes, such as lipases, peptidases, and phosphatases, required for obtaining various fermented dairy products with plant-derived and commercial strains of lactobacteria (Michaylova et al., 2007; Nomura et al., 2006; Venugopalan et al., 2010).

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00004-9

LAB have a generally recognized as safe (GRAS) status. They are important to the dairy industry and are essential for the production of a range of traditional and novel dairy products. It is well known that LAB (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Streptococcus*) are carriers of probiotic activity, which influences beneficially gastrointestinal tract diseases (Benyacoub et al., 2005; Cremonini et al., 2002; Marteau et al., 2002; Steidler et al., 2000). In addition to probiotic activity, a number of recent investigations have indicated their ability to produce different biologically active metabolites [lactic acid, conjugated linoleic acid, γ-aminobutyric acid, bacteriocin, reuterin and reutericycline, exopolysaccharides (EPSs), and bioactive peptides]. Food products supplemented with LAB strains possessing proven probiotic properties (different biological activities) are functional foods with undisputed benefits for the human health.

## 2 Microbial Diversity of LAB

Representatives of LAB can be isolated from almost all types of food including milk and dairy products, fresh and processed meats, fish, cereals, and vegetables. Some species of LAB are natural inhabitants of the gastrointestinal and urogenital tract, while others are specially adapted to various extreme conditions, such as alcoholic beverages or foods high in salt. Lately, the interest to LAB isolated from various nondairy sources has increased, due to their specific flavor-forming and metabolic activities (Fig. 4.1).

### 2.1 Traditional Sources

Traditional sources for the isolation of LAB are milk, dairy products, and fermented foods.

#### 2.1.1 Milk and dairy products

Milk and dairy products provide numerous nutritional and health benefits because of their composition and due to the microflora which they contain. Therefore, these products are

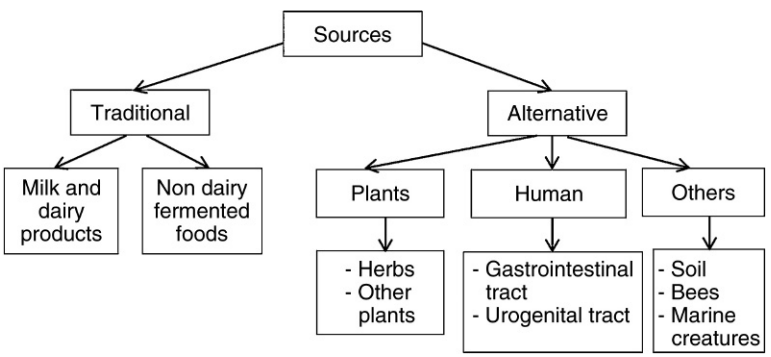


**Figure 4.1: Sources of Isolation of Lactic Acid Bacteria.**

sources for isolation of various species of LAB with specific characteristics and features. Raw milk (from cows, goats, buffalos, sheeps, camels, and donkeys), as well as human breast milk are sources for isolation of LAB, which aroused the interest of several research teams around the world (Akhmetsadykova et al., 2015; Aziz et al., 2009; Carminati et al., 2014; Jans et al., 2012; Khedid et al., 2009; Kim et al., 2006; Mehanna et al., 2013; Nomura et al., 2006; Ortolani et al., 2010; Perin and Nero, 2014; Randazzo et al., 2016; Sharma et al., 2013).

Aziz et al. (2009) have isolated LAB from buffalo, cow, and sheep milk, originating in Pakistan, and in all of the three types of milk the content of cocci prevailed over the number of rods. The authors have proved that the ratio among different species of LAB was as follows: in buffalo milk five species of LAB were found—*Lb. acidophilus* (25%), *Lb. delbrueckii* ssp. *bulgaricus* (21%), *Lc. lactis* ssp. *cremoris* (21%), *Lc. lactis* ssp. *lactis* (19%), and *S. thermophilus* (14%); in cow milk four species of LAB were found—*S. thermophilus* (34%), *Lc. lactis* ssp. *lactis* (28%), *Lb. delbrueckii* ssp. *bulgaricus* (28%), and *Lc. lactis* ssp. *cremoris* (10%); and finally, in sheep milk the following species of LAB were found—*Lc. lactis* ssp. *lactis* (36%), *Lc. lactis* ssp. *cremoris* (32%), *Lb. acidophilus* (22%), and *Leuconostoc* spp. (10%). *Lactococcus* and *Enterococcus* strains, with antimicrobial activity, have been isolated from goat milk, in Brazil (Perin and Nero, 2014). Sharma et al. (2013) have identified *S. thermophilus* from goat and cow milk, *Lc. lactis* from buffalo milk, *Lb. delbrueckii* from sheep milk (in India), which produced LA in high concentrations.

In Morocco, Khedid et al. (2009) have isolated from camel milk, not only representatives of the genera *Lactobacillus, Lactococcus*, *Leuconostoc*, *Streptococcus*, but also representatives of *Enterococcus* and *Pediococcus*. The dominant genera were *Lactobacillus* (37.5%) and *Lactococcus* (25.8%) and the dominant species were *Lb. helveticus*, *Lb. casei* ssp. *casei*, *Lb. plantarum*, *Lc. lactis* ssp. *lactis*, as well as *S. salivarius* ssp. *thermophilus*. The dominant genera of LAB isolated from camel milk in Kazakhstan were *Lactococcus, Lactobacillus,* and *Enterococcus* (Akhmetsadykova et al., 2015), while in milk from East Africa; the dominant species was *Streptococcus agalactiae* (Jans et al., 2012). In India *Streptococcus gallolyticus* was isolated from camel milk, which synthesized a high concentration of LA (Sharma et al., 2013).

Ten different species of LAB have been isolated from donkey milk, which were representatives of the following genera: *Enterococcus* (four species), *Streptococcus* (five species), and *Pediococcus* (one species) (Carminati et al., 2014). The prevailing LAB strains isolated from donkey milk in Italy were *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris,* and *Lb. paracasei* (Randazzo et al., 2016).

Human breast milk contains the necessary nutrients (carbohydrates, essential fatty acids, proteins, vitamins, and minerals) for infants (Serrano-Niño et al., 2016). It plays a major role in the development of newborns not only because of its various nutrients, but also due to the accompanying microflora. Commonly isolated bacteria belong to the genera *Streptococcus*,

*Enterococcus*, *Lactobacillus*, as well as *Bifidobacterium* and *Staphylococcus* (Jeurink et al., 2013; Martin et al., 2012). For that reason, human breast milk is an important source for isolation of LAB with beneficial health properties (Fernández et al., 2013; Pérez-Cano et al., 2010; Reis et al., 2016; Serrano-Niño et al., 2016). Mehanna et al. (2013) have isolated from Egyptian human milk representatives of the genera: *Lactobacillus* (*Lb. rhamnosus*, *Lb. plantarum*, *Lb. casei*, *Lb. fermentum*, *Lb. acidophilus*); *Enterococcus* (*E. faecium*, *E. faecalis*); *Lactococcus* (*Lc. lactis* ssp. *lactis*), and *Streptococcus* (*S. thermophilus*).

From various types of raw milk (cow, goat, buffalo, sheep, camel, and donkey) representatives mainly of the following genera have been isolated: *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus,* and in minor amounts—representatives of *Leuconostoc* genus, and from human breast milk—representatives of the genera *Streptococcus*, *Enterococcus*, *Lactobacillus*.

There are various types of fermented dairy products worldwide. Their diversity is determined by the type of milk used, its thermal processing, species of LAB forming starter cultures, fermentation conditions, as well as by the stages of the technological process. Fermented dairy products are manufactured from cow, sheep, goat, buffalo, donkey, and camel milk. They also could be used as sources for isolation of LAB (Caridi, 2003; Gao et al., 2012; Koleva et al., 2009; Mehmood et al., 2009; Nomura et al., 2006; Simova et al., 2002, 2009; Siragusa et al., 2007; Zamfir et al., 2006).

The major dairy products, which are used as sources for isolation of LAB are *fermented milks*. They could be classified according to their fermentation type: lactic (mesophilic and thermophilic), yeast-lactic (e.g., Kefir, Koumiss, acidophilus-yeast milk), and mould-lactic (e.g., Villi) (Walstra et al., 2006).

Products of mesophilic lactic fermentation are: traditional and cultured buttermilk, Nordic sour milks (Filmjolk, Nordic ropy milk, Ymer, lactofil), and cultured cream. They have mild acidic taste with an aromatic diacetyl flavor and from smooth to high viscous texture. These products are potential sources for isolation of LAB, belonging to the following genera *Lactococcus* and *Leuconostoc* (Walstra et al., 2006).

Products of thermophilic lactic fermentation are *yoghurt and yogurt-like products* (Bulgarian sour milk, dahi, katyk, laban, tarho, yiaourti, yaourt, etc.), from which the following species have been mainly isolated: *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (Bhattarai et al., 2016; Chammas et al., 2006; Mehmood et al., 2009; Walstra et al., 2006); *acidophilus milk—Lb. acidophilus* (Walstra et al., 2006); *probiotic fermented milk*—various LAB, including and bifidobacteria (Gad et al., 2014; Magdoub et al., 2015).

Yeast-lactic fermentation—products of this type of fermentation are: *Kefir*—the microflora of kefir is variable. *Lactococcus* (*Lc. lactis* ssp. *lactis* and *cremoris*, and *Lc. lactis* ssp. *lactis* biovar. *diacetylactis*), *Leuconostocs* (*Leuc. lactis* and *Leuc. cremoris*), and

*Lactobacillus* (*Lb. brevis*, *Lb. kefir*, sometimes also *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. acidophilus*) could form lactic acid, whereas yeasts, including *Candida*, *Kluyveromyces,* and *Saccharomyces* species, produced alcohol (Gao et al., 2012; Simova et al., 2002); *Koumiss*—from koumiss representatives of genus *Lactobacillus* and *Candida kefir* have been isolated (Danova et al., 2005).

Mold-lactic fermentation—a product of this type of fermentation is *Villi* (fermented milk product from Finland). *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, and the mold *Geotrichum candidum* have been isolated (Walstra et al., 2006).

*Cheeses* are another type of fermented dairy product potential source for isolation of LAB. They are the typical products of different regions and in some cases, the isolated LAB are adapted to high salt concentrations. Representatives of genera *Enterococcus*, *Lactococcus*, *Leuconostoc, Streptococcus,* and *Lactobacillus* have been isolated from different cheeses (Caridi, 2003; Hassanzadazar and Ehsani, 2013; Silva et al., 2015; Siragusa et al., 2007; Terzic-Vidojevic et al., 2014).

### *2.1.2 Nondairy fermented foods*

LAB are microorganisms used in the production of fermented milk products, as well as a number of fermented products of nondairy origin. They are among the main microorganisms used in food-fermented products and for this reason they could be isolated from a number of nondairy fermented products. Such products are:

- *Fermented beverages*: *Lactobacillus* spp., *Pediococcus* spp., and *Oenococcus oeni* have been isolated from wine (García-Ruiz et al., 2014); from *shochu* (traditional Japanese spirituous beverage)—*Lactobacillus satsumensis sp. nov.* (Endo, 2005)*; from* Ethiopian *borde* (low-alcoholic or nonalcoholic beverage, obtained from cereals)—representatives of the genera *Lactobacillus, Pediococcus, Weissella, Enterococcus* (Abegaz, 2014); from *boza* (a traditional low-alcoholic fermented beverage in some South East European countries prepared from various cereals, depending on the country where it is produced)—representatives of the genera *Lactobacillus, Leuconostoc, and E. faecium* (Kivanç et al., 2011).
- *Sausages:* From various types of fermented sausages (made of pork, beef, wildlife meat, poultry, lamb, etc.) mostly representatives of the genus *Lactobacillus* have been isolated, with a minor presence of representatives from other genera, such as—*Enterococcus, Pediococcus, Leuconostoc, Weisella, Lactococcus* (Albano et al., 2009; Dias et al., 2015; Federici et al., 2014; Fleck et al., 2012; Landeta et al., 2013; Marty et al., 2012; Wanangkarn et al., 2012, 2014).
- *Sauerkraut:* Plengvidhya et al. (2007) have isolated from sauerkraut representatives of the following genera—*Leuconostoc* (*Leuc. mesenteroides*, *Leuc. citreum*, *Leuc. fallax*,

*Leuc. argentinum*), *Lactobacillus* (*Lb. curvatus*, *Lb. plantarum*, *Lb. brevis*, *Lb. coryniformis*, *Lb. paraplantarum*, *Lb. mali*), *Pediococcus pentosaceus,* and *Weissella* spp. In sauerkraut *Leuc. mesenteroides* and *Lb. plantarum* had a dominating presence.

- *Pickles:* Yu et al. (2012) have isolated and identified, from traditional pickles (from 6 different regions of Sichuan province in China) *Lb. alimentarius* (16 strains), *Lb. brevis* (24 strains), *Lb. paracasei* (9 strains), *Lb. plantarum* (81 strains), *Lb. pentosus* (38 strains), *Lb. sakei* (8 strains), *Lb. spicheri* (1 strain), *E. thailandicus* (2 strains), *Leuc. lactis* (1 strain), and *Pediococcus ethanolidurans* (5 strains), proving that the dominant representative was *Lb. plantarum*, which has been isolated from almost all analyzed samples.
- *Fermented plants*: Representatives of the genus *Lactobacillus* as the predominant microflora have been isolated from olives (Abriouel et al., 2012; Hurtado et al., 2012); Kostinek et al. (2007) and Kpikpi et al. (2010) have isolated from fermented cassava (*Manihot esculenta*) and *kantong* (a product obtained from the fermentation of *Ceiba pentandra* seeds and flour from cassava), *Lb. plantarum* as the predominant species; from traditional fermented vegetable products in the Himalayas, Tamang et al. (2005) have isolated *Lb. brevis, Lb. plantarum, P. pentosaceus, P. acidilactici*, and *Leuc. fallax;* from traditional fermented vegetable products from Japan, Nomura et al. (2006) and Kimoto et al. (2004) have isolated *Lc. lactis* ssp. *lactis*; representatives of the genera *Lactobacillus* and *Pediococcus* have been isolated from fermented tea leaves (Miang) in Thailand (Tanasupawat et al., 2007); representatives of the genera *Lactobacillus* (76.2%), *Leuconostoc* (19.8%), and *Pediococcus* (4.8%) have been isolated from naturally fermented herbs used in the production of the traditional Turkish herb-cheese (Çakır, 2010).

## 2.2 Alternative Sources

### 2.2.1 LAB from plants

The demand for new solutions for improvement of starter communities provoked the idea to exploit the biodiversity in unique natural systems (plants). Each specified plant species provides a unique environment in terms of competitive microorganisms, natural vegetal antagonists, as well as accessibility, type, and concentration of the substrate in various physical factors. These conditions allow the growth of a typical epiphytic microflora by means of which a population emerges and a chain of fermentation processes commences after the plant material is prepared for fermentation.

#### 2.2.1.1 Medicinal plants/herbs

Medicinal plants are an important ecosystem for isolation of LAB.

Michaylova et al. (2007) have discovered that the following plant species—*Calendula officinalis, Capsella bursapastoris*, *Chrysanthemum, Cichorium intybus, Colchicum, Cornus*

*mas, Dianthus*, *Hedera, Nerium oleander, Plantago lanceolata, Rosa,* and *Tropaeolum* are suitable sources for isolation of *S. thermophilus*, while at the same time the species *C. officinalis, Cornus mas, Galantus nivalis,* and *Prunus spinosa* are suitable for the isolation of *Lb. bulgaricus*. Other representatives of the genus *Lactobacillus* have been also isolated from the surface of the herb—*Phyllanthus niruri* (Venugopalan et al., 2010), *Lb. paraplantarum* from tea leaves (*Camellia sinensis*) (Gharaei-Fa and Eslamifar, 2011), and *Lb. rhamnosus*—from the herb *Hottuynia cordata* Thunb. in Viatnam (Nguyen et al., 2013), as well as from *Panax gingseng* C.A. Meyer (Teneva-Angelova and Beshkova, 2016). *Lb. curvatus*, *P. pentosaceus,* and *Lc. lactis* have been isolated from the leaves of a Malaysian herb called *Polygonum minus* (Baradaran et al., 2012). Magnusson et al. (2003) have isolated representatives of the genus *Lactobacillus* (*Lb. plantarum*, *Lb. coryniformis,* and *Lb. sakei*) and of the genus *Pediococcus* (*P. pentosaceus* and *P. parvulus*), as well as *E. hirae* from the following herbs: *Hepatica*, *Coltsfoot*, dandelion, and clover. From different parts of plants—*Geranium sanguimeum* L. and *Hypericum perforatum* L. (in Bulgaria) have been isolated *S. thermophilus* and *E. faecium* (Teneva-Angelova and Beshkova, 2016). Coccus-shaped LAB—*S. thermophilus, Lc. lactis,* and representatives of the genus *Enterococcus* (*E. faecium, E. mundtii,* and *E. casseliflavus*) have also been isolated from various species of *Salvia* (*S. scabiosifolia* Lam., *S. officinalis* L., *S. blepharophylla* Brandegee ex Epling and *S. ringens* Sibth. & Sm.) (Teneva-Angelova and Beshkova, 2015).

#### 2.2.1.2 Other plant materials

Aside from medicinal plants and herbs, LAB could be isolated from various plants, such as sugar cane, grass, cereals, fruits, vegetables, and so on.

Representatives of the genus *Lactococcus* have been isolated from the leaves of sugar cane (Cock and de Stouvenel, 2006), from Napier grass in Japan (*Pennisetum purpureum*) (Kimoto et al., 2004; Nomura et al., 2006), as well as from other species of grass—*AberDart*, *Greengold,* and *Dunluce* (Alemayehu et al., 2014). Magnusson et al. (2003) have isolated representatives of the genus *Lactobacillus* (*Lb. plantarum*, *Lb. coryniformis,* and *Lb. sakei*) and of the species *P. pentosaceus* from different parts of plants (Lilac, Chestnut, and Rowan, as well as grass). In bean sprouts, mustard, and cress, Siezen et al. (2008) have identified LAB which belong to the genus *Lactococcus* and Hartnett et al. (2002) have identified *E. faecium* and *Lc. lactis,* isolated from barley, as well as *E. mundtii* and *E. faecalis* isolated from sorghum. Manini et al. (2016) have isolated *Leuc. mesenteroides, Leuc. citreum, Lb. brevis, Lb. curvatus, Lb. sakei, Lb. plantarum,* and *P. pentosaceus from spontaneously fermented wheat bran sourdough-like, and* Venturi et al. (2012) have isolated *Lb. sanfranciscensis*, from traditional sourdough.

Fresh fruits and vegetables could also be used as sources for isolation of LAB. Emerenini et al. (2013) have isolated representatives of the following genera: *Lactobacillus* (*Lb. plantarum, Lb. paraplantarum,* and *Lb. pentosus*), *Weissella* (*W. confuse*, *W. cibaria*,

*W. paramesenteroides,* and *W. kimchi*), as well as *P. pentosaceus* from tomatoes, citrus fruits, banana, fluted pumpkin vegetable (*Telfairia occidentalis*), and green vegetable (*Amaranthus spinosus*) in Nigeria. Trias et al. (2008) have isolated as follows: *Leuc. mesenteroides*, *Leuc. citreum*, *Lb. plantarum*, *Lc. lactis*, *W. cibaria,* and *E. mundtii* from fresh fruits and vegetables from Spain. Alemayehu et al. (2014) have isolated representatives of the genus *Lactococcus* from vegetables (fresh green peas, baby corn, broccoli, and cucumber). *Lb. plantarum*, *W. cibaria*, *Leuc. pseudomesenteroides,* and *Lc. lactis* ssp. *lactis* have been isolated from ripe mulberries in Taiwan (Chen et al., 2010).

### 2.2.2 LAB from urogenital tract

Scientific and research teams from various regions of the world are highly interested in LAB isolated from the urogenital tract of women, because of their important role in maintaining women's health, by improving the protection of their genital microflora against bacterial infections (Kaewsrichan et al., 2006; Voravuthikunchai et al., 2006). The diversity of LAB colonizing the urogenital tract of women could be the result of various geographical locations on the one hand, as well as due to their lifestyle and their surroundings on the other (Agboola et al., 2014; Antonio et al., 1999; Dimitonova et al., 2007; Jespers et al., 2012; Jin et al., 2006).

The species diversity of vaginal LAB from two populations of women, one in Uganda and one in South Korea has been examined. 128 strains of LAB from Korean women and 210 strains from African women have been isolated. These strains belonged to the following five genera—*Lactobacillus, Leuconostoc, Pediococcus, Streptococcus,* and *Weissella*. It was proven that *Lb. crispatus* was present in both populations, while *Lb. gasseri, Lb. reuteri,* and *Lb. vaginalis* were common only in the women from Uganda, and *Lb. fermentum* only in the women from Korea. Among the other LAB, *Weissella* was more common in Uganda, while *Pediococcus*—in Korea.

Lactobacilli play an important role in maintaining vaginal health of women (Jin et al., 2006). The strain of *W. kimchi*, which produced large quantities of $H_2O_2$ has been isolated from women in Korea (Lee, 2005). Vaginal LAB have been isolated from women in Nigeria and they were identified: *Lb. fermentum* (eight isolates), *Lb. jensenii* (nine isolates), *Lb. delbrueckii* (five isolates), *Lb. plantarum* (four isolates), *Lb. acidophilus* (three isolates), and *Lb. lactis* (three isolates). *Lb. jensenii* and *Lb. fermentum* were the most prevalent species of LAB (Agboola et al., 2014). Species belonging to the genus *Lactobacillus* have been isolated from Bulgarian women too (*Lb. fermentum, Lb. gasserri,* and *Lb. salivarus*) (Dimitonova et al., 2007).

In women of childbearing age (pregnant), the dominant species of lactobacilli were *Lb. crispatus, Lb. jensenii, Lb. iners, Lb. gasseri,* and *Lb. rhamnosus* (Kiss et al., 2007; Petricevic et al., 2014; Ravel et al., 2011).

### 2.2.3 LAB from gastrointestinal tract and feces

LAB exist in the gastrointestinal tracts of the human body and play an important role in physiological functions. *Lactobacilli*, *Bifidobacteria*, and Enterococci are normally associated with infant gut microbiota (Rodríguez et al., 2012). Sun et al. (2014) have isolated *E. faecium* (six isolates), *E. faecalis* (six isolates), *E. durans* (two isolates), *Lb. paracasei* (one isolate), *Lb. rhamnosus* (three isolates), and *Lb. plantarum* (three isolates) from newborn infants feces.

Di Cagno et al. (2009) have isolated fecal microflora of children suffering from coeliac disease. They have identified *Lb. fermentum, Lb. delbrueckii* ssp. *Bulgaricus*, and *Lb. gasseri* only in fecal samples of healthy children, while *Lb. brevis, Lb. rossiae*, and *Lb. pentosus* have been identified from fecal samples of children with gastrointestinal disorders, treated with a gluten-free diet.

### 2.2.4 Other alternative sources

Alternative sources for isolation of LAB may also include:

- *Soil*: These LAB represent five genera: *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc, Pediococcus*, and *Weissella* (Chen et al., 2005; Ekundayo, 2014; Fhoula et al., 2013).
- *Bees*: LAB have been isolated from honey, the gastrointestinal tract of bees, and bee pollen. Carina Audisio et al. (2011) have isolated *Lb. johnsonii* and *E. faecium* strains from *Apis mellifera* L. honeybee gut, and Belhadj et al. (2014)—*Lb. plantarum*, *Lb. fermentum*, *Lb. ingluviei*, *Lb. acidipiscis*, *Lc. lactis*, *P. pentosaceus*, and *W. cibaria* from raw bee pollen grains. *E. durans* (with antimicrobial activity) has been isolated from honey (Magnusson et al., 2003).
- *Marine creatures:* Representatives of the genera *Streptococcus*, *Leuconostoc*, *Pediococcus,* and *Lactobacillus* have been isolated from fresh and frozen fish and shrimps. The genus *Lactobacillus* is dominant and particularly of *Lb. plantarum* (Nair and Surendran, 2005). *Lb. plantarum*, *Lb. bulgaricus*, *Lb. brevis,* and *Lb. viridiscens* have also been isolated from the gut of the marine fish *Rastrelliger kanagurta* (Shubhankar Ghosh et al., 2013).

LAB are widespread microorganisms which can be found in any ecosystem and dairy and nondairy product. There has been a trend of increased interest in LAB isolated from plant sources due to their specific flavor-forming and metabolic activities, for enhancement of variety of food matrix.

## 3 LAB and Their Biogenic Metabolites for Human Health

LAB are the most important microorganisms that conduct many food fermentations. Milk is a natural source of a variety of beneficial nutrients and biologically active compounds with potential impacts on the human health. Among the most prominent representatives

of functional dairy products are certain fermented products. The health promoting effects of fermented dairy foods may be related to the activity of the selected LAB starters used in these products' production. Theoretically, LAB can exert beneficial effects through two mechanisms (Stanton et al., 2005):

- *Direct*: via probiotic LAB (the beneficial effect of live microbial cells);
- *Indirect*: via biogenics—lactic acid, conjugated linoleic acid, γ-aminobutyric acid, bacteriocin, reuterin and reutericycline, EPSs, and bioactive peptides.

### 3.1 Direct Mechanism of Beneficial Effect via Probiotic LAB (Live Microbial Cells)

Probiotic LAB are a direct source of beneficial health properties. The concept of probiotics was introduced in the early 20th century. It originates from the Latin word *"pro"* meaning *"for"* and the Greek word *"βίοτοζ"* meaning *"life"* (Hamilton-Miller et al., 2003). The definition of the term has evolved over the years (Granato et al., 2010). The origin of the first use can be traced back to Kollath (1953), who formulated the definition that "Probiotics are common in vegetable food as vitamins, aromatic substances, enzymes, and possibly other substances connected with vital processes." Parker (1974) defined probiotics as "Organisms and substances that contribute to intestinal microbial balance," and according to Fuller (1989) they are "Live microbial feed supplement which beneficially affects the host animal by improving microbial balance." Later, the Food and Agriculture Organization/World Health Organization (FAO/WHO) defined probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002).

The microorganisms should meet a number of predefined criteria in order to be considered as probiotics. Desirable properties characterizing them as probiotics (in fermented food products), resumed from Kearney et al. (2008) are:

- human origin
- GRAS status
- history of safe use in food
- documented health benefits
- antimutagenic and anticarcinogenic properties
- nonpathogenic
- tolerance to antimicrobial substances yet inability to tolerate other bacteria
- adherence to intestinal mucosa
- ability to reduce pathogen adhesion to surfaces
- antimicrobial activity against potentially pathogenic bacteria
- immunostimulation without proinflammatory effect
- acid tolerance
- human gastric juice tolerance

- bile tolerance
- phage resistance
- oxygen and heat tolerance
- desired metabolic activity
- ability to grow in milk
- good sensory properties
- retain viability and stability during food processing, storage, and following consumption

Microorganisms from many different genera are being used as probiotics (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus, Bifidobacterium,* and *Escherichia*). Representatives of genus *Lactobacillus* are the most commonly used probiotics in food, whereas bifidobacteria are used less, as they are sensitive to oxygen and have more strict growth requirements. Commonly used *Lactobacillus* species are *Lb. acidophilus* (Lee et al., 2015b), *Lb. casei* (Akoglu et al., 2015; Darjani et al., 2016), *Lb. gasseri* (Olivares et al., 2006), *Lb. johnsonii* (Kemgnag et al., 2016; Yamano et al., 2006), *Lb. paracasei* (Valerio et al., 2015), *Lb. plantarum* (Arasu et al., 2015; Haghshenas et al., 2014; Li and Gu, 2016; Özer et al., 2016; Yang et al., 2015), *Lb. reuteri* (Bustos et al., 2015; Flichy-Fernández et al., 2015), *Lb. rhamnosus* (Sul et al., 2007); *Bifidobacterium* species (Mayer et al., 2007; Pande and Gupta, 2015; Sul et al., 2007). *Lc. lactis* and some *Enterococcus* species have also been used (Lee et al., 2015a; Ohland and MacNaughton, 2010).

Some of the desirable properties characterizing microorganisms as probiotics are to be safe, to have GRAS status, as well as to have a history of safe use in foods (Mogensen et al., 2002). It is necessary for all probiotic strains not to be pathogens, to demonstrate tolerance to antimicrobial substances, but at the same time not to be able to transmit such resistance to other bacteria. Another desirable property of probiotic strains is the ability to adhere in the gastrointestinal tract, which enhances their probioticeffect (Lee and Salminen, 1995). Adhesion to the intestinal mucosa is important on the one hand for immune modulation, and on the other hand, for the exclusion of pathogens by stimulating their removal from the infected intestinal tract (Lee et al., 2000). In vitro studies were performed by investigating the adhesion of LAB to Caco-2 cells (Belguesmia et al., 2016; Piątek et al., 2012). Saxami et al. (2016) found higher adhesion of *Lb. pentosus* B281 and *Lb. plantarum* B282 to Caco-2 cells compared to the reference strain *Lb. casei* ATCC 393. Adherence properties of two strains *Lactobacillus* (*Lb. acidophilus* and *Lb. gasseri*), isolated from human vagina, were examined by Fernandez et al. (2003). The authors have proven that both strains adhered to Caco-2 cells through glycoproteins in *Lb. gasseri* and through carbohydrates in *Lb. acidophilus*, as well as that both strains were able to inhibit certain enteropathogens (*Salmonella*, *Listeria*, and *Campylobacter*) without interfering with the normal microbiota. The same authors have also found that strongly adherent *Lb. gasseri* inhibit the attachment of *Escherichia coli* O111 to intestinal Caco-2 cells under the condition of exclusion.

The microorganisms must confer health benefits to the host in order to be characterized as probiotics. They have to possess the ability to survive passage through the gastrointestinal tract (acid tolerance, tolerance to human gastric juice, and bile tolerance). The low pH and hydrolytic action of pepsin in the stomach are the first barrier for LAB to reach the small intestines, where once again the environment is very limiting. A higher pH (at around 8.0) and the presence of pancreatin and bile salts threaten the survival of LAB (Ranadheera et al., 2012). That is why the first step to check the probiotics potential of a newly isolated strain is in vitro studies on its viability in a gastrointestinal-like environment. *Lb. crispatus* (GI6, GI9, GI11, and GI16) and *W. koreensis* (FKI13, FKI21, and FKI29) were capable of surviving under low pH and bile salt conditions (Anandharaj et al., 2015). GI9 and FKI21 were able to survive at pH 2.0% and 0.50% bile salt for 3 h without losing their viability. All LAB strains exhibited inhibitory activity against the tested pathogens (*E. coli* MTCC 1089, *Pseudomonas aeruginosa* MTCC 2642, *Staphylococcus aureus* MTCC 7443, *Klebsiella pneumoniae* MTCC 7028, *Bacillus subtilis* MTCC 8561, and *Candida albicans* BS3) and were able to deconjugate bile salt. Moreover, *Lb. crispatus* GI9 (58.08 mg/cm$^3$) and *W. koreensis* FKI21 (56.25 mg/cm$^3$) exhibited maximum cholesterol reduction with bile salts.

Probiotic microorganisms should also be technologically suitable for incorporation into food products. They have to retain viability and efficacy in that food product as well as they should be capable of surviving industrial applications. The health benefits of probiotic LAB can manifest themselves when the concentration of viable cells in fermented milk products is not less than $10^6$ CFU/g (Karna et al., 2007).

## 3.2 Indirect Mechanisms of Health Enhancement

In the process of their development, LAB synthesize a number of metabolites (biogenic compounds) which have a positive impact on human health through their biological activities.

### 3.2.1 Lactic acid

During the fermentation by LAB, the main chemical change is transformation of lactose into LA. It has an antimicrobial effect and is mainly associated with food preservation (Pridmore et al., 2008). LA acts in a nontargeted manner and its contribution to probiotic functionality has not been investigated as extensively (O'Shea et al., 2012).

It is considered that the antimicrobial property of LA is due to two reasons. First, its accumulation lowers the pH and thus limits the growth of other microorganisms. Second, in this environment, the organic acids become undissociated and pass freely through the microbial cell membrane. As the cytoplasmic pH is generally higher than the outside environment, the weak acids dissociate and protons are released (Cotter and Hill, 2003). Therefore the cytoplasm is acidified. The acidic pH inside the cell causes deformation and damage to enzymatic activities, proteins, and DNA structure, thereby damaging the

extracellular membrane (Mani-López et al., 2012). At the same time, LAB are not affected by their metabolism and stay active in low pH environment. The mechanism by which LAB and other Gram-positive bacteria resist the acidic conditions is reviewed by Cotter and Hill.

Stanojević-Nikolić et al. (2016) reported that LA was more effective against Gram-positive bacteria than Gram-negative bacteria, studying nine bacteria (*E. coli*, *Proteus mirabilis*, *Salmonella enteritidis*, *Ps. aeruginosa*, *Staph. aureus*, *E. faecalis*, *L. monocytogenes*, *B. cereus,* and *B. megaterium)* and three yeasts (*Rhodotorula* sp., *Sacch. Cerevisiae,* and *C. albicans*).

### *3.2.2 Conjugated linoleic acid*

Conjugated fatty acids represent polyunsaturated fatty acids with conjugated double bonds, usually found in a mixture of positional and geometric isomers. Conjugated linoleic acid (CLA) gained a particular attention, thanks to its anticarcinogenic, antiobese, and antidiabetic activities (Nagao and Yanagita, 2005). In milk-derived products CLA (of which *cis*-9, *trans*-11–18:2 is the main isomer) is a natural component obtained after isomerization of linoleic acid by anaerobic rumen microorganisms (Ogawa et al., 2005). Moreover, dairy products can be additionally enriched in CLA through milk fermentation with specific LAB (Sosa-Castañeda et al., 2015). Many studies were performed to find CLA-producing LAB and also to enhance their ability to isomerize linoleic acid. The study on *Lb. plantarum* AKU 1009a revealed four enzymes involved in the synthesis of conjugated fatty acids, such as CLA (Kishino et al., 2013). These enzymes catalyze the hydration/dehydration (CLA-HY), oxidation of hydroxyl groups/reduction of oxo-groups (CLA-DH), migration of carbon═carbon double bonds (CLA-DC), and saturation of carbon═carbon double bonds (CLA-ER) (Ortega-Anaya and Hernández-Santoyo, 2016).

The CLA production in LAB is hindered by the inhibition of growth of LAB by linoleic acid (Ogawa et al., 2005). To overcome this problem, the use of LAB resting cells in media poor of linoleic acid was proposed. Otherwise cyclization and changes in monounsaturated fatty acid percentages were observed instead of conjugation (Vela Gurovic et al., 2014). Moreover, a better dispersion of linoleic acid is required (pretreatment with detergentor albumin) in a microaerobic conditions as well (Ogawa et al., 2005). The percentage of CLA, relative to total fatty acid produced by *Lb. pentosus* H16 increased 4 times, reaching 23.69% ± 0.79% when the resting cells were removed from agar plates and incubated without the addition of exogenous linoleic acid as a substrate (Vela Gurovic et al., 2014). Wei et al. (2014) studied the permeabilization of *Lb. acidophilus* cells with cetyltrimethylammoniumbromide enhanced the conversion rate of linoleic acid to CLA up to 86.4% (Wei et al., 2014).

Lactobacilli are the commonly investigated potential producers of CLA. Sosa-Castañeda et al. (2015) reported that out of 13 strains of *Lactobacillus* tested, only four-produced CLA in

skim milk supplemented with linoleic acid (13.44 ± 0.78–50.9 ± 0.26 μg/cm$^3$) and survived in simulated gastrointestinal conditions and to adhere to the intestinal mucosa of Wistar rats. The production of CLA also depends on animal's feed composition. CLA-enhanced caprine Coalho cheese was obtained after the addition of soybean oil to goats' diet and subsequent use of *Lb. acidophilus* La 5 as a probiotic adjunct culture. The CLA content (isomer C18:2 *cis*-9, *trans*-11) was 2.46–2.9 times higher than in the control cheese (dos Santos et al., 2012). The fatty acids profile of organic probiotic fermented milks produced by *B. animalis* ssp. *lactis* HN019 in coculture with *S. thermophilus* TA040 and *Lb. delbrueckii* ssp. *bulgaricus* LB340 showed 1.4 times higher amount of C18:2 conjugated linoleic acid and 1.6 times higher α-linolenic acids, compared to conventional fermented milks. The main difference in bacterial growth was the enhancement of *Lb. bulgaricus* growth in organic milk (Florence et al., 2012). *Lb. plantarum* AA1-2 and *Lb. plantarum* AB20-961 enhanced the CLA production in dry, spicy sausage (Özer et al., 2016).

The study on the effect of *Lb. rhamnosus* PL60 that produces *trans*-10, *cis*-12-CLA, on diet-induced obese mice showed a reduction of mice's body weight without reducing energy intake after 8 weeks of feeding with *Lb. rhamnosus* PL60 (Lee et al., 2006). Furumoto et al. (2016) proved enhancement of the cellular antioxidative responses using the derivatives of linoleic acid by *Lb. plantarum*, in vitro and in vivo.

### 3.2.3 γ-Aminobutyric Acid (GABA)

GABA is a ubiquitous amino acid with many biological functions associated to blood pressure regulation, neurotransmitter inhibition in the mammalian central nervous system, and inhibition of lung adenocarcinoma. It also improves the plasma concentration, growth hormones, and the protein synthesis in the brain. In addition, GABA has tranquilizing, diuretic, and antidiabetic effects (Dhakal et al., 2012; Diana et al., 2014a).

GABA is formed after irreversible α-decarboxylation reaction of L-glutamic acid by glutamic acid decarboxylase enzyme (GAD; EC 4.1.1.15) (Diana et al., 2014a). LAB represent the major producers of GABA although the yield is limited. After culturing *Lb. plantarum* NDC75017, l-monosodium glutamate as substrate, produced 0.315 mg/g GABA (Shan et al., 2015). *Lb. brevis* BJ20 converted the substrate (glutamic acid) entirely and yielded 2.5 mg/cm$^3$ GABA in fermented sea tangle solution (Lee et al., 2010). After optimization of production conditions, *Lb. plantarum* Taj-Apis362 originated from Asiatic giant honeybee was able to accumulate 737 mg/dm$^3$ GABA (Tajabadi et al., 2015). The addition of pyridoxal 5-phosphate (coenzyme of glutamate decarboxylase) in the culture medium of *Lb. paracasei* NFRI 7415, isolated from Japanese traditional fermented fish, has lead to the production of 31 g/dm$^3$ GABA (Komatsuzaki et al., 2005). *Lb. brevis* CGMCC 1306 highest yield was 54 g/dm$^3$ (Peng et al., 2013).

GABA producing LAB were also isolated from different types of cheese (Diana et al., 2014b; Franciosi et al., 2015; Siragusa et al., 2007). In Italian cheese *Lb. paracasei*

PF6, *Lb. delbrueckii* ssp. *bulgaricus* PR1 and *Lb. plantarum* C48 producing 99.9 mg/kg, 63.0 mg/kg and 16.0 mg/kg GABA, respectively (Siragusa et al., 2007), and *S. thermophilus* 80 mg/kg (Franciosi et al., 2015) were found. *Lc. lactis* ssp. *lactis* ULAAC-H13 was also able to produce GABA in concentration 0.32 mg/g cheese when used as coculture in Cheddar cheese production (Pouliot-Mathieu et al., 2013). It was found that GABA accumulates in the cheese proportionally to its aging.

### *3.2.4 Bacteriocins*

Bacteriocins are ribosomally synthesized peptides displaying antimicrobial activity against closely related bacteria. The persistent interest of researchers in LAB bacteriocins is prompted by their potential application as food biopreservatives, that is, they offer a successful prospective alternative strategy for inhibiting the growth of foodborne bacterial pathogens (Beshkova and Frengova, 2012). Moreover, the majority of bacteriocin-producing LAB are isolated from naturally fermented foods, and therefore expected to be safe in food applications (Deegan et al., 2006). Because of their proteinous nature, they are susceptible to digestive proteolysis, limiting in this way possible human immunological response. Nowadays, bacteriocin production is considered one of the traits of probiotics (Dobson et al., 2012). During the past few years, many reviews have been published on this subject (Balciunas et al., 2013; Beshkova and Frengova, 2012; Cotter et al., 2005; Heng et al., 2007; Oscáriz and Pisabarro, 2001; Parada et al., 2007; Perez et al., 2015; Zacharof and Lovitt, 2012).

Bacteriocins from LAB are important and a constantly growing group of antimicrobial substances that need to be properly classified. First, Klaenhammer (1993) proposed four classes of bacteriocins based on the existent structural, physicochemical, and molecular diversity. Subsequently, a simplified classification with only two classes was proposed: lantibiotics (class I) and nonlantibiotics-containing bacteriocins (class II) withdrawing Klaenhammer's class III (large heat-labile murein hydrolases) and class IV (the lipid- or carbohydrate-containing bacteriocins) (Cotter et al., 2005). Later, Heng et al. (2007) merged the two concepts, including the most recent developments within the bacteriocins and proposing four major classes summarized in Table 4.1.

The first reported bacteriocin from LAB is nisin (Hurst, 1981). It is produced by *Lc. lactis* ssp. *lactis* and belongs to the group of lantibiotics (class I). Nisin is also the best-studied bacteriocin so far and the only one in this class approved for food administration (Shin et al., 2016). However, its use in food formulations is impeded by the uncontrolled interactions with the complex food matrix. To overcome this problem, several biotechnological approaches were proposed including various immobilizations (Ji et al., 2014; Khan and Oh, 2015). The encapsulation of nisin in liposomal nanodelivery systems had a positive effect on the inhibition of *L. monocytogenes* CIP 82110 (Imran et al., 2015) and *L. monocytogenes* ATCC 7644 (Malheiros et al., 2012). Imran et al. (2015) reported better

**Table 4.1: Classification of bacteriocins from Gram-positive bacteria (Heng et al., 2007).**

| Class | Features |
|---|---|
| I | Lantibiotics, small (<5 kDa) peptides containing lanthionine and 3-methyllanthionine |
| Type A | Elongated amphipathic structures |
| Type B | Globular and compact structures |
| Type C | Multicomponent |
| II | Small (<10 kDa), nonmodified peptides (nonlantibiotic and noncyclic) |
| Type IIa | Pediocin-like peptides, possessing antilisterial activity |
| Type IIb | Two-peptides bacteriocins |
| Type IIc | All single-peptide nonmodified bacteriocins that do not fulfill the criteria of type IIa or type IIb |
| III | Large (>10 kDa) bacteriocins |
| Type IIIa | Bacteriolysins (bacteriolytic enzymes) |
| Type IIIb | Nonlytic bacteriocins |
| IV | Cyclic bacteriocins |

inhibitory activity of nisin mixture (free: encapsulated in soy and marine lecithin—1:1) against *L. monocytogenes* CIP 82110 in comparison to only free or encapsulated nisin. The treatment against *L. monocytogenes* ATCC 7644 in Minas frescal cheese stored at 7°C showed that at day 14 the count of the pathogen was 4.6 log CFU/g lower than the control and approximately 2 log CFU/g lower than free nisin (Malheiros et al., 2012). Nisin was also microencapsulated in alginate matrix and a prolonged inhibition against *Brochothrix thermosphacta* 7R1, a common meat spoilage bacterium, was recorded (Maresca et al., 2016).

Pediocins, plantaricins, enterocins are representatives of class II. Although the mechanisms by which they specifically recognize their target cells before permeabilization are not well understood, the main mechanism of inhibition of class II bacteriocins is also related to pores formation (Diep et al., 2007). Pediocin AcH/PA-1 produced by *P. acidilactici* PAC1.0 is the best-known pediocin so far (Papagianni and Anastasiadou, 2009) and after nisin, it is the second commercially produced pediocin (Yang et al., 2014a). Pediocins known as "antilisterial" bacteriocins are largely incorporated in food surface biopreservative formulations. Sawdust particle biocomposite film impregnated with pediocin PA-1/AcH (PLA/SP + Ped) was designed as a food-contact antimicrobial packaging on raw sliced pork and tested against *L. monocytogenes* ATCC19115. All treatments with PLA/SP + Ped significantly reduced the listerial population by about 1.5–2 log cycles from 1 to 14 days of storage at 4 ± 2°C (Woraprayote et al., 2013). Other antimicrobial films incorporated with pediocin that reduce the listerial population were also reported (Meira et al., 2015; Santiago-Silva et al., 2009) moreover, the application of pediocins in polymers for food preservation and packaging materials are reviewed by Espitia et al. (2016). Apart from their antilisterial activity, pediocin PA-1 produced by *P. acidilactici* J347-29 inhibited 84% of *O. oeni* strains (16 out of 19 strains) with $IC_{50}$ = 19 ng/cm$^3$ and 79% of LAB species tested (23 out of 29 strains) with $IC_{50}$ = 312 ng/cm$^3$ (Díez et al., 2012).

Several strains of *Lb. plantarum* were reported to produce class II bacteriocins—plantaricins. They are usually active in a broad pH range (pH 2.0—6.0 for plantaricin W; pH 2.0—8.0 for plantaricin K25 and pH 2.0—10.0 for bacteriocin ST71KS and plantaricin MG) and possess a high thermal stability (from 4 to 100°C for up to 60 min and 121°C for 15 min) (Barbosa et al., 2016; Gong et al., 2010; Martinez et al., 2013; Wen et al., 2016). Generally, *Lb. plantarum* bacteriocins exhibit a strong bacteriostatic activity against *L. monocytogenes* strains. Plantaricin W was reported to be active against *L. innocua* ATCC 33090 and *L. welshimeri* USP. The antimicrobial activity of plantaricin ZJ008 and plantaricin W was observed against *Staph. aureus* (Barbosa et al., 2016; Zhu et al., 2014). Moreover, plantaricin ZJ008 exhibited antimicrobial activity toward some Gram-negative strains (*E. coli* DH5α; *Vibrio parahaemolyticus* SCF16; *Shigella flexneri* DSM4782; *Ps. aeruginosa* B2). Plantaricin W was active against 3 out of 10 strains of *Enterococcus* spp. tested and 2 (*Lb. sakei* ATCC 15521 and *Lb. fermentum* ET35) out of 25 strains of LAB. It did not inhibit the tested Gram-negative bacteria (*Salmonella*, *E. coli,* and *Enterobacter)*. Unlike plantaricin W, plantaricin K25 possess inhibitory activity against *B. cereus* (Barbosa et al., 2016; Wen et al., 2016). Gong et al. (2010) purified a bactericidal bacteriocin from *Lb. plantarum* KLDS1.0391, named plantaricin MG with broad inhibitory spectrum. It was active against both Gram-positive (*L. monocytogenes* and *Staph. aureus*) and Gram-negative bacteria (*S. typhimurium* and *E. coli*).

*Enterococcus* spp. produce bacteriocins with diverse structures named enterocins. Some of them belong to class II. Maldonado-Barragán et al. (2009) purified enterocin C, from culture supernatant of *E. faecalis* C901, a strain isolated from human colostrums. The bacteriocin possessed two polypeptide chains and showed antimicrobial activity against both related and nonrelated to the producing strain as *Actinomycesneuii*, *E. faecalis*, *E. faecium*, *Facklamia hominis*, *Lc. lactis*, *Lb. paracasei*, *Leuc. mesenteroides*, *Propionibacterium acnes*, *Staph. caprae*, *Staph. epidermidis*, *S. anginosus*, and *S. intermedius.* Enterocin C inhibited most of the *E. faecium* and *E. faecalis* strains tested as indicators.

Until recently, the cyclic bacteriocins (belonging to class IV) were considered as rare, but nowadays the number of revealed N-to-C-terminally linked antimicrobial peptides has grown. The circular shape derives from posttranslationally modified bacteriocins initially synthesized as linear precursors, containing a leader sequence of variable size which is cleaved off during maturation (Gabrielsen et al., 2014; Pandey et al., 2013; Van Belkum et al., 2011). Generally, they are less susceptible to proteolytic cleavage, high temperature and pH, and, therefore, provide enhanced stability as compared to linear bacteriocins (Pandey et al., 2013). So far, this group is associated with few bacteriocins, being partially characterized and most of them are produced by LAB, that is, gassericin, acidocin, and enterocin AS-48. The latter is produced by *E. faecalis* and its mode of action is the most comprehensively studied (Grande Burgos et al., 2014; Maqueda et al., 2004). The lack of specificity between circular bacteriocin and host is the reason for their broad-spectrum. Nevertheless, it is limited to

Gram-positive bacteria. Enterocin AS-48 was reported to inhibit Gram-negative but much higher concentration is required for its action against Gram-positive bacteria. Moreover, a pretreatment is needed in order to disrupt the outer membrane (Cobo Molinos et al., 2008; Grande Burgos et al., 2014). Enterocin AS-48 was also extensively studied as a potential food biopreservative. Enterocin AS-48, produced by *E. faecalis* A-48-32, has proven to be very efficient when used alone or in combination with licensed chemical preservatives in vacuum or modified atmosphere packaging, in the control of *Lb. sakei, Brochothrix thermosphacta,* and *Staph. carnosus*. A reduction of 3–4 log units in CFU/g was achieved when 40 μg/g of the enterocin was applied (Baños et al., 2012). The toxicogenic psychrotrophic *Bacillus cereus* LWL1 viable cell counts in a model food system decreased rapidly when inhibited by enterocin AS-48 (in a concentration range of 20–35 μg/cm$^3$) (Grande et al., 2006). *Staph. aureus* in skimmed milk and in fresh cheese was also inhibited by enterocin AS-48 in a concentration-dependent mode (Muñoz et al., 2007).

Enterocin RM6 is also a cyclic peptide with 70 residues produced by *E. faecalis* OSY-RM6 and was proved to inhibit Gram-positive bacteria, including *L. monocytogenes*, *B. cereus*, and methicillin-resistant *Staph. aureus* (MRSA). Enterocin RM6 (final concentration in cottage cheese, 80 AU/cm$^3$) caused a 4-log reduction in population of *L. monocytogenes* inoculated in cottage cheese within 30 min of treatment (Huang et al., 2013a).

Among other cyclic LAB, the most studied is gassericin A isolated from *Lb. gasseri* showing antibacterial activity against a number of Gram-positive food-borne pathogenic bacteria (Kawai et al., 2004; Pandey et al., 2013; Van Belkum et al., 2011). Gassericin A produced by *Lb. gasseri* LA39 completely inhibited custard cream spoilage bacteria (*B. cereus*, *Lc. lactis* subsp. *lactis, Achromobacter denitrificans* and *Ps. fluorescens*) throughout 30 days of incubation at 30°C (Nakamura et al., 2013).

Todorov (2010) isolated five bacteriocin-producing LAB (*Lb. plantarum* ST69BZ, *E. faecium* ST62BZ, and *Leuc. lactis* ST63BZ, ST611BZ, and ST612BZ) from boza. All five bacteriocins exhibited bactericidal action against *E. faecium* HKLHS and *Lb. sakei* DSM 20017. Moreover, they inhibited the growth of *Enterococcus* spp., *E. coli*, *K. pneumoniae*, *Lactobacillus* spp., *Lc. lactis* ssp. *lactis*, *Listeria* spp., *Ps. aerouginosa*, *Staphylococcus* spp., and *S. caprinus*.

### 3.2.5 Reuterin and reutericycline

Reuterin (β-OH-propionic aldehyde, β-hydroxypropionaldehyde, β-HPA) is a nonprotein antimicrobial component, an intermediate product during the fermentation of glycerol to 1,3-propanediol by *Lb. reuteri* under anaerobic conditions. Reuterin exert a broad inhibition activity against many microorganisms, including protozoa, fungi, Gram-positive, and Gram-negative bacteria. Some recent investigations provide evidence that reuterin modifies the thiol-groups in proteins and small molecules, in this way inducing oxidative stress in cells (Schaefer et al., 2010; Stoianova et al., 2012). Additionally, reuterin is considered to

block the enzyme ribonucleotide reductase by acting either as a competitive inhibitor or as modifier of an unstable thiol group in the enzyme (Cleusix et al., 2007; Gálvez et al., 2014). Ribonucleotide reductase is needed in the generation of deoxynucleotides, required for DNA synthesis (Torrents, 2014). Reuterin is water soluble and stable over a wide range of pH. Unlike proteinaceous bacteriocins, reuterin is not sensitive to proteases, and is considered to be a suitable food biopreservation agent.

Reuterin produced by *Lb. reuteri* INIA P572 inhibited the growth of vegetative cells of *Clostridium perfringens* isolates (from ovine milk obtained in farms with diarrheic lambs) and *C. perfringens* CECT 486 (type A toxin producer) and the outgrowth of spores of all tested *C. perfringens* (Garde et al., 2014).

The inhibitory activity of reuterin obtained by *Lactobacillus reuteri* INIA P579 against three *L. monocytogenes* strains was assessed (Arqués et al., 2011). Montiel et al. (2014) reported a synergetic effect of reuterin and nisin against *L. monocytogenes* and *Staph. aureus*.

The combined inhibitory effect of reuterin under different pH conditions against *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* was also demonstrated (Langa et al., 2014).

Another inhibitory compound produced by *Lb. reuteri* (isolated from sourdough) is reutericyclin (Ganzle et al., 2000). It is structurally related to naturally occurring tetramic acids and represents a membrane-active antibiotic (Cherian et al., 2014; Gänzle, 2004; Höltzel et al., 2000; Lin et al., 2015). Several successful studies were carried out in order to chemically synthesize analogs with improved antibacterial properties (Böhme et al., 2005; Jones et al., 2014; Yendapally et al., 2008). Reutericyclin and its analogs exhibit both bacteriostatic and bactericidal activity against Gram-positive bacteria like *C. difficile* (Hurdle et al., 2009), drug-resistant *Staph. aureus* (Hurdle et al., 2009), and other food-related spoilage organisms and pathogens (Gänzle, 2004).

### 3.2.6 Exopolysaccharides

EPSs are another group of functional components produced by LAB. EPSs are heterogenous polysaccharides produced extracellularly principally by bacteria and microalgae (Patel et al., 2012). It is considered that the first bacterial EPS is isolated from *Leuc. mesenteroides,* a hindering LAB in wine (Nwodo et al., 2012). LAB are able to produce both homo- and heteropolysaccharides with diverse biological activities, such as immunomodulatory activity (Liu et al., 2011; Makino et al., 2006; Shao et al., 2014; Surayot et al., 2014), antitumor or anticancer activity (Dilna et al., 2015; Li et al., 2014b, 2015, 2016; Wang et al., 2014a,b, 2015), antioxidant activity (Dilna et al., 2015; Li et al., 2014a; Liu et al., 2011; Pan and Mei, 2010; Wang et al., 2015), and antibiofilm or protective biofilm activity (Kšonžeková et al., 2016; Wang et al., 2015; Zivkovic et al., 2015). The composition and the main functional characteristics of some of the recently reported exopolysaccharides (EXPs) from LAB are summarized in Table 4.2.

**Table 4.2: Main characteristics of recently reported EXPs from LAB.**

| Producer | EPS Type | Composition | Molecular Weight | Activity | References. |
|---|---|---|---|---|---|
| *Lc. lactis* ssp. *lactis* 12 | Hetero-EPS | Fru and Rha | 6.9 × $10^5$ Da | Antioxidant activity | Pan and Mei (2010) |
| *Lb. rhamnosus* KF5 | Hetero-EPS | S1—Glc:Ara: GlcN: GalN:Gal—2.03:1.29:1.25:0.72:0.61[a]; S2—Rha:Glc:Gal—1.73:1.47:1.00[a] | S1—1.36 × $10^4$ Da S2—1.23 × $10^6$ Da | Immunomodulatory activity | Shao et al. (2014) |
| *Lb. confusus* TISTR 1498 | Homo-EPS | (1→6)-α-D-glucan | After hydrolysis ≤70 × $10^3$ g/mol | After hydrolysis—immunomodulatory activity | Surayot et al. (2014) |
| *Lb. bulgaricus* OLL1073R-1 | Hetero-EPS | D-Glc: D-Gal 1:1.5[a] | 2.9 × $10^6$ g/mol | Immunomodulatory activity | Makino et al. (2006) |
| *Lb. paraplantarum* BGCG11 | ND | ND | Two fractions: F1—2.2 × $10^6$ Da F2—5.6 × $10^4$ Da | Protective biofilm formation | Zivkovic et al. (2015) |
| *Lb. reuteri* DSM 17938 | Homo-EPS | 1 → 4, 1 → 6 linked α-D-glucoses and branched (1 → 4,6) α-Glc | 6.5 × $10^5$ Da | Antibiofilm formation | Kšonžeková et al. (2016) |
| *Lb. reuteri* L26 | Homo-EPS | 1 → 3, 1 → 6 linked α-D-glucoses and branched (1 → 3,6) α-Glc | 8.2 × $10^5$ Da | Antibiofilm formation | Kšonžeková et al. (2016) |
| *Lb. plantarum* 70810 (c-EPS) | Homo-EPS | α-D-(1 → 6)-linked galactcosyl, α-D-(1 → 4)-linked galactcosyl, D-(1 → 2,3)-linked galactcosyl residues and a tail end of α-D-(1 → )-linked galactcosyl residues | 1.7 × $10^5$ Da | Antitumor/anticancer activity | Wang et al. (2014a) |
| *Lb. plantarum* 70810 (r-EPS) | Hetero-EPS | Glc:Man:Gal r-EPS1-18.21:78.76:3.03[a]; r-EPS2-12.92:30.89:56.19[a] | r-EPS1—2.0 × $10^5$ Da r-EPS2—2.0 × $10^5$ Da | Antitumor/anticancer activity | Wang et al. (2014b) |
| *Lb. plantarum* RJF4 | Hetero-EPS | Glc and Man | ND | Antioxidant activity; anticancer activity; cholesterol-lowering activity; antidiabetic activity (α-amylase inhibition) | Dilna et al. (2015) |
| *Lb. paracasei* ssp. *paracasei* NTU 101 *Lb. plantarum* NTU 102 | Hetero-EPS | 101EP (0.1 mg/$cm^3$), μmol/$dm^3$: Ara—92.1; Gal—1; Glc—106.3; Man—170.7; Fru 0.7; Mal—71.8 102EP (0.1 mg/$cm^3$), μmol/$dm^3$: Ara—80.3; Gal—40.3; Glc—113.0; Man—150.4; Fru—4.6; Mal— 67.2 | ND | Antioxidant activity; immunomodulatory activity | Liu et al. (2011) |
| *Lb. helveticus* MB2-1 | Hetero-EPS | Gal:Glc:Man LHEPS-1—1.33:2.75:1.00[a]; LHEPS-2—1.00:1.43:9.34[a]; LHEPS-3—1.17:1.00:2.96[a] | LHEPS-1-2 × $10^5$ Da; LHEPS-2—2 × $10^5$ Da; LHEPS-3—2 × $10^5$ Da; | Antioxidant activity; antitumor/anticancer activity | Li et al. (2014b, 2015) |
| *Lb. plantarum* YW32 | Hetero-EPS | Man:Fru:Gal:Glc 8.2:1:4.1:4.2[a] | 1.0 × $10^5$ Da | Antioxidant activity; antitumor/anticancer activity; antibiofilm formation | Wang et al. (2015) |

Abbreviations: Glc, Glucose; Fru, fructose; Gal, galactose; Rha, rhamnose; Man, manose; Mal, maltose; GlcN, glucoseamine; GalN, galactosamine.
[a]Approximate molar ratio.

Immunomodulatory EPS precursor is produced by *Lb. confusus* TISTR 1498 (Surayot et al., 2014). The native homopolysaccharide was unable to stimulate the RAW 264.7 cells to produce proinflammatory nitric oxide and cytokines in vitro while the partially hydrolyzed EPS significantly activated the macrophages cells and induced considerable immunomodulatory response. An acid EPS from *Lb. bulgaricus* OLL1073R-1 augments natural killer cell activity after oral administration in mice (Makino et al., 2006). *Lb. rhamnosus* KF5 produced two fractions of EPSs exhibiting stimulating effect on spleen cells proliferation (in a dose-dependent manner) (Shao et al., 2014).

EPS could also act as biofilm forming or preventing agent. An EPS produced by *Lb. paraplantarum* BGCG11 demonstrated the protective ability toward epithelial cell line HT29-MTX against the lysis, induced by *L. monosytogenes* (Zivkovic et al., 2015). The EPS seems to cover the epithelial cells by the formation of protective layer and in this way prevents from pathogenic action. Contrary, the EPSs produced by *Lb. reuteri* DSM 17938 and *Lb. reuteri* L26 Biocenol TM reduce the biofilm formation by some enterotoxigenic *E. coli* (Kšonžeková et al., 2016). When incubated with *E. coli,* both EPSs inhibited its adhesion to IPEC-1 cells.

Many studies demonstrate the multifunctional properties of EPSs produced by LAB. *Lb. plantarum* RJF4 produces heteropolysaccharide with in vitro cholesterol lowering (42.24%) and antidiabetic (40% α-amylase inhibition) properties. It exhibits an antiproliferative effect against MiaPaCa2-pancreatic cancer cell line and remains nontoxic to normal cell lines. The same EPS showed some antioxidant activity, too (Dilna et al., 2015). Liu et al. (2011) studied in vitro production of heteropolymers by *Lb. paracasei* ssp. *paracasei* NTU 101 (101EP) and *Lb. plantarum* NTU 102 (102EP). Both EPSs (101EP and 102EP) exhibit significant immunomodulatory properties and antioxidant activity (Liu et al., 2011). *Lb. helveticus* MB2-1 produces three heteropolysaccharides (LHEPS-1, LHEPS-2, and LHEPS-3) able to inhibit in vitro human gastric cancer cells (BGC-823 cells—human gastric cancer cell line) and human colon cancer cells (Caco-2 cells—human colon epithelial cancer cell line) (Li et al., 2014b, 2015). Moreover, these EPSs (separately or combined) have in vitro antioxidant activity according to four methods. The highest activity for all the tested methods was recorded when crude EPSs was examined (Li et al., 2014a).

Two types of EPSs are produced by *Lb. plantarum* 70810. Cell-bound EPS (c-EPSs) and released EPSs (r-EPS1 and r-EPS2). All of them were able to inhibit the proliferation of HepG-2 cells (liver cancer cell line), BGC-823 cells, especially HT-29 cells (colon adenocarcinoma cell line) to a different extent (Wang et al., 2014a,b). In addition, both r-EPSs possess antioxidant activity with r-EPS2 showing higher capacity than r-EPS1. The scavenging activity of EPS2 at 4.0 mg/cm$^3$ toward the hydroxyl radical was 69.81% and toward 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical −48.43%.

Hetero-EPS produced by *Lb. plantarum* YW32 also exhibits diverse activities. The EPS had antibiofilm activity toward *Shigella flexneri* CMCC (B), *Staph. aureus* AC1, *S. typhimurium* S50333 (inhibitory rate 44%−45%) and to a lesser extent toward *E. coli* O157 (inhibitory

rate 12.7%). Furthermore, an in vitro antitumor assay of the EPS showed that it had good inhibitory activity against colon cancer HT-29 cells. Antioxidant activity was also detected. At a concentration of 5 mg/cm$^3$, EPS had strong scavenging abilities toward hydroxyl (77.5%) and superoxide radicals (66.5%) (Wang et al., 2015). *Lc. lactis* ssp. *lactis* 12 produces heteropolysaccharide with strong antioxidant capacity. At 10 mg/cm$^3$, EPS exhibits similar scavenging activity toward superoxide anion (app. 80%) and slightly lower hydroxyl radical scavenging activity compared to Vit C. The antioxidant activity was confirmed by in vivo studies on mice. It showed increased serums antioxidant enzyme (SOD and CAT) activity and decreased malondialdehyde, a main index of lipid peroxidation, both in dose dependent manner compared with the control (Pan and Mei, 2010).

### 3.2.7 Bioactive peptides

Besides all the functionalities already described, LAB are also able to liberate biologically active peptides from proteins due to their complex proteolytic system, as *Lactobacillus* genus is the most cited LAB (de Castro and Sato, 2015; Pescuma et al., 2011; Sadat-Mekmene et al., 2011; Solieri et al., 2015; Wakai and Yamamoto, 2012). Many animal and plant proteins are constantly researched as sources of bioactive peptides but milk and dairy products remain the major source. The liberation of bioactive molecules from casein and whey protein can be resumed in three ways: (1) through hydrolysis by digestive enzymes, (2) through hydrolysis by proteolytic microorganisms, and (3) through the action of proteolytic enzymes derived from microorganisms or plants (Korhonen and Pihlanto, 2006) shown in Fig. 4.2.

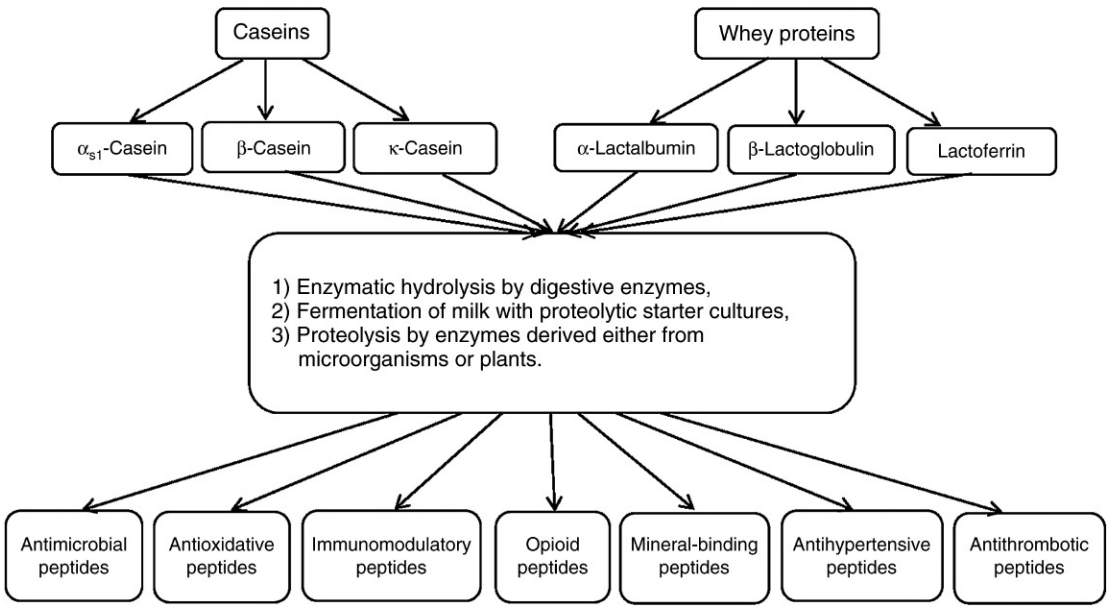


**Figure 4.2: Physiological Properties and Functionality of Milk-Derived Bioactive Peptides.**

The outcome of casein and/or whey proteins hydrolysis by LAB is a functionalized dairy product (Hafeez et al., 2014).

LAB release bioactive peptides with mainly immunomodulatory (El-Ghaish et al., 2011; Regazzo, 2012; Tellez et al., 2011, 2010), antihypertensive (Chang et al., 2015; Jauhiainen et al., 2010; Nejati et al., 2013; Qian et al., 2011; Solieri et al., 2015; Wakai and Yamamoto, 2012), antioxidant (Aleksandrova et al., 2013; Qian et al., 2011; Ramesh et al., 2012; Solieri et al., 2015), antimicrobial activity (Hayes et al., 2006), and mineral-binding activity (Aljewicz and Cichosz, 2015; Bergillos-Meca et al., 2015; Dimitrov et al., 2015) (Table 4.3).

High blood pressure is a common disorder in the Western lifestyle. It is a precondition for cardiovascular diseases. The angiotensin I-converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) has been classically associated with the renin-angiotensin system, which regulates the peripheral blood pressure. ACE raises the blood pressure by converting angiotensin I released from angitonsinogen by renin into the potent vasoconstrictor angiotensin II. ACE also degrades vasodilative bradykinin and stimulates the release of aldosterone in the adrenal cortex. Consequently, ACE-inhibitors may exert an inhibitory effect (Petrillo and Ondetti, 1982). Some food proteins contain special peptides that, after liberation, block the action of ACE. The best-studied milk-derived antihypertensive peptides are the tripeptides with amino acid sequence Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). The latest were isolated for the first time from fermented with *Lb. helveticus* milk. It was suggested that VPP and IPP act as ACE inhibitors in the aorta, where they may have a preventive role in the cardiovascular function (Yamaguchi et al., 2009). In addition, the long-term intake of fermented milk, containing VPP and IPP, may reduce arterial stiffness expressed as AIx in hypertensive subjects (Jauhiainen et al., 2010). The casein hydrolysis is initiated by LAB cell-envelope proteinase, resulting in oligopeptides formation (Griffiths and Tellez, 2013). The released peptides are further subjected to peptidase activities. Kilpi et al. (2007) demonstrated that the deletion of aminopeptidases PepN and PepX gen lead to a significant accumulation of ACE-inhibitory peptides. These peptidases are responsible for the hydrolysis of peptides (including VPP and IPP), which leads to lower ACEI activity.

VPP and IPP are also released by *Lactobacillus casei* PRA205 and *Lb. rhamnosus* PRA331 (isolated from cheese) during milk fermentation (Solieri et al., 2015).

ACE-inhibitory peptides are released either from fermented milk and cheese although the degree of hydrolysis of the latter is deeper. *Lb. helveticus* A1 was reported to release the peptide Ala-Leu-Pro-Met in Bulgarian white-brined cheese (Dimitrov et al., 2015). From Cheddar cheese produced with adjunct cultures *Lb. casei* ssp. *casei* 300 and *Lb. paracasei* ssp. *paracasei* 22 were also detected ACE-inhibitory peptides (Gupta et al., 2013). In Cheddar cheese (during ripening) by *Lb. acidophilus* LAFTIL10 were released many ACE-inhibitory

**Table 4.3: Bioactive peptides released from milk proteins by LAB.**

| Lactic Acid Bacteria | Precursor Protein | Peptide Sequence | Bioactivity | $IC_{50}$ | References |
|---|---|---|---|---|---|
| Cell-free extract of *Lb. helveticus* JCM1004 | Skim milk | VPP, IPP | ACE inhibitory | 9.13 ± 0.21 μM<br>5.15 ± 0.17 μM | |
| *Lb. jensenii* ATCC 25258 | β-CN | LVYPFPGPIHNSLPQN, LVYPFPGPIH; | ACE inhibitory | 71 μM<br>89 μM | Pihlanto et al. (2010) |
| *Lb. helveticus* A1 | β-CN, $a_{S1}$-CN | ALPM | ACE inhibitory | ND | Dimitrov et al. (2015) |
| *Lb. casei* C3 | β-CN, $a_{S1}$-CN | APFAK | ACE inhibitory | ND | Dimitrov et al. (2015) |
| *Lb. delbrueckii* ssp. *bulgaricus* J24 | β-CN, $a_{S1}$-CN | LGPVRGPFP | ACE inhibitory | ND | Dimitrov et al. (2015) |
| *Lb. acidophilus* LAFTIL10 | k-CN, $a_{s1}$-CN, β-CN | ARHPHPH, RPKHPIKHQ, RPKHPIK, RPKHPI, FVAPFPEVF, YQEPVLGPVRGPFPIIV | ACE inhibitory | 0.17 mg/cm$^3$<br>0.22 mg/cm$^3$<br>0.19 mg/cm$^3$<br>0.20 mg/cm$^3$ | Ong and Shah (2008) |
| *Lb. casei* PRA205 | k-CN, β-CN | VPP, IPP | ACE inhibitory<br>antioxidant activity | 54.57 μg/cm$^3$ | Solieri et al. (2015) |
| *Lb. rhamnosus* PRA331 | k-CN, β-CN | VPP, IPP | ACE inhibitory<br>antioxidant activity | 212.38 μg/cm$^3$ | Solieri et al. (2015) |
| *Lc. lactis* DIBCA2 | β-CN, k-CN, $\alpha_{S1}$-CN | LQSW, MFPPQSVLSLSQS, PEQSLVYP, LLYQEPVLGP, KPAAVRSPAQILQWQV, IHAQQK | ACE inhibitory | 5± 2 μg/cm$^3$ (for the fraction containing all the peptides) | Nejati et al. (2013) |
| *E. faecalis* strains | β-CN, $\alpha_{S1}$-CN | VVVPPF, VRGPFP, LHLPLP, VSKVKET, LHLPLPL, LQDKIHP, VRGPFPIIV, VLGPVRGPFP, LLRF, LKKYKVPQ | ACE inhibitory | 16.1–28.4 μg protein/cm$^3$ | Gútiez et al. (2013) |
| *Lb. acidophilus* DPC6026 | $\alpha_{S1}$-CN | IKHQGLPQE, VLNENLLR, SDIPNPIGSENSEK | Antimicrobial activity | | Hayes et al. (2006) |
| *Lb. casei* C3 | β-CN | SpLSpSpSpE | Calcium-binding activity | | Dimitrov et al. (2015) |

ACE, Inhibitory activity only; CN, casein.

peptides. They were identified as k-CN (f 96–102), $\alpha_{s1}$-CN (f 1–9; f 1–7; f 1–6; f 24–32) and β-CN (f 193–209) (Ong and Shah, 2008). In contrast to *Lb. casei* ssp. *casei* 300 and *Lb. paracasei* ssp. *paracasei* 22, most of the ACE-inhibitory peptides from *Lb. acidophilus* LAFTI L10 were accumulated at the early stage of ripening.

*Lc. lactis* DIBCA2 and *Lb. casei* FC113 showed the highest potential to release ACE-inhibitory peptides among 16 LAB strains used in milk fermentation (Nejati et al., 2013). The most potent ACE-inhibitory peptides released by *Lc. lactis* DIBCA2 were derived from β-casein, k-CN, and $\alpha_{s1}$-casein.

Milk fermented with *Lb. acidophilus, Lb. casei, Lb. jensenii,* and *Leuc. mesenteroides* strains, has also been reported to produce ACE inhibitory peptides. Two fractions were isolated from *Lb. jensenii* ATCC 25258 and identified as two β-casein peptides (Pihlanto et al., 2010).

*E. faecalis* strains are also able to release ACE-I peptides from milk (from α- and β-casein) (Gútiez et al., 2013).

Immunomodulatory peptides are peptide regulating cell-mediated and humoral immune functions. There are a variety of cells in the immune system and their external regulation is not well understood. In milk proteins, peptides with potentially immunoregulatory activity are encrypted, largely reviewed by Regazzo (2012). *Lb. acidophilus* ATCC 4356 and *Lc. lactis* ssp. *lactis* GR5 hydrolyzed casein to fractions that significantly decreased the basal NF-kB activity (involved in proinflammatory signaling pathway) in recombinant Caco-2 cell layers (Stuknyte et al., 2011). Four peptides (three derived from the hydrolysis of β-casein and one from the hydrolysis of α-lactalbumin) released by *Lb. helveticus* LH-2 in skim milk fermentation stimulated the in vitro production of TNF-α using murine macrophages RAW 264·7 cell line (Tellez et al., 2010). Moreover, these peptides showed an upregulation of TNF-α in mice and a decrease in *S. enteritidis* translocation compared to the control group (Tellez et al., 2011).

*Lb. acidophilus* and *Lb. paracasei* exerted an immunomodulating effect in the gut of BALB/c mice during the administration of Fresh cheese. *Lb. acidophilus* was mainly identified in the large intestine, whereas *Lb. paracasei*—mainly in small intestine (Medici et al., 2004).

Proteins and peptides also have excellent potential as food antioxidants by inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems (Elias et al., 2008). Casein hydrolysis with cell-free extract from *Lb. rhamnosus* NCDC24, *Lb. casei* ssp. *casei* NCDC17, and *Lb. paracasei* ssp. *paracasei* NCDC63 showed a significant ($P < 0.05$) increase in the radical scavenging activity (Ramesh et al., 2012). The reactive oxygen species levels within living yeast cells (used as indicator for the test) were significantly higher when a combined commercial yoghourt starter with either *Lb. delbrueckii* ssp. *bulgaricus* 287 (10-fold higher compared to commercial starter only),

and *Lb. delbrueckii* subsp. *lactis* 3559 (5-fold higher compared to commercial starter only), was used (Aleksandrova et al., 2013). *Lb. casei* and *Lb. rhamnosus* GG released peptides with antioxidant activities in Cottage cheese in terms of inhibition of DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals (Abadía-García et al., 2013). During milk fermentation by strain *Lb. casei* PRA205 or *Lb. rhamnosus* PRA331 (already reported to release ACEI peptides) an ABTS radical scavenging activity was detected (Solieri et al., 2015).

Milk is a rich source of minerals (mainly $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, and P) that must be first transformed into a soluble form. Certain LAB strains could increase mineral absorption by enhancing the release of casein phosphopeptides that act like chelating substances and are able to maintain these elements in a soluble state (Bergillos-Meca et al., 2015). *Lb. rhamnosus* HN001 significantly increased the availability of calcium, magnesium, and zinc from Dutch-type cheese after 6 weeks of ripening and potassium and phosphorus from cheese-like products as well (Aljewicz and Cichosz, 2015). *Lb. casei* C3 released calcium-binding peptide (fraction 15–20 of β-casein) in Bulgarian white-brined cheese (Dimitrov et al., 2015).

Casein-derived antimicrobial peptides (Hayes et al., 2006) and opioid peptides (Jarmołowska et al., 1999) by LAB were also reported.

## 4 Impact of Functional Food in Disease Prevention

LAB are very promising sources for novel products and applications. Food products supplemented with LAB strains with proven probiotic properties (having different biological activities) belong to the group of functional foods, which have undisputed human health benefits. The continuous search for new technologies and products with new functionalities emphasizes the potential of food fermentations, where the role of LAB is crucial for the manufacture of health.

The numerous definitions of functional foods have undergone a number of changes over the years. In 1984, the Japanese for the first time used the term "functional foods" and defined them as "Food products fortified with special constituents that possess advantageous physiological effects." The Japanese government allocated research funds for studying functional foods or food for specific health uses (FOSHU) (Kubomara, 1998). In Europe, researchers defined "functional food" using the following definition: "Food products can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases" (Martirosyan and Singh, 2015; Shimizu, 2003).

In the meantime, but mainly in the 1990s, a variety of terms, more or less related to the Japanese food for specific health uses, appeared worldwide. In addition to functional foods, these include more exotic terms such as "nutraceuticals," "designer foods," "pharmafoods,"

"medifoods," "vitafoods," and so on, but also the more traditional "dietary supplements" and "fortified foods" (Roberfroid, 2000). In 1989 was imposed the term, "nutraceuticals": "substance that is a food or part of a food that provides medical and/or health benefits, including the prevention and treatment of disease" or "a product produced from foods but sold in powders, pills and other medicinal forms not generally associated with food and demonstrated to have physiological benefits or provide protection against chronic disease" (DeFelice, 1995; Kalra, 2003; Roberfroid, 2000).

LAB are used as starters in beverages and fermented foods for a long time now because they can improve the nutritional, organoleptic, technological, and shelf-life characteristics. Recently, the use of functional starter cultures in food and beverage fermentation has been explored. These cultures have at least one functional property, contributing in the improvement of the fermentation process, enhancing the quality and safety of the end product, and conferring health benefits (Leroy and De Vuyst, 2004; Olivares et al., 2006).

Microorganisms considered as commercial probiotics, components of starter cultures in dairy products, are mainly representatives of *Lactobacillus* genus (*Lb. acidophilus*, *Lb. lactis*, *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. reuteri*, *Lb. delbrueckii* ssp. *bulgaricus*) and *Enterococcus* spp. (*E. faecalis*, *E. faecium*) (Shah, 2007). These species have also been incorporated into fermented foods as dietary adjuncts.

Probiotics have multiple and diverse effects on the host. Consumption of probiotic bacteria via dairy food products is an ideal way to reestablish the intestinal microflora balance. The main mechanisms of action of probiotic bacteria by which they improve mucosal defenses of the gastrointestinal tract include antimicrobial activity, enhancement of mucosal barrier function against ingested pathogens, and immunomodulation (Fig. 4.3).

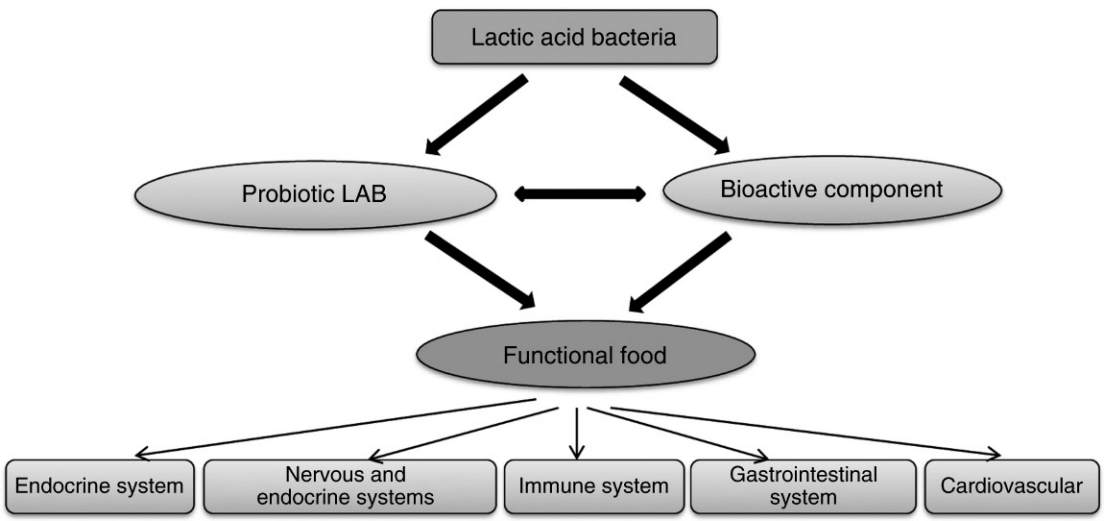


**Figure 4.3: Overview of LAB Application and Their Health Benefits.**

Probiotic LAB has potential health benefits in the following situations:

- *Diarrheal diseases* (infective, antibiotic associated, and *Clostridium difficile* associated diarrhea)—Probiotic LAB (mainly representatives of *Lactobacillus* genus)—*Lb. acidophilus*, *Lb. rhamnosus* GG, *Lb. reuteri*, *Lb. casei*, *Lb. delbrueckii* ssp. *bulgaricus*, were used for the treatment and prevention of different types of diarrhea. They reduce the severity and duration of diarrhea (Dinleyici et al., 2015; Gutierrez-Castrellon et al., 2014; Kale-Pradhan et al., 2010; Thomas et al., 2001).
- *Bowel diseases—Lb. rhamnosus* GG and *Lb. acidophilus* are probiotic LAB used in the treatment of inflammatory bowel disease (pouchitis, Crohn's disease, ulcerative colitis) and decrease of mucosal inflammations. They could stimulate the immune system of the gut and alleviates the symptoms of Crohn's disease and ulcerative colitis (Akoglu et al., 2015; Compare et al., 2015; Henker et al., 2008).
- *Prevention of colon cancer*—Probiotics may be attributable to a combination of mechanisms like the induction of pro- or antinflammatory and secretary responses that could inhibit carcinogenesis (Soccol et al., 2010). In vitro studies with *Lactobacillus* strains have shown antimutagenic activities (Rafter, 2003).
- *Helicobacter pylori*—A common chronic bacterial infection in human body, which causes many problems (gastritis, septic ulcers, etc.). Probiotics, such as *Lb. salivarius, Lb. casei* Shirota, and *Lb. acidophilus* and *Lb. johnsonii* have been shown to inhibit effectively the growth of *H. pylori* (Cats et al., 2003; Fujimura et al., 2012; Hamilton-Miller, 2003; Marteau et al., 2001).
- *Other diseases*—
  - *Lactose intolerance*: Probiotic LAB reduce the intolerance symptoms by improving lactose digestion.
  - *Blood cholesterol*: Strains of probiotic bacteria, such as *Lb. acidophilus, Lb. plantarum*, and *E. faecium* could significantly reduce blood cholesterol and increase the resistance of low-density lipoprotein oxidation, leading to a decrease in the blood pressure (Goel et al., 2006; Huang et al., 2013b; Liong, 2008).
  - *Atopic dermatitis*: Probiotics, such as *Lb. rhamnosus* GG can prevent or reduce the symptoms of atopic dermatitis (eczema) (Gerasimov et al., 2010; Isolauri et al., 2000; Yang et al., 2014b).

## 5 Conclusions

LAB isolated from various ecological systems or food products and possessing different biological activities and/or functional properties are reliable sources of natural, functional foods that meet consumers' nutritional requirements. They can be included in various diets to maintain human health and prevent diseases. More than a century ago, Prof. Mechnikov, the founder of gerontology, discovered a fact that LAB are not panacea but their daily usage is precondition for a better, healthy, and longer life.

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CHAPTER 5

# Development of Controlled Cocultivations for Reproducible Results in Fermentation Processes in Food Biotechnology

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## 1 Introduction

Definitions used in this text:

1. Pure culture: defined, single culture, proliferated under sterile conditions
2. Mixed culture: undefined culture of different microorganisms/organisms, under unsterile conditions, spontaneous fermentation
3. Coculture: defined mixture of different microorganisms/organisms, proliferated under sterile conditions

### 1.1 Development in Microbial Fermentation Processes

Mankind has used microorganisms in food technology for a very long time. Even before microorganisms were well understood, fermentation products have been consumed. In all parts of the world, many fermented food products have developed: including wine, beer, sauerkraut, kimchi, salami, cheese, fermented fish, coffee, cacao, sourdough bread, and many more. In all these examples, spontaneous fermentation of mixed cultures occurs. In most cases, microbial cultures originate from the applied substrates, the surrounding air, the water used, or the vessels and buildings in which the food is stored or matured. Knowledge of microorganisms began with the discovery of microorganisms by Antoni van Leeuwenhoek in 1683. In 1857, Louis Pasteur recognized that yeast cells are responsible for the alcoholic fermentation. Following this key discovery, fermentation technology developed very rapidly. Max Emil Julius Delbrück developed the first pure yeast culture in 1895. The first fed-batch fermentation process was developed in 1915. After propagation of pure yeast, the

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00005-0

fermentation of *Penicillium* sp. was conducted for the production of penicillin during the Second World War. Proliferation of microorganisms in pure cultures allowed for reproducible production in biotechnology and food technology in industrial scale. Today, beer and yoghurt, amino acids, enzymes for food or industrial application, and many drug substances are produced in pure culture fermentations. The utilization of one single microorganism under sterile conditions for the production offers the opportunity of a well-understood, controllable, and highly reproducible process for bioproducts synthesis. Today, the fermentation volume of sterile and pure cultures may reach volumes of up to 750,000 L. Especially in the pharmaceutical industry, reproducibility is strongly required to avoid the undesired side reactions due to altered metabolism of production microorganisms.

In the food industry, a reproducible fermentation process under sterile conditions offers advantages, such as automatable processes, constant flavor quality, consistent texture (mouthfeel), and predictable shelf life of food. Due to this advantage, the traditional wine production by spontaneous fermentation with mixed cultures was changed to a fermentation process inoculated with high concentrations of a single fermenting yeast strain. The high inoculation rate resulted in an inhibited proliferation of many natural yeasts and bacteria. Hence, the fermentation process could be better controlled. The disadvantage of this strategy was the poor flavor profile of the wine produced by the updated process. To overcome this, controlled cocultures of different yeasts and bacteria have been developed, resulting in a more well-balanced flavor profile, as well as a reproducible fermentation process. The strategy of coculture fermentations combines the advantages of reproducible pure culture fermentation processes with the flavor production of traditional mixed cultures. In many areas of food production, coculture fermentations are used or are being developed. In the following pages, we will summarize the actual examples of production and development in food biotechnology regarding controlled coculture fermentations.

Mixed cultures of microbes can be found everywhere in nature. In fact, pure cultures do not exist under natural conditions. Microorganisms can and do live in symbiosis with other microorganisms, or they can compete for the same environmental niche. In case of symbiotic coexistence, different microorganisms benefit from the combined enzymatic features in the mixed culture. Environmental conditions, such as pH or oxygen concentrations can be influenced by the mixed culture, enabling the existence of strictly anaerobic microorganisms in aerobic surroundings. The exchange of nutrients and other chemical substances can lead to the improved growth of microorganisms in coculture. Another example of a very successful symbiotic mixed culture is the wide family of lichen. There are more than 1500 lichens, which consist of coexisting fungi with green algae or cyanobacteria and potentially several other bacteria (Bates et al., 2011). These mixed cultures have proven to be in existence for at least 600 million years (Yuan, 2005). The prolonged existence of this symbiotic mixed culture shows the benefit for the partners. One more advantage in symbiotic cultures is the sustenance of vitamin B12 from bacteria to algae (Croft et al., 2005).

There are not only symbiotic coexistences in mixed cultures but also many different microbes compete for the same environment and the same growth substrates. Hence, different strategies have been developed to allow microbial species to protect their resources. Some bacteria produce large amounts of intracellular carbon storage molecules, for example, *Ralstonia eutropha* or oleaginous yeast. Other bacteria secrete large amounts of organic acids. As a result, the pH decreases and other bacteria are growth-inhibited, or even killed. Some microorganisms are secreting bacteriocides or fungicides, such as Nisin, Lactain, or antifungal proteins. Furthermore, microorganisms are able to communicate via quorum sensing (QS) molecules (Reuter et al., 2016). All these interactions indicate the complexity of coexistence in the microbial world. There is much research left to be done in order to achieve more insight into this promising field. Additional knowledge offers great opportunities for the development of new microbial substances in pharmaceutical and food technology by the use of mixed cultures or cocultures.

Examples for natural mixed cultures can be found in forest soil, in aerobic and anaerobic environments associated with natural water sources, plants, and the human skin. Besides the interaction between bacteria and plants via QS molecules or plant-induced degradation of QS molecules (quorum quenching) (Reuter et al., 2016), there are also interactions of bacteria with animal cells and humans (Ismail et al., 2016). An example of a very complex mixed culture is the digestive system of animals. In this environment, some microbial strains are able to degrade fructooligosaccharides, and the resulting monosaccharides can be utilized by many other bacteria. These microorganisms degrade polymeric substances, and they are able to synthesize vitamins and amino acids. Hence, these species are feeding their host.

## 1.2 Quorum Sensing

Microorganisms are able to communicate with members of their own species, as well as with other microbial species using so-called QS. The QS systems can also allow microbes to communicate with plants and even higher animals. Communication can proceed via different secreted chemical signal molecules or by direct interaction between the cells. *N*-Acylhomoserine lactone (AHL) is one of these secreted chemical messenger substances. It acts in the well-studied activation of bioluminescence in some Gram-negative bacteria. After secretion of AHL into the medium, it is taken up by the cells. AHL binds intracellularly to the LuxR proteins followed by the induction of the Lux operon and the expression of luciferase. Regulated genes are only induced when a high concentration of QS molecules are present, hence metabolic activities of the complete population are regulated by the cell concentration. This is only one example of autoinduction by the AHL within one microbial species. Other microorganisms are using different QS molecules. There are different AHL molecules within species of Gram-negative bacteria (Waters and Bassler, 2005).

There are QS molecules belonging to the autoinducer-2 group (AI-2). Examples are furanosyl borate diester (from *Vibrio harveyi*) and methyl-tetrahydroxytetrahydrofuran (from *Salmonella typhimurium*) (Waters and Bassler, 2005). The Gram-positive bacterium *Streptococcus* sp. secretes small-peptide pheromones for the interaction within and across species (Fleuchot et al., 2011). Other classes of QS molecules are γ-butyrolactones used by *Streptomycetes* sp. as autoinducing substances for the control of morphological differentiation and the production of secondary metabolites (Waters and Bassler, 2005). For yeast, some QS molecules like farnesol, tyrosol, phenylethanol, and tryptophol have been identified (Albuquerque and Casadevall, 2012). As previously mentioned, bacterial QS molecules can also react with plants. Sieper et al. (2014) reported the induction of genes for root development and systemic resistance in plants by the action of bacterial AHL. Furthermore, the host of commensal bacteria (e.g., animal model and/or human) is able to interact with bacteria via QS. Epithelial cells secrete QS molecules that mimic bacterial QS molecules, influencing the bacterial behavior and affecting the symbiosis between host and colonizing bacteria (Ismail et al., 2016). Similar results have been reported by Surette (2016).

The complex interactions of different microorganisms as described earlier offers the chance to develop new products and production processes in biotechnology, pharma, and food technology. Until recently, the utilization of mixed microbial cultures has been limited to the applications in environmental biotechnology (e.g., biogas production, bioremediation, or waste-water treatment) and traditional food production (e.g., fermented milk products, fermented vegetables, cacao beans and coffee beans, some alcoholic beverages, or fermented meat and fish). In all these examples, the mixed culture develops throughout the duration of fermentation. The initial microorganisms consume the easily degradable substances (monosaccharides, short-chain fatty acids, residual amino acids, etc.) and create environmental conditions that favor the proliferation of other microorganisms by secretion of products (organic acids or growth inhibiting substances), altering pH, limiting oxygen availability, or by other methods. The quality of produced food by mixed cultures is dependent on the growth substrate quality, which can be variable. Microorganisms associated with the substrate and the surrounding environmental conditions, such as temperature and humidity, and existing microorganisms can also affect the quality of the final food product. Fermented food products produced by mixed microbial cultivation or cocultivation and fermentation strategies for the controlled fermentation of cocultures will be summarized in the following. Furthermore, aspects of microbial interaction in food spoilage and risk assessment of microorganisms will be discussed.

### 1.2.1 Quorum sensing mechanisms in food production

One of the traditional fermentation processes in food industry is the sourdough fermentation. Other processes include the fermentation of vegetables resulting in kimchi or sauerkraut or the production of sour milk products like yoghurt or kefir. In all cases, organic acids are

secreted by the microorganisms involved in the production. Hence, the pH value decreases and the fermented food is protected against most forms of microbial spoilage. This is in large part because the organic acids produced have an inhibitory effect on microbial proliferation. In addition to this, organic acids can disrupt QS strategies. Almasoud et al. (2016) report the inhibition of QS of pathogenic *Escherichia coli* O157:H7 strain and *S. typhimurium* strains by lactic acid and malic acid. Due to the inhibited QS, virulence and survival of pathogenic bacteria in the food are reduced. Hence, utilization of organic acids as natural preservatives can increase food safety. These new findings indicate the importance of traditional food preservation by mixed culture or coculture fermentation processes, where organic acids are produced naturally. The influence of QS-based spoilage of fish is reported by Zhu et al. (2016). Hence, better understanding of regulatory functions involved in pathogenesis mechanisms may help to increase food safety.

Besides the aspect of food safety, the sensory properties of a fermentation product may also depend on QS. The molecule phenylethyl alcohol is produced by yeasts and exhibits a strong flavor but is also a QS molecule in yeast. The production of different flavor compounds is desired in many fermentation processes including beer, wine, or sourdough production. Even if a fermentation process has been conducted for several thousand years, there is still a demand to investigate the influence of different microorganisms on the flavor profile. Ripari et al. (2016) compared different single-strain fermentations with traditional fermentation processes and the chemical acidification during the production of white wheat sourdough. Newly isolated strains have been investigated regarding their production of volatile compounds. The authors observed very different flavor production profiles by the various individual strains. Over the duration of the fermentation, the pH profile and the rise of the dough were very different. The authors observed the formation of the flavor active compound phenylethyl alcohol during the sourdough fermentation with cocultures of different *Lactobacillus* strains and *Saccharomyces cerevisiae*. This substance has been described to act as a QS molecule for yeast, indicating the controlled metabolism of the yeast species during sourdough fermentation. The authors discuss the demand of further research in the field of QS in yeast to develop antifungal strategies, by potential inhibition of QS machinery. Some QS molecules seem to be produced only in cocultivation or mixed cultivation processes. Park et al. (2016) isolated 229 different bacterial strains of fermented kimchi. In the fermentation product, the QS molecule AI-2 was detected. However, when examined in monoculture, none of the isolated strains produced AI-2. The authors assumed that AI-2 production is the result of mixed fermentations only. Due to the influence of QS molecules on the formation of microbial virulence factors, biofilm formation, and food spoilage, further knowledge about QS, including the microbial inhibition of QS (quorum quenching), is required. Regulatory functions between different microorganisms must be determined. Thompson et al. (2015) reported the positive influence of elevated AI-2 concentrations in the gastrointestinal tract. Due to an increased concentration of AI-2, the population of Firmicutes was favored, and

the negative effects of antibiotic treatment could be minimized. Furthermore, the levels of AI-2 in the gastrointestinal tract have an effect on human health, due to the presence of these Firmicutes and one of their key fermentation products, butyrate. Many traditionally fermented food products prepared by mixed cultivation have been shown to contain AI-2 and/or active microorganisms that are able to produce AI-2 in the gastrointestinal system. QS-controlled mechanisms are not always activated with positive results. In some cases, virulence factors are expressed and secreted when the concentration of QS molecules reaches a threshold concentration. Cook et al. (2013) reported about the QS-controlled pathogenicity of streptococci. In this case, hydrophobic peptides act as QS molecules, not only between members of one microbial species but also via interspecies communication. The authors demonstrated that biofilm formation was controlled via QS molecules. Also in the pathogenic bacterium *Bacillus cereus*, QS is important for the secretion of toxins, hemolysins, and phospholipases. Several subgroups of *B. cereus* are able to communicate via QS across species. Better understanding of these regulations and communications can help to improve food safety and treatment of infected people.

All these findings indicate the importance of further research of cocultivations, mixed cultivations, and the occurring regulatory functions between the microorganisms. Furthermore, the development of controlled cocultivation processes should be promoted to increase the health benefits of industrially produced food products. A possible strategy for the controlled fermentation of cocultures has been published by Taniguchi and Tanaka (2004) (Fig. 5.1). This fermentation setup allows for controlled coculture fermentation without direct cell-to-cell interaction. Two bioreactors are connected via filters allowing interchange of fermentation fluid with all the requisite solubilized substances, while cells are retained in the bioreactor. In this way, QS mechanisms can be thoroughly investigated. Furthermore, relevant process parameters like pH value, oxygen availability, and temperature can be adjusted to the optimal values for each microorganism. Fermentation products of one culture can be utilized by the other culture. The disadvantage of this strategy is that direct cell-to-cell interactions are prevented. Hence, only QS-mediated gene activation can occur.

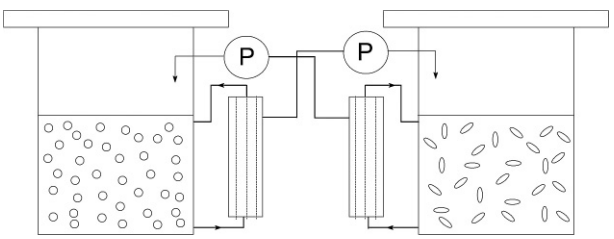


**Figure 5.1: Fermentation Setup for the Controlled Fermentation of a Coculture Without Direct Cell-to-Cell Interactions.**

*Adapted from Taniguchi, M., Tanaka, T., 2004. Clarification of interactions among microorganisms and development of co-culture system for production of useful substances. Adv. Biochem. Eng. Biotechnol. 90, 35–62.*

The development of a controlled cocultivation process in a single bioreactor requires a sophisticated strategy, based on many preexperiments, to determine optimal conditions for the production of the desired fermentation product. One possible strategy is presented in the Section 2.9.

### 1.3 Applied Detection Methods in Coculture Research

Metabolic processes and QS in mixed cultures and cocultures are of great interest for research in disease prevention and disease treatment, product development as functional food and safety with regard to the reduction of food-borne diseases. For these reasons and more, there is much research being conducted on QS and metabolic processes. Due to the complexity of metabolic pathways and interactions between microorganisms, highly sophisticated methods must be used to determine interactions between different microorganisms or between microorganisms and human cells. The complete metagenome of mixed cultures can be analyzed by high-throughput sequencing. This method has been applied in many published research accounts. Parente et al. (2016) developed an online database (http://www.foodmicrobionet.org/) to summarize the huge amount of public data related to the occurrence of microorganisms in dairy, meat, sourdough, and fermented vegetables from at least 33 different published studies. The number of incorporated publications on this topic is increasing steadily. The database allows easy access to the summarized data and offers a visualization of relationships between food matrices and identified microbes. Hence, it helps to cope with the increasing amount of data from modern analysis systems. Mayo et al. (2014) summarized the advantages of next generation sequencing in food production for the identification of beneficial or harmful microorganisms in food. Furthermore, they discussed the additional application of modern OMICS technologies, such as metabolomics, proteomics, and glycomics for the linkage between microorganisms and flavor formation. Trček et al. (2016) investigated the microbiome during vinegar production with denaturing high-pressure liquid chromatography (DHPLC) and next-generation sequencing. These examples demonstrate the increasing effort to gain additional knowledge about microorganisms or the complete microbiome that influences the food production process. Additional insight will help to increase food safety, allow for the development of controlled cocultivation processes, and may even help to gain further information about the links between food, microorganism, and human health. The application of these modern technologies also allows for the development of cultivation processes and the determination the enzymatic properties of microorganisms that are not culturable under standard conditions.

## 2 Application of Mixed Cultures and Cocultures in Food Technology

There are many examples of applied mixed cultures or controlled cocultures in food production, which will be summarized later. The wide variety of different products derived from microbial cocultivations is also presented in Fig. 5.2.

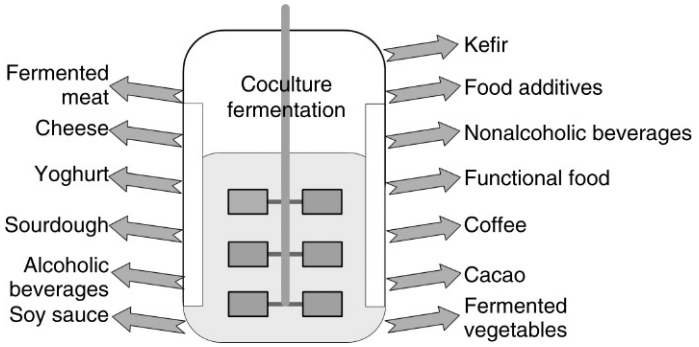


**Figure 5.2: Schematic Summary of Products Derived From Microbial Coculture Fermentation Processes in Food Technology.**

## 2.1 Coffee

There are different methods of coffee processing, but in all processes, a spontaneous mixed fermentation is conducted. In the dry processing, fruits are dried over a period of up to 20 days in the sun to achieve a residual moisture content of 12% (w/w). During the drying process, fermentation also occurs and aids in the removal of undesired fruit components from the beans. In the wet process, the pulp is removed from the coffee berries mechanically, and the berries are fermented in water. During this anaerobic fermentation process, which takes 24–48 h, solids are dissolved and can be washed away easily from the coffee bean. Beans are dried afterward to obtain a residual moisture content of 12%. In the semidry process, pulp is removed from the coffee beans and fermentation is conducted comparable to the dry process.

The three processes differ in fermentation conditions, as well as in the duration of fermentation. In the dry process, water availability is quite low and especially in the end of fermentation water activity is very low, promoting proliferation of yeasts. In the wet fermentation process, high amounts of water are present, enabling a rapid proliferation of bacteria and yeast. The dry process and the semidry process are at least partially aerobic fermentations while the wet process is an anaerobic fermentation, which results in the formation of different metabolites, flavor compounds, and flavor precursors.

These processes are spontaneous fermentation. This means, the quality of the end product relies not only on the substrate quality but also on the types of microorganisms that are prevalent on the fruits, in the air during the drying process, as well as the weather conditions. While altered flavor profiles may be a sign of manufactured food products, a reproducible quality is required for coffee production on an industrial scale. Therefore, the fermentation process should be controlled by inoculation with a microbial starter. Inoculation offers the possibility to avoid undesired proliferation of filamentous fungi and mycotoxin production. de Melo Pereira et al. (2014) report about the development of a suitable starter culture for the fermentation process. They isolated 144 yeast strains from a spontaneous coffee

fermentation and investigated whether these strains could cope with the stress conditions during the fermentation process. Nine yeast strains were selected for further experiments, comprising: *Candida* sp., *Saccharomyces* sp., *Hanseniaspora* sp., and *Pichia* sp. At the end, the authors suggest a strain of *Pichia fermentans* and *Saccharomyces* sp. for application in a preculture for coffee fermentation. They could show that flavor formation is strongly influenced by the application of these strains in wet processing. Evangelista et al. (2014a) conducted comparable developments for the dry process. They demonstrated that inoculation with selected yeast strains enables the production of specific flavor profiles. Furthermore, they presented data that indicated a reduction of microorganisms by washing prior to inoculation with the selected yeast species reduces the concentration of undesired organic acids and improves the sensory properties. The influence of different microorganisms for inoculation during the semidry process was also investigated by Evangelista et al. (2014b). They found that the overall evaluation of the sensory properties was not improved by the inoculation with the yeast compared to a spontaneous fermentation but they discuss the formation of coffee with a special and reproducible flavor by the application of a selected preculture.

### 2.2 Cacao Fermentation

During the production of cacao, the fermentation process with spontaneously developed mixed cultures is important for flavor production. It is the major processing step after harvesting the cacao fruit. A correct succession of yeasts, lactic acid bacteria, and acetic acid bacteria is required to achieve a high-quality product. During the fermentation process, plant cell walls are broken down, amino acids and reducing sugars are liberated and serve as precursors for flavor production via Strecker degradation and Maillard reactions during roasting processes. Ho et al. (2014) investigated the influence of prevalent yeast strains during the mixed fermentation of cacao beans. In these experiments, they inhibited proliferation of yeast and determined flavor production (esters, higher alcohols, aldehydes, and ketones). Without a mixed culture of different yeasts, cacao beans did not develop their typical flavor. In 2015, the same group published results about the influence of lactic acid bacteria on the fermentation of cacao beans. They compared fermentations with or without lactic acid bacteria and found no differences in flavor and quality of produced cacao beans (Ho et al., 2014). Even if lactic acid bacteria are not required for flavor production, it should be investigated if lactic acid bacteria increase food safety by the limitation of proliferation of undesired microorganisms due to low pH value, presence of lactic acid, possible secreted antimicrobial substances, and disturbed QS mechanisms (Section 1.2.1).

These findings indicate the importance of different microorganisms during the fermentation process. Further research is still required to achieve high quality cacao with pleasant aroma and reproducible flavor profiles. The impact of occurring microorganisms and the developing sequence during the 2–10 days fermentation process is investigated. There are many differences during processing steps that can influence the fermentation process.

Possible alterations are the duration of drying of the cacao fruits, soaking of beans prior to fermentation, and the fermentation process itself. Fermentation process can be conducted in heaps, in boxes, in barrels, on trays, or in baskets. Sizes of heaps or containers can vary and result in different temperature profiles and altered microbiota. Papalexandratou et al. (2011) compared microbial populations at different cacao farms in Ghana and Brazil with different methods of fermentation. The developing microorganisms were not dependent on the plantation, but large differences could be determined between heap fermentation in Ghana and box fermentation processes from Brazil. Due to the different microbiota, alterations in the concentrations of glucose, fructose, and mannitol as well as lactic acid, citric acid, and gluconic acid were also detected. Besides the fermentation process, the preparation of fermented beans also has a high influence on microbiota, produced metabolites, and cacao quality. Papalexandratou et al. (2011) found that a poor selection of beans results in a wide variety of homo- and heterofermentative lactic acid bacteria followed by the presence of undesired acidic flavor components. New breeding of cacao plants for increased resistance and higher productivity influence the succession of microbiota as well as the produced metabolites (Moreira et al., 2013). This alludes to the effect of substrate on spontaneous fermentation. Hence, alterations in substrates caused by different climate conditions, pretreatment strategies, or plant breeding will influence the flavor of the produced cacao. This is a disadvantage for industrial production of cacao with reproducible flavor profiles. To overcome this problem, cocultures were developed to achieve a reproducible fermentation process and a reproducible quality of cacao beans. Lefeber et al. (2012) presented results of an inoculation of cacao beans prior to fermentation with a controlled coculture of *S. cerevisiae* H5S5K23, *Lactobacillus fermentum* 222, and *Acetobacter pasteurianus* 386B. During fermentation these strains dominated, and reproducible qualities could be achieved independent of fermentation methods (heap or box) or the country (Ghana and Malaysia). For the development of a coculture based microbial starter, Papalexandratou et al. (2013) suggested a mixture of *Hansenula opuntiae* and/or *S. cerevisiae*, *L. fermentum*, and *A. pasteurianus*.

The development of microbial cocultures for the achievement of reproducible fermentation conditions was also reported by Meersman et al. (2015), and actual developments are summarized in a review article by De Vuyst and Weckx (2016).

### 2.3 Cheese

It is well accepted that microorganisms are expert biochemists, responsible for converting carbon feedstocks into product compounds. This tenet is most evident in cheese making, where microorganisms of all varieties are utilizing nutrients and compounds found in milk to produce a unique, valuable, and delicious product. Milk itself is a complex and nutritious agricultural product, and it provides a perfect medium on which microbial-mediated cheese conversions can occur. Cheese making is an 8000-year-old process, and has resulted in the

production of over 1000 varieties of cheese. The ingredients list for cheese is relatively simple: milk, microorganisms, rennet, and salt (Beresford et al., 2001). As suggested earlier, the milk and microorganisms bring complexity to this process; the milk because it is a natural beverage containing hundreds of different nutrients and bioorganic compounds, and the microbes because they have the cellular machinery needed to convert a subset of compounds in milk to necessary ingredients for cheese manufacture.

Microorganisms involved in cheese manufacture can be divided into different groups. Temporally, there are starter cultures and secondary flora. Starter cultures are either present in the milk (raw milk) or added at the beginning of cheese making and consist of a few well-characterized, industrial lactic acid bacteria (e.g., *Lactococcus*, *Lactobacillus*, and others). Additionally, microbial strains can be characterized as belonging to a group of defined starter strains (DSS) or wild microbes (Ayad et al., 2000; Beresford et al., 2001). The wild starter strains are by definition not well characterized. Also, it should be noted that most individual wild starter strains do not produce sufficient acid to make cheese. Thus, it is likely that a combination of wild strains in coculture is required for cheese production in most circumstances. Starter cultures can be mesophilic or thermophilic, depending on the cheese being produced. Cheeses like Cheddar, Gouda, and Blue use mesophilic starter cultures, whereas cooked hard cheeses like Parmesan and Gruyere use thermophilic starter cultures. Starter cultures of lactic acid bacteria are involved in acid production, an essential aspect of cheese making, and also contribute to cheese ripening. Secondary cultures contribute mainly to cheese ripening and are not involved in initial acid synthesis (Beresford et al., 2001). The ripening process is integral for the flavor development of cheese, and microbes play a central role in this. For example, the mold *Penicillium roqueforti* is responsible for the production of flavor compounds in blue cheeses, such as Stilton (Cantor et al., 2004). Also, the yeasts *Yarrowia lipolytica and Kluyveromyces lactis* are present in Stilton as secondary flora. Cheese produced with this flora tends to have flavor profiles associated with esters, ketones, and higher alcohols. These compounds are also associated with fruity (esters and alcohols) and "cheesy" (short-chain ketones) aromas (Price et al., 2014). Apart from the production of aroma compounds cocultures, particularly those of lactic acid bacteria and yeasts, have other benefits. The presence of lactose-fermenting yeasts in conjunction with *Lactobacillus acidophilus* has shown to reduce milk coagulation time due to increased acid production. Cocultures of this type have also shown to result in a higher number of viable lactic acid bacterial cells throughout the process, which is attributed to the stimulation of growth and viability by the yeast cells as well as the inhibition of organisms like *E. coli* and *B. cereus* during cheese production (Viljoen, 2001). The combination of wild strains and DSS has been used as starter cultures in pilot-scale cheese making. The cocultures of wild and defined strains produced unique flavor profiles, depending on the origin of the wild strains used. More specifically, the production of flavor compounds, such as methyl alcohols and methyl aldehydes, was shown to be enhanced in many pilot cocultures. However, the cocultures produced in this work were not always symbiotic. In a few cases, the wild strains introduced

bacteriocins, which resulted in the killing of the DSS. It is suggested that wild strains should be examined for their bactericidal activity prior to their use as part of a starter or secondary culture in cheese making (Ayad et al., 2000).

In cheese making, the microbiota must be monitored carefully, and the products (flavor and aroma) must be correctly attributed to the appropriate strain(s). To develop a clear picture of the cheese microbiome, a microbiologist can use phenotypic or molecular characterization. Phenotypic characterization of cheese microbiota must be preceded by cultivation, whereas for molecular characterization of flora, prior cultivation is optional and may depend on the strain being characterized. Karimi et al. (2012) examined techniques that have been used to selectively enumerate probiotic organisms present in various cheeses. Enumeration of probiotic bacteria in cheese includes enumeration of lactic acid bacteria, since these organisms are shown to be beneficial. The challenge is to enumerate each individual strain separately, and differential cultivation techniques can allow the microbiologist to do this. *Bifidobacterium* can be isolated on culture media containing 2 mg/mL lithium chloride. Taking advantage of the clindamycin resistance of some strains of *L. acidophilus* can allow for quantitation of these bacteria in cheese cultures. Thermophilic bacteria can be specifically cultivated at >40°C, and aerobic cultivation can allow for enumeration of organisms, such as *Streptococcus thermophiles* or anaerobic cultivation fosters enumeration of *Propionibacteria* (Karimi et al., 2012).

While it is important to enumerate the beneficial organisms that are associated with cheese production, there is much confusion about the health risks and benefits of raw milk cheeses. In the United States, retail sale of raw milk is generally not allowed, although on-farm sales of raw milk and raw milk products are acceptable, depending on the region of the country. However, the general view on raw milk is a negative one, and there is more information available on the risks of raw milk in the United States than the benefits. This is unfortunate because properly produced cheese from properly collected milk contains a diverse array of beneficial microorganisms. Over 400 species of bacteria, including lactic acid bacteria, yeasts, molds, and other organisms have been found in raw milk. Significantly, fewer organisms are found in raw milk cheese cores, where lactic acid bacteria have altered the conditions to make themselves numerically dominant. There is still an abundant variety of organisms present on the surface of raw milk cheeses, and all these organisms can impart many different flavor and aroma compounds. The general concern about raw milk and its products has to do with the presence of pathogenic organisms. There are compounds in the milk itself that act as antimicrobial agents, such as lactoferrin, the lactoperoxidase system, and the immunoglobulins. These can be effective in raw milk cheeses, but are rendered useless by pasteurization in other cheeses. Even raw milk cheeses are “safer” than raw milk itself, due to the decrease in pH, decrease water activity, and a ripening temperature of <15°C creating a hostile environment for most pathogens. Also, it should be known that raw milk contains an array of microbial strains that are antagonistic to pathogens. Lactic acid bacteria, first and foremost, are biopreservation agents as they deplete sugars while simultaneously lowering

the pH of the milk/cheese. As mentioned earlier, some species of lactic acid bacteria can produce bacteriocins, which can inhibit the most virulent of cheese pathogens (e.g., *Listeria monocytogenes, Staphylococcus aureus, S. typhimurium*). In addition to fighting and virtually eliminating pathogenic microbes, raw milk cheeses have shown to contain a balance of nutrients that allow for some gastrointestinal tract health benefits, mainly due to the activity of the wild microbial consortia present. It has been shown that raw milk cheese has a better balance of unsaturated lipids. In addition, unpasteurized cheeses still likely contain active growth factors and cytokines that may be beneficial for the development of gut epithelium and immune tolerance (Montel et al., 2014). On this last point, however, more evidence must be gathered to form a definite conclusion.

### 2.4 Yoghurt

Yoghurt is a dairy product that is often fermented with a defined mixture of starter culture bacteria. Specifically, the fermentation and interactions between *Streptococcus salivarius* (subsp. *thermophilus*) and *Lactobacillus delbrueckii*, when used as starter organisms, are well-documented. *S. salivarius* and *L. delbrueckii* stimulate each other's growth and readily exchange nutrients: *S. thermophilus* provides formic acid, folic acid, and fatty acids, while proteolytic activity by *L. delbrueckii* provides amino acids (Sieuwerts et al., 2010; Smid and Lacroix, 2013). The activity of this important microbial consortium results in the biochemical conversion of milk products to make yoghurt what it is, specifically: (1) conversion of lactose into lactic acid, (2) hydrolysis (proteolysis) of casein into peptides and amino acids, and (3) breakdown of lipids into free fatty acids. In conventional yoghurt, the proteolytic activity is performed by *L. delbrueckii*, allowing for *S. salivarius* feed off the proteolytic products and obtain the strain's amino acid requirements. Pilot tests of coculturing *L. delbrueckii* with a proteolytic strain of *S. salviarius* (Prt+ *S. thermophilus*) demonstrated that the two strains were not synergistic in their growth enhancing activities (Settachaimongkon et al., 2014). Coculturing of these two organisms has an effect on the concentration of aroma compounds, as well. Monocultures of either *S. salivarius* or *L. delbrueckii* had significantly lower concentrations of the aroma compound dimethyltrisulfide as compared to cocultures of the organisms (Smid and Lacroix, 2013).

To produce yoghurt, milk is heat treated (pasteurized) and then allowed to cool for the addition of starter culture, namely a mixture of *S. salivarius* and *L. delbrueckii*. The starter coculture ferments the lactose in the pasteurized milk, producing lactic acid as a result (www.milk.co.uk). Lactic acid gives yoghurt its characteristic tangy flavor. Another flavor compound that is produced during yoghurt fermentation is acetaldehyde, which is thought to be synthesized mainly by *L. delbrueckii* (Beshkova et al., 1998). As a result of acids being produced by the starter cultures, milk proteins coagulate and set, giving yoghurt its viscosity and creamy consistency. The gels made by the coagulated milk proteins are irreversible, in contrast to other food-based gels (Lucey, 2002).

As in cheese, the presence of lactic acid bacteria in yoghurt suggests that consumption of the product has health benefits. When containing live cultures of bacteria, yoghurt has long been used as a delivery for probiotic organisms, many of which played a role in fermentation of the product. It should be noted that the yoghurt starter bacteria, *S. salivarius* and *L. delbrueckii*, do not survive the passage through the gastrointestinal tract. Because of this, the trend is to add bacteria like *Lactobacillus casei*, *L. acidophilus*, and *Bifidobacterium* sp. to yoghurt to bolster its concentration and diversity of probiotic organisms (Ashraf and Shah, 2011).

Many studies have sought to demonstrate the health benefits of yoghurt consumption in a variety of ways. Yoghurt consumption has been suggested as a treatment for antibiotic-associated diarrhea (Beniwal et al., 2003), as well as for increasing the chances of eradication of *Helicobacter pylori* infection (Adolfsson et al., 2004). It has even been suggested that yoghurt has antitumor activity when consumed by a decrease in the inflammatory immune response (Perdigon et al., 1994), as well as potentially modulating cell proliferation during the development of colorectal carcinoma (Rachid et al., 2002). In "normal" (i.e., perceptively healthy) hosts, yoghurt was shown to stimulate immune cells that are associated with the gut (Perdigon et al., 1994).

### 2.5 Kefir

Kefir is a fermented beverage of ancient origin from the north Caucasus Mountains. It is prepared by the inoculation of milk (cow, sheep, goat milk, or other) with kefir grains (Altay et al., 2013; Satir and Guzel-Seydim, 2016). Kefir grains contain a coculture of bacteria and yeasts, as well as a water-soluble polysaccharide called kefiran. Kefiran adds a creamy texture and mouth feel to the final beverage product. Kefir grains are typically inoculated into pasteurized milk, incubated for ~24 h, and then removed. The "spent" kefir grains can actually be reused to inoculate a fresh milk culture, as they are still replete with primary culture organisms. In general, kefir grains contain a stable and specific microbiota that is encapsulated in polysaccharide and protein matrixes (Leite et al., 2013). These grains are a hotbed of microbial activity, with conditions specific to maintain a balance among the members of the coculture.

The kefir microbiota contains, among others, lactic acid bacteria and yeasts. These organisms are working in concert to promote each other's growth and survival. The yeasts in the culture are providing growth factors, vitamins, and amino acids to promote growth, whereas bacterial end products (i.e., organic acids, etc.) are used by yeasts for carbon and energy sources (Viljoen, 2001). Kefir is characterized by a flavor that is attributed to yeast activity, as well as the effervescence of the beverage is the result of $CO_2$ production. The main lactose-fermenting yeast found in most kefir grain samples are *K. lactis*, *Candida kefir* (*Kluyveromyces marxianus*), and *Debaryomyces hansenii.* Nonlactose fermenting yeast are also found, including *S. cerevisiae*, *P. fermentans*, and others (Leite et al., 2013). The bacterial

component of kefir grains includes *Lactobacillus* species, *Lactococcus* species, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, and others (Leite et al., 2012, 2013). The complex interactions among all the microbial species present in kefir grains are not well understood. These interactions are important, not only for the product, but also for the survival of each member of the coculture. It has been shown that, when the kefir bacteria are separated from the grain, the yeast grew at a poorer efficiency (Rattray and O'Connell, 2011). While under certain conditions, lactic acid bacteria could compete with yeast for nutrients for growth, in kefir culture, it is thought that the lactic acid bacteria stimulate the growth of yeasts by the production of vital nutrients (Leite et al., 2013).

Three different methods of producing kefir predominate. These methods vary, based upon the type of culture method used in production. In the artisanal process of kefir production, grains are inoculated into milk and fermented in a batch culture for up to 24 h. After this, the spent grains can be collected and reused. In a method known as the Russian method, fermentation in series is performed, using the percolate resulting from the initial fermentation. In the Russian method, sometimes the 1–7 days maturation phase is omitted, which results in a radically different flavor of beverage. In commercial fermentation of kefir, standardized inocula are used, mainly for homogeneity of flavor and mouthfeel of product. However, this type of commercial beverage, according to many, is not necessarily kefir, but a "kefir-type" beverage (Leite et al., 2013). Similar to yoghurt, kefir has been associated with antimicrobial properties and other health benefits. These benefits, however, are mostly anecdotal as few published accounts back up the claims.

### 2.6 Fermented Vegetables

There exist a wide range of fermented vegetables. In the Northeast Asian region, kimchi is a favorite product; in Germany, Sauerkraut and fermented cucumber; in Russia, Borscht; and in Great Britain, mixed pickles are produced. Also, pepper, onions, and carrots are being fermented. In Africa, lemons are processed by spontaneous fermentation. Fermentation of these vegetables and fruits has been developed to extend their shelf life, which was a necessity in the times before refrigerators and freezers were readily available. Today, these old technologies gain increasing interest due to their good digestibility, pleasant flavor, and health benefits. Also, the mechanisms of the improved shelf life have been investigated. Of course, the low pH value, low oxygen availability, high salt concentrations, and increasing concentrations of short chain organic acids are responsible for the resulting stability of the microflora present in the food products. Furthermore, it could be shown that certain lactic acid bacteria exhibit antifungal activity. Saladino et al. (2016) investigated an inhibition of *Penicillium expansum* and *Aspergillus parasiticus* by cell-free supernatants of lactic acid bacteria and bifidobacteria. For industrial production of kimchi, selected lactic acid bacteria have been developed as starter cultures to increase quality, functionality, and reproducibility. Also, the utilization of probiotic bacteria is desired to add functional and health benefiting

properties (Lee et al., 2016). In this context, the cocultivation of lactic acid bacteria and yeasts is of high relevance. Katakura et al. (2010) reported about the presentation of special surface proteins by lactic acid bacteria in the presence of yeast strains. These surface proteins are also responsible for the adhesion to intestinal epithelial cells.

## 2.7 Soy Sauce

Soy sauce is a traditional fermentation product that is produced in large scale and used worldwide due to its aroma and flavor-enhancing properties. As many other traditional fermentation products, soy sauce is produced by a mixed fermentation process based on two substrates: soy beans and wheat. Several microorganisms are incorporated in the two-stage fermentation process including fungi, lactic acid bacteria, and yeasts. Secreted enzymes from fungi hydrolyze polysaccharides and proteins in the first stage of fermentation. Hence the peptides, amino acids, and short chain sugars can be metabolized by bacteria and yeast in the second stage. During the second fermentation stage, many volatile flavor compounds are produced, especially by the yeasts. Strains of *Zygosaccharomyces rouxii*, *Candida versatilis*, *Candida etchellsii*, and *Pichia guilliermondii* have been identified as the most important producers of the desired flavor compounds (Wah et al., 2013). But there were more yeast found during fermentation as *Cryptococcus*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, *Trichosporon*, and *Zygosaccharomyces*. All of them can contribute to the total flavor of the final soy sauce. Bacterial fermentation partners were identified as: *Corynebacterium* sp., *Staphylococcus* sp., *Enterococcus* sp., *Lactobacillus* sp., *Weissella* sp., *Kurthia* sp., *Pediococcus* sp., and *Rothia* sp. (Yan et al., 2013). According to these authors, modern and culture-independent identification methods of participating microorganisms by PCR denaturing gradient gel electrophoresis and pyrosequencing have to be applied, in addition to the classical microbial quantification methods, to achieve sufficient knowledge for a reproducible fermentation process. Culture-dependent methods are insufficient because not all microorganisms from the complex mixed culture are able to proliferate under laboratory conditions. Some of them are capable of growing in mixed culture only. To achieve a reproducible industrial fermentation process, starter cultures of *Bacillus* sp. and/or *Aspergillus* sp. have been used in the first stage of fermentation, but the resulting fermentation product exhibits poor sensoric properties compared to traditional fermentation with a much broader range of microorganisms (Song et al., 2015). Due to the adverse effects of high salt intake on human health, attempts have been undertaken to reduce the salt concentration during the soy sauce fermentation process. When the salt concentration is reduced, undesired and even pathogenic microorganisms can proliferate during fermentation. Song et al. (2015) developed a fermentation strategy with two selected yeast strains to overcome this problem. Inoculation of low salt brine with *Torulaspora delbrueckii* and *P. guilliermondii* resulted in a fermentation process devoid of undesired microorganisms and a product with a comparable flavor profile as in high salt brine. This example shows that

the controlled cocultivation processes offer the opportunity to enhance the product without adverse effects on the sensoric properties. A variation in flavor and an inhibition of pathogenic microorganisms can be achieved by increased fermentation temperature (Wei et al., 2013).

Modern fermentation processes in food technology and even in biotechnology have developed from the traditional fermentation processes by mixed fermentations to sterile cultivation of monocultures. Advanced techniques will continue to improve understanding and quality of traditional fermented food products. Enhancing the quality and product properties requires the controlled cultivation of cocultures. The fermentation process of soy sauce is a very good example for this development, and is summarized by Zhu and Tramper (2013).

### *2.8 Alcoholic Beverages*

Alcoholic beverages have been produced worldwide since several thousand years. This suggests that mankind has utilized microorganisms for alcoholic beverage production for a long time, well before there was any knowledge about participating microorganisms. It can be assumed that all kinds of traditional alcoholic beverages have developed from nonsterile mixed cultures. Even today, many alcoholic beverages are still produced by mixed fermentation. Some examples of beverages derived from mixed fermentation or coculture fermentation processes are presented in Table 5.1.

Different kinds of wine, based on many fruits, are still produced by spontaneous fermentation. The required microorganisms are introduced to the process via the substrate. For example, fresh cut apples were analyzed regarding their naturally associated microbial population, and more than 200 different yeast strains could be identified from 25 apple samples (Graça et al., 2015). This high number of yeast strains indicates a great variety of microorganisms at the onset of wine fermentation process. During the course of wine fermentation, a selection of anaerobic, alcohol-tolerant microorganisms predominates but in the meantime a variety of different bacteria and yeasts contribute to the final flavor of the wine. A strong influence of different non-*Saccharomyces* yeast strains on flavor formation has been reported by Belda et al. (2016). Gobbi et al. (2013) observed different metabolic interactions between participating yeasts and bacteria, and these interactions strongly influenced the flavor of the wine. For the production of wine from grapes, there has been a development of the application of starter cultures of pure yeast strains to achieve a stable, reproducible fermentation process. Wines produced by this method were comparably poor in flavor (Capece et al., 2013) and the development of precultures with several different microorganisms has been conducted to achieve a stable, reproducible fermentation process without a reduction in key flavor substance concentrations (Terrell et al., 2015). Furthermore, a starter culture consisting of *S. cerevisiae* and *L. plantarum* has led to an improved malolactic fermentation, which resulted in improved quality of the wine (Berbegal et al., 2016).

**Table 5.1: Examples of alcoholic beverages produced by mixed cultures and/or cocultures.**

| Product Name | Substrate | Participating Microorganisms | Country |
|---|---|---|---|
| Arrack | Coconut | Yeasts, acetic acid bacteria | Sri Lanka, India, Philippines |
| Berliner Weisse | Barley, wheat | *S. cerevisiae*, *Lactobacillus* sp., *Brettanomyces bruxellensis* | Germany |
| Boza | Wheat, sorghum, corn | *S. cerevisiae*, *Lactobacillus* sp., *Leuconostoc* sp. | Turkey, Bulgaria, Romania, Albania |
| Burukutu | Sorghum | *Saccharomyces* sp., *Leuconostoc* sp., *Candida* sp., *Acetobacter* sp. | Nigeria, Benim, Ghana |
| Busaa | Corn | *S. cerevisiae, Lactobacillus* sp., *Penicillium damnosus* | Nigeria, Ghana |
| Bushera | Sorghum | *Lactobacillus* sp., *Weissella confusa* | Uganda |
| Cider, apple wine | Apples | *Kloeckera apiculata* and *Metschnikowia pulcherrima*, *Saccharomyces* sp., *Brettanomyces* sp., *Lactobacillus* sp. | France, Germany, Great Britain |
| Doro | Sorghum | Yeast and bacteria | Zimbabwe |
| Lambic-Bier | Wheat | *Kluyveromyces apiculata*, *Saccharomyces* sp., *Brettanomyces* sp., *Pediococcus* sp., *Enterobacteria* sp. | Belgium |
| Kaffir-Bier | Corn | Yeasts and lactic acid bacteria | South Africa |
| Khaomak | Rice | *Rhizopus* sp., *Mucor* sp., *Saccharomyces* sp., *Hansenula* sp. | Thailand |
| Kwass | Wheat or bread | *L. casei*, *L. mesenteroides*, *S. cerevisiae* | Russia |
| Mahewu | Corn or wheat | *Streptococcus lactis*, *L. plantarum* | South Africa |
| Mead | Honey | *S. cerevisiae*, *Pichia* sp. | Scandinavia |
| Merissa | Sorghum | *Lactobacillus* sp., acetic acid bacteria, *S. cerevisiae* | Sudan |
| Pito | Corn, sorghum | *Geotrichum candidum*, *Lactobacillus* sp., *Candida* sp. | Nigeria, Ghana |
| Rum | Sugarcane | *S. cerevisiae*, *Candida* sp., *Pichia* sp., *Hansenula*, *Lactobacillus* sp., *Leuconostoc* sp. | Worldwide |
| Sake | Rice | *Saccharomyces sake*, *Lactobacillus* sp. | Japan |
| Seketeh | Corn | *Saccharomyces* sp., *Lactobacillus* sp., *Bacillus subtilis*, *Aspergillus* sp., *Mucor rouxii* | Nigeria |
| Tapai pulut | Rice | *Hansenula* sp., *Chlamydomucor* sp., *Endomycopsis* sp. | Malaysia |
| Tequila/Mescal | Agave | *S. cerevisiae, K. marxianus, Zymomonas mobilis* | Mexico |
| Tupay | Rice | *Saccharomyces* sp., *Mucor* sp., *Rhizopus* sp., *Aspergillus* sp., *Leuconostoc* sp., *L. plantarum* | Philippines |
| Togowa | Sorghum | *Lactobacillus* sp., *Pediococcus* sp., *Weissella confuse, Issatchenkia orientalis*, *Candida* sp. | Tanzania |
| Wine | Grapes | *Saccharomyces* sp., *Kluyveromces* sp., *Brettanomyces* sp., acetic acid bacteria, lactic acid bacteria | Europe, Australia, Africa, USA |

Also, in beer production, mixed cultures and cocultures are applied. Famous examples are the Belgian Lambique (Lambic) beers that are produced by mixed fermentations. They are produced in traditional open fermentation vessels and flavor formation is strongly affected by microorganisms from the environment. This offers the advantage of the production of many different flavored beers, but reproducibility is not guaranteed. The transfer of the fermentation process to a different location may result in an altered flavor profile. The development of

a defined starter culture with relevant microorganisms can help to overcome this problem, enabling a location-independent and reproducible fermentation process.

### 2.9 Nonalcoholic Beverages

An increasing awareness in health and well-being leads to an enhanced demand for nonalcoholic and naturally produced beverages. The application of cocultures for the development of new beverages is very promising. Traditionally, Kombucha is a nonalcoholic beverage derived from a cofermentation process by acetic acid bacteria and yeast based on different teas and sugar. The disadvantage of the natural fermentation process is the presence of a thick biofilm that is produced during fermentation that complicates cleaning and sterilization of the fermentation vessel.

In the following, an industrially applicable fermentation process with lactic acid bacteria, acetic acid bacteria, and a yeast strain will be described, based on the results from Bader (2008). Beer wort was the substrate for the fermentation process. The desired product was a nonalcoholic, flavorful beverage produced by a cocultivation process. After the selection of suitable strains in pure culture, optimal process parameters (oxygen availability, pH value, temperature, inoculation rates, time of inoculation, and fermentation time) had to be identified in order to enable the cocultivation with the desired flavor formation. The combined metabolic capacities of the individual strains drastically improved flavor formation, as compared to pure culture fermentation.

One reason for this is the observed metabolic activity of a selected *Gluconobacter oxydans* strain. This strictly aerobic bacterial strain exhibited maltase activity, even under anaerobic conditions (Fig. 5.3).

Combining the achieved knowledge about optimal process parameters, the tolerance of fermentation products resulting from the fermentation process and the flavor production resulted in the fermentation strategy presented in Fig. 5.4. In the first phase, gluconic acid and the biomass, which included the maltase activity, had been produced. In the second stage, fresh beer wort was added, and during the following anaerobic biotransformation, the maltose was hydrolyzed, and glucose accumulated in the culture. At the beginning of the anaerobic stage, three selected yeast strains of *K. lactis* and *Lactobacillus* sp. were added. The high glucose concentration improved the flavor formation due to highly expressed acetyl transferases ATF1 and ATF2 (Verstrepen et al., 2003). Both enzymes catalyzed the intracellular ester formation, resulting in a pleasant, fruity flavor. Simultaneously in this fermentation, lactic acid was produced.

This controlled coculture fermentation resulted in a pleasant, fruity beverage on the base of beer wort, without the addition of fruit components or aroma substances. Production was conducted in a volume of 400 L (maximum). It is only one example of the potential of controlled coculture fermentation processes in food technology.

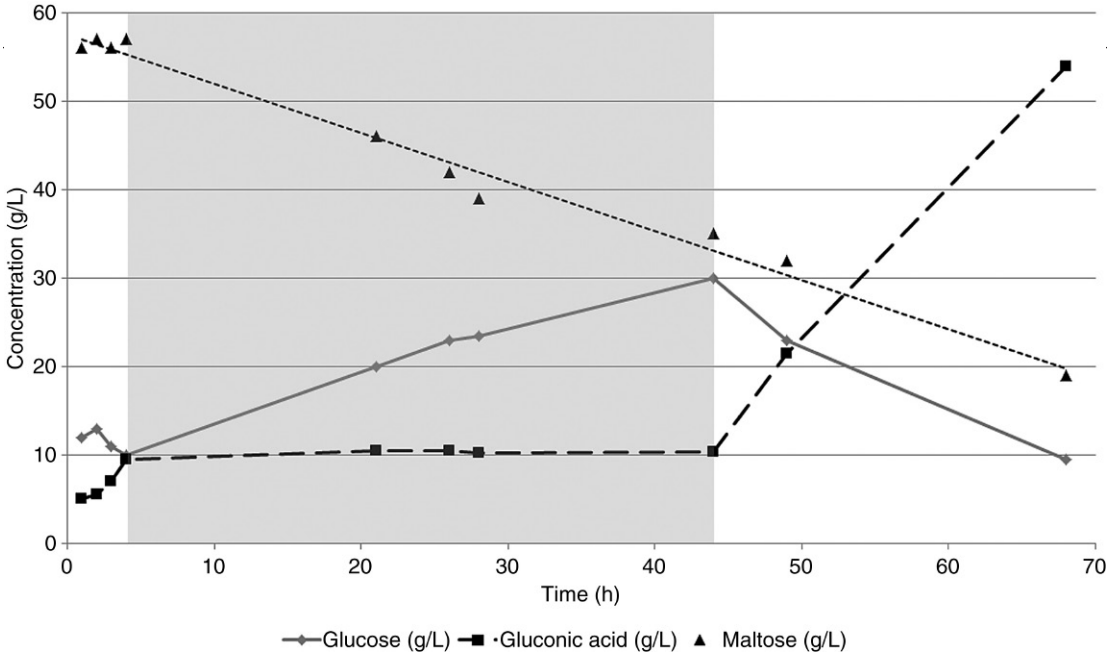


**Figure 5.3: Fermentation of *Gluconobacter oxydans* in 12% (w/v) Beer Wort at a Temperature of 26°C; Aerobic Fermentation Until 5 h, Anaerobic Conditions Until 44 h (*gray*), Followed by Aerobic Fermentation.**

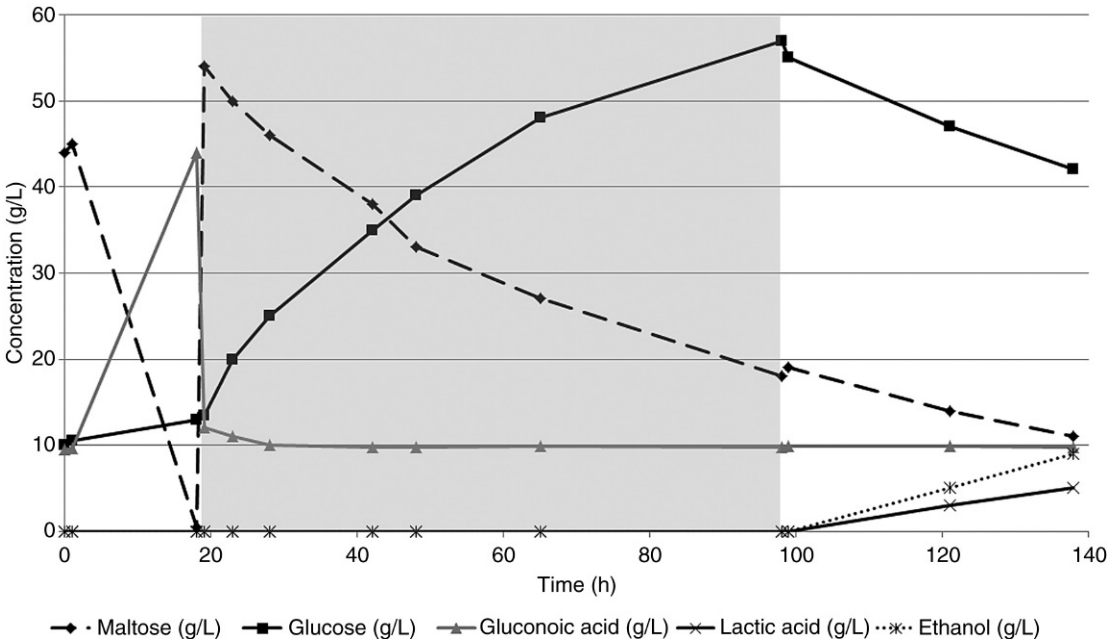


**Figure 5.4: Beverage Production by a Controlled Coculture Fermentation.**
Aerobic fermentation of *Gluconobacetr oxydans* until 19 h, afterward, addition of new beer wort and anaerobic fermentation until 98 h, inoculation with *Kluyveromyces lactis* and *Lactobacillus* sp. at 98 h; temperature = 26°C.

## 2.10 Sourdough

Flour is a nonsterile product, and many different microorganisms have been identified from spontaneous sourdough fermentation. De Vuyst et al. (2014) summarized data from 40 references from 26 countries and found the most dominant yeast species: *Candida humilis*, *Pichia kudriavzevii*, and *S. cerevisiae* and the most dominant lactic acid bacteria: *L. fermentum*, *Lactobacillus plantarum*, and *Lactobacillus sanfranciscensis* are present in sourdough cultures. The existing microbial culture is also affected by the antimicrobial properties of substrate components, for example, presence of polyphenols. Hence, there is a very complex interaction between microbial strains and the fermentation substrate, especially during the early stage of fermentation where many other bacteria and yeasts have been found. In the later stages, lactic acid bacteria and one or two yeast strains dominate. Overall, more than 60 different lactic acid bacteria have been identified till now (De Vuyst et al., 2014).

Different microorganisms are applied in mixed culture or in coculture for the production of sourdough. The sourdough fermentation exhibits several advantages compared to bread produced with nonfermented flour: the shelf life of the bread is increased due to the produced organic acids and consequently the decreased pH value. Digestibility is improved due to the reduced quantity of undesired polyphenols. High level of amino acids, phytase, antioxidative compounds, and soluble fibers are present in sourdoughs (Rizzello et al., 2016). Furthermore, additional flavor components are produced. Russo et al. (2016) reported more than twofold increase in Vitamin B2 production during fermentation with selected lactic acid bacteria, indicating the possibly enhanced vitamin concentration in sourdough-based baked goods.

For the utilization of rye for breadmaking, the acidification by the lactic acid bacteria is required to achieve the desired rise of the dough. Without acidification, the starch would be degraded by amylases, and there would be no carbon dioxide in the dough. Therefore, a result of no acidification would be only a flatbread with a very low loaf volume. There is an increasing interest in sourdough fermentations due to fact that sourdough fermentation can help to improve the quality of gluten-free products. Even though the application of sourdough fermentation processes has been used for a very long time, there is still a lot of actual research required in this field. The complex interactions between the microorganisms, QS, and the production of antimicrobial substances by fermentation partners are subjects of current investigations. Furthermore, consumers demand for more alternatives to industrially produced baked goods with improved sensorial properties, increased amount of health vitamins, amino acids, or probiotics. Sourdough fermentation can contribute to the development of these baked goods with increased nutritional functions. Fritsch et al. (2016) reported about the benefits of sunflower as an alternative base for bread production. The required reduction of polyphenols has been conducted by fermentation with selected lactic acid bacteria.

In commercial sourdough fermentation processes, starter cultures of the desired yeast and lactic acid bacteria are added to achieve a reproducible and rapid fermentation process. In many processes, *S. cerevisiae* and *L. sanfranciscensis* are used, but as discussed earlier, there are valid reasons to incorporate additional microbial strains to produce baked goods with functional properties.

### 2.11 Fermented Meat and Fish

Microbial cocultures are used for the production of several fermented meat and fish products that are consumed worldwide. One example of fermented meat is an Italian sausage (salami), which has an annual economic value of € 925 million (Montanari et al., 2016). Besides aforementioned salami, there are other fermented meat products like sucuk, chorizo, saucisson, pepperoni, and bubanita (Benkerroum, 2013). During fermentation, aroma compounds are produced from microbial activity on fatty acids and proteins. Furthermore, the microorganisms prevent proliferation of undesired or even pathogenic microorganisms. Hence, the shelf life of the meat product is increased, even under high temperature conditions. Traditionally, mixed cultures were responsible for the fermentation. Today, in many cases, starter cultures are being used to increase reproducibility and to decrease the risk of pathogenic bacteria. In many processes, lactic acid bacteria are used due to the resulting decrease in pH-value and the prevention of germination of endospores of potentially pathogenic strains of *Bacillus* sp. and *Clostridium* sp. Fermentation of the sausage is controlled by environmental conditions, as well as the ingredients present in the sausages or even the diameter of the sausages (Montanari et al., 2016). These parameters influence the aroma production. Of course, the applied microorganisms also drastically influence the aroma profile. Domínguez et al. (2016) investigated the influence of different cocultures on the quality of fermented sausages. They found that the utilization of starter cocultures restricted the proliferation of undesired Enterobacteriaceae, which were found in the product that was derived from spontaneous fermentation.

Özer et al. (2016) investigated the ability of several *L. plantarum* strains to produce conjugated linoleic acid during sucuk fermentation. During their work, they selected one strain that demonstrated an increased conjugated linoleic acid production content during mixed culture fermentation, when was applied as a starter. Kantachote et al. (2016) selected microbial strains for the purpose of reducing the concentration of biogenic amines and cholesterol in a Thai fermented pork sausage (Nham) during fermentation with a coculture of *Pediococcus pentosaceus* HN8 and *Lactobacillus namurensis* NH2. Both examples of current research indicate the potential of improved food production by selected strains in coculture fermentation.

Besides sausages, microbial fermentations are also applied for the production of ham or during the dry aging of meat. Proteolytic and lipolytic enzymes contribute to the desired

structure of the produced end product. Additionally, flavor formation is catalyzed by the microorganisms, and undesired germs are restricted. The same goals are being pursued during the fermentation of fish. The traditionally conducted fermentation of fish in China is dependent on the microbiota in the environment. Therefore, starter cultures have been developed to increase food safety. Zeng et al. (2014) isolated naturally occurring strains for improved mixed fermentation with decreased formation of biogenic amines during the fermentation of fish. Comparable work has been reported by Saithong et al. (2010). These authors developed a coculture of *L. plantarum* and *L. reuteri* to suppress pathogenic bacteria and to increase the rate of fermentation of a traditional Thai fermented fish. Cocultivation of both strains was necessary to prevent pathogen bacteria during the fermentation process.

The presented results showed that an increase in food safety is achieved by applying cocultures as starter cultures in meat and fish fermentation. Growth of pathogenic bacteria can be inhibited and biogenic amines can be reduced. Furthermore, the fermentation process can be accelerated.

## 2.12 Food Additives

There is a high demand for flavor substances and pigments for application in food, feed, beverages, cosmetics, and pharmaceutical products (Gupta et al., 2015). Due to the increased consumer awareness about natural products, natural flavors and pigments are preferred. Historically, many flavor substances and pigments are extracted from plant material. The disadvantages of this method are the limited availability of desired plant material and the high price of the products.

Biotechnological processes offer the possibility to produce natural flavor substances. Biotechnologically produced flavor substances may be declared "natural" when the substrates and the products are also found in nature or in food (Vandamme and Soetaert, 2002). In food production processes, the advantage of flavor production is applied in many instances that improve the flavor of the fermented substrate. These fermentation processes can serve as source of potential microbially produced aroma components. Longo and Sanromán (2006) summarized more than 100 important substances that are relevant for the aroma of food products. Especially, cocultivation processes allow the production of a wide variety of flavor substances. One example has been presented in Section 2.9.

Examples for the utilization of coculture processes for the production of flavor substances are presented later. The cocultivation of *Trichosporon asahii* and *Paenibacillus amylolyticus* with the carotene lutein resulted in the production of components of tobacco flavor (Rodriguez-Bustamante et al., 2005). The cocultivation of both microorganisms enables the rapid transformation of the toxic intermediate β-ionone to the nontoxic end products 7,8-dihydro-β-ionone, 7,8-dihydro-β-ionol, and 3-hydroxy-β-ionone (Maldonado-Robledo et al., 2003).

Another report of the utilization of a coculture, consisting of *Geotrichum* sp. and *Bacillus* sp., for the production of tobacco flavor components has been published by Sanchez-Contreras et al. (2000).

One example of natural coloring agents used in food industry is the carotenoids. Besides their color, they exhibit antioxidant properties with the advantage of the prevention of undesired oxidation reactions that result in off-flavor formation in food products. Natural sources of carotenoids are plants, and carotenoids can be produced by extractions, but they can be also produced de novo by microbial coculture fermentation processes. Biotechnological production processes compete economically with the extraction processes from plants. Hence, the utilization of a low-cost substrate is beneficial. One example for the production of carotenoids using a low-cost substrate is the cocultivation of *Rhodutorula glutinis* and *Debaromyces castellii* on corn syrup. In this coculture, *D. castellii* is responsible for the hydrolyzation of the corn syrup-derived polymers, and *R. glutinis* synthesizes the carotenoids (Buzzini, 2001). Whey filtrate can also be used as an inexpensive substrate for carotenoid production by coculture fermentation. Simova et al. (2003) reported about the utilization of this substrate with *R. rubra* and *L. casei*. Lactose was hydrolyzed by the lactic acid bacteria, enabling the utilization of liberated monosaccharides by the yeast. Similar results have been published by Frengova et al. (2003).

It can be assumed that additional coculture processes will be developed for the production of food additives due to an increasing demand of natural flavors and pigments. One promising substrate for the flavor production could be lignin, due to its complex structure and wide variety of cleaving products. This substrate may require specialized microbial strains for hydrolyzation and other microbial partners for the flavor production based on the hydrolysis product. One example of a flavor substance derived from lignin is vanillin which can be produced biotechnologically (Carroll et al., 2016). As described earlier, the coculture process may prevent the accumulation of toxic cleavage products during this fermentation process.

### 2.13 Functional Food

Microbial processing, especially by cocultures, offers the possibility to increase the concentration of vitamins and amino acids, and to reduce undesired components like phenolic substances, to improve flavor, to increase the antioxidative potential, to prevent proliferation of undesired microorganisms, and to incorporate living microorganisms in food products. Because of these possibilities, the application of controlled cocultures offers the possibility to develop food with additional health benefits. The application of living microorganisms is found to be very promising for the development of new functional food.

Microorganisms strongly affect the human health and as a result of this strong influence, the coexistence and interaction between humans and microorganisms continues to be investigated

by many groups, in many research projects. The following paragraphs provide only a rough overview about the actual research in this field. Some examples are presented as a result of the strong connection between food industry, food intake, microbiome in the intestinal system, and health.

Several authors have reported about the influence of microorganisms on the development of severe human diseases. Erdman and Poutahidis (2015) summarized the effect of a complex mixed culture of several hundred (possibly even more than 1000) different bacterial strains on the development of cancer. Some of the existing bacteria can trigger colonic and also prostate gland and mammary, tumor development in mouse models (Poutahidis et al., 2013). Also in mouse models, a cancer preventing effect of gut bacteria was found. Some of the bacteria can stimulate the immune system. In this way, the resistance against mammary cancer could be increased (Lakritz et al., 2014). Furthermore, a transgenerational effect of dietary microbes on cancer formation and development was reported by Poutahidis et al. (2015). The authors suggested the intake of beneficial bacteria aids in the reduction of cancer development. This claim must be investigated in further experiments to determine whether these results can be transferred from a mouse model system to humans.

The influence of the microbial mixed culture in the intestinal system on the development of inflammatory bowel disease is summarized by Wlodarska et al. (2015). In other review articles, further connections between microorganisms and human health are presented. Yurkovetskiy et al. (2015) wrote about the influence of microorganisms on autoimmune responses and interaction with hormones. Also, allergies were found to be influenced by microbial mixed cultures. Scientific results in this regard are presented in the review article of Fujimura and Lynch (2015). There is also a strong connection between food intake, quality of food, and gut microbiome. The microbiome influences the development of obesity, which can be the reason for further sicknesses as cancer or cardiac disease. The metabolic function of microorganisms on obesity is reviewed by Dao et al. (2015). Besides the already mentioned illnesses, the development of diabetes also seems to be dependent on the microbiome (Guelden et al., 2015). However, not just the health is affected by the microbiome; the development of the human brain is also influenced by the complex microbial mixed culture in the human intestinal system (Sampson and Mazmanian, 2015).

Due to the enormous complexity of the microbiome, it is very difficult to determine the exact influence of single strains on the health status of humans. Furthermore, it is assumed that the influences on the human body are caused by microbial communities, not just by single strains. Food intake and other external factors including stress or drug intake may influence the microbiome (Yurkovetskiy et al., 2015). An actual strategy to treat humans with a disorder in the intestinal microbiome is to transplant complete microbial cultures from healthy people. In mouse model, this technique has been proven such that the transfer of the microbiome between twins may result in decreased body weight of the former obese twin

(Ridaura et al., 2013). One of the interesting question arises is if the transplanted microbiome has to be adapted to the new host to reduce the risk of undesirable side effects. In order to find the answer to this question, many studies are still ongoing. Due to the very complex interactions between microbiome and the human body, much more research is required in this promising field related to nutrition, microbiome, and health. High-throughput technologies and modern analysis techniques are being used to identify the mechanisms of interaction between a microbiome and its host. In recent years, much research has been performed on this topic, and many results are being published, a fraction of which are mentioned in this chapter.

Additional knowledge in this subject may help to prevent diseases. In Europe, further knowledge may also lead to the acceptance of health claims related to probiotic functions by the European Food Safety Authority (EFSA).

As of now, no health claims for a beneficial effect of probiotic microorganisms have been approved. There are still too many unanswered questions regarding the interaction of single microorganisms or cocultures with the microbiome and the human body. Furthermore, a detailed characterization of the probiotic microorganisms is required. For the production of probiotic compounds, it has to be determined if fermentation conditions, processing conditions, and storage conditions influence the probiotic properties. The development of defined cocultures as probiotics has a huge impact on the development of new functional food and also on medicine. Legislation about the advertising of health benefits of food is very strict in many parts of the world to protect consumers from unproven promises or even undetected side reactions. Furthermore, the development of functional food requires answers to the open question, if a food with proven health benefiting properties is still food or if it is a drug. Also, the dose of active components taken up by the consumer is difficult to quantify, due to very different quantities and variations of ingested food.

## 3 Conclusions

Industrial production of many fermented food products has led to the development of reproducible fermentation processes by single cultures. The disadvantage of this production method is the reduced aroma production and the decreased variety of individual fermentation products.

Controlled coculture fermentation offers many possibilities to produce food products with improved properties including digestibility, flavor, and perhaps even health benefits. This kind of fermentation combines the benefits of traditional, uncontrolled mixed fermentation with the required reproducibility for industrial food production. The foods that are produced by these methods meet consumer demands with a variety of added flavors. As summarized in this publication, reproducible coculture fermentation processes can be developed and utilized for food production.

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CHAPTER 6

# Enumeration and Identification of Probiotic Bacteria in Food Matrices

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## 1 Introduction

The use of probiotic bacteria in food products is expected to be of high therapeutic value, which depends on the dose of probiotics. Based on clinical studies, it is claimed that $10^6$–$10^9$ CFU/g of the product is the minimum number of cells of probiotic bacteria, which may have therapeutic values. So, the high number of cells of probiotic bacteria need to be maintained throughout the shelf life of the food, as the lower concentration of bacteria will have weak impact on the human body and will be less effective (FAO/WHO, 2002). Viability is generally considered as a prerequisite for optimal probiotic functionality (Maukonen et al., 2006). Many studies have shown that the viability of bacteria is not a simple question whether the cells are dead or alive. Traditionally, plate counting has been the method of choice for viability determination, but there are obvious disadvantages, such as relatively long time is required for the growth of colonies. The viable plate count method can be frustrated by clumping, inhibition by neighboring cells, and composition of the growth media used. Also, the media used for enumeration are not as selective (Davis, 2014; Karimi et al., 2012).

Therefore, there is a need to identify probiotic bacteria in food at strain level and also needed for qualitative (viable cells of probiotic bacteria) and quantitative analysis (adequate number of cells). Food acts as a complex and dynamic environment to which physicochemical parameters directly affect the growth of microorganisms. Enumeration and identification of probiotic bacteria at the strain level of the environmental matrix, that is often microbial heterogeneous, proves to be quite a laborious and costly process. Furthermore, the identification of bacteria at the strain level is necessary for testing probiotics strains introduced to distinguish between naturally occurring in the test environment, and to their use in the industry, for example, for the protection of patented strains (Amor et al., 2007).

To use nutrition or health claims on the packaging of their products, the producers of probiotic food should confirm the presence of this strain in food in sufficient numbers


http://dx.doi.org/10.1016/B978-0-12-811443-8.00006-2

along with the supporting documentation of its prohealth effects. In traditional methods for identification of bacteria, such as *Lactobacillus* and *Bifidobacterium*, the cell morphology observation, analysis of fermentation products, and possible use of carbohydrate substrates, tend to be still insufficient. The researchers emphasize the lack of reproducibility and high dependence on the culture conditions (Tannock, 1999a). Culture methods are also inadequate because in the dairy industry especially, the product in addition to probiotic bacteria contains starter culture bacteria cells, with similar nutritional requirements.

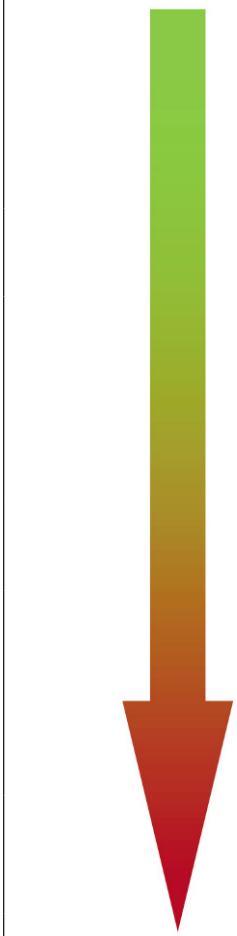
Presently, there is no standard technique reported to selectively enumerate probiotic bacteria in the presence of starter and nonstarter lactic acid bacteria (LAB) in food. To enumerate and to correctly identify the probiotic bacteria in mixed population, selective media should be used that would allow the growth of the organisms of interest and inhibit other microorganisms encountered in a particular food product (Davis, 2014) and also other advanced techniques should be used to identify the species. According to the FAO/WHO (2002), classification of strains should be made with a reproducible method of using a unique genetic or phenotypic characteristic.

The aim of the work is to provide different methods of enumeration and identification of probiotic bacteria in food according to the culture-dependent and culture-independent techniques, such as phenotyping, biochemical, physical, immunological, and molecular biology methods. In particular, the direct identification potential of molecular tools, such as DNA, RNA, and peptide analysis will be highlighted.

## 2 Methods of Enumeration of Probiotic Bacteria

In purpose to accurately enumerate live probiotic bacteria, scientific consensus on the definition of a viable microbial cell is paramount. The scientific community typically considers a cell "viable" if it reproduces to form a colony on an agar plate that supplies key nutrients for the strain. Standard culture techniques are commonly used to quantify probiotic strains, but cell culture only measures replicating cells (Davis, 2014). In addition, many possible probiotic effects of bacteria depend on activity rather than culture-ability, and even dead cells can have some probiotic effect, such as immunomodulation. The existence of cells in viable but nonculturable (VBNC) or active but nonculturable (ABNC) state, that is, transition of bacteria in adverse living conditions in the sleep state, was indicated for the first time by Roszak et al. (1984). The term VBNC describes unsatisfactory cells in physiological state, that is, the cells are alive, but do not divide, which means the cells do not exhibit the ability to grow and create colonies on culture media. Stressed or injured cells may reestablish replication capacity through repair mechanisms, opposite to dead bacteria (Lahtinen et al., 2008; Olszewska and Łaniewska-Trokenheim, 2011). Because standard culture-dependent methods enumerate only replicating cells, so culture techniques may underestimate the number of viable organisms that contribute to the functional capacity of the probiotic

**Table 6.1: Major physiological states of probiotic strains.**

| Physiological State | | Phenotype |
|---|---|---|
| Viable (live) |  | Intact cytoplasmic membrane, functional synthesis of protein, and other cell components (nucleic acids, polysaccharides, etc.) and energy production necessary to maintain cellular metabolism and eventually, growth and multiplication. |
| Culturable (replicating) | | Capable of division; will form a colony on agar plate or proliferate observably in liquid medium. |
| Nonreplicating (in stationary phase; inhospitable conditions for replication; injured) | | Will not form a colony on an agar plate nor proliferate observably in the liquid medium; but may have active physiologic activity and intact cytoplasmic membrane. Cells may be inhibited by the medium or injured but capable of repair (Le et al., 2008). |
| Starving | | Cells undergo remarkable decrease in the metabolism, but remain fully culturable (Mahdi et al., 2012). |
| Dormant (viable but not culturable) | | In a state of low metabolic activity and unable to divide or to form a colony on an agar plate without a preceding resuscitation phase. A protective response. Also seen in "postacidification" (Lahtinen et al., 2008; Shah, 2000). |
| Irreparably damaged cells | | Will not grow with vigor under any conditions due to progressive metabolic decline. These cells may be irreparably injured (Le et al., 2008). |
| Nonviable (dead) | | No metabolic activity. |

*Source: According to Davis, C., 2014. Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. J. Microbiol. Methods, 103, 9–17.*

preparation once constituent microbes reach the anatomical niche in the host to which they are well-adapted. Moreover, other various states have also been identified in probiotic strains (Table 6.1).

Davis (2014) proposed that the definition of live, viable probiotic microbes should be extended to encompass all microbes in the population that are metabolically active and/or have intact membranes. These strains still possess the capability of exhibiting beneficial function(s) when reacclimated to the host environment. Instead of identifying only those cells that are live as those that are capable of forming septae and accumulating biomass to

form a colony when isolated on appropriate artificial media, the new operational definition encompasses the various states that an organism may need to go through before progeny are formed. Obtaining information about all individual bacteria and their physiological status is relevant (Kramer et al., 2009).

Standardized methods are available only for a limited number of species in certain dairy products, such as publications from the International Organization of Standardization (ISO) regarding enumeration standards for *Lactobacillus acidophilus* (ISO 20128/IDF 192:2006) (ISO, 2002) and *Bifidobacterium* (ISO 29981/IDF 220:2010). There is no one single medium and/or set of techniques for isolation of the strain, which is applicable to all probiotic strains (Vinderola and Reinheimer, 1999). Moreover, the International Scientific Association for Probiotics and Prebiotics recognized that culture-based analysis of strains can underestimate the number of viable cells and fails to account for the impact of bacterial growth modes (Champagne et al., 2011).

### 2.1 *Selective Enumeration of* Bifidobacterium

Lactobacilli MRS (Mann–Rogosa–Sharpe) agar is probably the most widely used base plating medium for pure cultures of bifidobacteria and lactobacilli. The MRS agar is frequently used as a reference medium when *Bifidobacterium* is the only live culture present (Blanchette et al., 1996). For the determination of *Bifidobacterium* counts, 0.05% L-cysteine.HCl was added to MRS medium as a reducing agent to provide more strict anaerobic conditions necessary to stimulate bifidobacterial growth (Van de Casteele et al., 2006).

Also NPLN components are widely used for the selective enumeration of bifidobacteria. NPLN-agar and MRS-LP media have been prepared by the supplementation of lithium chloride and sodium propionate or by the supplementation of lithium chloride, sodium propionate, and L-cysteine.HCl (Ashraf and Shah, 2011; Ibrahim et al., 2010). Neomycin sulfate and nalidixic acid are included as growth inhibitors of Gram-positive and Gram-negative rods, respectively, and also lithium chloride is commonly used as a selective agent in bifidobacterial enumeration (Roy, 2001).

The choice of culture medium and methodology for the selective enumeration depends strongly on the product matrix, the target microbe, and the diversity of the background microbiota in the product (Van de Casteele et al., 2006). In study of Darukaradhya et al. (2006) numerous selective media were tested for the enumeration of *Bifidobacterium* spp. in combination with starter cultures and other probiotics in Cheddar cheese. Differences were noted in the selectivity and recovery of bifidobacteria among media. The media MRS Ox-Bile (Bibiloni et al., 2001), WCM (Wilkins Chalgren Mupirocin) agar (Rada and Koc, 2000), and NPNL (neomycin, paromomycin, nalidixic acid, and lithium chloride) agar (Wijsman et al., 1989), were all found to allow the growth of *Lb. acidophilus* strains, while AMC (Arroyo, Martin, and Cotton) agar (Arroyo et al., 1995) inhibited the growth of

*Lb. acidophilus*, it did not inhibit the growth of starter and other probiotics. According to the study of Darukaradhya et al. (2006), the most appropriate media for selectively enumerating *Bifidobacterium* spp. were reinforced clostridium agar with aniline blue and dicloxacillin (Phillips et al., 2006).

Reinforced clostridial medium agar is now a preferable medium, and a few studies point to the value of this formulation (Champagne et al., 2011; de Carvalho Lima et al., 2009; Van de Casteele et al., 2006). Reinforced clostridium agar with aniline blue and dicloxacillin especially seems to work better (give higher CFU numbers) than MRS for stressed cells, which warrants its recommendation (Karimi et al., 2012).

### 2.2 *Selective Enumeration of* Lactobacillus

Several media have been suggested for the enumeration of *Lb. acidophilus* group.

Tharmaraj and Shah (2003) found that MRS agar, under aerobic or anaerobic incubation at 43°C for 72 h, could be used to count *Lb. acidophilus* (except DS 910), if *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are not present in the product. de Carvalho Lima et al. (2009) proposed the best media, that is, MRS agar with bile salts (MRSB) and best incubation conditions, that is, 37°C or 42°C, aerobiosis for enumerating *Lb. acidophilus*.

Nutrient agar supplemented with salicin and MRS medium supplemented with clindamycin were used for counting *Lb. acidophilus* (Van de Casteele et al., 2006). Phillips et al. (2006) for enumeration of *Lb. acidophilus* strains tested MRS agar with bromocresol (BC) green and clindamycin (MRS-BC). Similarly Darukaradhya et al. (2006) have found that the most appropriate media for selectively enumerating *Lb. acidophilus* in probiotic Cheddar cheese were reinforced clostridium agar-bromocresol (RCA-BC). RCA was prepared following the manufacturer's recipe with the pH of the agar adjusted to 6.2.

Lankaputhra and Shah (1996) proposed the use of MRS-maltose (MRSM) agar for selective enumeration of *Lb. acidophilus* in the presence of yoghurt organisms in a product, which does not contain *Bifidobacterium* spp. They also proposed the use of MRSM agar for enumerating total counts of *Lb. acidophilus* and *Bifidobacterium.* IDF (1995) also proposed pour plating in modified MRS agar, prepared as a basal medium containing maltose for counting *Lb. acidophilus*.

*Lb. acidophilus* could be enumerated on BA-sorbitol agar at 37°C for 72 h or MRS agar at 43°C for 72 h or BA-maltose agar at 43°C under anaerobic incubation (Tharmaraj and Shah, 2003). Özer et al. (2008) used MRS-D-sorbitol agar in the count of *Lb. acidophilus* LA-5. As the heat treatment caused deformation in the structure of D-sorbitol, it is better to employ membrane sterilization.

Kneifel and Pacher (1993) developed an agar medium, designated as X-Glu agar, for the selective enumeration of *Lb. acidophilus* in yoghurt-related milk products containing mixed

microflora of the lactobacilli, streptococci, and bifidobacteria. The detection principle was based on the specific visualization of the β-D-glucosidase activity of *Lb. acidophilus* via a chromogenic reaction of 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, which is incorporated into a Rogosa agar medium at 40 μg/mL.

Selective enumeration of *Lactobacillus casei* as well as other closely related *Lb. paracasei, Lactobacillus rhamnosus* was also well investigated.

A selective medium known as *Lb. casei* (LC) agar has been developed by Ravula and Shah (1998) for the enumeration of *Lb. casei* populations from commercial yoghurts and fermented milk drinks that may contain yoghurt starter bacteria (*S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*), *Lb. acidophilus*, *Bifidobacterium* spp., and *Lb. casei*. LC agar inhibited the growth of *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, and bifidobacteria, and thus the medium could be used to selectively enumerate *Lb. casei*. However, Vinderola and Reinheimer (2000) indicated that differential enumeration of *Lb. casei* could be performed on MRS-LP agar when it appears with *Bifidobacterium. Lb. casei* yielded round white creamy colonies on media with diameter ranging from 1.7 to 2.4 mm and bifidobacteria yielded small round colonies ranging from 0.7 to 1.2 mm in diameter (Vinderola and Reinheimer, 2000).

MRS-LC medium was also used for selective enumeration of *Lb. paracasei* (Van de Casteele et al., 2006). In another study by Tharmaraj and Shah (2003), *Lb. casei* grew in MRS-NaCl (4%), MRS-LiCl (0.5%) at 37°C under anaerobic incubation and LC agar.

*Lb. casei* was enumerated using MRS-vancomycin agar (1 mg/L of final concentration) (Ong and Shah, 2009). Also, RCA with BC green and vancomycin (RCABV) agar (at pH 5.5) was used for enumerating *Lb. paracasei*, *Lb. casei*, and *Lb. rhamnosus*. In another study, the MRSAC medium [i.e., MRS medium of which the pH was adjusted to 5.2 with acetic acid (99%)] was used for the enumeration of *Lb. rhamnosus* (Van de Casteele et al., 2006).

### 2.3 Selective Enumeration of Other Lactic Acid Bacteria

The MRS agar at pH 5.2 (MRS 5.2) was recommended for the enumeration of *Lb. delbrueckii* subsp. *bulgaricus* when the incubation was carried out at 45°C for 72 h, although occasional growth of *Bifidobacterium* spp. was recorded (Dave and Shah, 1996; Lankaputhra and Shah, 1996).

Tabasco et al. (2007) proposed the use of MRS-containing fructose (MRSF) for this purpose, which comprised of MRS fermentation broth, exclusive of glucose or meat extract and enriched with 0.2% Tween 80 and supplemented with 1% fructose, 0.8% casein acid hydrolysate, 0.05% cysteine, and 1.5% agar. The method was found to be differential against *Lb. acidophilus*.

M17 agar is recommended by the International Dairy Federation for selective enumeration of *S. thermophilus* from yoghurt. The M17 medium supplemented with lactose was used for enumeration of *S. thermophilus* under aerobic incubation at 45°C (Ashraf and Shah, 2011).

## 3 Methods for Identification of Probiotic Bacteria

Identification is a process of determining the affiliation of an isolated microorganism to one of the specific taxa. It should be carried out up to the strain level—it is necessary to determine the exact genus, species, and the particular strain. This allows the assignment of beneficial health effects, creates the possibility of oversight, and execution of epidemiological studies. It is recommended to use both phenotypic tests and genetic typing [molecular genetic techniques, e.g., polymerase chain reaction (PCR)], as well as to use nomenclature in accordance with the International Code of Nomenclature. In addition, strains of bacteria should be deposited at the International Collection of Pure Cultures and marked with an appropriate code (FAO/WHO, 2002).

The process to prepare microorganisms for identification is same for all analyses. However, direct identification is the best. Right after homogenizing, the first step is to enrich the sample, with nutrients that will step up the development of a certain group of microorganisms and limit the growth of others. If organisms are in poor condition, that is, their cell membranes are damaged, it is necessary to perform a preincubation of isolated microorganisms on substrates enriched by nutrients commonly used by them (preenrichment step). This will help the organisms to rebuild and restore their bioactivity (Forsythe, 2011).

### 3.1 Phenotypic Identification Methods

Basic bacteriological methods include: (1) microscopic examination of microorganisms (cell morphology) and (2) examination of the following:

- culture (colony morphology on solid substrates: colony growth, colony size, colony shape, colony elevation, colony edge, colony transparency, colony fluorescence, colony color and surrounding, colony structure, colony odor; morphology and split growth on slanting agar, growth in stab culture; growth on agar slope, growth on liquid substrates);
- physiological and biochemical features (i.e., ratio to oxygen, ratio to $CO_2$; determining minimal, optimal, and maximal growth temperatures; resistance to temperature; ability to produce dyes; proteolytic features; acid- and gas-producing features; ability to reduce methylene blue; ability to produce catalase, urease, hydrogen sulfide, and ammonia; changes in milk, ability to ferment carbohydrates, ratio of microorganisms to carbon

sources, ability to reduce nitrates to nitrites, methyl red test on Clark's substrate, indole production, hemolysis, movement ability, ability to produce spores);
- determining the sensitivity of microorganisms to antibiotics and other drugs as well as determining the sensitivity of microorganisms to bacteriophages (Hansen and Nielsen, 2014).

However, it is impossible to identify species or strains of the genus *Lactobacillus* solely on the basis of the colony appearance or cell morphology. These features allow an initial differentiation of bacteria present in a product before identifying them with other phenotypic or genotyping methods (van Belkum, 2012). Tests, such as assessment of cell motility, Gram staining, as well as tests for oxidase or catalase are screening tests, which can be used to determine affiliation of a given microorganism to a wide group of LAB. However, the expert group of FAO/WHO (2002) recommends testing phenotypic traits as a complement to more advanced identification methods.

### 3.2 Biochemical Methods

Biochemical methods for identifying microorganisms are based on determining the organisms' ability to assimilate, ferment, or decompose certain compounds. Identification follows an inoculation of samples isolated from food on a solid selective substrate with an appropriate chemical composition, which allows only some species or strains of bacteria to grow. Multiple passages for the next selective substrates eventually give a complete answer about a microorganism, which appeared in the examined sample, that is, its identification is the next step (Sreenivasulu et al., 2015).

Biochemical methods are used not only to identify, but also to provide data on the number of microorganisms present in the test environment. They help to identify only living organisms and the speed of assays largely depends on the ability of microorganisms to multiply. Many researchers emphasize on high sensitivity of biochemical analyses and their low execution cost. Compared to the other methods, their major drawback is long waiting time for results. The assay time falls within a period of several days for most of the microorganisms. However, it requires a lot of intensive work to prepare a medium for growth of bacterial cultures, inoculate plates, incubate them, and count colonies, as well as to characterize biochemical features (de Boer and Beumer, 1999).

The problem associated with the need to prepare substrates for many biochemical tests has been solved by the use of rapid biochemical tests in the form of ready kits. These are strips or plates which have pits or little cells constituting mini test tubes. Each of them is filled with a different substrate with a dehydrated culture and, potentially, an indicator. The individual sets differ in structure of plates and type of selective substrates, the choice of which depends on the level of knowledge about the microorganisms present in the tested food. After inoculation

of microorganisms on each of the little cells, 24 h are enough to get data necessary to identify the organism. Interpretation of the results is carried out by the software provided by the test manufacturer. Many researchers emphasize on the fact that these methods often have low reproducibility, and the results are ambiguous in many cases, which is associated with their insufficient discriminative power.

Tests, such as API (bioMeriéux) and Biolog (Biolog Inc.) can be distinguished in the market. The principle of functioning of these tests is to determine the ability of microorganisms to assimilate, ferment, or decompose certain compounds. API 50 CH is used to study the metabolism of 49 hydrocarbons and their derivatives (heterosides, polyalcohols, uronic acids) by microorganisms. In conjunction with API 50 CHL Medium, they allow for identification of microorganisms from the genus *Lactobacillus* as well as a few related types (e.g., *Aerococcus*, *Carnobacterium*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*) (Tammerman et al., 2003). In addition, microbiologists have a fully automated system, VITEK 2 Compact (bioMeriéux), at their disposal. It is used for the identification and drug susceptibility testing of microorganisms previously grown on solid substrates. The results are available after 18 h of incubation under suitable conditions.

Brolazo et al. (2011) demonstrated the use of the CHL API 50 kit in the identification of strains of *Lb. acidophilus*. As a result of the identification of three identical strains belonging to this species, different results in biochemical tests or no reaction result were obtained in all the samples included in the kit (Brolazo et al., 2011). By contrast, Boyd et al. (2005) observed that one-third (33 out of 97) of strains identified with the use of API 50 CHL, was not specifically matched to the species. Moreover, authors speculate that erroneous identification may result from inaccuracies in the APIweb base itself (Boyd et al., 2005). In addition, it was observed that the analysis of phenotypic patterns of species of the genus *Lactobacillus* (*Lb. casei*, *Lb. zeae*, *Lb. rhamnosus*), using the API 50 CHL, showed incorrect assignment of the *Lb. casei* strain as *Lb. rhamnosus* (Tynkkynen et al., 1999). Identification performance when using API 50 CHL may be affected by factors, such as acidification process identified with the process of growth and fermentation, oxygen, as well as changes in density of a bacterial suspension.

The Biolog Microplate kit (Biolog, Inc., Hayward, CA) has been developed to quickly identify the species of the following genera: *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, *Lactococcus*, *Megasphaera*, *Pectinatus*, *Pediococcus*, *Peptostreptococcus*, *Propionibacterium*, and *Weissella*. The system is based on an assessment of the efficiency of the oxidation of reactants in the processes of bacterial metabolism. Tetrazolium chloride and formazan are used as indicators, which in the course of redox processes produce colored products (Williams et al., 2000). At the same time, a metabolic fingerprint of a strain is obtained due to the observation of biochemical reactions with the use of 95 different carbon sources. The data are collected by Biolog Automatic Instrument

Reading and analyzed by the Biolog MicroLog software connected to a database (Biolog, Inc., Hayward, CA) to determine the affiliation of the tested strain to the given taxon. The kit itself is designed for an explicit identification up to the species level.

Unfortunately, similar to API 50 CHL, the application of Biolog Microplate is also limited. In the study by Moraes et al. (2013), where these tools were used, no strain of the genus *Lactobacillus* was correctly identified. Strains, identified by genotyping method belongs to the *Lb. plantarum* species, were classified by the Biolog system to the genus *Pediococcus*. By turn, API 50 CHL classified most of them to *Enterococcus* (Moraes et al., 2013). This confirms limitations associated with biochemical methods for bacterial identification, especially when it is important to classify the bacteria correctly, not only up to the species level, but also up to the strain level.

However, the use of Biolog Microplate allows an analysis of biochemical abilities within a single species. Specific strains of *Lb. plantarum*, used in the test by Di Cagno (2009), were different, for example, in terms of their ability to ferment glycerol, D-malic acid, D-galacturonic acid, inosine, D-sorbitol, and D-ketobutyric acid (Di Cagno, 2009).

Although Biolog Microplate offers a wide range of physiological tests, currently, an unambiguous identification of the genus *Lactobacillus* with its use does not seem to be possible (Tamang, 2008). However, it can be a useful tool to verify the results of other phenotypic or genotypic studies and to determine physiological needs and fermentation potential of a particular strain (Tamang, 2008).

In view of the uncertainty of results in biochemical identification methods, the most reasonable thing to do is to carry them out in combination with the other methods.

### 3.3 Biophysical Methods

Biophysical methods are based on studying the chemical composition of microbial cells using physical techniques, such as electrophoresis, chromatography, or spectroscopy.

Studying protein profiles is based on the assumption that each species of organism has a unique composition of cellular proteins. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis method involves electrophoretic separation of all the proteins on appropriately dissected polyacrylamide gel. Separation is performed after the step of incubation of microorganisms in the substrate supplemented with amino acids labeled with radioactive isotopes (e.g., methionine with sulfur isotope S35), which then allows to visualize the separated proteins on a film. Detection can also be carried out by a densitometer, which examines absorbance or emission of light incident on the gel (Domig et al., 2003). Protein profiles of microorganisms are also used by the AMBIS system that identifies the species with the use of electrophoresis and β radiation. The results are read by suitable software supplied by the manufacturer, with reference to the organisms from the database. They are read in 8 h, approximately.

In the study by Tammerman et al. (2003), 268 out of 323 isolates were successfully identified to the species level by means of electrophoresis in denaturing conditions. Based on a morphological evaluation, the unidentified isolates were classified as yeasts or lost during cultivation in MRS substrate. By turn, an analysis of protein profiles of microorganisms isolated from spontaneously fermented aubergine allowed the identification of 149 isolates by matching the image of electrophoretic separation of proteins of the test strains to the image of indicative protein strains (Sánchez et al., 2003).

In recent times, considerable interest is brought out by an identification method, which uses Raman spectroscopy with Fourier transform as well as infrared (IR) spectroscopy with Fourier transform (FT-IR). The technique that uses Raman spectroscopy allows a direct identification of microorganisms present on the surface of a given sample by measuring the inelastic scattering of photons. This spectroscopy is complementary to IR, which consists of measuring the vibration of molecules induced by radiation in the wavelength range of IR light. Identification of microorganisms carried out with these techniques is based on the analysis of the resulting spectrum and comparing it with the spectrum of the control sample (e.g., product on the surface of which microorganisms are not present). These methods seem to be promising because of the possibility of a direct analysis of microbial environment. Moreover, they are distinguished by high sensitivity and discriminative power. An additional advantage is the possibility of complete automation of assays. However, their use requires relatively expensive equipment, and the received signal is often distorted by all kinds of interference (Yang and Irudayaraj, 2003). The principle of the functioning of the FT-IR method is based on the differentiation of bacteria by testing their cellular components, that is, fatty acids, proteins, and cell membranes, as well as polysaccharides and nucleic acids (Dziuba et al., 2007). In 1950s, researchers have already started to use these methods to study biological samples. However, due to unsatisfactory results, they discontinued using them (Dziuba et al., 2007). Thanks to the possible automation of reading and analysis of results, today they are becoming much more effective and convenient.

The use of the Raman spectroscopy method allows for a rapid identification of microorganisms isolated from various foods and feeds. This makes it possible to distinguish starter cultures from other microorganisms that are the result of contamination of the product. For instance, *Lb. kefiri* has various surface properties related to the structure of the S-layer as compared to other strains of *Lactobacillus*, which is important to clarify functional capacities of a starter culture (fermentation characteristics, etc.) (Mobili et al., 2009). The FT-IR spectroscopy allows for an identification of intact cells from encapsulated probiotics added to thermally processed products, such as cereals (Prabhakar, 2011).

Prabhakar (2011) modified the FT-IR method in combination with a hydrophobic membrane to identify more than 40 starter cultures as well as other cultures present in Swiss cheeses. Hydrophobic filtration membranes have a unique grid pattern, which separates bacterial colonies from each other that prevents the strains to grow and mix. The evolved model took

into account the classification of the biochemical similarity between strains that resulted in specific clusters being isolated. The models showed significant discrimination in the spectral region from 1100 to 900 $cm^{-1}$, related to the extension of the vibration signal of polysaccharides in cell walls. The advantage of this method is the possibility to identify a microorganism to the strain level. Moreover, the study by Samelis (2011) demonstrated reproducibility of identification results, using FT-IR as well as genotypic methods (Samelis, 2011).

Spectroscopic methods also include mass spectrometry coupled with matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) used by VITEK MS (Biomerieux). It is analyzed that there is a spectral distribution of proteins directly in bacterial cells. A tested material sample is combined with a matrix. Then, a metal plate is applied and subjected to a laser beam. The matrix absorbing laser light is ionized during evaporation. The ions appearing in the electromagnetic field goes into the mass spectrometer, where the TOF of ions is measured. The ions are separated according to their mass to charge ratio, allowing finding out their numerical amounts. Measurement occurs at the end of the tube in which the particles are separated.

The effectiveness of identification using MALDI-TOF is varied. It is difficult to determine the species affiliation of the strains of *Lb. casei* and *Lb. paracasei*. To conduct an effective identification of these strains, it is necessary to use the genetic methods. MALDI-TOF does not allow for an effective distinction between species (Sato et al., 2012). A common mistake is assigning strains of *Lb. casei* to *Lb. zeae* or *Lb. paracasei*. However, an effective differentiation between *Lb. delbrueckii* subspecies was observed when using MALDI-TOF (Duskova et al., 2012). MALDI-TOF MS is a fast and simple tool for the identification of LAB, but the costs of purchase and exploitation related to this method are very high and often beyond the reach of in-plant small laboratories (Herbel et al., 2013). It is, however, important to remember that in case of the differentiation of closely related organisms, it is necessary to use this method combining with other methods, such as the rRNA 16S sequencing (Carbonnelle et al., 2012).

### 3.4 Immunological Methods

The basic phenomenon used in immunological methods is to create a stable connection between antigens and antibodies specific to them. Protein antibodies generated during the defense process are designed to initiate the process of inactivation of the antigen.

Most immunological methods are based on the labeling of one of the substrates in the antigen–antibody reaction with a marker that enables subsequent detection (these are the so-called immunochemical methods). These markers may be radioisotopes, enzymes, chemiluminescent systems, fluorescent molecules, or colloidal gold. Immunoblotting and agglutination tests are also commonly used to identify microorganisms. The former consists

in transferring the compounds separated during electrophoresis in agarose or polyacrylamide gel onto an active membrane. Particles imprinted on the membrane contain antigens and are incubated together with a labeled antibody. In contrast, agglutination tests are based on the phenomenon of making the strontium units (agglutinates) visible after the reaction of antibodies with corresponding cell antigens, such as erythrocytes or bacteria. The results of agglutination reaction are read under a microscope, through a magnifying glass, with the naked eye, or with the use of nephelometric or turbidimetric assays (Hansen and Nielsen, 2014).

The most commonly used technique for immunological identification of microorganisms is enzyme-linked immunosorbent assay (ELISA). It involves a reaction of an antibody with an antigen labeled with an enzyme or an antigen with an antibody labeled with an enzyme. The enzymes used when marking are catalysts of colored reactions with appropriate substrates. The degree of conversion, which depends on the amount of antibodies or antigens associated with an enzyme, is measured using a spectrophotometer. In this way, it is possible to get additional information on the amount present in the antigen sample. Most markers are used as enzymes and substrates in the following configuration: phosphatase with *para*-nitrophenyl phosphate, and horseradish peroxidase with tetramethylbenzidine. The antigens or antibodies used in the ELISA method are immobilized on a solid substrate, which is a polymer (mostly polystyrene) microplate with small wells. This allows for an easy separation of the formed immune complexes from the rest of the solution that greatly facilitates the analysis (McCarthy and Colworth, 2003).

The ELISA method comes in many variations among which three basic ones can be distinguished: (1) direct competitive ELISA, (2) indirect competitive ELISA, and (3) sandwich ELISA.

In competitive methods, antibodies are used in connection with only one epitope of an antigen. To identify a microorganism, it is important to anchor antigen-specific antibodies onto a solid substrate. After cleaning the plate from unbound antibodies, it is then introduced into a solution containing the labeled antigens present in a given quantity, as well as antigens derived from the test sample. Marked antigens and those originating from the sample will compete to join the antibodies. After this stage, the plate is cleaned from unbound antigens. Then, it is transferred to a solution containing the substrate forming a colored product in the reaction catalyzed by the enzymes bound with the antigen. The degree of the reaction being performed is measured with a spectrophotometer and indirectly reflects in an inversely proportional manner with the amount of the antigen present in the sample. The course of study may be reversed, that is, antigens are anchored on a solid substrate. Then, a certain quantity of labeled antibodies together with the antigens contained in the sample is added to the solution. Antigens immobilized on the substrate and antigens derived from the sample will compete with each other to join the antibody. Then, the plate purified from unbound antibodies is transferred to a solution in which the reaction with the substrate of a

colored reaction catalyzed by the enzyme linked to the antibody is carried out (Immer and Lacorn, 2014).

The indirect competitive method differs from the direct one in a way that two sets of antibodies are used in the indirect one. First-row antibodies recognize an epitope belonging to a given antigen, and second-row antibodies as well as other antibodies recognize an epitope belonging to the first-row antibodies. In practice, first the following reaction is carried out: the antigens anchored on the solid substrate together with the first-row antibodies that recognize the epitope in the presence of antigens derived from the microorganism that is to be identified. Then another reaction is carried out: the resulting complex with the second-row antibodies, which join the first-row antibodies. This solution allows for the use of many antibodies recognizing different epitopes of the antigen without the need to identify them individually. In addition, the second step of the reaction constitutes a kind of a confirmation that the performed tests are correct (Immer and Lacorn, 2014).

The sandwich ELISA test is named so due to its methodology, which consists in covering the antigens with two layers of antibodies in such a way that the whole system consists of two layers of antibodies between which there is a layer of antigens. In the first step of the test, the reaction carried out results in bringing together the test antigens with the antibodies trapped on a solid substrate. The second step results in introducing the antibodies recognizing another epitope of the test antigen to the complex. These antibodies are labeled with an enzyme. Using this solution, the selectivity of the method is greatly increased. Immunological methods are fast, sensitive and selective, and mostly inexpensive. They are often semiquantitative that constitutes an additional advantage. They can be used to carry out a large number of samples and often do not require long preparation (McMeekin, 2003).

An additional advantage of immunological methods for the identification and detection of probiotic bacteria is their ability to detect them by means of specific substances produced by these bacteria, that is, adhesins or bacteriocins. When using an appropriate antibody, it is possible to quantitatively and qualitatively determine the amount of the produced substance (Goktepe, 2005).

The PCR coupled with ELISA is an interesting method to identify probiotic bacteria. The marking principle is based on the formation of colored products of the amplification reaction that can be visualized under appropriate conditions depending on the used dye. The set containing digoxigenin and horseradish peroxidase is frequently used. The digoxigenin-labeled DNA, amplified by PCR, is subject to hybridization with a specific probe bound to the substrate. After the unbound DNA is washed away, it is followed by the visualization based on the enzymatic color reaction. Tamminen et al. (2004) compared the discriminatory power of the PCR–ELISA method with API 50 CHL medium. It was demonstrated that the PCR–ELISA modification allows obtaining faster results than with the biochemical API CHL 50 method, while maintaining the reproducibility of the results. The authors suggest that the

effectiveness of an immunological method in this case depends on the sequence of the probe used for hybridization (Tamminen et al., 2004).

### *3.5 Molecular Biology Methods Based on PCR*

Molecular identification methods are based on the study of the genome of microorganisms. Each organism has a unique set of genes, specific to their species or strain, and that generates a specific phenotype. The main advantage of molecular methods is distinguished by the lack of impact of environmental conditions on the genome. Only the level of expression of certain genes is changed, that may also be studied using molecular techniques. Genetic identification methods have revolutionized the use of the PCR, which greatly facilitated the molecular research, thanks to a simple and selective proliferation of a given portion of the genome. Depending on the technique used, genetic identification methods can be divided into methods based on molecular hybridization and PCR (Bagheripoor-Fallah et al., 2015).

Molecular techniques have so far been mainly used in medical research. Those associated with the identification of microorganisms in food began only in the 1990s, and their implementations into routine control have so far been realized to a small extent (Van Der Vossen and Hofstra, 1996).

Gene sequences encoding ribosomal RNA 16S, 23S, and 5S occurring in bacteria and 18S, 26S, and 5.8S occurring in fungi proved to be very useful fragments of the genome of microorganisms. It was found that these genes are evolutionarily highly conserved, separated with very diverse and highly polymorphic fragments. Bacterial genes encoding rRNA of each of the subunits of ribosomes are concentrated in a single operon defined as rrn (Fig. 6.1) (Tannock, 1999b). The rrn operon system does not always have to be the same, and the number of copies in the bacterial chromosome is not fixed.

The gene encoding rRNA, which belongs to a small subunit of the bacterial ribosome (16S rRNA) is composed of fragments, which are both highly conserved (present in many families of bacteria) and evolutionarily very different. Evolutionarily highly conserved sequences are usually common for bacteria of the same type, and may even be identical to a wider


**Figure 6.1: Generalized Model of the rRNA Operon of Bacteria Showing the 16S–23S Spacer Region.**

Some bacteria have two tRNA genes while others have either one or none. $P_1P_2$, Promoters; $T_1T_2$, terminators. *After Tannock, G.W., 1999b. Probiotics: A Critical Review. Horizon Scientific Press, Wymondham, UK.).*

phylogenetic range (Tannock, 1999b). There is a total of nine highly diverse "V" regions occurring in this gene, of which usually only a few have such a diverse sequence so that it can be possible to properly identify a given microorganism. Both genes, rRNA S16 and rRNA S23, may prove to resemble two different species of bacteria too strongly, and then they will not provide adequate discriminating power.

Sequencing of the fragments amplified by PCR with starters complementary to species-specific sections of 16S–23S rRNA is carried out using the Sanger method, pyrosequencing, or sequencing in real time by a single molecule (SMRT—*single molecule real time*) (Luo, 2012). This additional analysis of the exact design of the test section of the genome allows specifying the result of the electropherogram obtained from the separation of PCR products (Bested et al., 2013). The obtained sequences are comparable to those generally available, that is, BLAST (NCBI) or Megalign Suite (Lasergene DNAStar) (Luo, 2012).

Researchers distinguish the so-called ITS region ("intergenic-transcribed walk" in bacteria and "internal-transcribed sequences" in fungi) as one of the best elements differentiating bacteria and fungi. In the case of bacteria, these are small DNA fragments comprised between encoding rRNA genes of the large and small ribosomal subunits, and also between genes encoding the rRNA 23S molecule as well as. The first of these involve an additional short gene or a few genes responsible for the tRNA sequence (Tannock, 1999b). It was shown that the rrn operon is present in the bacterial genome in the number of multiple copies, which may vary in terms of ITS length. This is due to the presence of tRNA genes that may be present in each copy in a different configuration and quantity. It was also shown that the ITS region is one of the most diverse regions in the rrn operon (Tannock, 1999b). ITS-based analysis allows obtaining a certain identification result after 3 h of isolation (Luo, 2012).

Although sequencing of the 16S–23S rRNA is an effective method, it is very time consuming, and sometimes also requires allocation in modified conditions, such as in a denaturing/thermal gradient (DGGE/TGGE) or by pulsatile electrophoresis (Herbel et al., 2013).

Repetitive sequences (repeated sequences) also constitute useful fragments in molecular typing. There are two classes of repetitions in bacteria. These are simple repetitions, which consist of short sequences (1.5 nucleotides) that occur close together in a "head-to-tail" configuration as well as complex repetitions, that is, long ones, among which there are transposons, insertional elements, minisatellites, macrosatellites, long tandem repetitions, and separated repetitions. REP and ERIC repetitions are examples of commonly used repetitive sequences in bacteria. REP repetitions are nongene sequences also known as palindromic units. They consist of 38 nucleotides and their sequence consists of highly conserved fragments that exhibit symmetry relative to each other (palindromity). Additionally, these fragments are separated by short stretches of highly variable sequences. After rewriting the single-stranded RNA, this arrangement forms a stable structure called "stem-loop" in which the stem constitutes palindromic sequences located symmetrically with respect to each other,

and the loop includes a five-nucleotide, centrally located, and highly variable fragment (Versalovic et al., 1991). REP sequences are present predominantly in the form of clusters and they usually contain 3, 4, and 5 elements separated by fragments always composed of sequences made of not longer than 25 base pairs. Such clusters are called "REP elements." REP sequences are dispersed throughout the bacterial genome and are present in an amount of usually about five elements arranged in 100–200 clusters. They are located between the genes located in operons or at the end of operons (Koh-Luar et al., 1997).

Enterobacterial repetitive intergenic consensus (ERIC) sequences, also called intergenic repetitive units (IRU), are located between genes, present in most Gram-negative and some Gram-positive bacteria. These sequences are made of 127 base pairs in length. Similarly to REP sequences, they exhibit palindromity due to which, after rewriting the mRNA, they take a structure of a "hairpin" (Koh-Luar et al., 1997).

Performing a quantitative PCR in real time (qPCR) allows simultaneous detection and identification of microorganisms in the product and detection of their amount based on the amount of the single-stranded DNA in the sample complementary to probes (Bustin et al., 2012). By using such suppressing systems associated, for example, with the SYBR Green (TaqMan) dye, it is possible to detect species-specific regions flanked by hybridization with the universal probes (Castoldi, 2013). As applying a molecular probe forms a hairpin structure, they are not fluorescent, if nonhybridized.

The qPCR method is rapid, and its result is independent of a culture's conditions. In addition, the test is sensitive, and the accuracy of the method allows determining the amount of microorganisms and their phylogenetic affiliation to a very high extent. Compared to culture techniques, qPCR is more rapid and provides the possibility to detect a small population of bacteria within a dominant microflora.

PCR, thanks to the use of the specific conditions of the separation, also allows detecting and identifying microorganisms comprising complex biological environments and cultures grown in the nonoptimal conditions. This method can be particularly beneficial for the assessment of microbial probiotic products in which there is often a great diversity of microorganisms. The *denaturing gradient gel electrophoresis* (DGGE) separation technique is based on the difference between melting temperatures of different DNA sequences based on the content of individual nucleotides. The use of this technique allows separating products of the same length on the basis of differences in the DNA sequences because a double-stranded DNA segment migrating in a gel treated with denaturant stops when it reaches its melting temperature, that is, at the moment of denaturation (Logan et al., 2003). Then it becomes possible to carry out a comparative analysis between DNA samples and the DNA reference strain separated in the same gel. The separation result also has a semiquantitative nature and allows determining the level of dominance of particular strains in the environment (Chen, 2011). The study by Hassan and Chaudhry (2013) showed that the analysis with the

DGGE separation allowed to detect a greater number of species (e.g., *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. rhamnosus*, *Lb. acidophilus*, *Lb. casei*, *Lb. acidophilus*) than culture research, and the results did not depend on the environmental conditions (Hassan and Chaudhry, 2013).

A disadvantage of the PCR–DGGE method is its limited efficacy in identifying species present in a sample in an amount below 1% of the overall population of microorganisms (Chen, 2011). Moreover, it happens that because of similarities in images of electrophoretic separations of closely related species, such as *Lb. casei* and *Lb. paracasei*, identification by this method may have limited efficacy (Liu, 2012). In the case of this method, a greater discriminatory power can be also observed in a method, which is based on the use of starters complementary to *cpn60* and *rpoB* gene segments rather than those encoding 16S rRNA (Herbel et al., 2013). Therefore, researchers suggest that this method should not be used as the sole identification of probiotic microorganisms and it requires confirmation by sequencing 16S rRNA (Fontana, 2010; Herbel et al., 2013).

Random amplification of polymorphic DNA fragments, that is, random amplified polymorphic DNA (RAPD)–PCR, is based on the use of a single, short starter oligonucleotide of the length of a few nucleotides. The starter is composed of any random sequence of nucleotides, and therefore should be attached to the matrix in many places, including sequences which are not fully complementary. The effect of the amplification is the creation of a dozen to several dozens of products, which are then subjected to electrophoretic separation. It should be remembered that amplification will occur only when the length of the fragment of the matrix, bounded by the starters, will be no longer than a few thousand base pairs (Venturi et al., 2012). Due to the length of the starter, it is necessary to retain the specific hybridization conditions, that is, low temperature (35−40°C) and elevated concentration of $Mg^{2+}$ ions (Venturi et al., 2012).

Thanks to the possibility of random selection of the starter's sequence, in this method, it is not necessary to know the DNA sequence of a microorganism being identified. In turn, fragments obtained using RAPD–PCR, are classified as second class of genetic markers with high polymorphism (Venturi et al., 2012). The analysis of the results is carried out by introducing the resulting pattern of stripes (the so-called "fingerprint" of the microorganism) to a computer database, and an appropriate software adjusts the results to identical ones in the database. In a case of a small amount (10–15) of amplification products, the results can be analyzed visually (Venturi et al., 2012). It is recommended to separate the products of the amplification reaction with the RAPD–PCR method with the use of pulsed field gel electrophoresis (PFGE). This allows to separate relatively large DNA fragments, while improving the strength of the discriminatory power. This technique makes it possible to distinguish between even closely related species and strains, as well as to carry out an analysis of changes on the substrate of the genome (Venturi et al., 2012). Moreover, it is relatively easy and inexpensive, even for inexperienced users.

The results of Di Cagno et al. (2010) showed that the discriminatory power of the RAPD–PCR method is nearly 20% higher than the Biolog System, while slightly less than the amplified fragment length polymorphism analysis (Di Cagno et al., 2010). Gosiewski et al. (2012) observed, however, that set of the Ready-To-Go-RAPD type did not distinguish between strains of the *Lb. plantarum* species derived from the human microbiome, but also to some extent it discriminated these species from the plant sources (Gosiewski et al., 2012).

The only way to observe structural genetic changes very accurately, as well as to separate comparative individual units for functional genomics, is to sequence the entire genome (WGS—whole genome sequencing) (Herbel et al., 2013). It is currently possible to apply modern methods of sequencing, so that the process becomes more and more automated and fast. These modern methods are generally less expensive than those previously used, and they have a much higher throughput as well (Soon et al., 2013). With the sequencing of the entire genome, it is also possible to track evolutionary changes in the environments, which consist of LAB. Furthermore, differentiation of genetic strains can be observed within a single species, which is particularly important in the identification of potential probiotic bacteria (Herbel et al., 2013).

### *3.6 Genotyping Methods Based on Hybridization*

Genotypic methods conducted on the basis of the process of hybridization allow conducting complex and automated identification techniques. Fluorescent in situ hybridization (FISH), microarrays, and Southern blot are the most popular methods.

The FISH method allows to effectively identify microorganisms seen under a microscope by using specific fluorescent dyes. Diamidino-2-phenylindole (DAPI) is the most commonly used one. This substance selectively intercalates with pairs of A–T alkali in cellular DNA. By using an epifluorescence microscope, glow emitted by the dye stimulated with ultraviolet light can be observed. DAPI can be combined with other fluorescent dyes, so that not only a genetic matter, but also other cell structures can be visualized in only one marking (Joux and Lebaron, 2000).

The FISH technique is based on the hybridization of sections of rRNA, which is located in immobilized cells, with an oligonucleotide probe complementary to 16S rRNA. The probe is marked with a fluorescent dye, which bonds with rRNA and visualize the location of complementary sections in the cell. Currently, probes complementary to rRNA are used because its quantity in cells is significantly higher than the number of copies of the genes included in rDNA. This allows for an increase in the sensitivity of the technique, and due to a hybridization process carried out in situ, problems related to the direct examination of RNA isolated from cells are reduced. Moreover, as the mRNA half-life period is much shorter

than that of DNA, rRNA can also be used as an indicator of cellular activity (Miks and Warmińska-Radyko, 2008).

Probes used in the FISH technique are classified according to their properties as universal ones, specific to the selected classes of microorganisms, and the nonsense ones. Currently, new sequences of oligonucleotide probes that could be used as standard ones to identify types and species of LAB are being looked for (Miks and Warmińska-Radyko, 2008).

FISH technique carried out through the use of dyes, such as DAPI, additionally allows for a simultaneous assessment of bacteria's physiological state, their number, and their taxonomic affiliation. Thus, the time of the comprehensive study of microflora is significantly reduced. Visualization of cells of the in situ fermentation microflora in a food product, which is possible when using a nonspecific probe, currently constitutes an interesting trend resulting from the properties of FISH (Ercolini et al., 2004).

The use of the FISH method for the identification of LAB has already been tested by the researchers. Machado et al. (2013) identified 36 strains of different species belonging to the genus *Lactobacillus* using a modified FISH method, based on a probe composed of peptide nucleic acid. Moreover, 20 strains of different taxonomic affiliation were identified. The method was observed to be highly sensitive and specific—100% and 95%, respectively. Moreover, the authors conducted a successful attempt to assess the number of potentially probiotic bacilli of the genus *Lactobacillus*, which is extremely important when producing probiotic foods (Machado et al., 2013).

In turn, Lebeer et al. (2011) used the FISH technique to identify the potentially probiotic bacteria of the genus *Lactobacillus* isolated from gastrointestinal tracts of different hosts. Probes targeted to mRNA were used. During the study, with the use of FISH, it was observed for the very first time that probiotic bacilli form a biofilm (Lebeer et al., 2011).

Flow cytometry is a diagnostic method that allows assessment of the size, color intensity, and fluorescence intensity of tested microorganism cells. Flow cytometry has been discovered to enhance the fluorescence microscope and has a high yield. A typical flow cytometer consists of a hydraulic system, a light source, an optical system, an electric circuit system, and data analysis tool.

There are two main types of cytometry: stationary cytometry and flow cytometry. Microbial cells for the analysis of food samples by flow cytometry are in suspension, eliminating the initial stages of filtration products. Cells in suspension are stained with a fluorescent dye, respectively. Passing the camera flow, cytometric cell intersect the laser, which emits light. The emitted laser beam is scattered by contact with the microbial cells. Front detector and the detector side measure the distraction. In addition, the cytometer measures the fluorescence emitted by the sample. Optics distributes different fluorescent spectra that are subsequently converted into a digital signal. With cytometry multicell charge different fluorescent dyes,

so they can be distinguished from each other on the basis of color. The resulting signals are transformed into electrical impulses, amplified, and transmitted to a computer for further processing, storage, and analysis. The results are presented in graphs or histograms dot (Olszewska et al., 2016).

Cytometric analysis has many advantages. First, it allows you to evaluate the test sample in terms of both quality and quantity. It is less time-consuming and labor-intensive than traditional methods of analysis, because it allows the analysis of single cells in an automated manner and in a short time. Furthermore, they used fluorescent dyes to allow examination of the various parameters of the cell and further interactions between molecules in the cell. First of all, viable cells and dead cells are distinguished regardless of the capacity of cell proliferation. It allows the determination of the cell able to VBNC, which makes this method much more accurate than traditional breeding methods. Speed of a single analysis using a flow apparatus is several thousand cells per second (Davis, 2014).

Uses of flow cytometry in food research are very broad and are not designed for only one type or species of microorganisms. Raymond and Champagne (2015) examined the accuracy of flow cytometry in the opinion of the population of probiotic bacteria *Lb. rhamnosus* R0011 form of dried and placed in a food matrix, which was chocolate. It has been found that the use of flow cytometry microbiological is 2 times more accurate than traditional methods. Comas-Riu and Rius (2009) evaluated the degree of lysis of bacterial cells at various stages of formation of the cheese. Olszewska et al. (2015) determined the effect of sodium chloride on the physiology of cells of a strain of LAB of the *Lactobacillus brevis* species, which was isolated from food (fermented cabbage). It was found that the *Lb. brevis* strains showed a significant tolerance to sodium chloride and over a range of knowledge can be useful in screening and the usefulness of *Lactobacillus* in the food industry.

Cytometric analysis is now an invaluable method of testing microbiological research and food processing. By using cytometry, it is possible to directly test the samples, skipping, or shortening of the incubation period, which greatly accelerates the acquisition results.

DNA microarrays are the most modern techniques of rapid identification of microorganisms. Each microarray constitutes a collection of molecular probes complementary to the target nucleic acid sequences present in the microbial cells, which are possible to identify. These probes are permanently connected with the substrate in a particular order and specifically bind only their homologous DNA sequences present in the test sample. With the simultaneous use of multiple probes, it is possible to shorten the time of analysis and reduce operations related to hybridization of nucleic acids.

cDNA microarrays and oligonucleotide microarrays can be distinguished. This division is associated with different origins and types of probes located on the solid substrate.

Immobilization of a probe may be conducted on membranes, glass coating, as well as silicone.

So far, DNA microarrays have been successfully implemented in the food industry to detect pathogens in food samples. Moreover, they allow an identification of a significant number of microorganisms contained in the environmental samples of significant diversity. These microarrays are usually based on the 16S rRNA sequence. Therefore, they provide accurate information about the diversity of a studied microflora, but often only to genus level. "GutProbe," which was developed for efficient identification of individual species present in foods and dietary supplements, is an interesting tool produced in the need for identification of probiotic bacteria (Patro et al., 2015). This array contains 92 whole genomes and 229 plasmids, which enables an optimal representation of the majority of microorganisms present in human intestines as well as in food products. The use of such probes in standard tests of probiotic products can significantly reduce costs associated with the use of molecular and microbiological techniques (Patro et al., 2015).

### 3.7 CRISPR-Based Technologies

Almost 60% of bacteria and 40% archaea have an acquired immune system against bacteriophages; this is called clustered regularly interspaced short palindromic repeats (CRISPR) mechanism. This mechanism depends on the leader sequence, CRISPR array and CRISPR-associated protein responsible genes (Cas genes) in bacteria.

CRISPR was recognized in 2007 in LAB (Barrangou et al., 2007). CRISPRs represent a family of DNA repeats, which typically consist of short and highly conserved repeats, interspaced by variable sequences called spacers, often times adjacent to *Cas* genes (Makarova et al., 2011).

CRISPRs, together with Cas genes, form the CRISPR/Cas immune system, which provides adaptive immunity against phages and invasive genetic elements. The immunization process is based on the incorporation of short DNA sequences from virulent phages into the CRISPR locus. Subsequently, CRISPR transcripts are processed into small interfering RNAs that guide a multifunctional protein complex to recognize and cleave matching foreign DNA (Barrangou and Horvath, 2012).

Recent studies have established that CRISPR provides acquired resistance against viruses (Barrangou et al., 2007; Horvath et al., 2008) and allow microbial populations to survive phage predation (Kunin et al., 2008; Tyson and Banfield, 2008), possibly via an RNA-interference-like mechanism (Sorek et al., 2008).

So far, out of 2605 analyzed and sequenced bacterial genomes system, CRISPR/Cas was detected in 1174 strains. It was found that this system is present in the genomes of many species of LAB include: *S. thermophilus, Lb. acidophilus, Lb. brevis, Lb. casei, Lb.*

*delbrueckii* subsp. *bulgaricus, Lb. fermentum, Lb. helveticus, Lb. johnsonii, Lb. paracasei, Lb. rhamnosus, Lb. salivarius* (http://crispr.u-psud.fr/crispr). LAB have relatively small genomes, approximately 1.7–3.3 Mbp in size, containing 1700–3000 genes (Makarova et al., 2011).

Comparative and functional CRISPR-based genotyping genomics analyses have provided novel and critical insights into the numerous LAB functionalities, phylogenetic diversity, and evolutionary processes, notably with regards to their ability to catabolize nutrients, enhance human health, and adapt to their respective habitats. The presence or absence of a CRISPR array may also be used to differentiate strains, but is only reliable when it correlates with the phylogeny of the organism. In order for adequate spacer comparison to occur, within a given CRISPR locus, the most ancient spacers must share a common origin, which then diverges over the course of the array. In other words, there must be some shared and some disparate spacers in order to effectively type strains based on array content. Thus, the process is largely contingent on having had active spacer acquisition machinery at some point in evolutionary history, although degeneracy of CRISPR arrays can also add to polymorphisms in spacer content. Of course, CRISPR-based typing also depends on the presence of CRISPR-Cas systems in the genomes of genera and species of interest (Selle and Barrangou, 2015). To date, CRISPR-based typing schemes have been effectively employed in foodborne pathogens, such as *Salmonella* (Shariat et al., 2013) and *Escherichia coli* (Toro et al., 2014; Yin et al., 2013), but also in the industrial fermentation starter cultures, such as *S. thermophilus* (Horvath et al., 2008), probiotics, such as *Lb. casei* (Broadbent et al., 2012), and spoilage organisms, such as *Lactobacillus buchneri* (Briner and Barrangou, 2014). Emerging CRISPR-based typing methods open new avenues for high-resolution typing of a broad range of bacteria, especially probiotics.

Table 6.2 and Fig. 6.2 show the comparison assessment of selective discussed enumeration and identification methods.

**Table 6.2: Comparison of enumeration and identification methods of probiotic species.**

| Methods | Cost | Time to Obtain the Results | Specificity |
|---|---|---|---|
| Nucleic acid–based | | | |
| • FISH | + | + | ++ |
| • Flow cytometry | +++ | ++ | +++ |
| • Sequencing | ++ | ++ | +++ |
| • PFGE | + | + | +++ |
| • CRISPR/Cas | + | ++ | +++ |
| • PCR-based techniques | + | ++ | ++ |
| Culture | Inexpensive | +++ | + |
| Biophysical | + | ++ | + |
| Immunological | + | ++ | + |
| Biochemical | + | ++ | + |

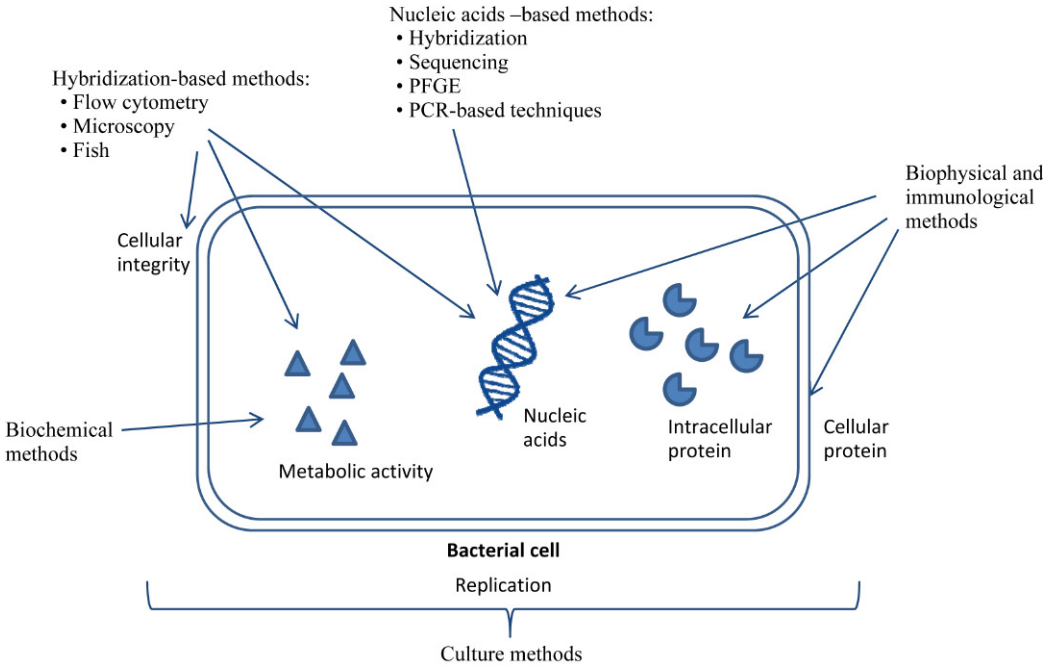



**Figure 6.2: Summary of Specificity of Methods That Could be Used for Probiotic Bacteria Enumeration and Identification.**

## 4 Conclusions

Currently, the techniques used to identify microorganisms are often inadequate to quickly assess the microbiological state of food. The increasing turnover of food products in the world requires introduction of modern methods that would ensure not only adequate speed of marking, but would also demonstrate adequate sensitivity, selectivity, and versatility. In addition, the aim is to reach a complete automation and computerization of such analyses that would significantly reduce time-consuming and laborious work. Unfortunately, these methods are usually expensive or require appropriate equipment and staff training.

Identification of microorganisms is a basic criterion for probiotics set by the FAO/WHO Expert Group. Defining an organism up to its strain level is obligatory for safety reasons, because individual species or types of bacteria may significantly vary in terms of their features. FAO/WHO recommends molecular biology techniques for identification, considering them to be the most precise ones. DNA–DNA hybridization, considered as a replacement for the use of DNA sequences encoding 16S rRNA, is found as the preferred methods to be a reference one. Moreover, gel electrophoresis in alternating electric field (PFGE) or polymorphism analysis of DNA fragments amplified by PCR using starters of arbitrarily chosen sequences (RAPD) are considered golden standards.

Recent investigations show that genome sequencing can be considered the "gold standard" for identification and typing of bacterial strains in terms of resolution. However, genome sequencing seems to be costly, analytically challenging, and time-intensive process, which is not suitable for high-throughput or rapid applications. Recently, repetitive-element PCR-based genotyping using high-resolution microfluidics has proven to be rapid and reliable in strain differentiation, but identification of strains requires a database of fingerprint data for comparison. 16S rDNA sequencing, although not a typing tool, is relatively fast and affordable for rough identification of bacterial genus and species, but can be unreliable for applications that require resolution down to the species level. By contrast, CRISPR array genotyping offers a rapid, affordable, and high-resolution means of typing bacterial strains within species that carry them (Barrangou and Horvath, 2012).

More and more authors claim that flow cytometry appears as a promising tool for use in the manufacturing of product containing probiotic strains (Davis, 2014). Flow cytometry provides insight regarding microbial fitness and metabolic activities during bioprocessing of product formulations. This could improve processes optimization involving strains for commercial use, prediction of microbial performances along with the whole process, and the presence/absence of activity during storage could benefit the quality control of probiotic products during their shelf life (Sohier et al., 2014).

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## Further Reading

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CHAPTER 7

# *Improvement of Ripened Cheese Quality and Safety With* Thymus mastichina *L. Bioactive Extracts*

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## 1 Introduction

The increasing awareness of consumers regarding food quality and safety issues, namely the presence of synthetic additives and ingredients with potential side effects on health and environment (Bearth et al., 2014) is fostering the search for natural and environmentally friendly alternatives (Gyawali and Ibrahim, 2014). In cheese production, antibiotics (e.g., natamycin, nisin) (Ollé Resa et al., 2014; Pintado et al., 2010; Ramos et al., 2012) or curing salts are commonly used to control microbial growth. However, there is some controversy about the use of such products (Carocho et al., 2015; Martínez et al., 2013; Rencüzoğullari et al., 2009; Streekstra et al., 2015).

The use of herbs and spices in several foodstuffs is an ancient practice based on the empirical grounds regarding the ethnobotanical and ethnopharmacological knowledge (Tarakci and Temiz, 2009). Recently, extensive work has been done on the study of the properties of the bioactive compounds present in the plant extracts. The research also focuses on the full understanding of composition, mechanisms of action, toxicological aspects, and potential health benefits of bioactive compounds (Tajkarimi et al., 2010). In this context, the innovation and the economical, industrial, and social interests are based on the optimization of the extraction and concentration procedures and also on the validation of such extracts efficacy about the safety and quality of the food products to which they are added.

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00007-4

### 1.1 Thymus mastichina *L.*

*Thymus* spp. are normally used as spices and/or medicinal herbs (Barros et al., 2011; Evans and Edward, 1989). *Thymus mastichina* L. belongs to the *Lamiaceae* family and is endemic in the Iberian Peninsula. In Portugal, it is ubiquitous except in calcareous soils, blossoming in April–June (Franco, 1983). The essential oils (EOs) isolated from *T. mastichina* L. are rich in 1,8-cineole (57.8%) and limonene (10.8%) (Miguel et al., 2004a,b). However, the authors found a plant group in which linalool (73.5%) was the main component of the oil extract, indicating the presence of, at least, two *T. mastichina* chemotypes. Other species of the genus *Thymus* (e.g., *Thymus vulgaris*, *Thymus capitatus*, *Thymus thymoliferum*, and *Thymus serpyllum*) are also commonly used in foods due to their antimicrobial properties (Bounatirou et al., 2007; Ehivet et al., 2011; Oussalah et al., 2006, 2007).

In Portugal, *T. mastichina* L. is known as *Tomilho bela luz* or "pure salt." The bioactive compounds present in *T. mastichina* are well characterized (Fernandes, 2010; Gordo et al., 2012; Miguel et al., 2004a,b). Besides the flavoring properties, several authors refer to its antimicrobial/antifungal activity (Ballester-Costa et al., 2013; Elshafie et al., 2015; Faleiro et al., 2003; Fernandes, 2010; Peñalver et al., 2005; Pina-Vaz et al., 2004), antitumor properties (Nikolić et al., 2014), as well as to its anti-inflammatory, and antioxidant properties (Albano and Miguel, 2011; Barros et al., 2010; Delgado et al., 2014; Fraternale et al., 2003; Méndez-Tovar et al., 2015). These properties not only contribute to enhance the organoleptic properties of foods, but also improve the shelf life of products to which they are added. However it is also referred the considerable intra- and interpopulation variations among *Thymus* spp. regarding EOs composition and their bioactive properties (Salgueiro et al., 1997; Zeljković and Maksimović, 2014).

Concerning the antimicrobial properties, Faleiro et al. (2003) investigated the chemical composition of *T. mastichina* EOs and their antimicrobial activity against *Escherichia coli*, *Proteus mirabilis*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes,* and *Candida albicans.* The antimicrobial activity of EOs was compared to that of pure linalool, 1,8-cineole, and a mixture of both in the ratio 1:1. It was concluded that the antimicrobial activity is related to more than one component and possible antagonistic and synergistic effects may occur.

### 1.2 Bioactive Compounds in the Food Industry

In the food industry, the use of antioxidants is a common practice that envisages the retardation of oxidative reactions and thus increasing the shelf life of food products. The use of synthetic antioxidants has been the subject of some controversy, specifically regarding health issues. The use of natural antioxidants is envisaged as an excellent alternative for the replacement of synthetic products (Delgado et al., 2014).

Several cheeses include the addition of plant extracts in their recipes to gain particular sensory characteristics (Tarakci and Temiz, 2009). In most of the cases, this results from empirical ancient practices and more recently, several works refer to the resultant potential benefits concerning the organoleptic properties of the cheeses and the intention to control spoilage or pathogenic microorganisms (Dagdelen et al., 2014; Honório et al., 2016; Kavaz et al., 2013; Olmedo et al., 2013). Govaris et al. (2011) evaluated the positive effect of thyme and oregano EOs on the inhibition of *L. monocytogenes* and *E. coli* O157:H7 in Feta cheese and evidenced a stronger antibacterial activity of both EOs against *L. monocytogenes.* Han et al. (2014) report a 2.5 Log CFU reduction of inoculated *L. monocytogenes* in Mozzarella cheese packed in emitting sachets containing thyme oil. It was also mentioned that the presence of EOs also reduced the development of lactic acid bacteria and lowered the total microbial counts. de Carvalho et al. (2015) reported the inhibitory effects of *T. vulgaris* EOs against *S. aureus*, *L. monocytogenes* in *Coalho* cheese. However, they also concluded that its addition should be cautiously considered as the negative effect on the mesophilic starter coculture. Akarca and Tomar (2016) refer to the improved characteristics of Mozzarella cheese with added spices, including *T. serpyllum*.

Otoni et al. (2016) extensively reviewed the recent trends in antimicrobial food packaging systems based on emitting sachets and absorbent pads. The advantages, drawbacks, and the antimicrobial compounds, including a variety of plant EOs and their main active compounds, incorporated in these systems were discussed. Atarés and Chiralt (2016) reviewed the applications of EOs as additives in biodegradable films and coatings for active food packaging. It was concluded that EOs may provide the films with antioxidant and/or antimicrobial properties and that the oil composition and the specific interactions with the polymer, determine its effectiveness as an active ingredient. Finally, Nguyen Van Long et al. (2016) presented an extensive review on active packaging with antifungal properties. It is pointed out that the major problem to overcome is the impact of EOs on the organoleptic properties of the product, and more studies are required to determine the compatibility between the EOs and the kind of product to which they are added. A clear increase of the free mold and yeast shelf life of food products, such as cheese and bread was demonstrated.

## 2 T. mastichina *L. Extracts Applied to Ripened Cheese: A Case Study*

This case study refers to the efficacy of *T. mastichina* L. bioactive extracts applied to cheese as an alternative to the salt used in cheese formulations, to commercial synthetic products used for spoilage control in cheese surfaces during ripening and to fumigation agents in ripening chambers.

### 2.1 *Antioxidant Activity of* T. mastichina *L. Extracts*

Two types of *T. mastichina* L. extracts were produced. For the preparation of the extracts, 25 g of the dry plant was submitted to the decoction with 1 L of water or maceration with ethanol.

**Table 7.1: Antioxidant capacity and total phenolic compounds present in the aqueous and ethanolic extract of *Thymus mastichina* L. (*,**$P < 0.001$, compared to control).**

| Methods | AE | EE |
|---|---|---|
| ABTS (IC50 µL/mg) | 16.8 ± 3.2* | 24.9 ± 4.0 |
| DPPH (IC50 µL/mg) | 38.6 ± 1.2** | 81.6 ± 1.9 |
| Total phenolic compounds (nmol GAE/mg) | 257.0 ± 23.5** | 9.2 ± 3.8 |

AE, Aqueous extract; EE, ethanolic extract.

For the aqueous extract (AE), after heating at 100°C for 15 min, the solution was cooled and filtered under vacuum through a membrane (0.2 µm pore diameter) before freeze drying and storage at −18°C. The yield of the extract (dry mass of extract/mass of dried plant) was in the order of 20%. In case of the ethanolic extract (EE), the solution was macerated stirred for 1 h, filtered in the same conditions and stored frozen.

The antioxidant capacity of both extracts (Table 7.1) was evaluated by means of two colorimetric procedures: the ABTS method described by Miller and Rice-Evans (2012) and the DPPH method described by Sharma and Bhat (2009). The total phenolic compounds were determined using the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007).

From Table 7.1, the higher antioxidant capacity and total phenolic compounds of AE are evident when compared to EE (Carvalho et al., 2013). These results favor the utilization of AE in view of the increase of the nutritional and antioxidant capacity of foods (Carvalho et al., 2014; Ferreira et al., 2014).

### 2.2 T. mastichina *L. Aqueous Extract as a Salt Substitute in Ripened Cheese*

*T. mastichina* L. is popularly known as "pure salt," a name that emphasizes its character as a flavor enhancer. Thus, the utilization of AEs of *T. mastichina* L. may be regarded not only as a vehicle to improve the antioxidant activity in foods but also as a salt substitute.

Ripened cheese normally presents considerable amount of salt, considered as a factor that may limit its consumption by people suffering from hypertension. The use of *T. mastichina* L. as a salt substitute in cheeses was not reported in literature. Therefore, a series of tests were developed with the intention to evaluate the use of AE of *T. mastichina* L. applied to cheese curds on the physicochemical, nutritional, and sensory characteristics of the cheese.

Ripened cheeses with and without addition of AE of *T. mastichina* L. were produced and compared. Two variations of the salting process were also introduced; salting in brine (B) and dry salting (S) on the surface of the curd. Hence, four types of ripened cheese were evaluated and compared: conventional cheese with dry salting (C.S) at the surface, and in brine (C.B), cheese with added AE of *T. mastichina* dry salted (AE.S), and in brine (AE.B).

### 2.2.1 Cheese production

Raw milk was normalized to 3.5% ± 0.1% fat. After coagulation, the curd was cut in pieces and whey was drained. The curd was divided in two portions. To one, the diluted AE was added and thoroughly homogenized, while the other worked as a control. Each curd portion was molded and allowed to drain for 24 h under refrigerated conditions (<8°C). After drainage, half of the cheeses with added AE were placed in brine (23.06% NaCl) for 10 min (half of the time used for conventional process) and the other half was dry salted on the surface with half of the amount of salt used in the conventional process. The remaining cheeses, with no AE added, were submitted to the conventional brine salting during 20 min (half of the cheeses) and dry salting (the other half). All cheeses were ripened for a period of 45 days in a ripening chamber at 9.2 ± 0.58°C and 92.4% ± 3.1% RH.

### 2.2.2 Physicochemical and sensory evaluation of cheeses

Cheeses were evaluated for their moisture (NP 3544, 1987), fat (NP 2105, 1983) and salt contents (AOAC, 1997a). Humidity in defatted cheese, pH, titratable acidity (AOAC, 1997b) andwater activity ($a_w$) were also determined.The cheese hardness, was measured by means of a Stable Micro Systems texture analyzer, according to the method applied by Henriques et al. (2013).

In Fig. 7.1, it can be observed that the salting method did not affect the cheese moisture content. Cheeses with added AE of *T. mastichina* L. present slightly lower amounts of

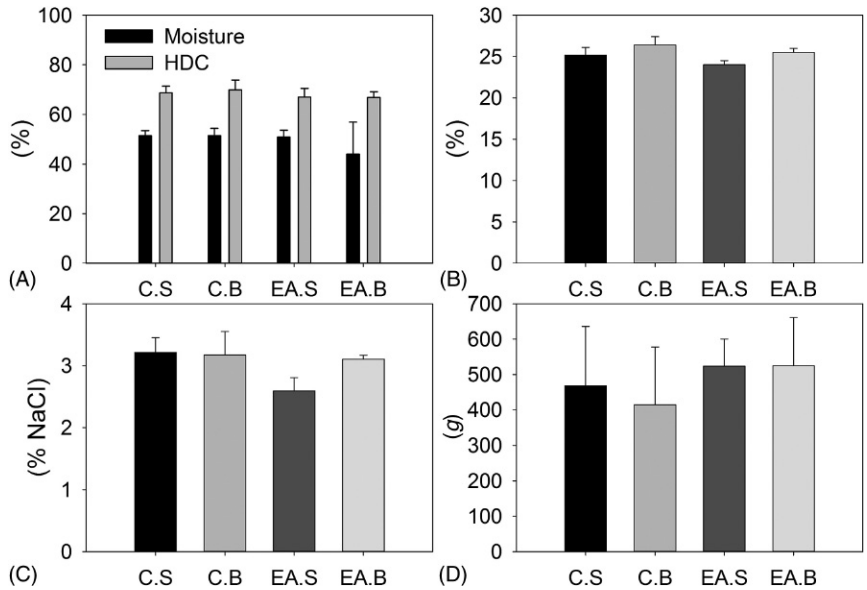


**Figure 7.1: Compositional Analysis and Texture Analysis of the Cheeses After 45 days of Ripening.**
(A) Moisture content and humidity in defatted cheese (HDC) (%); (B) fat content (%); (C) salt content (% NaCl); and (D) hardness (*g*). *C.S*, Conventional cheese with dry salting; *C.B*, conventional cheese salted in brine; *AE.S*, cheese with AE with dry salting; *AE.B*, cheese with AE salted in brine.

**Table 7.2: Titratable acidity (% lactic acid), pH, and color parameters ($L^*a^*b^*$ e $\Delta E^*$) of the cheeses after 45 days of ripening.**

| Types of Cheeses | Titratable Acidity | pH | Color Parameters | | | |
|---|---|---|---|---|---|---|
| | | | $L^*$ | $a^*$ | $b^*$ | $\Delta E^*$ |
| C.S | 0.30 ± 0.02 | 5.68 ± 0.33 | 76.54 ± 10.15 | −0.10 ± 3.58 | 2.14 ± 2.43 | 23.54 ± 10.63 |
| C.B | 0.64 ± 0.14 | 5.40 ± 0.24 | 81.51 ± 9.95 | −10.55 ± 5.03 | 9.11 ± 1.50 | 23.15 ± 9.97 |
| AE.S | 0.46 ± 0.01 | 5.46 ± 0.22 | 70.3 ± 10.98 | 6.57 ± 6.29 | 14.54 ± 1.85 | 28.47 ± 10.56 |
| AE.B | 0.48 ± 0.14 | 5.52 ± 0.20 | 75.58 ± 10.21 | 3.19 ± 4.79 | 15.32 ± 3.89 | 23.49 ± 10.18 |

C.S, Conventional cheese with dry salting; C.B, conventional cheese salted in brine; AE.S, cheese with AE with dry salting; AE.B, cheese with AE salted in brine.

humidity particularly in the case of AE.B (Fig. 7.1A). The lower water content results in the higher hardness values observed for this cheese type (Fig. 7.1D). Levels of humidity in the defatted cheese were presented in the range of 61%–69%, and so were classified as semisoft cheeses (NP 1598, 1983).

The amount of salt present in AE.S cheeses is significantly lower when compared to all other cheeses. AE.B cheeses present levels of salt similar to the conventional cheeses. This result indicates that the reduction of the time of immersion in brine did not diminish the amount of salt absorbed by the cheeses. These, somewhat unexpected, values may be the result of the small dimension of the cheeses (120 g), indicating a high rate of salt absorption (Carvalho et al., 2014).

Titratable acidity, pH, and cheese color are summarized in Table 7.2. According to the percentage of lactic acid and pH in the samples, it can be postulated that the AE may have a regulatory effect in the activity of the lactic acid bacteria in cheese during ripening, because the samples present values between the ones obtained for conventional cheeses.

Cheese color was evaluated using the Hunter CIEL$^*a^*b^*$ system, concerning individual coordinates and color difference ($\Delta E^*$) by means of a colorimeter HP-2132, Zheijang Top Instruments Co., calibrated with a yellow standard plate ($L_s^* = 98.9$; $a_s^* = -2.5$ e $b_s^* = 5.6$). Darker cheeses (lower $L^*$ values) were obtained with the addition of *T. mastichina* L. AEs comparing with the conventional cheeses. The effect of AE addition to the cheese is, however, more evidenced by the changes in $a^*$ and $b^*$ values. AE.S and AE.B cheeses present positive $a^*$ values (red direction) when compared to the conventional cheeses. The same occurs with $b^*$ (more positive values) indicating a more intense yellow color of the cheeses containing AEs of *T. mastichina* L. When global color variation ($\Delta E^*$) is concerned, the AE.S are the cheeses that showed higher differences from the yellow pattern. No significant changes were observed in the water activity of the cheeses with values ranging from 0.85 to 0.90, typical for this kind of cheeses.

Triangular tests, using an untrained panel of 21 persons, were performed in order to detect differences between conventional cheeses and cheeses with AE. Only 47.6% of the panelists were able to detect differences between the samples, which indicated that there were no

significant differences. In the preference test, 57.1% of the consumers preferred cheeses with AE. These results encourage the use of such extracts in cheese.

It could be concluded that the addition of AEs of *T. mastichina* L. in cheese may offer the possibility to produce cheeses with lower salt content and increased level of antioxidants. On the other hand, this will allow the valorization of an autochthonous plant. In our view, it is possible to further reduce the amount of salt in cheeses while maintaining its sensory characteristics. Further studies will envisage the reduction of salt to levels lower than 1%, which represents a significant improvement of the nutritional characteristics of cheeses. One of the aspects to be addressed is the need to modify the method of application to prevent the loss of extracts with the drainage of the curd. The use of concentrated extracts after partial drainage of the curd may overcome this problem. Finally, it is believed that such extracts can be also applied to several food products, such as cured meat products.

## 2.3 T. mastichina *L. Ethanolic Extract as Natural Antimicrobial in Ripened Cheese*

In conventional cheese production, natamycin is normally used to limit the microbial growth. Despite being generally regarded as safe (GRAS), the use of such product is limited to conventional cheeses and not allowed in several Protected Designation of Origin (PDO) products. Producers of PDO cheeses have to rely on good manufacturing practices and on intensive labor to control the development of molds on cheese surfaces. Even so, microbial contamination represents the major challenge for the producers. EE of *T. mastichina* L. were essayed as a possible alternative to decrease or even avoid spoilage microbial growth in traditional cheeses.

### *2.3.1 Antimicrobial efficacy of T. mastichina L. EE in vitro and in vivo tests*

After collection and characterization of the major types of molds, isolated from the cheese surfaces (using a cotton swab technique) and from the ripening chambers (by a sedimentation technique using open Petri-dishes containing plate count agar (PCA) or potato dextrose agar (PDA), a series of tests were performed in order to compare the efficacy of the EE of *T. mastichina* L. and of natamycin.

*Mucor* sp. and *Penicillium* sp. were grown in Petri dishes with PDA in the presence of EE of *T. mastichina* L. or natamycin (N) and compared with the negative control without inhibitor (C). The diameter of the colonies was recorded over 5 and 7 days at 25°C. From Figs. 7.2 and 7.3, the efficacy of EE in both the mold strains, chiefly concerning the inhibition of *Penicillium* sp., is evident (Carvalho et al., 2014; Ferreira et al., 2014).

In order to validate the results obtained in vitro, the extracts were essayed in raw milk cheeses submitted to a ripening period of 45 days at 11°C and 85% RH (Fig. 7.4).

No fungal growth was observed on the cheese surface treated with EE 8 days after application (Ferreira et al., 2014). However, in cheese with no treatment, there was a considerable growth

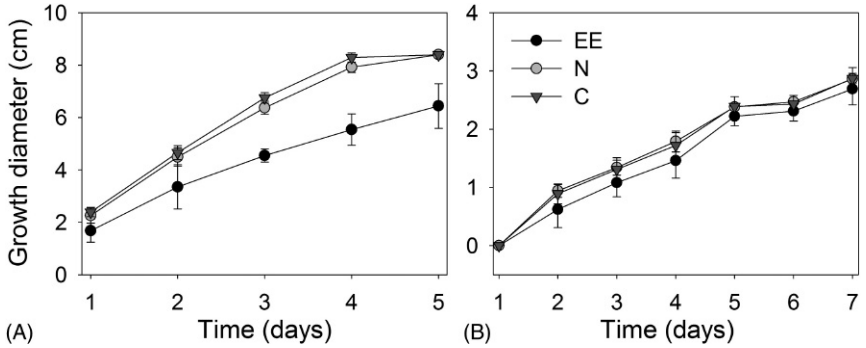


**Figure 7.2: Evolution of the Diameter of the Colonies.**
(A) *Mucor* sp. and (B) *Penicillium* sp. in the presence of *T. mastichina* L. ethanolic extract (EE) and natamycin (N) compared with the negative control (C).

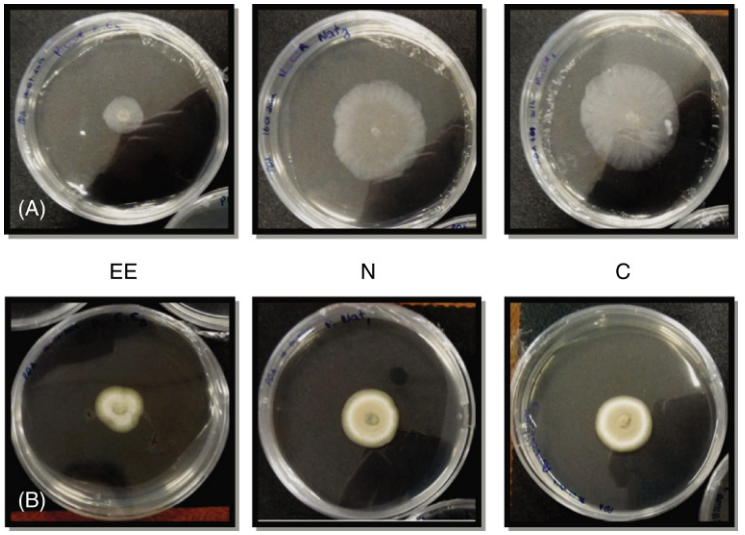


**Figure 7.3: Colony Growth Diameter.**
(A) *Mucor* sp. after 2 days; (B) *Penicillium* sp. after 4 days, in the presence of *T. mastichina* L. ethanolic extract (EE) and natamycin (N) compared with the negative control (C).

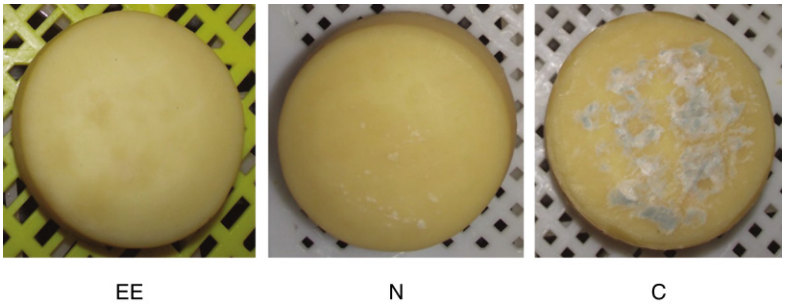


**Figure 7.4: Aspect of the Rind of the Cheeses 8 Days After the Application of the *T. mastichina* L. Ethanolic Extract (EE), Natamycin (N) and Compared With the Negative Control (C).**

of fungi. These results allow us to infer that the extract prevents spoilage mold growth on cheese surface, confirming the in vitro results. This represents several economic benefits for producers by decreasing handwork required for cheese washing during ripening.

### *2.3.2 Evaluation of the microbial profile of the cheeses*

The evaluation of the counts of several groups of microorganisms was performed after 1, 8, 16, 24, and 40 days after treatment. The tests consisted of the quantification of *Staphylococcus* spp. in Baird-Parker Agar Base (HIMEDIA MO43) according to ISO 6888, 1983, *Pseudomonas* spp. in Pseudomonas Agar F (PAF, DIFCO-0448-02-8) with due adaptations of ISO 13720, 2010, *Enterobacteriaceae* in Violet Red Bile Glucose Agar (VRBGA, HIMEDIA M581) after incubation at 37°C for 24 h, in accordance with Ramos (2011), and finally the quantification of yeasts and molds using (PDA, LIOFILCHEM-610102), incubated at 25°C for 5 days.

From Fig. 7.5, it can be observed that the EE of *T. mastichina* L. showed similar or even better results when compared to natamycin (Ferreira et al., 2014). For both inhibitors, and

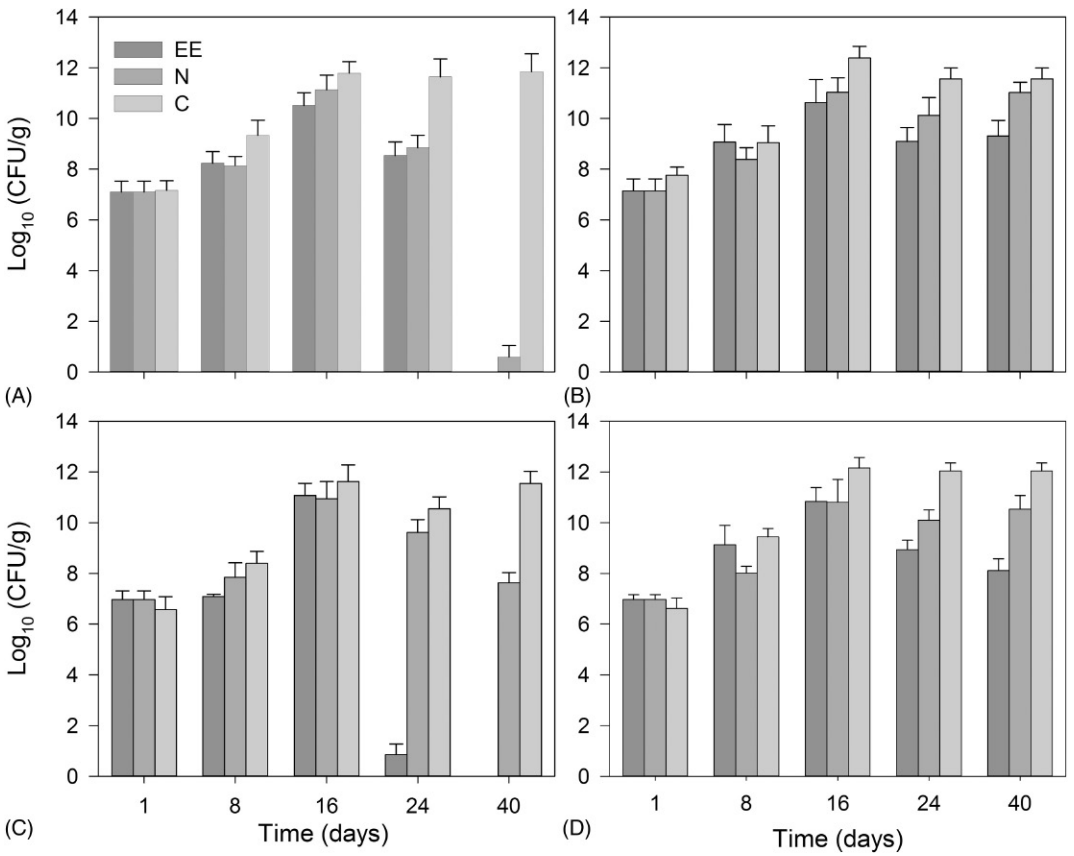


**Figure 7.5: Microbial Counts Over the Ripening Period.**
(A) *Staphylococcus* spp.; (B) *Pseudomonas* spp.; (C) *Enterobacteriaceae*; (D) yeasts and molds ($Log_{10}$ CFU/g) of cheeses treated with *T. mastichina* L. ethanolic extract (EE), natamycin (N), and negative control (C).

concerning *Staphylococcus* spp. the results reached almost zero CFU by the end of the ripening period. Concerning *Enterobacteriaceae*, an exponential growth was observed during the first 16 days, but after that, microbial counts reveal a reduction of 3 Log cycles for cheeses treated with natamycin and a total reduction in the cheeses treated with the EE. This fact can be explained by the increase in microbial competition promoted by the species that have higher replicative effectiveness in the environmental conditions to which they are subject at the end of the maturation period. According to several studies, EOs or extracts, in general, are more active against Gram-positive bacteria (Dagdelen et al., 2014; Govaris et al., 2011) as *Staphylococcus* sp., probably due to the protective role of the outer membrane found only in Gram-negative bacteria (Burt, 2004), like *Pseudomonas* spp. This can also explain the higher counts of *Enterobacteriacea* and *Pseudomonas* spp. compared with *Staphylococcus* spp. at 40 days of ripening in cheeses treated with natamycin. A screening for the antimicrobial activities performed by Atta et al. (2015) showed that natamycin antibiotic produced by *Streptomyces lydicus* AZ-55 was found to exhibit a wide spectrum of antimicrobial activities over Gram-positive, Gram-negative bacteria, and unicellular and filamentous fungi. However, it was also concluded that the inhibition was greater on Gram-positive and smaller on fungi.

With regard to *Pseudomonas* spp.and yeasts and molds; all have a similar growth pattern. It was observed that in the first 16 days, cheeses treated with EE had similar or slightly higher counts than the cheeses treated with natamycin, but this trend is reversed until the end of the maturation period. This reduction ranges from one to two orders of magnitude.

Ripened cheese samples without any treatment, as expected, presented a more pronounced microbial development for all the studied media. Although, at the beginning of maturation, all cheeses exhibited similar microbial counts, in the case of the control cheeses, there was a significant increase of microorganisms that remains at higher levels in the last days of ripening. In turn, the cheeses that received the treatment with EE showed the lowest contamination level.

The application of the EE in cured cheese production, both by conventional or organic production, yields a final product free of synthetic antibiotics, preventing the increase of antibiotics resistance by spoilage microorganisms. This fact represents an increase in food safety for consumers. The benefits for producers come from the fact they have a better and competitive product with less production costs.

### 2.3.3 *Efficacy of* T. mastichina *L. EE in ripening chambers*

The efficacy of the *T. mastichina* L. EE was also tested as a microbial inhibitor in ripening chambers. The microbial load of the air of the ripening room (Fig. 7.6) was evaluated before the nebulization of the chamber with EE and after 2 and 4 days, according to the method cited by Abelho (2012). The evaluation of the microbial load was made in Plate Count Agar (PCA, LIOFILCHEM-610040) for total aerobic microorganisms and in (PDA, LIOFILCHEM-610102) for yeasts and molds.

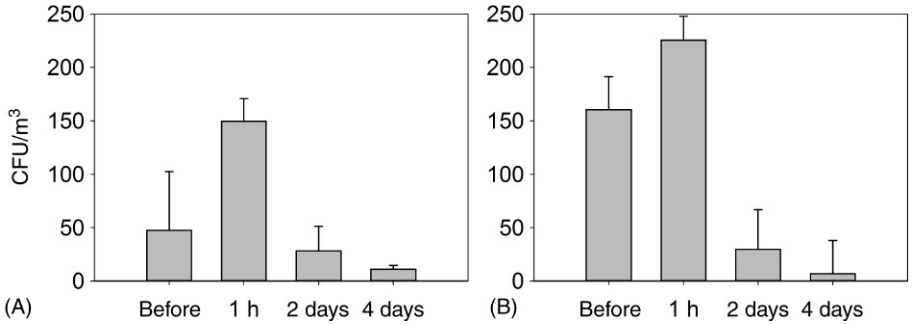


**Figure 7.6:** Counts of (A) total aerobic microorganisms, (B) yeasts and molds (CFU/$m^3$) of the air of the ripening chamber, before nebulization and 2 and 4 days after nebulization.

Before nebulization, the microbial load of the air was of 47 and 160 CFU/$m^3$ for total aerobic microorganisms and yeasts and molds count, respectively. One hour after the nebulization, both counts reach maximum values but suffer a drastic reduction on day 2 and 4. The increase in the load of microorganisms registered in the first hour results from the sedimentation that occurred after nebulization. This indicates that if the intention of use of EE extracts is the air chamber sterilization, the cheese samples should be introduced inside the room at least 1 h after nebulization, preventing the deposition of microorganisms at the cheese surfaces. From Fig. 7.6, it can also be concluded that *T. mastichina* L. EE presents higher efficacy over yeasts and molds. It was observed that after 4 days of application of the product, the microbial counts reached the minimum values.

*T. mastichina* L. EE, characterized by its antimicrobial properties, still presents disinfection properties of various surfaces that may be in contact with foods (e.g., manipulation surfaces), food preparation utensils, or even food products and/or their packaging.

### *2.3.4 Potential and limitations of the* T. mastichina *L. ethanolic extract*

Taking into account the different approaches of the utilization of the EE of *T. mastichina* L. presented in this chapter, and based on the promising results achieved, it is possible to conclude that its use can have an important and positive impact in the food industry, especially in the cheese production. Its performance as antimicrobiological agent gets close and sometimes even exceeds the antimicrobial activity of natamycin against some specific microorganism strains. Thus, producers in general may take advantage of the benefits of a natural and biological product that can be used directly in the cheese production or as fumigation agent in curing chambers.

More research is needed in order to evaluate the inhibitory effect of the EE on the microbiota responsible for the sensorial attributes of the cheeses at the final ripening stages. Nevertheless, one of the main difficulties envisaged is associated with its approval as a new food additive, including the determination of the maximum allowable concentrations

and dosages. Certification processes are usually time consuming and costly. They demand the implementation of in vivo toxicity tests, which may require long periods up to the commercialization of the product.

## 3 Conclusions

The *T. mastichina* L. AE allowed the reduction of the salting period and consequently the sodium content up to 28.0% compared to the traditional ripened cheeses.

The EE decreased in vitro *Mucor* sp. growth by 33.2% while natamycin only decreased its growth by 4.7%. In cheese, its antimicrobial activity was higher over spoilage yeasts, *Pseudomonas* sp. and *Staphylococcus* sp., whereas for *Enterobacteriaceae* showed similar efficiency to natamycin over 40 ripening days. The use of *T. mastichina* L. EE as a fumigation agent in ripening chambers, after 4 days of treatment, has reduced by 95.7% of the presence of yeasts and molds and by 76.9% of the total aerobic count.

Therefore, the use of *T. mastichina* L. EOs, EEs, and AE can be regarded as natural substitute of natamycin (or other synthetic antibiotics) used in the cheese production.

## Acknowledgments

This work was supported by program CENTRO2020, Operation Code CENTRO-01-0246-FEDER-000020, Grant nº6644: Lab2factory.

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CHAPTER 8

# Potential of High Hydrostatic Pressure to Improve the Production of Plants Used as Food

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## 1 Introduction

Using plants as a food source can be speculated to be dated back as human beings appeared on our planet Earth. Since then till today plants are an important and irreplaceable food resource for all human beings (Bennett, 2010). It is a well-known fact that this will be also true for the future, because human beings are heterotrophs and are not able to produce their own food like autotrophs, such as plants (Mauseth, 2008). Thus, we unfortunately depend on foods that we obtained from our external environment (Reece and Campbell, 2011).

All human beings need nutrients, which are components of food, to survive and grow. These nutrients can be classified under two main classes: macronutrients and micronutrients. Macronutrients mainly used for synthesizing other metabolites or providing energy, while micronutrients mainly act as cofactors for enzymes that take role in the metabolism. Metabolism is defined as the sum of all biochemical reactions that occur in the body for building up or repairing tissues and regulating the body processes (Whitney and Sharon, 2015).

As being perfect autotrophs plants produce macro and micronutrients for themselves by taking compounds from the soil through their roots and from atmosphere through their leaves, respectively. They also have the capacity of storing energy in the chemical bonds and using energy of sunlight during photosynthesis. As a result, plants are good resources for these nutrients because they not only produce food for themselves to survive, but also store these nutrients in some of their parts, such as in their roots, stems, leaves, fruits, and seeds for future use (Mauseth, 2008). As heterotrophs, human beings explore and find these nutritious parts of the plant and consume them as food.

When all the plant parts, where nutrients are stored, taken into account, fruits and seeds have the first level of importance among other parts of plants as a food resource for humans

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00008-6

(Ros, 2010; Salunkhe and Desai, 1984). Fruits are important for human nutrition as they are able to supply many macro- and micronutrients, such as carbohydrates, lipids, proteins, minerals, vitamins, particularly vitamin C and B-complex vitamins, dietary fiber, pigments, and some secondary metabolites (Salunkhe and Desai, 1984). On the other hand, seeds, such as the cereals, legumes, and nuts are nutrient dense foods, which have relatively low water content. Although a combination of several important nutrients are stored in seeds, they are accepted as an important source of nutrients as proteins, carbohydrates, and lipids are abundantly found in their composition, in addition to minerals, fibers, and several secondary metabolites (Ros, 2010).

Even though roots, leaves, and stems have relatively less nutrients as compared to fruits and seeds, these plant parts are equally important for human nutrition as they contain proteins, carbohydrates, lipids, vitamins, minerals, fiber, and water (Whitney and Sharon, 2015).

In addition to nutrients, plants also contain several secondary metabolites, which are also important in terms of human nutrition and health. Although the difference between primary and secondary metabolites is not very clear, primary metabolites are accepted as metabolites, which directly take up their role in growth, development, and reproduction. On the other hand, secondary metabolites are compounds, which do not involve such processes, but have some ecological roles, such as preventing the plant against stress conditions, defend the plant against bacterial, fungal, or herbivore attacks, attracting pollinators by producing a pleasant flavor or scent, etc (Altuner, 2014). Secondary metabolites are important for humans as being natural medicines having several antiinfective properties, such as antibiotic, antifungal, antiviral, anticancer, antioxidant, antidiabetic, antiinflammatory, insecticidal (Zhao, 2007), and capacity of being used as flavorings, fragrances, and food additives (Hussain et al., 2012).

As plants contain macronutrients, micronutrients, primary, and secondary metabolites, they are used as an essential component of human diet since early times either accidental or purposeful (Eaton and Konner, 1985).

It is proposed that early human societies were hunter-gatherers and collected their food by hunting and gathering, or in other words by foraging (Marlowe, 2005). It was suggested that one of their main food resources was wild edible plant parts. According to several evidences, it was thought that human beings were using several easy methods to preserve food for later (Huyck, 2012). But these methods were not enough to protect food for a long time, so these early human societies were thought to be frequently foraging for food. As they did not invent any method to produce their own food, in order not to fail to supply the food they required to survive, they needed to keep moving from one area to other time by time (Marlowe, 2005).

The very first trials of plant cultivation were just sowing seeds of some plants. Several excavations in diverse area, such as Jordan, Iraq, and Turkey uncover the earliest archeological evidences about early grain farming (Gibbons, 2016). As early grain farming

became successful, it caused some changes in habits of these humans, such as staying at the same place until harvesting of crops instead of their nomadic behavior (Snir et al., 2015). Early farming would possibly become favorable throughout the societies started to be experienced in grain farming as a reason of finding food easier than moving frequently from one area to other, for the society, especially after increased population.

According to the scientific studies, the very first evidence about trials for plant cultivation was about 23,000 years ago (Snir et al., 2015). Simple cultivation processes then were replaced with much more sophisticated agriculture processes, step by step, which created more powerful populations, because agriculture was a driving force to push humans live together in groups, to produce, and consume food they need (Mazoyer and Roudart, 2006).

In the ancient times, agriculture solved problems of gathering food for a population. But then, the main objective of food production was shifted from just producing enough food for the population to increasing productivity, palatability, and in some cases, the nutritive value of food crops (Bennett, 2010). Scientists worked to find different methods about modifying cultivars that will improve plant production and fit the recent needs of human population (Bennett, 2010).

This chapter is aimed to present the potential of high hydrostatic pressure (HHP) in improving plant production and expand the perspective of the scientists mainly working in this field.

## 2 Plant Production

To discuss an improvement in plant production, we should first understand some details about plant production chain.

Plant production chain is a typical food supply chain, which is mostly known as "farm to fork" production (Greer, 2005). A plant production chain can be divided into five main steps, which are production, processing/packaging, distribution, retailing, and consuming.

In plant production chain, the first step, namely production, starts with sowing, cultivation, and harvesting (Lehari, 2006). These three steps are the most important steps in improving plant production, thus we need to analyze these steps in details.

### 2.1 Sowing

Sowing as a term means the process of planting seeds. There are three main parameters related to sowing process, which may directly affect the plant production. These are depth of sowing, time for sowing, and the density of plants (Kurata and Kozai, 1992).

In addition to these parameters, several other factors may also affect germination and seedling emergence (Sayuti and Hitchmough, 2013), such as the quality of the seeds used during sowing (Alderson, 1987); dormancy, which is important in ability of germination of seeds

(Baskin and Baskin, 2001); application of several presowing methods before sowing the seeds (Khan, 2010); availability of water in the environment, which can be accepted as water stress (Hegarty, 1978); ambient temperature (Thompson and Grime, 1983); light (Fenner and Thompson, 2005); predation (Kirkpatrick and Bazzaz, 1979; Wilby and Brown, 2001); and pathogens (Kirkpatrick and Bazzaz, 1979; Wilby and Brown, 2001).

### 2.1.1 Depth of sowing

There are several studies in the literature showing that depth of sowing is a very critical parameter of plant production.

For example, Tamet et al. (1996) sowed *Daucus carota* (carrot) seeds to different depths to test the effect of depth of sowing on seed germination and determined that this factor is effective on the emergence force and also on the early growth in seed germination. Another data as an output of this study, which is important for us to discuss later, as we will be talking about the effect of HHP on seed germination, were the hypocotyl length of carrot seeds had correlation with the mean sowing depth for carrots and also the growth force was maximum at this heterotrophic growth phase (Tamet et al., 1996).

In another study, Harris (1996) observed again the effect of depth of sowing by using *Sorghum bicolor* (great millet, durra, jowari, or milo) and showed that seedlings have arisen much more quickly from the seeds sowed at shallow depths, when conditions are appropriate. But on the other hand, seeds sowed at deeper depths grow vigorously, when they are compared to the seeds sowed at shallow depths (Harris, 1996).

This information is very important to understand the reaction of seeds against HHP processing, which will be discussed later.

### 2.1.2 Time for Sowing

It is previously shown that time of sowing is another critical parameter in plant production. It is proposed as an important factor in controlling the size of plants (Chavasse, 1977). Early or late sowing may block the growth of seedlings, which directly affects not only the yield, but also the quality of the plants (Rashed and Darwesh, 2015).

Rashed and Darwesh (2015) conducted a study by using *Coriandrum sativum* (coriander) and they proposed that early sowing of coriander seeds will begin to produce flowers earlier than normal, but these plants are sensitive against extreme cold and frost. Sowing the coriander seeds later will affect the production of plants adversely not only in terms of the yield, but the quality of the plants as well.

The main idea behind deciding the optimum time for sowing is choosing the right time, which is matching with the climatic requirements of the seeds, such as environmental temperature, soil moisture, etc.

On the other hand, time of sowing is also effective in the crop phenological development, which is a key parameter in conversion of biomass into economic yield effectively (Khichar and Niwas, 2006).

Phenological development is important in all plant activities, such as leafing, flowering, and fruiting (Rosenzweig et al., 2008). Analyzing phenological changes has a capacity of providing key information to be used in predictive models for plant activities in plant populations (IPCC, 2014; Morellato et al., 2016; Rosemartin et al., 2014).

### *2.1.3 Density of plants*

The number of seeds present in a unit area directly affects the number of sprouts growing in the same place. Thus, it is a very critical parameter that should be considered while spreading seeds around (Menzies, 1988; Menzies et al., 1985). It was previously demonstrated that plants growing too closely will have fragile stems and roots and they tend to be very thin in their general structure. The main reason for that was explained as one plant may act as a sun shade to another, which is trying to grow up within spitting distance, so that the leaves of the plant trying to grow in dim light may have several complications (Kurata and Kozai, 1992).

On the other hand Zhang et al. (2016) showed that the number of plants produced in the same area also affects the amount of the grain production, and the nutritional composition of the grains, such as their protein content.

### *2.1.4 Seed quality*

Seed quality is one of the important parameters, which plays a key role in production of plants (Alderson, 1987). The term seed quality consists of several subparameters, such as genetic and physical purity of seeds, germination capacity, seed viability, vigor, and freedom from any seed-borne diseases and pests (Hill et al., 1997; Loch and Boyce, 2001; Thomson, 1979).

To test the seed quality, some standard protocols are commonly used, such as International Seed Testing Association (ISTA, 1999) and the North American-based Association of Official Seed Analysts (AOSCA, 1999a,b).

To put forward the effect of HHP on improving plant production, we should accept that the quality of seeds chosen is high according to one or more standard protocols mentioned earlier.

### *2.1.5 Seed dormancy*

Seeds are the key units in reproduction of a flowering plant, which are capable of developing into another new plant, thus seeds are important in survival of plant species. After the seeds are produced, they mostly stay in a dry state until they find suitable conditions to germinate and form a new generation. Seed dormancy plays an important role in the survival of plant species by allowing seeds to overcome some unfavorable periods for germination and

forming seedlings (Bentsink and Koornneef, 2008). So taking seed dormancy into account, it is important in plant production.

### 2.1.6 Water availability (water stress)

There are two main reasons for plants to be affected by water stress, which are the limiting amount of water and high transpiration rates. The primary reason of water stress is water deficiency as a result of drought, high salt concentration in the soil, etc (Lisar et al., 2012).

Seeds require water uptake especially during germination and there are several evidences, which prove that reduced water availability inhibits seed germination and the primary root emergence (Demir and Mavi, 2008; Eneas Filho et al., 1995).

In seed germination process, the first step is imbibition of water, which initiates embryo growth. Imbibition step consists of three phases (Bradford, 1986, 1990). The first phase is the phase in which a rapid uptake of water into seeds is observed. This step takes place right after the presence of water in the environment. It is followed by a second phase, where internal water is kept nearly constant with a very little change, which is also known as a plateau phase or a lag phase. In the last step of imbibition, an increase in internal water content is observed with the growth of radicle. It was previously shown that the second step is the most important step in the regulation of seed germination. After keeping the water content constant, the growth of the embryo is initiated. Thus, it can be proposed that any factor that affects the length of Phase II, will directly affects germination time. We need to keep this information in our mind to understand the reaction of seeds against HHP processing, which will be discussed later.

Several factors, such as dormancy, very high and very low ambient temperature, which extends the length of Phase II increases the time to need germination, whereas some other factors decrease the Phase II and drastically decrease the time for germination. So, it is logical for a seed to have longer Phase II, which means having longer germination time, those are under osmotic stress or water stress (Hegarty, 1978).

As the radicle growth is initiated, it penetrates into any enclosing tissues and starts to grow. The germination step is accepted to be ended right after a seedling growth is observed. So, if the seed germination time is shorter, seedlings appear quicker, which means they start to grow earlier than the seeds having longer germination time.

### 2.1.7 Ambient temperature

The effect of ambient temperature on germination and seedling growth is different in the seeds of different species (Sayuti and Hitchmough, 2013; Vassilevska-Ivanova and Tcekova, 2002). This depends mostly on the prevalent climatic conditions in the habitat of the plant (Grubb, 1977), in another words, the ambient temperature is important in matching up with the minimum and maximum temperature requirements for germination, seedling growth, or any other physiological process in the plant growth, such as leaf formation, flowering, etc.

For example, for the species adapted to cool climate, germination can occur mostly at low temperatures, whereas the species adapted to a warmer climate may germinate at much higher temperatures (Palazzo and Brar, 1997; Shimono and Kudo, 2005).

In general, it is accepted as plants adapted to temperate areas, germinate between 0°C and 35°C, but for plants adapted to tropical areas, germinate between 10°C and 45°C (Vassilevska-Ivanova and Tcekova, 2002).

O'Connor and Bredenkamp (1997) proposed that for most of the C4 grasses, daily mean temperature, which is higher than 25°C is required for germination.

On the other hand, *Watsonia* species, which are adapted to winter rainfall areas, need 10–20°C ambient temperature for germination, whereas the species adapted to summer rainfall areas need 15—25°C ambient temperature for optimal germination (Ascough et al., 2007).

### *2.1.8 Light*

The light requirements of seeds for germination is the key factor, which determines the right time and right place for germination and this is thought to be linked to an adaptation for survival of plant species (Motsa et al., 2015). This requirement prevents seeds to germinate in the place and times, which are not favorable to establish seedlings, such as at the wrong depth of sowing or at the wrong season (Fenner and Thompson, 2005). Some seeds have no preference of light and they both germinate easily in light or darkness, whereas some others prefer light or darkness to germinate (Chanyenga et al., 2012)

### *2.1.9 Presowing treatments*

Poor germination, yield, and quality are common concerns for plant producers. Several types of presowing seed treatments are usually applied To improve seed germination, time and efficiency, and to increase the total yield and its quality (Iqbal et al., 2012).

Presowing treatments can be classified under several categories, such as biological treatment, chemical treatment, mechanical treatment, water treatment, inorganic salt treatment, thermal treatment, electrical treatment, and combinations of those.

In biological treatments, the main aim is to increase hydration of seeds by using several types of biological compounds (Ashraf and Foolad, 2005). An example for this type of treatment is coating sweet corn seeds with bacteria, and then soaking the seeds into warm water to increase the seed moisture content to about 35%–40% (Tzortzakis, 2009). It was also proven that treating seeds with several bacterial strains, such as *Azospirillum*, *Pseudomonas,* and *Azotobacter* could affect germination and seedling growth (Shaukat et al., 2006). Soaking seeds into bacterial solutions have been shown to increase the germination rate and time, and vigor in carrot, cucumber, pea, beet, and tomato (Siqueira et al., 1993). The yield for wheat seeds has been increased up to 30% after inoculation of

seeds by *Azotobacter*, where up to 43% after *Bacillus* inoculation (Bakonyi et al., 2013; Kloepper and Beauchamp, 1992).

The chemical treatments mostly depends on using chemicals, which can act as an oxidative agent that soften the seed coat and cause an increase in the penetrability of this coat for $O_2$, $CO_2$, $H_2O$, etc. It was previously shown that absolute ethyl alcohol, concentrated sodium hydroxide and sodium bicarbonate solutions and concentrated sulfuric acid, hydrochloric acid, salicylic acid, and nitric acid can be used for this purpose (Asha Rani and Prasad, 2014; Rego et al., 2014). For example, to overcome seed dormancy and improve germination, sulfuric acid was shown to be effective in *Cotinus coggygria* (European smoketree) seeds (Olmez et al., 2009), *Tamarindus indica* (Tamarind) seeds (Muhammad and Amusa, 2003), and *Parkia biglobosa* (African locust bean) seeds (Aliero, 2004), and ethyl alcohol can be used in *Euphorbia heterophylla* (fireplant) seeds (Kern et al., 2009). Several hormones, such as gibberellic acid and indole-3-acetic acid are also another type of chemical treatment, which improves germination (Asha Rani and Prasad, 2014).

In mechanical treatment, damage is done by mostly creating cracks on seed coats or removing the entire seed coat to help water or gases permeate easily. Most common methods used in this treatment are rubbing seeds against sand papers gently, using several types of mechanical scarifiers and piercing a needle or a knife to make small incisions (Asha Rani and Prasad, 2014). Ren and Tao (2004) used mortar and silica as a mechanical scarification to grind 10 different *Calligonum* seeds to rupture their testa layer. They observed that mechanical scarification increases percentage of germination.

Another option in improving seed germination is treating seeds with hot or cold water before sowing. The idea of treating seeds with water or in other words soaking seeds before sowing is to shorten the lag phase of germination and to enhance establishing seedlings (Sabongari and Aliero, 2004). For example, most leguminous plants have a hard seed coat, which need to uptake moisture for germination and a presowing treatment with hot water is advised for such plants (Singh et al., 1991). A study conducted by *Acacia auriculiformis* (earleaf acacia), which is a leguminous tree in subfamily Mimosoideae of Fabaceae family, proved that treatment of seeds with hot or cold water increases the germination percentage. In this study, a germination percentage of untreated seeds were observed to be 43%, but an improvement in germination percentage was observed after treating seeds by hot and cold water as 83% and 52%, respectively (Azad et al., 2011).

To improve vegetable production, inorganic salt solutions have been successfully used as presowing agents (Heydecker and Coolbear, 1977; Yoon et al., 1997). Sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium sulfate, magnesium sulfate, and potassium nitrate are some of the salts which are used for this purpose (Govahi et al., 2007; Panuccio et al., 2014; Tobe et al., 2004). Govahi et al. (2007) used potassium nitrate as a presowing agent to improve *Beta vulgaris* (sugar beet) production and found that

potassium nitrate application increases the percentage of germination from 42% to 64%. On the other hand, they also observed that a combination of this salt with 0.05 mM of acetyl salicylic acid is better than using potassium nitrate alone, which increased percentage of germination from 42% to 89%.

Application of dry heat on plant seeds has been proved to increase the imbibition and germination to some extent. Depending on the type of seed, the optimum temperature application changes from one seed to another. While application of 60–80°C cause an increase in one type of seed, this can cause a serious damage on others. This type of treatment is mostly used for seeds, which are adapted to areas receiving high temperature during summer months (Nigam and Joshi, 1970).

In addition to these presowing treatments there are several new applications, such as electrical treatment, micro DBD plasma, high voltage nanosecond pulsed plasma, and electromagnetic field to improve the germination.

As it was summarized earlier, in addition to other steps in plant production, a presowing treatment step is mostly added before sowing to improve the plant production (Lehari, 2006). Thus, application of HHP can be accepted as a mechanical type presowing treatment, which may have a potential of improving plant production.

### 2.2 Cultivation

Cultivation or, in other words, horticulture is a branch of agriculture, which especially works on the science of growing plants. Productivity of horticulture process can be influenced by several parameters, such as the seed type used, the technology used in cultivation, climatic factors, edaphic factors, and the use of treatments to reduce the effects of stress conditions (Böhme et al., 2010).

The effect of seed type on yields, quality, nutritional value, and resistance to insects, diseases and environmental stresses of plants, and application of some presowing treatments to improve plant production were earlier discussed in detail.

The technology of cultivation is basically choosing cultivation strategies suitable for plant type, soil type, and environmental conditions (Bati et al., 2012).

Climatic factors are abiotic factors, which includes light intensity, temperature, relative humidity, air, and wind. Any change in these parameters will directly affect plant production.

Edaphic factors are factors, which are directly related to physical and chemical composition of soil. Physical parameters include texture, structure, moisture levels, pore space of the soil, and soil permeability. Chemical composition of soil includes pH, salinity, cation exchange capacity, organic matter, and carbon–nitrogen ratio (Dighe et al., 2009).

All these factors are important in improving the plant production, but since we will be talking about the potential of HHP on improving plant production in the later sections, there is no need to go into details about these parameters.

### 2.3 Harvesting

The last stage of the first step of plant production chain is harvesting, which can be explained as the process of collecting a ripe crop from the fields. On gathering crops from the fields, several different techniques may be applied, such as labor-intensive harvesting, especially used on relatively smaller fields, whereas harvest mechanization is mostly used on larger fields (Thompson and Blank, 2000). As the plant is collected from the field or any plant part is separated from its parent plant, it begins to deteriorate. Thus, handling procedures and environmental factors, such as temperature and relative humidity, is very critical, which directly affects the final quality of crop (Arah et al., 2015). So, any improvement in these parameters may also improve the crop yield, but this is out of focus of this chapter.

## 3 High Hydrostatic Pressure

HHP processing is a novel food-processing technology, which is one of the nonthermal food-processing methods and also known as a cold pasteurization technique.

In HHP applications, food samples are subjected to 100–800 MPa or in some cases even at higher pressures up to 1000 MPa (Altuner et al., 2012a; CFSAN, 2014). The potential of using pressure as a processing parameter has a long history dating back to the 19th century (Rendueles et al., 2011; San Martin et al., 2002). The very first observation on the effect of high pressure on food was about increasing the shelf life of food products, such as milk, fruit, and other foods after pressurization in 1899 by Hite (1899) (Rendueles et al., 2011; San Martin et al., 2002). But the application of HHP, especially in the food industry is quite recent and has taken place in the past 2 decades (Considine et al., 2008; Devlieghere et al., 2004; Farr, 1990; Rendueles et al., 2011).

Since HHP is a nonthermal food-processing technology, it has reduced effects especially on the nutritional properties and quality of processed foods in contrast to thermal food treatment processes (Tiwari et al., 2009), because it does not require any heating process, which may cause reversible or irreversible damages on especially biomacromolecules, such as enzymes (Altuner et al., 2005).

The process of application of HHP is isostatic, in which the pressure is transmitted uniformly. On the other hand, the transmission of pressure through the food sample is instantly and adiabatic. The applied pressure is transmitted uniformly and in all directions (Altuner and Tokuşoğlu, 2013). Thus the efficiency of the process does not depend on the shape or size

of food (Rendueles et al., 2011). In addition to this, pressure applied on a sample is directly transmitted through a pressure-transmitting medium, which is usually water (Oey et al., 2008; Rawson et al., 2011).

Temperature variation due to increase of pressure in water, used as a pressure-transmitting medium, is little, which is approximately 3°C per 100 MPa depending on the composition of the food (Smelt, 1998; Wilson et al., 2008). Little temperature change is extremely important in preventing deformation and heating of food, which may cause a deterioration in food's organoleptic properties (Rendueles et al., 2011).

Although HHP processing has several advantages, it is a well-known fact that after certain pressure values; HHP may cause several irreversible changes, such as cellular deformation, cellular membrane damage, and protein denaturation (Altuner, 2002; Altuner et al., 2014; Le Noble, 1988; Richard, 1992; Zhang et al., 2005).

In food sciences, HHP is used for several purposes. The most common use is sterilization of food samples and to provide coagulation and gelation in food samples as well (Bertucco and Vetter, 2001).

On the other hand, there are several successful applications of HHP in some different areas rather than food processing, such as pressure application on biological materials, were discussed by several researchers (Alpas and Bozoglu, 2002; Altuner and Tokuşoğlu, 2012; Altuner et al., 2012a,b; Bozoglu et al., 2004; Buzrul and Alpas, 2004; Kaletunc et al., 2004; Peñas et al., 2008; Peter et al., 1998). Although application of HHP on biological materials investigated intensively in last few decades, pressure was firstly proposed as a thermodynamical parameter, which can be used on biological samples, at the end of the 19th century by Regnard (1884), Royer (1895), and Hite (1899) (Rivalain et al., 2010).

Even though the potential of using HHP on biological samples were proposed in 1890s, scientists started to focus on this especially between 1910 and 1955, which yield great contributions to this field (Rivalain et al., 2010).

Particularly in the last 3 decades, there was a great increase of interest in the application of HHP in biology or biology-related fields, such as, biochemistry, molecular biology, biotechnology, and bioengineering. With these studies, a huge amount of information was collected regarding biological applications of HHP (Rivalain et al., 2010).

One of the successful applications of HHP on the biological material is using this to increase the percentage of germination, to decrease the germination time, and to improve the microbial quality of seeds and sprouts (Peñas et al., 2008, 2010).

To understand how HHP effects on seeds and sprouts, we need to put forward some basic definitions regarding HHP.

## 3.1 What is Pressure?

In a sample thermodynamic system, such as a closed container, the particles present in the container collide to the inner walls of the container. These collisions will cause a net force on the walls of this system. Thus, the sum of these collisional forces can be defined as pressure.

Pressure is an important parameter in thermodynamics, which is closely related with other variables of thermodynamics, such as volume, temperature, entropy, etc.

As a basic definition, pressure is the amount of force acting per unit area, which is symbolized as $p$ or $P$. As a mathematical equation, pressure can be defined by the following formula, in which $p$ represents the pressure, $F$ is the normal force applied to the surface, and $A$ is the area of the surface:

$$p = \frac{F}{A}$$

The official SI unit for pressure is Pascal (Pa). The atmospheric pressure, which is the pressure applied by the weight of air in the atmosphere at sea level has a mean value of 101,325 Pa. So, it is obvious that the Pascal unit represents a very small pressure unit. Thus, the Megapascal (MPa), which equals to $10^6$ Pa, is the common pressure unit used in HHP studies.

## 3.2 Static and Dynamic Pressure

There are two main types of fluid systems, which are static systems and dynamic systems (Heeley, 2005). As the name implies, in a static fluid system, the fluid is static, which means it is not moving. On the other hand, in dynamic fluid systems, the fluid moves.

Mainly two types of pressures can act on objects underwater, which are known as static and dynamic pressure (Southard, 2006).

In a static fluid system, a uniform static fluid is distributed constantly throughout the container. In such a system, the pressure at any point depends only on the depth. In addition to this, the pressure at any point is independent from the shape of the container. This type of pressure is known as the static pressure.

A static pressure can be isostatic or nonisostatic (Rivalain et al., 2010).

As a definition, isostatic means a state of equilibrium between two forces. Thus, isostatic pressure is referred to a pressure where the pressure value is the same in all the directions at a point in a system. Hydrostatic pressure is a good example for isostatic pressure (Rivalain et al., 2010).

Nonisostatic pressure is the type of pressure, where a pressure gradient is observed. One of the reasons of a nonisostatic pressure is the fluid in the system which is not homogeneous.

So, when a pressure is applied, a nonhomogenous pressure is generated due to the nonhomogeneous compressibility of the medium (Rivalain et al., 2010).

Dynamic systems are much more complex than static systems, where the pressure acting on an object depends on several parameters. As a result of this, measuring pressure on an object at a point in the system is more complex, when it is compared to a static system. In a dynamic system, three main types of pressures are observed, which acts directly on an object. The first pressure is the pressure acting on the object equally in all directions due to its depth, which is the static pressure that has been mentioned in static systems. As it was stated previously that in a dynamic system, fluid is not static, it is moving. Thus, the second pressure acting on the object is the pressure depending on the flow of the fluid, which is called as dynamic pressure. The third pressure is the total pressure that is the sum of the static and the dynamic pressures acting on the object (Rivalain et al., 2010).

### 3.3 Hydrostatic Pressure

The HHP is an isostatic pressure between 50 MPa and 500 MPa. We will discuss its effect on improving plant production later.

The equipment used to apply pressure on plant seeds is a static system and pressure applied is an isostatic pressure that acts with the same value in all directions on plant seeds.

### 3.4 Mass Transfer Theory

There are several reasons, which affect the efficiency of HHP, but the most important factor can be understood by analyzing the mass transfer theory.

The mass transfer theory proposes that the rate of mass transfer is directly proportional to the increase in pressure. In other words, cells processed under pressure present an increase in the permeability, especially through their membranes (Ahmed and Ramaswamy, 2006; Altuner et al., 2012b; Dornenburg and Knorr, 1993; Yan, 2002; Zhang et al., 2005). On the other hand, it is also known that HHP increases the rate of dissolution. As the pressure increases, a large differential pressure is observed between the interior and the exterior of cell membranes. This is the main driving force that is responsible for a rapid permeation (Zhang et al., 2005). Due to this rapid permeation process, more water can easily enter in the cells, which has a great importance in processing of seeds under pressure. As it was discussed before in seed germination process, imbibition of water is the first step and this step has three phases. In these phases, sequentially water enters into seeds, internal water is kept constant, and due to an increase in the internal water content, a growth in radicle is observed. Since the permeability is increased under pressure, the uptake of water will be much quicker and due to this, the internal water amount can be kept constant easily. As it was discussed before, the growth of embryo can be initiated after internal water content kept constant and any factor,

which may affect the length of this phase will directly affect at the time for germination. Thus, the internal water content can be established constant quicker under pressure, it is logical that HHP may shorten the germination time.

### 3.5 Main Factors That Characterizes Pressure

Pressure as a thermodynamical parameter used in HHP processing can be characterized by several factors, namely its energy, densification effect, and chemical reactivity (Rivalain et al., 2010). Among others, understanding the amount of energy generated by HHP application has great importance to understand why such a high pressure does not cause severe changes to some extent.

Before going into details, it is better to check the average bond energies of some common covalent bonds found in living organisms. The energies for these covalent bonds are given in Table 8.1 (King, 1994).

On the other hand, the energy generated by compressing 1 L of water due to an increase of pressure from 0.1 MPa, which is the atmospheric pressure to 400 MPa at 20°C, is only 19.2 kJ (Mertens, 1995). This amount of energy is comparatively below the energy required to break down the covalent bond having the lowest energy. Thus, it can be proposed that with generating relatively low energy, HHP can only affect chemical interactions, which are very weak compared to covalent bonds.

It is important to mention that this is true for systems, where the pressure-transmitting medium is water due to its degree of compressibility. The energy generated by pressure application will be higher, if the pressure-transmitting medium is gas rather than water (Demazeau, 2006; Wentorf, 1961). As a general rule, whether the pressure-transmitting medium is gas or liquid, the energy generated as a result of pressure application is lower than the energy needed to break down a chemical bond (Rivalain et al., 2010).

Although it is out of our focus, it will be good to mention about the amount of energy generated as a result of a temperature change, to compare the energy generated by pressure. At atmospheric pressure (0.1 MPa = 1 atm) an increase of 5°C, from 20 to 25°C, at the same

**Table 8.1: Average bond energies (kJ/mol).**

| Single Bonds | | Multiple Bonds | |
|---|---|---|---|
| **Bond Type** | **Energy** | **Bond Type** | **Energy** |
| H—H | 432 | C═C | 614 |
| H—O | 456 | N═O | 607 |
| H—C | 411 | C═N | 615 |
| H—S | 363 | | |
| C—O | 358 | C≡C | 839 |

medium, namely water, will generate about 20.9 kJ energy (Rivalain et al., 2010). As the energy generated by a temperature change is higher than the energy generated by a pressure change are different, especially biological samples exposed to temperature and pressure will react unlike each other.

Here, it is also important to mention how the character of the pressure-transmitting medium may affect the energy generated due to a pressure change. It is obvious that the compressibility, in other words, the degree of densification, is different for media, which has a nature of gas, liquid, or solid. As the degree of densification is related with the density of the medium, the difference in the degree of densification in different medium can be understood by looking at the scientific definition of the term density. Density of any substance is the quantity of mass per unit volume. So, densification is the process where the volume is decreased, while keeping the mass constant.

HHP can lower the volume of both pressure-transmitting medium and the sample under pressure and this volume change will cause densification.

Densification process will also trigger another phenomenon called the chemical reactivity. Due to a decrease in the volume during densification process, particles in the sample will condense. As the particles are condensed and the distance between the particles in the sample is decreased, it is logical to see an increase in the chemical reactivity and the kinetics of a reaction (Schettino and Bini, 2007).

As a result, it can be concluded that because the application of HHP does not cause generation of high energy, it does not cause unwanted changes on the samples processed to some extent.

## 4 Application of HHP on Plant Seeds

As it was mentioned earlier, HHP find itself a wide range of application area, whereas today it is mostly used in processing of food samples.

Application of pressure on plant seeds is not a very new issue. The main aim of these applications was mostly in improving germination time and rate, and increasing microbiological quality of seedlings.

De Vries (1915) was one of the first scientists applied pressure on plant seeds. *Oenothera cockerelli* seeds were used in this study, but the pressure applied was relatively low pressures, such as 6–8 atmospheres for 2 to 3 days. As a result of this study it was observed that application of pressure both decreased the germination time from 5–3 days and increased the germination percentage from 2% to 72%.

In contrast to De Vries (1915), Davies (1926) applied high pressure (2000 atmospheres) on *Medicago sativa* (*MS*) and *Melilotus alba* (*MA*) seeds. The seeds of these two plants

have problems in germination, as they have a water-resistant seed coat, which cause delay or failure during germination process. To improve the germination time and germination percentage of these seeds, traditionally, several mechanical methods, such as scarification by sand paper or using $H_2SO_4$ to destroy and increase the permeability of the seed coat, were used.

As a result of applying 2000 atmospheres of pressure at 18°C on *MS* seeds, about 50% improvement in germination percentage was observed, whereas the improvement is higher than 200% in *MA*. On the other hand, application of 500 atmospheres for 2–8 h did not present an improvement in the germination when the results were compared to the results obtained by the seeds applied with 2000 atmospheres for 1–10 min for *MS* and 5–20 min for *MA*. As a result of the study, it was proposed that application of high pressure for a short time was much more successful than the application of low pressures for a long time. On the other hand, it was also observed that the application temperature is an important parameter, whereas the application of pressure at 18°C gave better results as compared to the applications at 0°C.

Peñas et al. (2008) also studied the effect of pressure on *Vigna radiata* (mung bean) (*VR*) and *Medicago sativa* (alfalfa seeds) (*MS*) with the combination of application time and temperature parameters. They observed that application of pressures on *VR* seeds up to 250 MPa with an increase in the temperature had no effect on the germination capacity. On the other hand, they observed that the viability of *MS* seeds affected positively when temperature was increased 10–40°C, but negatively affected when pressure was increased from 100 to 400 MPa.

Shimizu and Kumakura (2011) tested the effects of HHP ranging between 0.1 and 400 MPa in combination with three different temperatures, namely, 4, 25, and 35°C, on the germination percentage and germination time of garden cress (*GC*), leaf mustard (*LM*), and radish (*RA*). They observed that *GC* seeds were found to be the most resistant seeds against pressure application in all pressure and temperature combinations. The shortest germination time for *GC* was founded to be of only 2 days. Although the percentage of germination was decreased during the first germination time, the germination percentage was increased over 90% on the 7th day of germination. The exception for this was for pressure and temperature combination of 400 MPa and 35°C. In this study, the most sensitive seeds for pressure application were found to be *RA* seeds. At the lowest temperature, which was 4°C, the germination percentage was found to decrease to about 25% for only 50 MPa pressure application. Although a slight recovery was observed at higher pressures, such as 350 and 400 MPa, this low germination percentage was still retained for application of higher pressures. Similar results, where decreased germination is recovered as a reaction against an increase in pressure was also observed in some other previous studies, in which sesame seeds were treated by HHP at 25°C (Wuytack et al., 2003). Shimizu and Kumakura (2011) also showed that *LM* seeds presented a close trend to the reaction of *GC* seeds against pressure. It was observed that 400 MPa

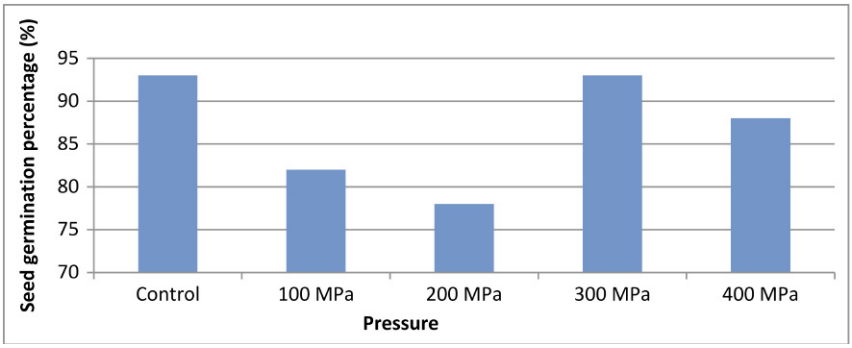


**Figure 8.1: Percentage of Seed Germination in Garden Cress.**
*Adapted from İşlek, C., Altuner, E.M., Çeter, T., Alpas, H., 2013. Effect of high hydrostatic pressure on seed germination, microbial quality, anatomy-morphology and physiological characters of the garden cress (*Lepidium sativum*) seedlings. High Press. Res. 33, 440–450.*

considerably affected germination time and percentage negatively. In addition to pressure, an increase in the temperature from 4 to 35°C was also found to decrease germination percentage from 70% to 50% on the 7th day.

İşlek et al. (2013) applied HHP on garden cress (*GC*) with a range of pressures between 100 to 400 MPa for 10 min at 30°C. In terms of germination percentage, they observed a slight decrease at 100 and 200 MPa, when compared to unpressurized seeds. The germination percentage at 300 MPa was equal to the germination rate for unpressurized control seeds and also slight decrease was observed at 400 MPa (Fig. 8.1).

Alexandre et al. (2014) applied only one set of parameters in terms of pressure and application time, which is 50 MPa for 10 min on pea seeds (*PE*). In this study, they have tested the effect of HHP on *PE* seeds, which were preprocessed by imbibition for different processing times. As a result of the study, they have observed that the percentages of germination for all seeds were decreased. But as no higher pressures were tested, it is not clear whether a recovery could be observed in higher pressures or not.

In another recent study conducted by İşlek et al. (2015), *Capsicum annuum* (pepper) (*CA*) seeds are exposed to HHP with different pressures, such as 50, 100, 200, and 300 MPa for 5 min at 25°C and pressurized and unpressurized *CA* seedlings were compared in terms of their percentage of seed germination and the mean germination time. As a result of the study, it was observed that the germination percentage was increased from 77.5% to 89.7% as a result of applying 200 MPa on *CA* seeds. On the other hand, mean germination time was decreased from 9.8–8.3 days (Fig. 8.2).

Mori et al. (2012) applied very high pressures, such as 5.5 GPa on seeds of *Brassica oleracea* var. *italica* (broccoli), *Brassica rapa* var. *perviridis* (turnip leaf) and *Brassica rapa* var.

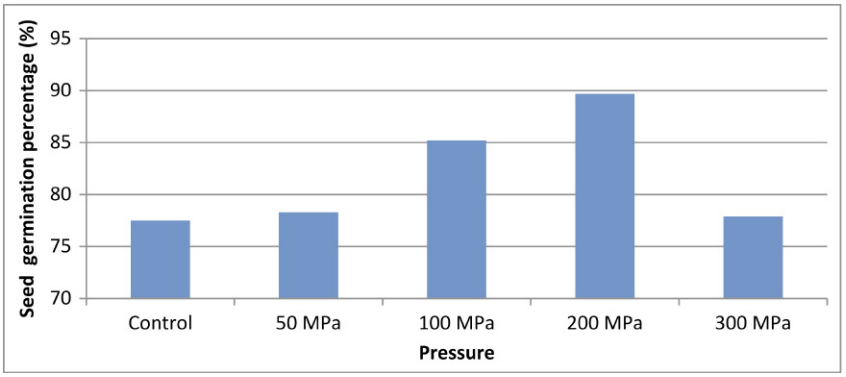


**Figure 8.2: Percentage of Seed Germination in Pepper.**
*Adapted from İşlek, C., Altuner, E.M., Alpas, H., 2015. The effect of high hydrostatic pressure on the physiological and biochemical properties of pepper (*Capsicum annuum *L.) seedlings. High Press. Res. 35(4), 396–404.*

*nipposinica* (potherb mustard). As a result of the study, they observed that although there was a decrease about 20%–30% in the germination percentage, the sprouts grew up as rapidly and tall as unpressurized samples.

Latest research regarding the application of HHP on plant seeds, especially focused on not only the germination time and rate, but also on some other parameters, such as microbiological quality of sprouts emerged from HHP applied seeds.

The reason of focusing on sprouts was due to their value of being used as cheap and good sources of dietary supplements. Sprouts are accepted as healthy foods according to their nutritional composition and availability (Kurtzweil, 1999; Mwikya et al., 2001). On the other hand, sprouts may cause an important problem of causing food-borne outbreaks (Bremer et al., 2003). As sprouts are mostly consumed fresh or half-cooked, consuming contaminated sprouts may cause food-borne outbreaks (Bremer et al., 2003; Feng, 2014; Peñas et al., 2010; Taormina et al., 1999). The high microbial load on sprouts also affects the shelf life (NACMCF, 1999). Several studies proved that the final microbial load of sprouts mainly depends on the insufficient decontamination of seeds before germination (Scouten and Beuchat, 2002). The main reason for that is during the germination process, nutrients present in the seeds will release, which makes a suitable place for microorganisms to reproduce. Furthermore, released nutrients with several optimal parameters, such as temperature, pH, $a_w$ (water activity), for reproduction of the microorganisms present on the seeds before germination process, will increase the microbial load on sprouts immediately during germination (Feng, 2014; Peñas et al., 2010).

Some recent studies proved that HHP application on seeds would increase the microbial quality of sprouts.

Peñas et al. (2008) used sprouts emerged from *VR* and *MS* seeds after HHP application to put forward the change in the microbial load in terms of total aerobic mesophilic bacteria (*TAMB*), total coliforms (*TC*) and fecal coliforms (*FC*), yeast and moulds (*Y&M*) populations. In general, it can be proposed that both *VR* and *MS* showed similar reaction in terms of microbial load on sprouts after HHP application. *TAMB* load on both plant sprouts reduced as a result of both temperature and pressure increase. The highest log reduction was observed for application of 400 MPa at 40°C. Total load of *TC* decreased as a response to an increase in pressure applied. Thus, the log reduction was the lowest for 100 MPa, whereas the highest was for 400 MPa. The response for a change in application temperature is slightly different than the log reduction of *TAMB*. As the application temperature was increased from 10 to 25°C, the log reduction was decreased, but after this point till 40°C, an increase in log reduction was observed. The log reduction of *FC* in *VS* and *MS* responded slightly different from each other. In *VR*, the log reduction of *FC* increases as both the application temperature and pressure increases. On the other hand, in *MS*, the log reduction of *FC* decreases as the application temperature increases from 10 to 30°C, but the response against an increase in pressure was an increase in log reduction too. The log reduction of *Y&M* in both plant sprouts were similar to each other, as the application temperature and pressure increased, the log reduction also increased. But it can be possible to derive from the results that *Y&M* on *VR* were more sensitive to an increase in pressure for all application temperatures. For *MS*, as the temperature increased, the sensitivity of *Y&M* to pressure change was increased, which means that in *MS*, the log reduction of *Y&M* was not much affected by an increase in pressure at 10°C, whereas they become very sensitive to an increase in pressure change at 40°C.

İşlek et al. (2013) also analyzed the effect of HHP on the microbial load on *GC* sprouts by looking at the change in *TAMB*, *TC*, *FC,* and *Y&M*. As a result of the study, it was observed that the microbial load on sprouts was significantly decreased as the pressure increases from 100 to 300 MPa. Except for *TAMB*, after application of 300 MPa for 10 min at 30°C, the microbial loads in terms of *TC*, *FC*, and *Y&M* was observed as zero. On the other hand, although the pressure was increased to 400 MPa, a recovery of microorganisms except *FC* was observed, where microbial load in terms of *TAMB* still continued to decrease.

There are some studies in the literature, which analyzed the effect of HHP on further parameters of seeds and sprouts rather than looking only germination time, germination percentage, and microbial load.

For example, Kadlec et al. (2011) tested the effect of HHP processing on the changes of α-galactosides in grain legume seeds during germination. In this study, 500 MPa pressure was applied on *Pisum sativum* (pea), *Cicer arietinum* (chickpea), *Lens esculenta* (lentil), and *V. radiata* (mung bean) (green gram) seeds for 10 min. It is a well-known fact that germination is a process, which is extremely effective to lower the α-galactosides contents in the grain legume seeds. As the seeds processed through HHP to increase the microbial quality, it was

found that α-galactosides are decomposed as well. Due to this decomposition, seeds were proposed to be on the market longer than unpressurized seeds. On the other hand, keeping the sensory qualities as good as the fresh seeds after being processed by HHP was presented to be a great advantage.

İşlek et al. (2013) analyzed the effect of HHP on fresh and dry weights, chlorophyll a and b, total phenolic compounds concentrations in seedlings, and the anatomy–morphology characteristics of *GC*. As a result of the study, it was observed that fresh weights of the seedlings were dramatically decreased in 100 MPa applied seeds, but a recovery was observed for 200 MPa, where 300 MPa was the highest fresh weight, when it is compared to all other samples including unpressurized control samples. The dry weights of the seedlings of 100 MPa applied seeds were remained the same as the unpressurized control samples, a slightly decrease was observed for 200 MPa applied seeds, but in 300 MPa, the dry weights were the highest among others. It was previously known that fresh and dry weights of seedlings are closely related to the growth rates, which means an increase in growth rate, can be understood by an increase in fresh and dry weights (Hurdzan, 1974). Thus it can be concluded that pressure application on seeds triggered and increase in the growth rates of seedlings, emerged from pressure applied seeds.

İşlek et al. (2013) proved that chlorophyll a and b content of the seedlings were lower than the control seedlings in 100 and 200 MPa, but both of these parameters were increased in 300 MPa, which are higher than control seedlings. Kacar et al. (2009) mentioned before that an increase in the ratio of chlorophyll b to a, is related to a decrease in the rate of photosynthesis. As a result of the study, it was observed that the rate of photosynthesis was decreased in seedlings of both 100 and 200 MPa applied seeds, but 300 MPa was induced an increase in the rate of photosynthesis. Another interesting result obtained from this study was regarding the total phenolic compounds in seedlings. In the study, it was observed that the amount of total phenolic compounds in seedlings of 200 and 300 MPa applied seeds were quite higher than control samples. Similar results were observed by Knorr (1994) before. Knorr (1994) presented that 50 MPa pressure increases the amount of anthocyanins in *Vitis vinifera* (common grape vine). These results are good evidences showing that HHP may affect several metabolic pathways, which may increase the development of some secondary metabolites as a response.

The maturation in seedlings can be identified by looking at the lengths of hypocotyls, which can be defined as the stems of germinating seedlings. İşlek et al. (2013) showed that the length of hypocotyls emerged from 300 MPa applied seeds are higher than unpressurized control samples. Higher hypocotyls mean ready for harvesting earlier.

İşlek et al. (2015) tested the effect of HHP on *Capsicum annuum* (*CA*), the seedlings in terms of their chlorophyll a and b content, proline content, total protein, carotenoid, malondialdehyde (MDA), glucose, fructose, and phenolic compounds concentrations. In this study, they have used chlorophyll a to b ratio to present the resistance of *CA* seeds to

different pressure applications. As a result of the study, they founded that *CA* seeds were resistant to pressures up to 200 MPa, but this resistance decreases in 300 MPa. The results about carotenoid content was found in correlation with the results obtained for chlorophyll a and b content and this correlation was explained as the accumulation of carotenoids and was acted as an oxidative stress protector in pressure applied *CA* samples. Thus, in the carotenoid accumulation, the plant could help to resist to pressure change up to 200 MPa. Some other proofs about establishing a resistance mechanism in *CA* seedlings, where 50, 100, and 200 MPa pressure was applied, were proposed by analyzing the change in free proline content and total glucose and fructose content. It was found that the free proline content was increased as the pressure increased from 50 to 200 MPa. It was previously shown that free proline accumulates as a result of a stress condition to establish a resistance mechanism (Matysik et al., 2002). İşlek et al. (2015) also found that the total glucose and fructose content were increased due to an increase from 50 to 200 MPa. In general, carbohydrates are produced in photosynthesis and an increase in total carbohydrates can be related to an increase in photosynthesis (Köksal et al., 2001; Matysik et al., 2002). Several environmental conditions may affect the synthesis of carbohydrates, so the results about an increase in both proline content and total glucose and fructose content were thought to be supported the idea about the plant that was under stress after pressure application and it tried to establish a resistance mechanism against this stress condition. In addition to these parameters, it was also observed that the total protein content was also increased. These proteins are proposed to be defense-related proteins or some new proteins synthesized as a response of pressure application. The idea of the pressure application as being a stress condition for *CA* seeds were also supported by two other parameters, which were MDA and total phenolic compounds content. MDA for unpressurized control samples were observed to be 2.258 nmol/g T.A, where it was 7.354 nmol/g T.A for the seedlings emerged from seeds processed by 200 MPa. Also total phenolic compounds were found to be 647.28 GAE/mL for unpressurized control samples and 1175.13 GAE/mL for the seedlings emerged from seeds processed by 200 MPa. These differences in both MDA and total phenolic compounds for unpressurized control samples and seedlings emerged from seeds processed by 200 MPa shows that pressure application can be accepted to be a stress condition in which plants started a resistance mechanism to protect themselves.

## 5 Conclusions

According to the previous studies, it is very clear that seeds processed by HPP respond to this effect and this response can be proposed to be an improvement in plant production.

The most important benefit in processing the seeds by HHP is that it helps in increasing the germination percentage, decreasing the germination time, and improving the microbial quality of the sprouts emerged from pressure processed seeds. But before discussing why and how, an improvement can be observed in plant production due to pressure application, we

need to accept that the quality of seeds used in the process should be high as it was mentioned previously.

The reason for an improvement in germination percentage and a decrease in germination time is most probably related to be establishing internal water content of the seeds much quicker under pressure. This is probably either due to an improvement on the seed coat as mechanical presowing treatments do or by increasing the permeability so that water can easily penetrate into the seed. As it was discussed before, when the internal water content can be established quicker, it may shorten the germination time and increase the germination percentage. So, if the germination time for seeds is shorter, seedlings appear quicker, which means they start to grow earlier than the seeds having longer germination time.

On the other hand, according to previous studies, the growth rates of seedlings emerged from seeds processed by HHP are higher than the seeds in which no pressure applied. As the growth rates are higher, the length of their hypocotyls is higher than the control seeds. This is very important point, because this means that pressurized seeds are ready for harvesting earlier than the unpressurized seeds, which would lower the costs for the production. An improvement in the growth rate may depend on several factors, which were discussed in the previous sections.

The first reason for the improvement in the growth rate is very basic. As seeds processed by HHP germinated earlier as compared to the seeds unpressurized, they will start to grow quicker, which make them ready for harvesting earlier.

The second reason for the improvement in the growth rate may be due to the response of the plant against a stress condition. As it was stated before, the rate for photosynthesis increases as a response against pressure application. An increase in photosynthesis would cause the improvement in the growth rate.

The third and may be the most speculative reason would be related to the response of a seed against the depth it was sowed. As it was discussed before, seeds sowed at deeper depths grow vigorously, when they are compared to the seeds sowed at shallow depths (Harris, 1996). Plant seeds may understand, whether they are sowed at deeper depths or shallow depths with the pressure on them. If they are sowed at deeper depths, they need to increase the length of their hypocotyls longer as quick as possible, so that they can reach to sunlight, before the nutrients deposited in the seed finish and to start the process of photosynthesis, as soon as possible. So, the seeds, which were processed by HHP, would react as they were sown at much deeper depths and in order to survive they may increase their growth rate to reach the sunlight. Although this reason seems speculative, it is logical if we remember the study conducted by Tamet et al. (1996) using *Daucus carota* (carrot) seeds that we have discussed in detail before. This study proved that the length of carrot seed hypocotyls had correlation with the mean sowing depth and also the growth force was maximum at this growth phase.

As a result, increasing the germination percentage and decreasing the germination time can be accepted as an improvement in plant production.

As it was discussed before, improving microbial quality of the seedlings is extremely important, because seedlings are mostly consumed fresh or half-cooked, consuming contaminated seedlings may cause food-borne outbreaks (Bremer et al., 2003; Feng, 2014; Peñas et al., 2010; Taormina et al., 1999). So, increasing microbial quality of seedlings is important for the health of the consumers. Thus, an improvement in the microbial quality can also be accepted as an improvement in the plant production. The reason of why application of HHP increases the microbial quality of seedlings depends on the response of the microorganisms to pressure, which is out of focus of this chapter.

As it was previously discussed in detail, in addition to the germination percentage, germination time, and microbial quality, the effect of HHP on some other parameters, such as fresh and dry weights, chlorophyll a and b, total protein, total glucose and fructose content, and phenolic compounds concentrations were also studied.

As it was shown before, application of pressure may increase both the fresh and dry weights. This is another point presenting that HHP may improve plant production, because increasing fresh and dry weights mean to increase the yield of food per harvest. In addition to fresh and dry weights, it was also proved that there will be an increase in total protein and total glucose and fructose content. This can definitely be accepted as an improvement as some nutritional characteristics of plants, such as protein and carbohydrate content could be improved.

The last point which should be discussed is an increase in total phenolic compounds in plants due to HHP application. Phenolic compounds are some secondary metabolites synthesized by plants. As it was discussed before, secondary metabolites have a great importance with their pharmacological importance, which can be used as an antioxidant, antimicrobial, anticarcinogenic, immunomodulatory, etc. In addition to these properties, some of the secondary metabolites are also responsible from some organoleptic properties, such as taste and smell. Using HHP, an increase in total phenolic compounds is noticed; this may be accepted as an improvement in plant production, because it is possible to increase the plant's pharmacological and organoleptic properties.

As a result, it can be concluded that HHP has some potential of improving plant production, but it is clear that the optimal parameters for improving the production of different plant types are different from each other. So, further researches should be conducted to identify these optimal parameters for different plants.

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CHAPTER 9

# Corrosion in Electronic Sensors Used in Manufacturing Processes Decrease the Quality in the Seafood Industry

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## 1 Introduction

In the Baja California State located in the northwest of Mexico, there are various companies, in which nearly 60% are the electronics industries, 25% are food industries, and the rest uses electronic devices as electronic sensors in the industrial machinery and industrial equipment. In any industrial plant, the production planning is of great importance in the development of their manufacturing processes and expansion of these, which contemplates the cost elements: raw materials, labor (wages operators) and indirect charges: depreciation of machinery, buildings, transportation systems, and furniture, mainly (Garetti and Taisch, 2012). Tijuana city of the Mexican Republic is located in the zone where temperatures in summer ranges higher than 35°C and reaches near 0°C in winter (Badilla et al., 2011c). A study from 2014 to 2015 suggests that seafood industry generates millions of dollars and has a good influence on the global market. The major quantity of the industrial processes of the food industry that is located in this zone of Mexico, contains electronic sensors, and is exposed to climatic variations and concentration levels of air pollutants, being the principal are the sulfurs, chlorides of the sea breeze, and the nitrogen oxides (Badilla et al., 2010a). The climatic variations considered as a meteorological phenomenon, in combination with the air pollutants generates the atmospheric corrosion, which deteriorates the metallic surfaces of the electronic sensors and originates malfunction of these. This originates unnecessary costs by the presence of atmospheric corrosion, causing the damage of the electronic sensors of industrial systems, industrial machinery, and industrial equipment in indoors of the manufacturing processes (Gao et al., 2015). For this reason, an investigation was done, based on the costs generated by the presence of corrosion phenomenon, to determine the

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00009-8

intensity of the effects on costs and deterioration of the metallic surfaces of the electronic sensors to evaluate the corrosion process. For this analysis, an information of levels of CR was obtained and correlated with climatic factors pollutants [relative humidity (RH) and temperature] and air ($H_2S$, $SO_X$, $Cl^-$, and $NO_X$, mainly), using the gravimetric method with the metallic probes of copper, tin, nickel, and silver, that are the materials mostly used in the electronic sensors. Copper and silver found to be the most affected materials. The generation of corrosion observed at this stage of the study, occurred even with the company controlled environments, showing small spots of electrochemical phenomenon. To determine the pollutants that caused the deterioration of metals, the technique of Auger Electron Spectroscopy (AES) was used and images at nanoscale showing the levels of corrosion (Badilla and Tesis, 2008).

### 1.1 Food Industry

The food industry is the industry that is responsible for all the processes related to the food chain. They included the concept stages of transportation, receipt, storage, processing, preservation, and food service for human and animal consumption. The raw materials of this industry mainly consist mainly of vegetable products obtained from agriculture activities, animal products as livestock, and fungi products (Nestle, 2013). Thanks to the progress of science and food technology, this industry has seen a remarkable growth and is now increasing the number of possible foods available in the diet. The increased production has been linked with a progressive effort in monitoring hygiene and food and depending of the laws of the countries trying to regulate and unify the processes and products.

### 1.2 Processes of the Food Chain

Production processes can be classified into six different processes: food transport, food reception, food storage, food process, food preservation, and food service, which are mentioned subsequently (Badilla et al., 2013; Vasconcellos, 2003).

### 1.3 Food Transport

In this process, all procedures seeking food safety during transportation from the place of production to storage or processing are included. In this field, the engineering concepts are applied to maintain proper temperatures and avoid environmental pollution (Badilla et al., 2013; Vasconcellos, 2003).

### 1.4 Food Reception

It's just a part of the link in the food chain with the receipt of goods. It is a critical checkpoint in the quality assurance systems, seen that if decomposed raw materials are received and

if they are not managed with care, can cause damage to the health. The criteria for the acceptance and rejection of goods, very often, are decided by the professional judgment of quality inspectors applied at this stage (Badilla et al., 2013; Vasconcellos, 2003).

### 1.5 Food Storage

This step was made to evaluate the time of exposure of food when is stored in polluted environments. To prevent the rapid deterioration of the food, was applied some regulations as the controlled rotations of food and the storage temperature and relative humidity in the storage locations of the food. They generally are used for storage in silos, warehouses conditioners to a specific industry type, with hermetic places, outdoors and cold rooms, principally (Badilla et al., 2013; Vasconcellos, 2003).

### 1.6 Food Processing

This stage is as varied as the amount of food in the world. Each food has its different processing, and quantity or complexity of the procedures varies according to the type of food. Normally, the food-processing diagram depicting process flow charts and control systems depends on the quality assurance (Badilla et al., 2013; Vasconcellos, 2003).

### 1.7 Food Preservation

The usual processes of food preservation are aimed at the initial transformation of food to obtain a different product and transformed to present a longer shelf life. Some of the processes of conservation are: using salt, dehydration, refrigeration, freezing, pasteurization, sterilization and acidification, essentially (Badilla et al., 2013; Vasconcellos, 2003).

### 1.8 Food Service

Within the food industry, there are establishments that are responsible for preparing food for service and consumption. In these establishments gastronomic there are techniques to prevent the rapid deterioration of the food, and is included restaurants, canteens, school canteens, industrial canteens, hotels, hospitals, cruises, and street food sales. This group of establishments includes restaurants, canteens, school canteens, industrial canteens, hotels, clinics, hospitals, cruises, and street food sales (Badilla et al., 2013; Vasconcellos, 2003).

### 1.9 Seafood Industry in Mexico

The type of industry has great interest because the seafood contains some important vitamins, nutrients, and supplements in the development of the human body in any period of the age. From 1990´s, the food industry increased in the Mexican Republic,with the General Agreement on Tariffs and Trade at global market, and was made a restructuration

of the seafood industry in our country (Badilla et al., 2012; Badilla et al., 2011b). In 1994, Mexico signed the Free Trade Agreement (NAFTA) with Canada and the United States and thus the seafood industry increased the productivity and competitiveness of the Mexican economy. Currently, the principal cities in the Mexico country with seafood industries are Veracruz, Mazatlán, Tijuana, Los Cabos, Acapulco, and Cancun; where these industrial plants are creating a high quantity of jobs to the Mexican population.

### 1.10 Nutritional Properties of Seafood

The seafood is an important key of the nutrition process, being the fish one of the most consumption in the majorly of the world. Within the term seafood, we can distinguish two groups, being the crustaceans (principally, prawns and lobsters) and the second group are the mollusks (essentially, mussels, clams, cockles, razor clams, oysters, scallops, snails, octopus, squid, and cuttlefish) (Vasconcellos, 2003).

### 1.11 Nutritional Benefits

Seafood has water from 75% to 80% and also protein as high biological value, in each 100 g of edible portion of 18–20 g that are knotweed as nutrients. It is also composed of minerals, such as calcium, magnesium, phosphorus, potassium, sodium, zinc, iodine, iron, and chlorine; and vitamins, being the most abundant group B and lesser amounts vitamin A and vitamin D (Badilla et al., 2013). The seafood has polyunsaturated fatty acids, which is considered very healthy for our body and provides low cholesterol, generating positive effects in the health of people with low concentrations of fat. It also contains purines that are responsible for raising the level of uric acid in the blood. In 100 g of crabs, 115 g of purines are found while out of 100 g, 87 g of oysters are purines, reducing the energy value, contributing on average 80 calories per 100 g (Badilla et al., 2012).

### 1.12 Health Benefits

For all the nutritional characteristics mentioned, we can say that the seafood is very beneficial to health. Thanks to its content of polyunsaturated fats that reduce the risk of blood clots, protect us from cardiovascular diseases, and also reducing bad cholesterol. On the other hand, seafood provide high biological value proteins, necessary for the proper growth and development, containing calcium which is beneficial for proper bone health and preventing osteoporosis (Mahaffey et al., 2011). Its iodine content prevents diseases, such as goiter. Because of its low calorie level, seafood can be included in diets suitable for reducing the body weight. Some people with health problems, such as shellfish allergy, high uric acid, pregnancy, young children, and women of childbearing age (the last three by its high content of heavy metals, such as cadmium, lead, or mercury) eat seafood with some limits (Bath et al., 2013). The life cycle of seafood has its best period between the

months of November to March (autumn–winter). Instead, during the warmer months (May–August), its flesh is softer, loose flavor and weight, and will generate health problems. The crustaceans should have a pleasant smell and remain intact. If purchased alive, they must move and their eyes should be black and shiny. Never submit detachments between head and tail. The mollusks should have tightly closed shells and the cephalopods should have the following characteristics: smooth, firm, unblemished and pearly skin color. To make consumption healthy, it is ideal to combine and switch to seafood with other meats and fish (VKM, 2014). The recommendation is to include seafood at least 3 times a week, being a normal ratio from 125 to 150 g of raw weight. It is recommended as accompaniments of vegetables, salads, or rice to obtain a varied and balanced dish. To limit the intake of calories, it is important to select cooking methods, such as steaming, grilling, boiling, or baking. The quality and safety of fishery products and aquaculture are increasingly perceived as key competitive advantages in access to different markets. The marketing of fishery products and aquaculture have evolved significantly, in recent years, in terms of new formats and products that are considered as a healthy alternative to meat by consumers. Fresh fish rapidly loses its original quality due to microbial growth and enzymatic processes. The principal deterioration of seafood is caused by the exposition of hard waters, and presence of polluted environments. The breakdown of proteins by microorganisms generates unpleasant odors (Wennberg et al., 2012).

### *1.13 Corrosion in the Food Industry*

The basic function of an electrically conductive material of the electronic sensors, used in the food industry, is to conduct electricity. The electrical conductivity of the contact materials can be greatly reduced by the presence of corrosion. To avoid corrosion, protective coatings are used but still with aggressive environments, some metallic surfaces deteriorate by corrosion (Samuels and Young, 2003). Another phenomenon that leads to the increased contact resistance is fretting corrosion, which occurs in improper connections that causes short circuits and power failures or sometimes fire due to electrical overload. Also, fretting corrosion and the mechanism of degradation of the material surface results in the increased contact resistance (Connolly, 2006). Fretting corrosion occurs when there is a relative movement between the electrical contacts with stationary metal surfaces. This occurs in industrial plants and in the company investigating the seafood type. The presence of corrosion generate deterioration in the electrical connections and connectors and with this the presence of electrical failures. Gold is one of the materials commonly used for very high performance electrical contacts and due to its high corrosion resistance and stable electrical behavior, its cost is high. There are scientists who have investigated different ways to minimize the consumption of gold for electrical contacts and improve the performance of this metal. Other materials used in the corrosion protection of the conductive surfaces are tin, nickel, silver, and palladium (Badilla et al., 2011b).

## 1.14 Functionality of Electronic Sensors

The efficiency of the industrial operations is based on the productivity and quality of industrial equipments and machines of the electronics industry. The main features to show the effectiveness of a manufactured product are appearance of the product, low cost, ease of operation, and safety (Badilla et al., 2007). And electronic equipment used in storage areas, production, and transportation devices are exposed to environmental factors in the interiors of industrial plants, influenced by the environment outside. Climatic factors, such as relative humidity (RH) and temperature in combination with sulfates, affects the operation of electronic systems and thus affecting the business economy. The seafood industry accounts for much of the productive sector worldwide, so it is of great importance in the economy of each country where these businesses are being installed and are dedicated to the manufacturing of seafood products to consume. There are a lot tools used to evaluate the numeric information, statistical methods, such as the graphs for inventory control analysis of parts of industrial machinery and raw materials, in addition to assessments operative yielding and productive performance. The electronic sensors used in the food industry contain a variety of macro, micro, and nanoelectronic devices that perform the functions for industrial machinery to operate properly and its ability determines the operative yield of the equipment and systems used in the manufacturing processes (Veleva et al., 2008). If the functionality of some of these electronic components decreases, other devices are affected, and thus industrial machines fail to operate at peak performance. Some factors that industrial equipment and machines are by the presence of corrosion and this decrease the operative yielding of these. This electrochemical phenomenon occurs by the effect of condensation caused by the temperature and RH levels above and influences the formation of water films on metal surfaces that can be visible or invisible. This causes damage to the surface of the connections and connectors of electrical and electronic equipment forming copper sulfide (Cu) as corrosion product after removing the degraded material. Based on that, the surface mass of Cu is lost, reducing the electrical resistance, and generating faulty electrical conductivity or may cause a short circuit that can cause fire in the company (Badilla et al., 2010b). The deterioration of metal surfaces of electrical and electronic components of industrial machinery is due to the aforementioned climatic factors and the presence of sulfides in the interiors of industrial plants electronic rotation.

## 1.15 Electronic Sensors

An electronic sensor is a transducer that varies its output voltage in response to a magnetic field. For example, the Hall effect sensors are used for proximity switching, positioning, speed detection, and current detection applications. In its simplest form, the sensor operates as an analog transducer, directly returning a voltage. With a known magnetic field, its distance from the Hall plate can be determined. Using group of sensors, the relative

position of the magnet can be deduced (Moncmanova, 2007). Electricity transmitted through a conductor produces a magnetic field that varies with the current, and Hall sensor can be used for measuring current without breaking the circuit. Typically, the sensor is integrated with a permanent magnet core or wound around the conductor to be measured. Often, a Hall sensor is combined with circuitry that allows the device to operate in a digital (on/off), and can be called a switch in this configuration. This sensor is commonly used in the industrial applications, such as the pneumatic cylinder, computer printers and to detect the lack of open and covered with paper. When high reliability, used in keyboards is required. The laser displacement meters are used throughout the automotive industry to control the position of the moving parts in a vehicle and providing continuous feedback on operating conditions (Rocak et al., 2005). As a result, they allow designers and engineers to maximize the vehicle performance and improve operator safety. Automotive applications are typically hostile environments and sensors have to withstand extreme temperatures, shock, vibration, and high. With current market demands the electronic sensors are used in a lot applications and is evident considered as a high technology. Sensor technologies are chosen to provide the optimal performance and reliability in specific applications (Badilla et al., 2011a).

### *1.16 Air Pollution*

The air pollutants mentioned earlier, causes aggressive environments and are generated by external companies, such as geothermal power plant generating electricity to the city of Tijuana city sources, to the border cities in the state of California of the United States of America. The sulfides used as gases can penetrate holes, cracks, and air-conditioning systems and even sometimes by the filters that are placed in construction companies. Because of this situation, connections, devices, and parts that require electrical energy to perform activities on industrial machines, reduce their operational life and stop working much before than planned by the supplier of such parts. Companies always have proper planning in each production department with regard to inventory control of parts of equipment and systems for the electronics industry (Badilla et al., 2010a). Occasionally, by the presence of electrochemical phenomenon, it is necessary to replace the damaged parts of industrial machines before the end of its lifetime. This create an imbalance and some times the lack of prevention of electronic parts of industrial equipment and machines, can generates malfunction of these and can stop the manufacturing processes. Because of that, yield decreases and thereby company faces economic losses, which is a major concern to the operating personnel, specialized management, and owners of industries (Badilla et al., 2013). This has occurred in certain companies of the city of Tijuana city. No profit in the electronics industries has dismissed their employees as companies are not able to bear the costs of production and sales. For this reason in some times, this situation can originated by the government of the region to be part of leaving unprotected labor source.

In order to have control of this type of situation, specialists develop security plans. But while this is solved, which is very common in all companies in the region, tools, such as graphs that are used. Graphs are used with the aim to always providing the necessary replacements and to prevent the operating equipment and systems of these companies to stop (Veleva et al., 2008).

### 1.17 Atmospheric Corrosion

The atmospheric corrosion is undoubtedly the most visible of all processes of corrosion, as rusty bridges, flag poles, buildings, and monuments outdoors. The large segment of the paint industry engaged in the manufacture and application of products for the protection of metals and large-scale operations in the galvanizing industry, indicative of the importance of controlling the atmospheric corrosion (Badilla et al., 2012). Economic losses caused by the atmospheric corrosion are tremendous and therefore the disappearance of a significant portion of the metal produced was found. Consider, for example, agricultural machinery, steel structures, fences, metal exposed in buildings, car mufflers or agencies, and the myriad of metallic elements are discarded when they become unusable as a result of corrosion. These are direct losses by corrosion. Atmospheric corrosion has been reported to account for more failures in terms of cost and tonnage than any other type of material degradation processes. This particular type of material degradation has recently received more attention, especially in the aviation industry since the Aloha incident in 1988, when a Boeing 737 lost an important part of the top of the fuselage in flight at 7300 m (Badilla et al., 2011b). All general types of corrosion attack in the atmosphere occur. In presence of humidity environments, in the metal is corroded is formed electrolytic cells to deteriorate the electrical connections and connectors. Therefore, the calculation of the potentials of the electrodes on the basis of the ion concentration, determination of polarization characteristics, and other electrochemical operations are not so simple in atmospheric corrosion. Is necessary consider the majorly of factors in the generation of electrochemical corrosion to applied methods to prevent it (Badilla et al., 2010b).

### 1.18 AES Analysis

It is an important technique at nanoscale, used in the seafood industry, even knowing that the testing equipment are very expensive to detect bacteria and fungi in food, are necessary in their manufacturing processes with complex functions (Van Ingelgem et al., 2003). Once the chemical composition of elements and compounds in the samples is determined, one can observe the spatial distribution of these, to meet the contaminants of greater and lesser impact reacted with the metal of the electronics sensors that are used by the industrial systems, industrial equipment, and industrial machinery (Badilla et al., 2011a). The generation

of corrosion is promoted. A distinction between the different compounds is possible to determine the oxidation state of the metal ions participating in the corrosion products.

### 1.19 Operative Yielding of Industrial Equipment and Machinery

The operative performance of the industrial systems and industrial equipment of the seafood industry using electronic sensors is of great importance in the economic profits or losses. When in the manufacturing areas, the activities are developed with good functionality, its generates expected results in operations daily, weekly, monthly, seasonal and annual (Shank and Govindarajan, 2005). The operative performance is part of the yield where machines and hand work are part of this type of industrial plants. Analysis with graphs as statistics information, information on the behavior of manufacturing processes can be obtained to identify the causes of possible events with difficulties in their activities and to further increase the productivity and competitiveness. It is useful to know about the operative performance of the industrial systems, industrial equipment, and industrial machinery out of which the maximum benefit of the operations is obtained. All electronic sensors used in the seafood industry, usually show electrical failures (EF) in certain periods, continuous or discontinuous, of the year due to the wide variety of electronic devices and components and as these act as primary connections and connectors, hence deteriorate faster and easier. Sometimes an electronic sensor may remain without operated for short or long period of time, it is a matter of concern for operating personnel, specialized management, and owners of companies (Ballesteros, 2007). The electrical failures that occur in industrial equipment and machines. In the industrial systems, industrial equipment, and industrial machinery of the seafood industry, a study was conducted on three types of areas where you have installed electronic equipment and where the corrosion phenomena are present (Badilla et al., 2013).

### 1.20 Atmospheric Corrosion in Indoors of the Seafood Industry

The higher levels of RH 70% and temperature 35°C are the main factors of atmospheric corrosion in indoors industrial plants in arid environments, such as the city of Tijuana city, where sometimes there is no airflow. This creates a major change in the valence state of metals, such as Cu assets deteriorate faster. Corrosion is caused by the chemical reaction from exposure to metal sulfides, $NO_X$, ammonia, and organic compounds mainly being sulfides that generate a greater effect on the deterioration of metallic materials (Badilla et al., 2011c). Copper is a metal with low cost and easy to manage, and for this reason it was specially analyzed. When oxide films are formed in the surface of Cu by the presence of atmospheric corrosion, occurred the electrical failures. The use of copper in the electronics sensors, which are used in the seafood industry, is of great interest for its excellent electrical and thermal

properties, and so are used in the exposed environmental conditions of such industries. In the corrosion process, the physicochemical properties of Cu are changed. The objective of this investigation was to determine the principal factors of the presence of atmospheric corrosion that generate the malfunction of industrial equipment and machines and the rapid deterioration of food in the region of Mexico evaluated with the climatic and pollution parameters evaluated. This was to apply some methods to prevent if (Badilla et al., 2012). An important aspect of corrosion is rates air quality (RAQ) of air pollutants, such as sulfides in this city. These are overcome every year, as it promotes quick and easy corrosion on the electronic sensors used in the seafood industry and installed in indoors. These indices are proposed by the Secretariat of Environment and Natural Resources (SEMARNAT) and the Environmental Protection Agency (EPA) (Badilla et al., 2013). Corrosion is an important factor in the maintenance costs and premature failure of electronic devices and equipment factor. Life prediction of structural and functional materials performance is vital for safe, reliable, and efficient operation of aircraft, ships, bridges, buildings, and other infrastructure systems. The determination of the first signs of corrosion damage and corrosion assisted is essential for life prediction and corrosion prevention. It helps reduce major repairs and catastrophic losses. The use of sensors to find corrosion is the best choice for which early detection and control is possible. Sensors corrosion, corrosion detectors, and systems corrosion control are becoming more popular these days because it can be used to increase product life and reduce the cost of maintenance of metal structures. The combined actions with the increased miniaturization of electronic systems and the explosive increase in availability, it is expected that corrosion and deterioration of electronics will become a serious social problem with unpredictable consequences. The mechanisms of corrosion in electronic components have undergone extensive studies. Since electronics is mainly indoors or inside enclosures, the mechanisms leading to corrosion problems are not easy to define. The problems are compounded by the fact that these systems are manufactured by a series of complex processes and consist of a variety of different materials. The miniaturization and the requirement of high component density have resulted in smaller components, closer spacing, and thinner metallic paths. Thus, the effect of polarization potentials and small defects are magnified (Badilla et al., 2011b).

## 2 Methodology

One of the concerns of business owners in the seafood industry and management is to avoid economic losses. This occurs when phenomena referred to as the process of corrosion deteriorates the metal materials equipment and industrial machines. At present, this situation generates electrical failures causing a decrease in the operational performance. The deterioration of electrical connectors and connections of these materials is showing up in the first month of the study, a low corrosivity, indicating a slight damage that can be expanded as time evaluation. That is why there is a need for evaluating the effects of corrosion by levels

in the electronics industry with costs generated by this phenomenon. Based on this, metallic specimens are evaluated, requiring weight to obtain mass loss on an analytical balance to the nearest of 0.0001 g of reliability. To determine the concentration of sulfate within industrial plants technique sulfation dishes were applied inside the company. The operative performance analysis for the industrial systems, industrial equipment and industrial machinery of the seafood industry was investigated, various activities were developed and appropriate tools for the study presented later were used and includes the following:

1. An evaluation of the electrical failures of the industrial systems, industrial equipment, and industrial machinery of the seafood industry was done, with the Excel program and subsequently studied by MatLab, which is a specialized statistical method (Walsh et al., 2010).
2. A mathematical correlation was made with the MatLab program on climatic factors and air pollutants mentioned earlier, with specialized devices to monitor the temperature and humidity in time periods; daily, weekly, monthly, seasonal, and annual, using the same programs mentioned in the previous section. Also the assess levels of intensity of deterioration of electronic equipment were analyzed causing electrical faults by the presence of corrosion and publicize the adverse effects on the cost of the electronics industry, by daily, weekly, monthly, seasonal, and annual periods from 2014 to 2015, to determine the degree of effect on the deterioration of copper materials.
3. An analysis of the metallic specimens mentioned earlier were used for the analysis with gravimetric method to determine the weight loss of each metal evaluated in the first month of the study, correlated with the minimum, maximum, and average values of RH and temperature environments in the interior of the industry were analyzed, in the current period, based on the ASTM standards (ASTM Standards, 2000) and ISO standards (ISOA, 2006; ISO, 2005).
4. The corroded metals were analyzed by AES technique (Philips ESEM XL). With this technique important information from chemical pollutants reacting with the metal surface was obtained. Even in the first month analysis, the morphology of corrosion products showed data without effecting much on the deterioration of metals, but significant to evaluate the future corrosion process. Corrosion products were observed under an optical microscope by MBE before being cleaned.
5. An economic analysis of financial losses was made due to the presence of corrosion that was occurred.

## 3 Results

The level of corrosion presented by the metallic surfaces of the electronic sensors evaluated, in the first month of the study, was not very significant. It may worsen the process by generating the decrease in the operating performance of equipment and industrial machines

with electronic devices. With this investigation are founded the principal methods to prevent the presence of atmospheric corrosion and the deterioration of food. Mainly, this can occur with customer losses compliance with guarantees for the sale of defective electronic products, breach of contracts with customers, and higher repair costs of equipment and industrial electronic machines. That is why, the project was made with the aim of assessing the presence of corrosion in the company analyzed.

### 3.1 Evaluation with ABC Graph of Electrical Failures of Industrial Machines

The industrial systems, industrial equipment, and industrial machinery, had variation of the rates at different times of the investigation. In times of day, power failures occurred with a slightly higher than the standard level, from the 10 a.m. in the months of June, July, and August and from 1 p.m. in December, January, and February. This happened in this way by the effect of condensation, as in summer, the visible or invisible film of water is formed and evaporates faster than in winter period. Tables 9.1–9.3 show the analysis with the graphs in according to the types of areas where electronic devices are installed and are placed as a test in the electronic sensors. Such representations are the causes of higher level to the lower intensity for schedule analysis; daily, weekly, monthly, seasonal, and annual. Both the axial machines are the radial causes of its defective inoperability, only with the difference in the direction of the installation of electronic components on the electronic board of manufactured products, such as televisions and computers in the enterprise resembled where he developed the study. Various types of causes were evaluated and represented in the tables in an organized manner to assess the graphs. Tables 9.1–9.3 show the rates of electrical faults

**Table 9.1: Statistical evaluations of electrical failures of industrial machines that use electronic sensors in axial position in the industrial systems, industrials equipment, and industrial machinery (August–December 2014).**

| Causes of Improper Operation | Periods | | | | | |
|---|---|---|---|---|---|---|
| | Hourly | Daily | Weekly | Monthly | Seasonally | Annually |
| Improper alignment of installation of electronic components | 1 (35%)<br>10 a.m. | 1 (33%)<br>Jueves | 1 (32%)<br>Semana 03 | 1 (36%)<br>Enero | 1 (37%)<br>Invierno | 1 (37%)<br>2011 |
| Deficiency in supply of air compressed to electropneumatic system of the machine | 2 (26%)<br>12 a.m. | 2 (25%)<br>Lunes | 2 (25%)<br>Semana 49 | 2 (26%)<br>Diciembre | (28%)<br>Invierno | 2 (25%)<br>2011 |
| Faulty operation in the feeder unadjusted of the electronic components | 3 (18%)<br>05 p.m. | 3 (28%)<br>Martes | 3 (29%)<br>Semana 07 | 3 (20%)<br>Febrero | (23%)<br>Invierno | 3 (25%)<br>2011 |
| Bad communication of the computer program with the industrial machine | 4 (13%)<br>02 p.m. | 4 (15%)<br>Jueves | 4 (13%)<br>Semana 30 | 4 (12%)<br>Agosto | 4 (11%)<br>Verano | 4 (14%)<br>2010 |
| Mismatch of the detection systems of electronic devices bad installed | 5 (14%)<br>04 p.m. | 5 (12%)<br>Viernes | 5 (12%)<br>Semana 25 | 5 (13%)<br>Julio | 5 (14%[a])<br>Verano | 5 (12%)<br>2010 |

[a]Axial position.

**Table 9.2: Statistical evaluations of electrical failures of industrial machines that use electronic sensors in radial position in the industrial systems, industrials equipment, and industrial machinery (August–December 2014).**

| Causes of Improper Operation | Periods | | | | | |
|---|---|---|---|---|---|---|
| | Hourly | Daily | Weekly | Monthly | Seasonally | Annually |
| Improper alignment of installation of electronic components | 1 (31%)<br>11 a.m. | 1 (32%)<br>Jueves | 1 (29%)<br>Semana 04 | 1 (30%)<br>Enero | 1 (31%)<br>Invierno | 1 (29%)<br>2011 |
| Deficiency in supply of air compressed to electro-pneumatic system of the machine | 2 (25%)<br>12 p.m. | 2 (23%)<br>Martes | 2 (24%)<br>Semana 47 | 2 (26%)<br>Diciembre | (26%)<br>Invierno | 2 (26%)<br>2011 |
| Faulty operation in the feeder unadjusted of the electronic components | 3 (20%)<br>03 p.m. | 3 (19%)<br>Martes | 3 (21%)<br>Semana 08 | 3 (20%)<br>Febrero | (20%)<br>Invierno | 3 (20%)<br>2011 |
| Bad communication of the computer program with the industrial machine | 4 (13%)<br>02 p.m. | 4 (15%)<br>Viernes | 4 (16%)<br>Semana 29 | 4 (13%)<br>Agosto | 4 (15%)<br>Verano | 4 (15%)<br>2010 |
| Mismatch of the detection systems of electronic devices bad installed | 5 (11%)<br>05 p.m. | 5 (11%)<br>Viernes | 5 (10%)<br>Semana 26 | 5 (11%)<br>Julio | 5 (08%[a])<br>Verano | 5 (10%)<br>2010 |

[a]Radial position.

**Table 9.3: Statistical evaluations of electrical failures of industrial machines that use electronic sensors of final test in the industrial systems, industrials equipment and industrial machinery (August–December 2014).**

| Causes of Improper Operation | Periods | | | | | |
|---|---|---|---|---|---|---|
| | Hourly | Daily | Weekly | Monthly | Seasonally | Annually |
| Missing component on board not detected by the electrical test equipment | 1 (30%)<br>10 a.m. | 1 (31%)<br>Jueves | 1 (29%)<br>Semana 05 | 1 (31%)<br>Febrero | 1 (30%)<br>Invierno | 1 (29%)<br>2011 |
| Inadequate communication equipment to the computer and does not detect proper positioning of electronic components | 2 (24%)<br>11 a.m. | 2 (24%)<br>Martes | 2 (25%)<br>Semana 48 | 2 (25%)<br>Enero | 2 (25%)<br>Invierno | 2 (25%)<br>2011 |
| Missing information in test equipment appropriate value of installed components on the board | 3 (19%)<br>04 p.m. | 3 (18%)<br>Lunes | 3 (20%)<br>Semana 09 | 3 (19%)<br>Diciembre | 3 (21%)<br>Invierno | 3 (19%)<br>2011 |
| Lock test equipment program for low electrical conductivity and necessary to rekindle programming system | 4 (15%)<br>03 p.m. | 4 (15%)<br>Viernes | 4 (17%)<br>Semana 29 | 4 (14%)<br>Julio | 4 (15%)<br>Verano | 4 (16%)<br>2010 |
| Detection template components installed on the board with defects caused confusion in programming | 5 (12%)<br>05 p.m. | 5 (12%)<br>Jueves | 5 (09%)<br>Semana 27 | 5 (11%)<br>Agosto | 5 (09%[a])<br>Verano | 5 (11%)<br>2010 |

[a]Final test.

for each period evaluated, showing that the winter was the most problematic situation of low operating performance of the industrial machinery company.

In Table 9.1, an hourly evaluation was conducted, which shows that the highest percentages of power failures occurred in the morning shift, whereas the lowest rates were down in the evening hours. This indicated that during the night and early morning, the effect of condensation was generated with the increase of at least 1–2°C, in the morning, corrosion originated in the electrical connections and connectors and electrical and electronic systems of the industrial plant. In the daily evaluations were evaluated the electrical failures in summer and winter periods with RH higher than 80% and temperatures higher than 30°C. Weekly periods with higher rates of electrical failures were in winter and in summer, HR levels were higher than 80%, regardless of the temperature values. The same was true when analyzing the monthly period where the winter was key in generating electrical failures of industrial machinery and the highest rates of the electronic failures on an annual basis were in 2014. It was because every year in the city of Tijuana, RH increases in summer for at least a small percentage, from 1% to 3% and in winter, decrease in at least 1°C in temperature, was observed. These small variations modify the outer atmosphere of companies and have varied environments within them. This coupled with air quality levels exceeding in greater numbers each year and generate easy and quick aggressive environments in the interiors of companies causing economic losses. The same happened in the radial or vertical component installation of machines and the electrical test of the products manufactured in the company. With this investigation the company evaluated improved its manufacturing processes. According to the graph analysis, the electrical failures with higher incidence created greater concern for the staff of the company, it was the first three data for each table, where the percentages indicated. It should be noted that based on the evaluations with the graphs, other electrical failures were presented on a smaller scale and took only these data to be the highest occurrence and indicated by the company to be assessed and be organized information, based on tables and graphs. The goal of the company was to meet with rates of electrical failures levels close to or greater than 50% to determine the highest incidence and thus making improvements to the production processes.

### 3.2 Analysis of Atmospheric Corrosion

The presence of atmospheric corrosion in the indoors of the seafood industry generates deterioration in metallic surface of the electronic sensors evaluated, taking in some areas of industrial plant with specialized detection systems of particles and gases. Some air pollutants penetrate the air-conditioning system, which is widely used in the city of Tijuana, for cracks, crevices, den walls, and ceilings. This occurred in a seafood industry of this city, where a study was conducted, air pollutants were penetrated to the clean areas of seafood industries. Table 9.4 shows the air pollutants and their

**Table 9.4: Concentration levels of air pollutants in the Tijuana city (2015).**

| Min., Max. (μg/m³)/Months | January | February | March | April | May | June |
|---|---|---|---|---|---|---|
| CO ($1 \times 10^6$) | 9.35–16.55 | 9.13–15.89 | 8.88–13.76 | 8.34–12.66 | 7.99–9.79 | 7.31–8.13 |
| $NO_x$ ($1 \times 10^6$) | 0.033–0.097 | 0.028–0.088 | 0.031–0.082 | 0.026–0.068 | 0.021–0.049 | 0.017–0.044 |
| $O_3$ ($1 \times 10^6$) | 0.04–0.16 | 0.04–0.14 | 0.03–0.11 | 0.03–0.1 | 0.02–0.08 | 0.01–0.06 |
| $SO_2$ ($1 \times 10^6$) | 0.05–0.17 | 0.04–0.14 | 0.04–0.11 | 0.03–006 | 0.01–0.04 | 0.01–0.03 |
| **Min., Max. (μg/m³)/Months** | **July** | **August** | **September** | **October** | **November** | **December** |
| CO ($1 \times 10^6$) | 5.68–7.77 | 6.93–8.42 | 7.51–9.05 | 8.92–11.11 | 9.54–13.44 | 11.22–17.89 |
| $NO_x$ ($1 \times 10^6$) | 0.016–0.049 | 0.022–0.056 | 0.028–0.063 | 0.041–0.079 | 0.056–0.085 | 0.061–0.095 |
| $O_3$ ($1 \times 10^6$) | 0.01–0.05 | 0.02–0.08 | 0.03–0.09 | 0.04–0.1 | 0.04–0.12 | 0.06–0.14 |
| $SO_2$ ($1 \times 10^6$) | 0.01–0.04 | 0.02–0.06 | 0.03–0.06 | 0.03–0.08 | 0.04–0.119 | 0.04–0.13 |

Average values considered by the SEMARNAT and EPA: CO = 9 ppm/8 h; $9 \times 10^6$ μg/m³; $NO_X$ = 0.053 ppm/annual average; $0.053 \times 10^6$ μg/m³; $O_3$ = 0.08 ppm/8 h; $0.08 \times 10^6$ μg/m³; $SO_2$ = 0.03 ppm/annual average; $0.053 \times 10^6$ μg/m³.

concentration levels in monthly periods. The analysis period was from 2014 to 2015, with the highest concentration in 2015 where the pollutants mentioned were generated as compared to 2011. This table shows the levels of concentration of pollutants evaluated in monthly periods indicating the higher rates in the winter period due to the phenomenon of the greenhouse in the city of Mexicali. All contaminants showed the same trend. The protruding was $SO_2$, where ranges were presented within the rates established by the EPA and SEMARNAT in the month of June 2015. The NOx not exceed only in may, June, and July of 2015, of the Aiq Quality Standards (AQS). CO and $O_3$ have influencein the generation of atmospheric corrosion with less effect. AQS is chageud by ECA.

### *3.3 Evaluation of Climatic Factors*

Table 9.5 showed the indices of external and internal climatic factors that affected the interior of the seafood industry. These were the important factors in the generation of

**Table 9.5: Concentration levels of humidity and temperature (2015).**

| Months (HR, T) | January | February | March | April | May | June |
|---|---|---|---|---|---|---|
| % (min., max.) | 14.8–90.2 | 14.7–94.5 | 11.0–95.8 | 10.6–71.6 | 9.5–82.8 | 9.6–91.1 |
| °C (min., max.) | 6.3–29.1 | 6.0–30.9 | 8.6–32.6 | 11.1–33.4 | 14.2–42.4 | 19.6–45.8 |
| **Months (HR, T)** | **July** | **August** | **September** | **October** | **November** | **December** |
| % Min, Max | 9.5–90.7 | 13.7–90.1 | 9.3–88.9 | 8.7–90.8 | 14.2–94.5 | 15.1–90.4 |
| °C Min, Max | 25.5–48.1 | 25.9–46.3 | 21.7–45.0 | 14.9–41.2 | 5.6–29.5 | 2.1–25.9 |

HR, Hazard ratio.

corrosion in the electronic sensors. In this investigation in the food company, are detected with a specialized hygrometer the RH and temperature values. It was detected in the clean room considering the weather outside the company causing an effect on the indoor climate of the company.

The RH and temperature values obtained from the weather outside the company are shown in Table 9.2, with a little smaller scale inside the clean room in monthly periods of 2015. In this table, indexes observed in the climate inside the room areas of the food industry were evaluated, with higher or close to 70% levels and 35°C in the maximum values and the minimum temperatures near 0°C, which are important in generating the corrosion factor. In periods of high humidity in the summer outside the company, the air conditioning does not work properly and sometimes generates humidity in the clean room, causing electrochemical corrosion phenomenon.

### 3.4 Corrosivity Levels in Marine Environments

Corrosivity levels represents the degradation grade (DG) of materials according to the correlation of RH, temperature, and air pollutants concentration mentioned earlier. Corrosion Rate (CR) was calculated from the atmospheric pollution in the indoor of electronics industry, indicating the air pollutants that caused major grade of damage to copper, namely, sulfur dioxide ($SO_2$) and the ion chlorides ($Cl^-$) in both cities. In both cities, RH was correlated with the major CR was 30%–50% with temperatures of 25–40°C. In summer, CR was different than in winter in both environments (Figs. 9.1 and 9.2). The electronics system of car corrodes at high humidity levels. Atmospheric corrosion is an electrochemical phenomenon that occurs in the wet film formed on the metal surfaces by climatic factors in Tijuana. Fig. 9.1 shows the correlation of ranges of RH and temperature in the concentration of air $SO_2$ in the summer period, which is the air pollutant with more effect in the generation of corrosion. The RH and temperature ranges were from 30% to 75% and 20–35°C with CR from 30 to 100 $\mu g/m^2$.year. Fig. 9.2 showed the evaluation of Corrosivity levels indicating that levels of RH and temperatures ranges from 40% to 75% and 20–35°C, and CR from 10 to 160 $\mu g/m^2$.year.

### 3.5 Gravimetric Analysis

The evaluation of metallic specimens by the gravimetric method, showed relevant data in the first month and the process of continuous analysis until June 2014. The information obtained is a fundamental part in the characterization of internal ecosystem of industrial plant and correlated these results with expenses not considered at the start of production planning. Locating the strategic locations of the installation of metal specimens was fundamental to achieve and characterize the maximum area of the company factor. After the first month of exposure, the specimens of the aforementioned metals were removed and weighed to obtain

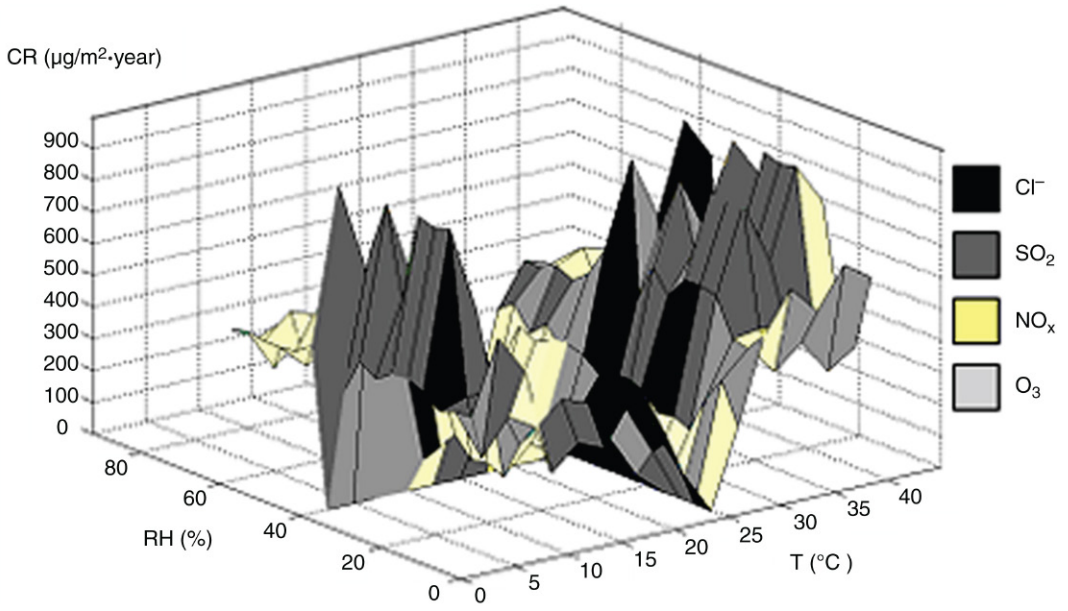


**Figure 9.1: Correlation of Corrosion Rate (CR) With Relative Humidity (RH) and Temperature of Electronic Sensors After 1 Year of Exposition to Arid Environment in the Tijuana City in the Summer Period (July 2015).**

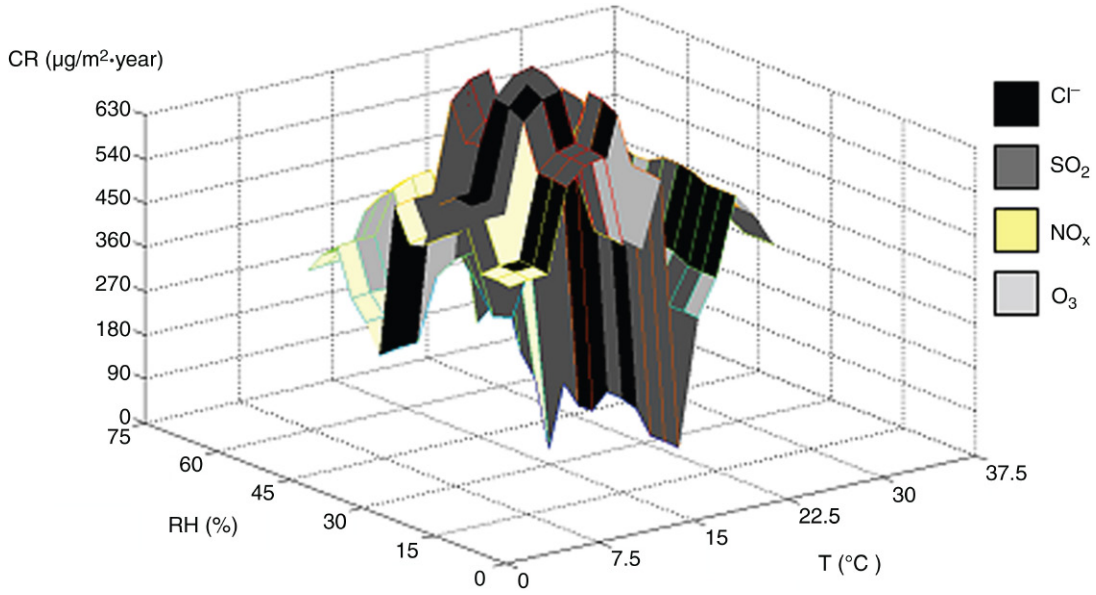


**Figure 9.2: Correlation of CR With RH and Temperature of Electronic Sensors After 1 Year of Exposition to Arid Environment in the Tijuana City in the Winter Period (December 2015).**

**Table 9.6: Gravimetric evaluations of metallic probes in July 2013.**

| Metals | Corrosion rate (mg/m$^2$·year) |
|---|---|
| Copper | 80 |
| Tin | 18 |
| Nickel | 12 |
| Silver | 92 |

the weight gain generated by the corrosion process. Later they were cleaned and the final weight was obtained to evaluate the corrosion rate with the mass loss. ASTM standards were used to evaluate the corrosion rate of metals. Table 9.6 shows the results of the CR of each metal tested, according to the period of exposure is evaluated, and the process of study continues for the following month. Mostly CR was the silver, followed by copper, carbon steel, tin, and nickel. The CR obtained from each metal, does not mean that problems are immediately generated, but future short, medium, or long term, could have serious repercussions. Silver and copper were the materials most damaged, that was corroborated in the practice applications and with the mathematical simulation.

### 3.6 AES Analysis

The AES technique was used to determine the types of corrosion. The AES spectra evaluated the surface analysis in three points of different areas of Cu surface of the electronic sensors. Fig. 9.3 shows two images of the specimens of Auger map. The nanophotographs of corrosion products covering the complete surface of Cu in the Tijuana city (Fig. 9.3A) formed the protective film, and thus the CR was slow. Fig. 9.3B shows a sample Cu installed in Tijuana, with isolated spots. Auger analysis showed the presence of $Cl^-$ and $SO_X$, which reacted with the Cu surface, made with the technique in the vacuum chamber showed in Fig. 9.3B, which requires two steps: using a mechanical pump oil to reduce the ambient pressure to 50 m Torr and then using a turbo molecular pump with the mechanical pump combined with the mechanical pump to reduce the pressure at 1 Torr nano.

Auger spectra of Cu samples were generated by an electron beam with a voltage of 5 keV, which shows the chemical composition of corrosion products in the samples of the Tijuana city in the summer period (Fig. 9.3A) and winter period (Fig. 9.3B). In both cities, the peaks of Cu, sulfates, chlorides, carbon, and oxygen appear in the spectra at different levels of intensity of kinetic energy. For the analysis of data, the program Origin Pro 6.1 was used. The spatial resolution of this technique was 100 nm and a resolution of 1 nm in depth. The depth profiles were obtained from samples tested in both periods, showed in Figs. 9.4 and 9.5. The technique of depth profiling is defined by alternating cycles of ion $Ar^+$sputtering method, to remove a thin layer of 5–10 Å of air pollutants, and its characterization in some regions. In Fig. 9.4, the chlorine and sulfur located between the carbide particles were bombed during

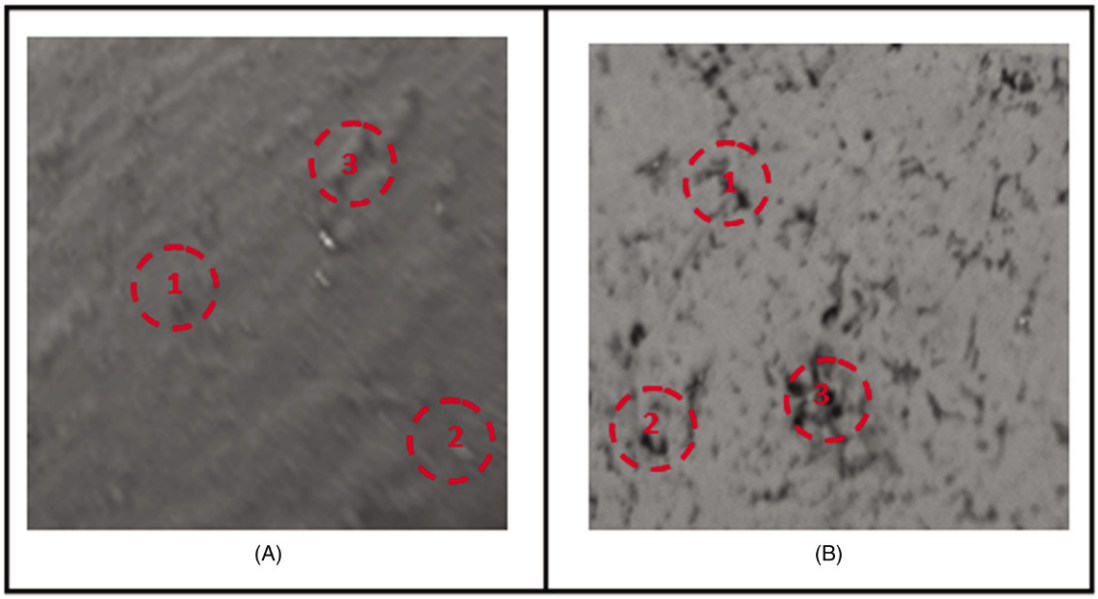


**Figure 9.3: Auger Map of Corrosion Products in the Tijuana city.**
(A) Summer (B) Winter periods (2015).

the first cycle of sputtering method (10 Å). A small proportion of chloride and sulfur persisted with more carbide particles (point 2). In Fig. 9.5, the depth profile indicates a low presence of sulfur between the carbide particles. Figs. 9.4 and 9.5 show the analysis of winter of 2015 because it was the period with major presence of the corrosion in the electronic sensors evaluated.

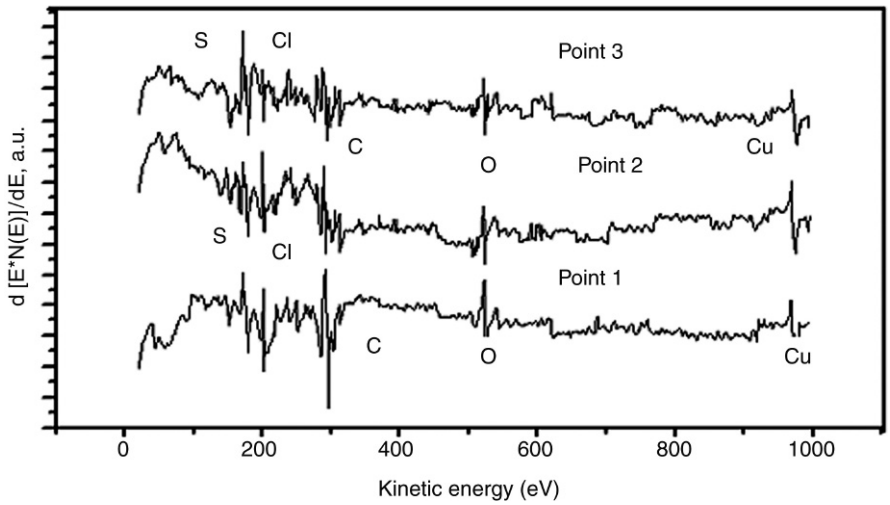


**Figure 9.4: Auger Electron Spectroscopy (AES) Analysis of Corrosion Products After 6 Months of Exposure in the Winter Period (2015).**

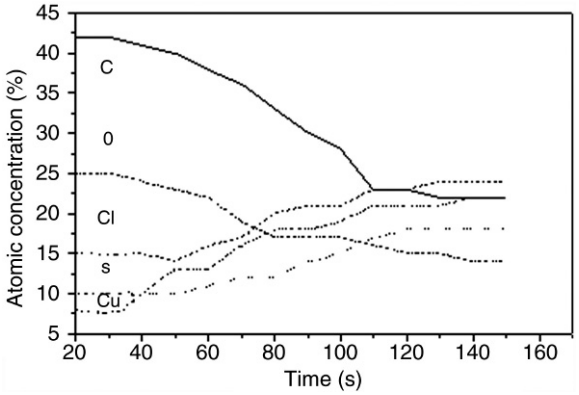


**Figure 9.5: Depth Profiles of Corrosion Products After 6 Months in the Winter Period (2015).**

### 3.7 Formation of Thin Films on the Cu Surface

The mechanism of film formation process is in the form of deposits covered by oxides on the surface of Cu formed principally for humidity and temperature that provide the time of wetness (TOW). The TOW is very important because it allows to know whether a surface is completely covered by thin film of water visible or nonvisible, maintaining a wet surface, and generate oxidation on the Cu surface of the electronic sensors.

### 3.8 Cost Analysis Corrosion

The appropriate use of economic resources in a business leads to be the part of success in its development expansion. This is part of the procedure in the initial budget. The presence of unexpected costs leads to a financial imbalance in industrial plants, so it is required to quickly locate the causes of these unnecessary expenses. One of the factors contributing to such expenses is the presence of corrosion in the electronics industry, which generated worldwide economic losses, and thus led to the closure of companies. In this research, an analysis of atmospheric corrosion is performed, which has a period of 1 month after starting. Previous studies in other regions of Mexico and other countries, the concentration levels of pollutants $H_2S$, $SO_2$, and $NO_X$ with RH and temperature variations correlated to obtain the VC of the aforementioned metals. Copper and silver were the most damaged. Based on that, a simulation was performed in MatLab program of possible expenses corrosion (%) that could be generated in a period of 24 months (from 2014 to 2015) and electrical failures (%) that could occur, as shown in Fig. 9.6.

In the aforementioned chart in Fig. 9.6, shown in dark blue, levels of low intensity over expenditure and electrical failures in the first 4months of the simulation, and as the months go analysis shown in blue green color have a higher level of intensity, that high costs and power failures originate, and thus the possibility of increased economic losses. This simulation

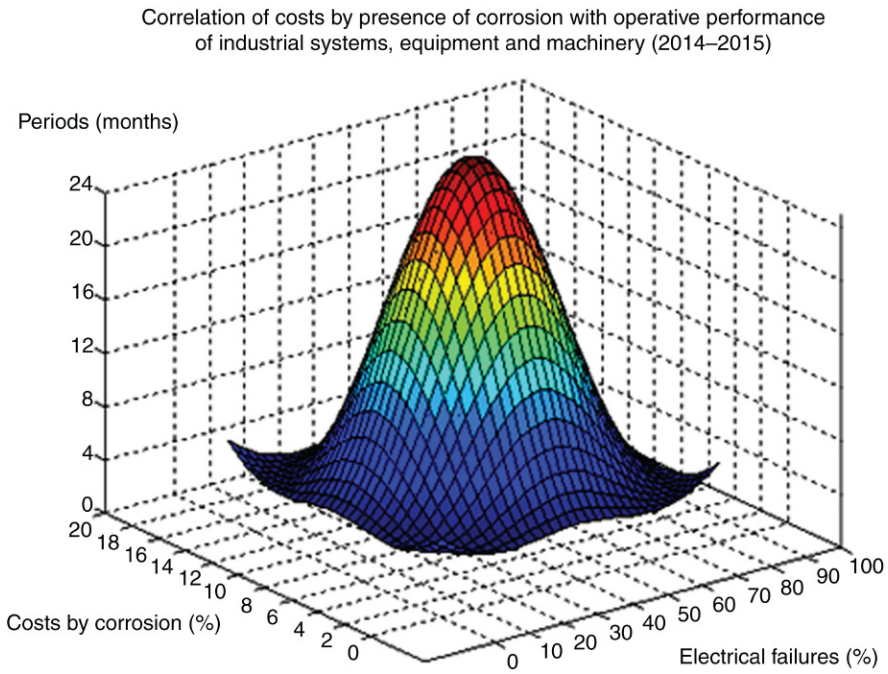


**Figure 9.6: Correlation Analysis of the Unnecessary Costs by Presence of Corrosion (2014–15).**

analysis is to consider what can happen but it is considered appropriate to take control of this electrochemical phenomenon. This analysis also includes costs that can be generated by environmental damage made by authorities in this area.

## *4 Conclusions*

Microcircuits, connectors, and electrical contacts used in the electronics industry are very susceptible to corrosion, which occurs in indoor conditions in the electronics sensors used in industrial systems, industrial equipment, and industrial machinery used in the food industry in the Tijuana city. The presence of corrosion results in the presence of unnecessary expenses required to carry out to avoid future complications in the manufacturing process. The exposure of these electronic components in uncontrolled environments inside industrial plants, corrosion causes deterioration in the materials of these electronic devices. The corrosion products in the electronic sensors evaluated, even microgram scale, can disrupt the current flow of electrons between the electrical contacts, having a component and thus results in useless power failure. The formation of the corrosive metal film depends on factors, such as nature of the material, surface roughness and composition, temperature, and contaminants adhered to the surface. The complex nature and composition of the film of moisture determine the interaction of the interface formed by the variety of variables,

generating corrosion in the electronics industry and irreparable compromise in the reliability of electronic devices. This research is still in progress since December 2014, the five main metals used in the electronics industry were analyzed. Based on ASTM standards, the rate of corrosion of each metal installed in the interiors of a company that supported the study was evaluated. The evaluation was performed to obtain a balance between the industrial activities and ecosystem conservation in this city. The graphs used in the manufacturing processes in the electronics industry are the fundamental part in the detection of the main causes that creates stoppages for the concerned personnel of the industrial plants. In this study, the most important options, which created the low operating performance of industrial machinery company allowed the study, were identified. The effect of corrosion on the deterioration of electrical connections and connectors of electrical and electronic equipment and systems of the company was an important aspect in the generation of electrical failures. This causes in certain times, the highest incident and keep alert to specialized personnel. The time period analysis allowed having a better view of quick and practical solutions of the causes, and thus preventing to further continue. The graphs' represented an important tool for evaluating all options of electrical failures of the electronic sensors, evaluated them and how to solve them as soon as possible.

## Acknowledgments

The authors thank the Directorate General of Higher Education Technology (DGEST) for permitting to perform research activities in the Technological Institute of Mexicali (ITM) and an industrial plant located in the Tijuana city for the development of a project.

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CHAPTER 10

# Biotechnology of Ice Wine Production

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## 1 Introduction

Ice wine is a kind of dessert wine or naturally sweet wine produced from frozen grapes obtained after natural freezing while the grapes are still on the vine. A major difference with other high quality sweet wines, such as Sauternes or Tokaji, is that the grape is not botrytized. Variations include artificial ice wines produced from running must concentrated by cooling. The origin of ice wine probably occurred by chance, when winemakers tried to produce wine from frozen grapes after unsuitable climatic conditions in northern areas at the end of maturity. Ice wine is believed to have been first produced in the Franconia region in 1794 after a vineyard's grapes froze while the monks in charge were waiting for the permission to harvest them. The first fully documented *Eiswein* harvest dates back to 1830, when wine growers left grapes on the vine to freeze, assuming they could be used as an animal fodder. However, once they pressed the grape they realized the quality of the must and the extreme sweetness. The fermentation process must have proved difficult because of the high sugar content and it is quite likely that it was impossible to fully ferment the sugars so that as a result the wine remained sweet, but it is likely that they liked it very much and perceived it as high-quality wine.

Ice wines are produced in Canada, Germany, Austria, Switzerland, and China. They are known as ice wines in English, *Eiswein* in German, 冰酒 (Bīngjiǔ) in Chinese, *Vin de glace* in Luxembourg, and *vi de gel* in Catalan. The greatest producers are Germany and Canada. Ice wines are really expensive because 3–4 kg of grapes are used to produce a small 375 mL bottle, equating to a glass per vine or a drop per grape berry; however, the magic conjunction of a strongly concentrated must in sugar acidity and aromatic compounds together with a complex and delicate fermentation process produce an enological jewel.

In many countries, ice wines are strongly connected with heroic viticulture, extreme climates, and even big slope mountain viticulture, making vineyard management and soil conditioning really difficult. But there are also connections to protect excellent natural environments with sustainable/organic viticulture.

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00010-4

## 2 Market

Currently, the main global producer is Canada (8500 hL, 2015) and the main world market is China. Canadian ice wines generally come from Ontario's Niagara Peninsula and the Okanagan Valley in British Columbia, but also Nova Scotia and Quebec (www.winesofcanada.com). Year after year, Canada's wineries are awarded gold medals throughout the world for their quality ice wines and Canadian winemakers strive to produce better wines, challenging themselves and each other to produce this liquid gold. Only ice wine approved by the appellation of origin system called Vintners Quality Alliance (VQA) is allowed to be produced in Canada and several requirements set by the VQA must be met, which includes the prohibition of artificial freezing and sugar addition during the entire ice wine ("icewine" in the VQA terminology) production process (Setkova et al., 2007). Thus, one of the most important things is to distinguish real ice wines from the fraudulent products that have begun to emerge in the market recently. The main market for Canadian ice wines is China with sales of more than 65,000 L, followed by the United States of America and South Korea with about 22,000 L (Agriculture and Agri-Food Canada, 2010). The price of Canadian ice wines in China ranges from €30 to more than €400 in premium categories.

Due to the lower yield of grapes and the difficulty of processing, ice wines are more expensive than table wines. They are sold in half-bottles, with the average price being around $45 per bottle. Currently, with a gradually increased understanding of ice wine, the Chinese market is steadily expanding. It has been reported that China has overtaken France as the world's largest consumers of red wine in 2014, having consumed 1.86 and 1.80 billion bottles of red wine, respectively, and considering the special preference for dessert wines, it is probable that China is also the world's main consumers of ice wines.

Production of ice wine in China is slightly higher than 700,000 L, about 40% of the global production. The price of ice wines ranges from €49 to €92 for 375 mL bottles in the Gansu province and from €21 to €115 in Changyu (Golden Ice Wine Valley, Liaoning province). Chinese ice wine history is not more than 20-years old and as such its exportation is not fully developed. In Gansu, Qilian exports ice wine to Japan and Malaysia, but only a very limited amount of about 1000 L. Changyu Golden Ice Wine Valley has exported to Germany, Australia, the United Kingdom, the United States, and other European countries, but the export volume is also very limited, just 3000–5000 L each year.

## 3 Regions for Ice Wine Production

There are few places suitable for producing ice wine in the world, traditionally only three countries including Germany, Austria, and Canada. The main ice wine producing countries are Germany and some regions in Austria, Ontario in Canada, Niagara Peninsula, and British Columbia's Ken Root Valley (Fig. 10.1).

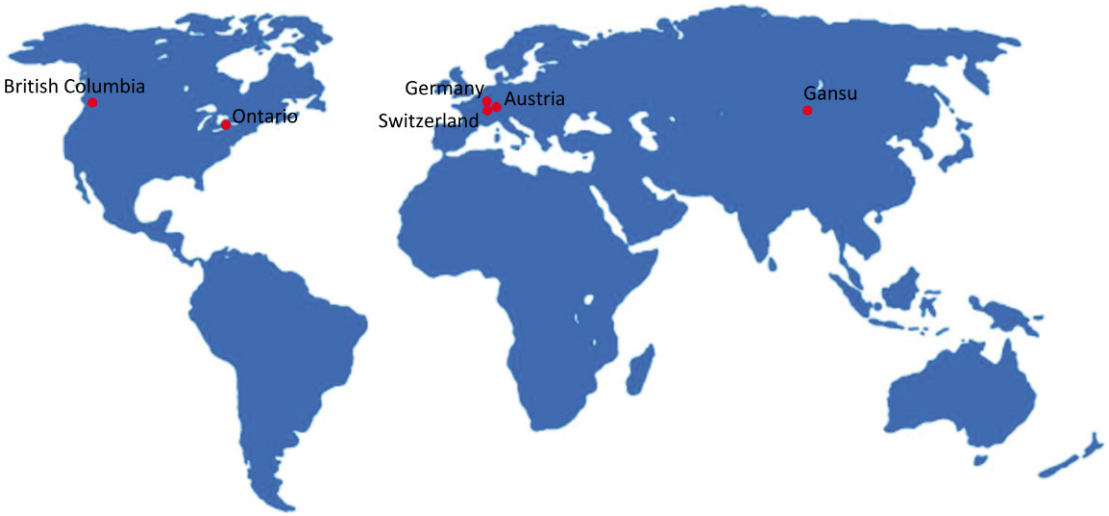


**Figure 10.1: Countries and Regions Producing Ice Wines.**

Germany is the home of ice wine, originating in the Franconia region. At present, the main regions that produce ice wine in Germany are Pfalz, Mosel, Rheingau, and Rheinhessen. Although Germany is the home of ice wine, it cannot produce it year after year. *Eiswein* is part of the *Prädikatswein* quality category in German wine classification. The *Pradikat* level is determined by the level of sugars in the grape at harvest, and chaptalization is not permitted. Grapes used must be of a *Beerenauslese* quality. The main difference between Canadian and German ice wines is in the varieties of grapes, with Riesling being one of the most important for the production. Also, German generally has lower alcoholic volumes with 6%–9% (v/v) being the most common.

Ontario (Canada) has been producing ice wine since 1972 with three VQA wine appellations—Niagara Peninsula, Prince Edward County, and Lake Erie North Shore; the Niagara Peninsula is also divided into 10 subappellations. The Inniskillin winery produced its first vintage in 1984, the first commercial production of Canadian ice wine, but in 1972, a German immigrant made ice wine at Okanagan using Riesling grapes. In 2015, Canadian ice wine production was 850,000 L (Wine Country Ontario, 2015).

Switzerland ice wines are produced in Valais among Europe's highest vineyards, the plots range in altitude from between 650 and 1150 m. Swiss production is very small but is of high quality.

Austria is also famous for its ice wine production, and has four ice wine producing areas including Vienna, Wachau, Styrie, and Burgeland. Wachau is the most popular ice wine producing area in Austria.

In recent decades, China has developed its ice wine producing regions. There are several ice wine-producing regions, which includes the Qilian Mountain in Gaotai (Gansu Province), Huanren and

Tonghua in Liaoning Province, Meri Snow Mountain's Lancang River Valley in Yunnan, and Yili, Xinjiang. The two most famous ice wine producing regions are the Qilian Mountain in Gansu and Huanren in Liaoning. The Qilian producing region, located in the central area of Hexi Corridor in Gansu Province at northern latitude 47 degrees, is flat, and the elevation is 1400 m. Area of 300 Ha is devoted to ice wine production in Gansu. It has a very extreme climate, with very cold temperatures in winter (reaching −25°C), making it necessary to cultivate the vineyard using buried viticulture. At −20°C, the permanent wood of the vine dies and it is necessary to protect the plants against extreme cold. For this purpose, plants are within a trench below ground level and the plant is covered by earth over the colder months of winter (Fig. 10.2).

**Figure 10.2: Vineyard at Qilian Employing Buried Viticulture Techniques (Gansu province, China).**

There is enough sunshine, the average daily temperature is 14.9°C, the climate is dry, the annual precipitation ranges from 66.4 to 104.4 mm, the relative humidity of sandy soil is 52%, heat changes fast, and water from the Qilian Mountain is used for irrigation. This production region uses the Qilian Mountain as a natural protective screen. The region has optimal conditions for organic production because of the low incidence of pests and rot diseases due to very low environmental humidity and extreme temperatures in winter. As there is no disease and industrial pollution, it is a favorable region for ice wine development, and the annual production of ice wine is 330,000 L. The Huanren ice wine producing region in Liaoning is located near northern latitude 41 degrees, known as the Liaoning Huanlonghu Region, it has a plantation area that is 380 m high, has fertile soil, the vineyard is on a gentle slope, the summer is hot but there is a large temperature difference between the day and night, the winter is cold but not dry. As such, it has three ideal conditions "a cold climate, water, and sunshine" to develop the grape, and is called the golden ice valley by wine experts. The region produces 400,000 L wine annually.

Michigan has been turning frozen grapes into ice wine since 1983. New York's Finger Lakes region also produces ice wines in the United States of America. The climate is not always ideal for ice wine. Some places get cold enough a few years in a decade, but this is not generally the case. The production is quite small but the wines are of a very good quality and come with a high price tag. In 2016, President Obama served Grand Traverse County at an official reception dinner at the white house. It is necessary for temperature to be at −8°C to collect frozen grapes for Michigan ice wines.

Luxembourg also produces *Vin de glace*. Quality labels *Vin de Glace* (also *Vendanges Tardives* and *Vin de Paille*) were regulated in 2001 following very explicit quality criteria, such as sugar concentration, varieties, and handmade harvests. The harvest of the grapes for this wine occurs at around 6 a.m., following a night of frosty temperatures with a minimum of −7°C. Several hours of this low temperature are needed one night before the grapes can be gathered. This is why, the harvest of the grapes for this wine is only possible in December or even in January. Under the control of the Wine Growing Institute (Institut vini-viticole, IVV), three domains harvested grapes: Alice-Hartmann (Wormeldange), Schumacher-Lethal (Wormeldange), and Madame Aly Duhr (Ahn) (Grand Duchy of Luxembourg official site, 2016).

Other countries with notable ice wine industries include Croatia, Slovenia, Slovakia, the Czech Republic, Hungary, and Romania (Schreiner, 2001). In warm regions, altitude can play a role in making ice wines, even though some areas are hot and dry, the Spanish winery Altolandon, with vineyards at 1100 m (3600 ft.) made an ice wine with the Petit Manseng variety in January 2014 after temperatures dropped to −8.5°C.

## 4 Varieties and Harvesting Conditions

Because of the high residual sugar and alcohol content in ice wine, there is a real need for high acidity to balance taste to avoid cloying, so traditional ice wines are generally made from aromatic and acidic grape varieties. Although cold weather conditions result in an absolute loss in acidity (potassium tartrate crystallization), juice concentration due to ice formation could provide sufficient acidity) (Jackson, 2008). The aromatic varieties often used for white ice wine are Vidal Blanc, Riesling, Silvaner, Gewürztraminer, Italian Riesling, etc., while Cabernet Franc, Merlot, Syrah, Cabernet Sauvignon, etc., for red ice wines.

Traditional ice wines are made from aromatic and acidic white Riesling, Gewürztraminer, Vidal Blanc, and Sylvaner varieties. In Canada and the United States, Vidal Blanc (*Vitis vinifera* Ugni blanc × Rayon d'Or 4986), a white hybrid grape variety is widely used. It is Canada's leading grape variety. It is a late-ripening variety with great cold hardiness. The clusters can hang on the vine after frost, until almost 30% of its weight is lost due to dehydration. It can be harvested more than 40 days after reaching maturity. Vidal Blanc is a thick skin variety so it is more protected than more sensitive *V. vinifera* against the cold temperatures before harvest. Vidal Blanc is readily available, inexpensive to grow, and easy to manage.

For red grape varieties, Cabernet Franc is used frequently in Canada for ice wine production bit Merlot, Pinot noir, and even Cabernet Sauvignon varieties can also be used. The Niagara-on-the-Lake region was the first to produce Syrah ice wine in 2004. They also started with Sangiovese in 2007.

Austrian red ice wine is made with Zweigel (St. Laurent × Blaufränkisch), which ensures large and heavy clusters hang on the vine for a long time after reaching maturity. White ice wine is made from Scheurebe, Grüner Veltliner, Riesling, Gewürztraminer, and Welschriesling.

The ice wine in China's Huanren producing region is mainly made from white Vidal grapes and red Beibinghong grapes. The ice wine in the Qilian producing region is mainly made of white Vidal and Italian Riesling grapes, and red Merlot grapes (Fig. 10.3). Grapes are frequently harvested at night, and squeezed and fermented in low temperatures.

Beibinghong was selected from the hybrid of the female parent Zuoyouhong, which is the hybrid of (*V. amurensis* Zuoshaner and *V. vinifera* Tchervine muscat) F1 × *V. amurensis* 74-1-326, and the male parent 86-24-53, which is the hybrid of (*V. amurensis* 73040 and *V. vinifera* Ugni blanc) F1 × *V. amurensis* Shuangfeng in 1995 (Song et al., 2008). This cultivar is resistant to extreme cold and serious fruit diseases: downy mildew, white rot, and anthracnose (Liu and Li, 2013). Because of its strong resistance to cold, earth covering treatment, which is common in buried local viticultural practice in Northeastern China for international varieties (such as Vidal, Riesling, and Cabernet Franc), is not required.

**Figure 10.3: Frozen Merlot Grapes at Quilian (Gansu, China).**

Therefore, the vinicultural management of Beibinghong is less labor intensive. Moreover, Beibinghong berries have relatively thick skins and high acidity, which is similar to cultivars typically used in ice wine production. In recent years, Beibinghong, widely cultivated in the Changbaishan region (Jilin province) and the Huanren region (Liaoning Province), was the most popular cultivar for red ice wine production (Yi-Bin et al., 2016).

Some vinicultural practices will increase many key aroma compounds in ice wine. After thinning at veraison, the volatile compounds in Vidal and Riesling ice wines increased significantly (Bowen and Reynolds, 2015a,b). Delaying the harvest of Riesling grapes increases many key aroma compounds in ice wines, such as: 1-octen-3-ol, ethyl benzoate, ethyl octanoate, *cis*-rose oxide, and β-ionone (Khairallah et al., 2016), and ethyl isobutyrate, ethyl 3-methylbutyrate, 1-hexanol, 1-octen-3-ol, 1-octanol, *cis*-rose oxide, nerol oxide, ethyl benzoate, ethyl phenylacetate, γ-nonalactone, and β-damascenone in Gewürztraminer (Lukić et al., 2016). Early harvesting produces higher concentrations of esters and aliphatic compounds.

Birds are a big problem for ice vine plots because the delayed harvest makes the sweet grapes really attractive to them. Moreover, at these times, seeds used as food by birds became scarce so they eat bunches of the fruit, destroying the entire crops. This is normally controlled by covering canopies with thin nets. Wild boars may also jeopardize bunches in some regions. Environmental conditions can affect the quality of grapes; wind, rain, hail, or even warm temperatures during harvest make the collection of frozen grapes or grapes at suitable temperature (below −7°C) impossible. Pests and mold can also degrade grape quality, making it difficult to produce high quality ice wines.

Harvesting can be done using mechanical vine harvesters or by picking grapes by hand (Fig. 10.4). Manual harvesting aids in the selection of bunches on the plot with the ability to reject those affected by pests or rotting. A mechanical process is useful to harvest at night when low temperatures are a priority. It is also faster and lower in cost.

**Figure 10.4: Frozen Vineyards and Harvests by Hand at Huanren (Liaoning province) and Qilian (Gansu province, China).**

## 5 Pressing Technology and the Wine-Making Process

For sugar solutions, a temperature of around −20°C is necessary to completely freeze water and sugar. When temperatures are in the range between −8 and −15°C, water is frozen as pure ice crystals, and the resulting liquid is highly concentrated syrup of sugar with little water. So, if the grapes are frozen at this temperature, the syrup can be removed by pressing with a high concentration degree (25–42°Brix), with most of the water remaining in the pomace as ice crystals. Normally, ice wines are pressed in hydraulic basket presses and it

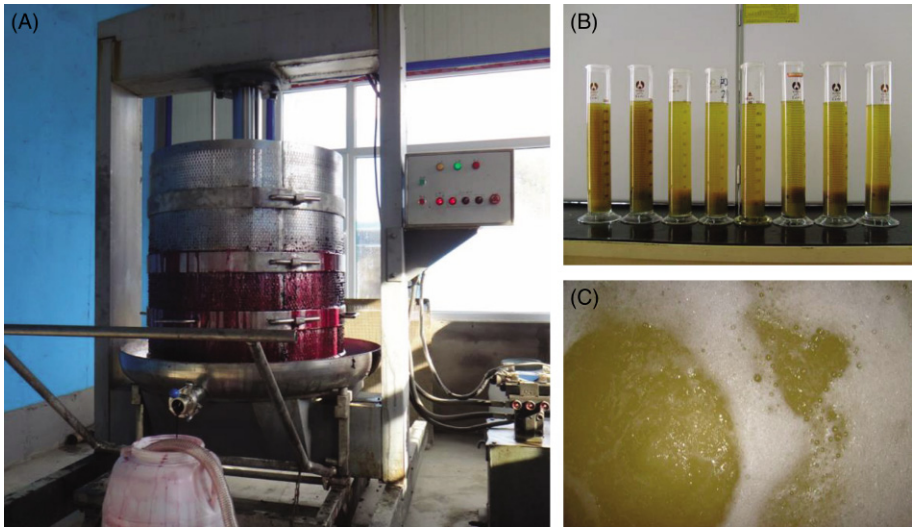


**Figure 10.5:** (A) Pressing of frozen merlot grapes, (B) Settling of syrupous white must, and (C) Must fermentation.

takes a long time (several hours) because of the hardness of the frozen grape (Fig. 10.5A). It is beneficial to use prechilled presses to keep grape temperature. Pneumatic presses are not frequently used even when it is the most suitable technology for conventional white wines, because it is difficult to extract must at the working pressure range of these presses, usually below 2 bar. Ontario presses at $T^a$ between −8 and −12°C, and Germany not higher than −7°C. It is usually said that one grape yields a single drop of ice wine must. The yield of pressing is about 10%–15%, which produces sugar concentrations that are generally higher than 25°Brix (Table 10.1). The pressure can reach up to 95 bar and frequently higher than 20 bar. Pressing takes more than 2 h and grapes must remain frozen. After pressing, water remains in the pomace as ice crystals. Pressing process also concentrate acids (malic and tartaric), reaching acidity values frequently higher than 10 g/L.

The wine-making process has several key steps (Fig. 10.6), such as pressing because it is necessary to keep temperatures below −7 to −8°C during the process to keep water in the pomace and to obtain a very concentrated must. Settling is a very slow process due to the syrupous and thick consistency of must because of the high concentration levels of sugar (Fig. 10.5B). Also, fermentation is a very difficult stage that is prone to sluggish fermentations because of the high concentration of sugar and extreme osmotic pressure (Fig. 10.5C). After fermentation, the next steps include typical stabilization processes and bottling—usually in small half bottles or even in 200 mL batches.

### *5.1 Cryoconcentration*

Some wine producers try to emulate the natural ice wine traits by freezing grapes or musts. Defenders of this technique in warm countries or regions like California, Australia, New

**Table 10.1: Main regional regulations in ice wine production.**

| Country | Region | Production (L) | Maximum Temperature at Harvest (°C) | Must Yield (%) | Juice Concentration (°Brix) | Varieties | Residual Sugars (g/L) | Acidity (g/L) | Maximum Volatile Acidity (g/L) | Ethanol [% (v/v)] |
|---|---|---|---|---|---|---|---|---|---|---|
| OIV | | | −7 | | >25 | | | | 2.1 | >5.5 |
| Austria | | | −7 | | 29 | White<br>Grüner Veltliner<br>Red<br>Zweigelt | | | 2.1 | >5.5 |
| Canada | Ontario (three appellations: Niagara Peninsula, Prince Edward County, and Lake Erie North Shore) | 850,000 | −8 | 10–15 | 35–38 | White<br>Vidal<br>Riesling<br>Chardonnay<br>Gewurztraminer<br>Red<br>Cabernet Franc | 180–320<br>Average 220 | >10 | | 8–13 |
| China | Gansu | 330,000 | −8 | 15 | 35–38 | White<br>Vidal<br>Italian Riesling<br>Red<br>Merlot | 146–180 | 8.1–9.2 | 1.1–1.5 | 11.5–11.8 |
| | Huanren | 400,000 | −8 | 15 | 33 | White<br>Vidal<br>Red<br>Beibinghong | 140–180 | 8.0–10.0 | 0.8–1.5 | 11.0 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Germany | Mosel<br>Rheingau<br>Rheinhessen<br>Pfalz | 45,000 | −7 | | >28 | White<br>Riesling<br>Red<br>Merlot<br>Pinot noir | >100<br>>250 | >10 | 2.1 | >5.5 |
| Luxembourg | Moselle | | −7 | 10 | 35–40<br>Minimum 28 | White<br>Riesling<br>Pinot Blanc<br>Pinot Gris | >125 | | 1.8 | |
| Switzerland | Valais | | −12 | | 39 | Red<br>Pinot noir<br>Eyholzer Roter (Hibou) | | | | 12 |
| United States | Michigan and the Finger Lakes (NY) | 5,000[a] | −8 | <20 | 36–40 | White<br>Vidal<br>Riesling<br>Red<br>Cabernet Franc | | | | 12.5 |

[a]6 Michigan Wineries in 2002.

*Source: From Wine Country Ontario, 2015. Available from: http://winecountryontario.ca/media-centre/icewine; Bowen, A.J., 2010. Managing the quality of ice wines. In: Reynolds, A. (Ed.), Managing Wine Quality, first ed. Woodhead Publishing, Cambrige, UK, pp. 523–552 (Chapter 18); Commission Regulation (EC), 2008. No 423/2008. Off. J. Eur. Union 8; OIV, 2003. Definition of Ice Wine. Resolution OENO 6/2003. International Organization of Vine and Wine. Paris, France; Robinson, J., 2001. Available from: http://www.jancisrobinson.com/articles/icewine-worth-the-money-and-the-hassle.*

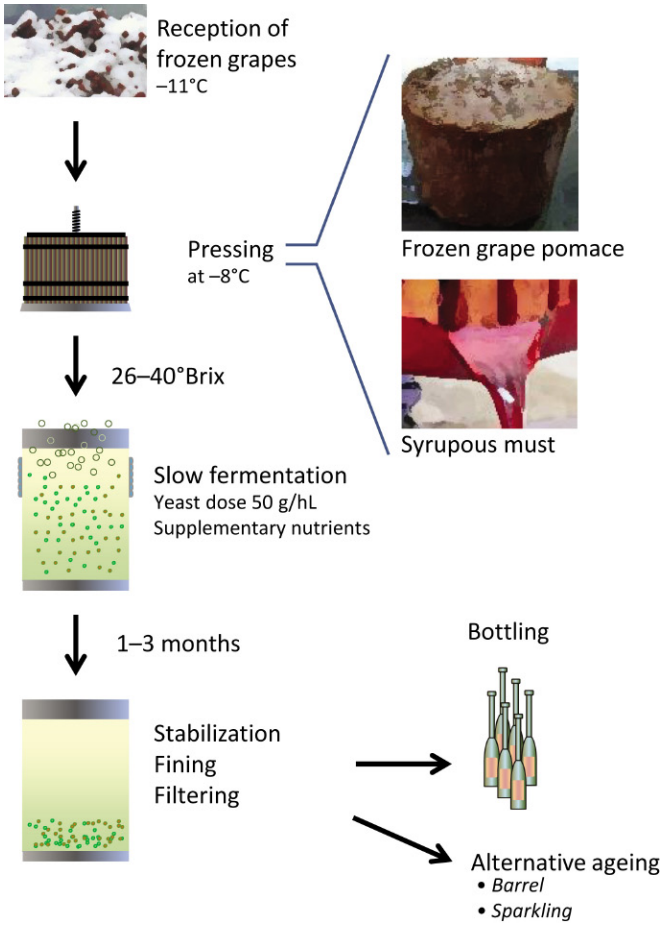


**Figure 10.6: Chart Flow for Ice Wine Making.**

Zealand, Spain, and even Argentina, say that the way in which the grape is frozen is not of importance and that the artificial process using cooling technology also allows the preservation of grapes from microbial alterations and pests. However, traditional producers say that not only the cold is necessary but that grapes mature under these aggressive conditions to develop the full aromatic complexity of a traditional ice wine.

## 6 Fermentation and Biotechnologies

The ecology of grapes under ice wine harvesting conditions shows a prevalence of *Aureobasidium pullulans* and *Rhodotorula glutinis* (Alessandria et al., 2013). Also, spontaneous fermentations were mainly driven by *Hanseniaspora uvarum*, *Metschnikowia fructicola,* and *Saccharomyces cerevisiae* at the midend of the fermentation process.

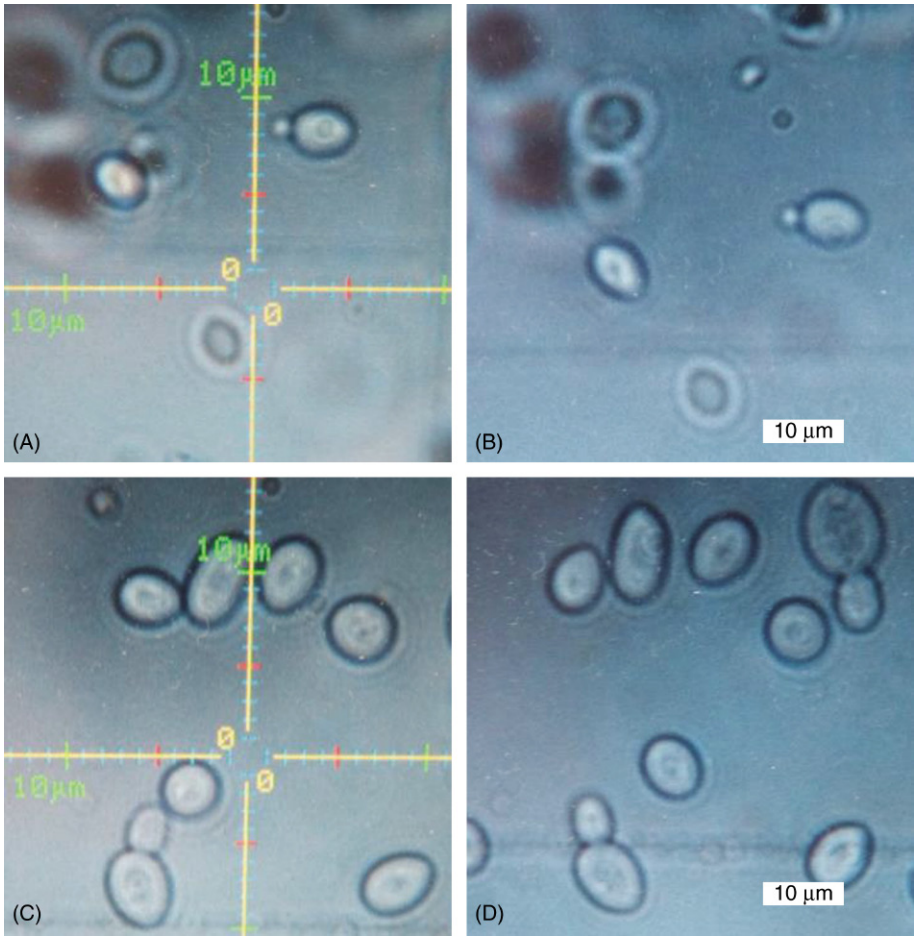


**Figure 10.7:** *Saccharomyces cerevisiae* fermenting concentrated must at 38°Brix (A–B) and running must at 22°Brix (C–D). Smaller size, lower turgency, and less budding percentage.

Ice wine fermentation is a complex and difficult process due to the high sugar content in the majority of the regions, with typical levels reaching higher than 30°Brix. There are reports that Riesling juice cannot reach 10% (v/v) of ethanol if the initial sugar concentration is higher than 42°Brix (Pigeau et al., 2007). Moreover, a sugar concentration of above 52.5°Brix could make the must unfermentable. During the making of ice wine, yeast cells are under very limitative conditions. It is frequent to observe smaller sized cells than those in normal wines (22–23°Brix), and osmotic pressure also affects yeast by reducing budding percentage and plasmolysis can be observed in the shape of the cells (Fig. 10.7). Buds are smaller, and take more time to evolve into adult cells, slowing the fermentation rate.

When yeasts are under hyperosmotic stress due to very high sugar concentrations, they produce glycerol and accumulate it inside the cytoplasm to balance external osmotic pressure.

It also means a higher excretion of glycerol to the fermentative media (Fig. 10.8) that usually reach 10 g/L. The over production of acetic acid is another typical consequence of hyperosmotic stress with values ranging from 0.8 to 2.3 g/L.

Fermentation of must from frozen grapes takes a long time (>6 weeks, sometimes several months) and values of some fermentative intermediates are normally higher than for conventional wines (Fig. 10.9). Cell growth is inhibited by hyperosmotic conditions in standard must fermentations (24–26°Brix), the cell counts can easily overcome log 8 CFU/mL,

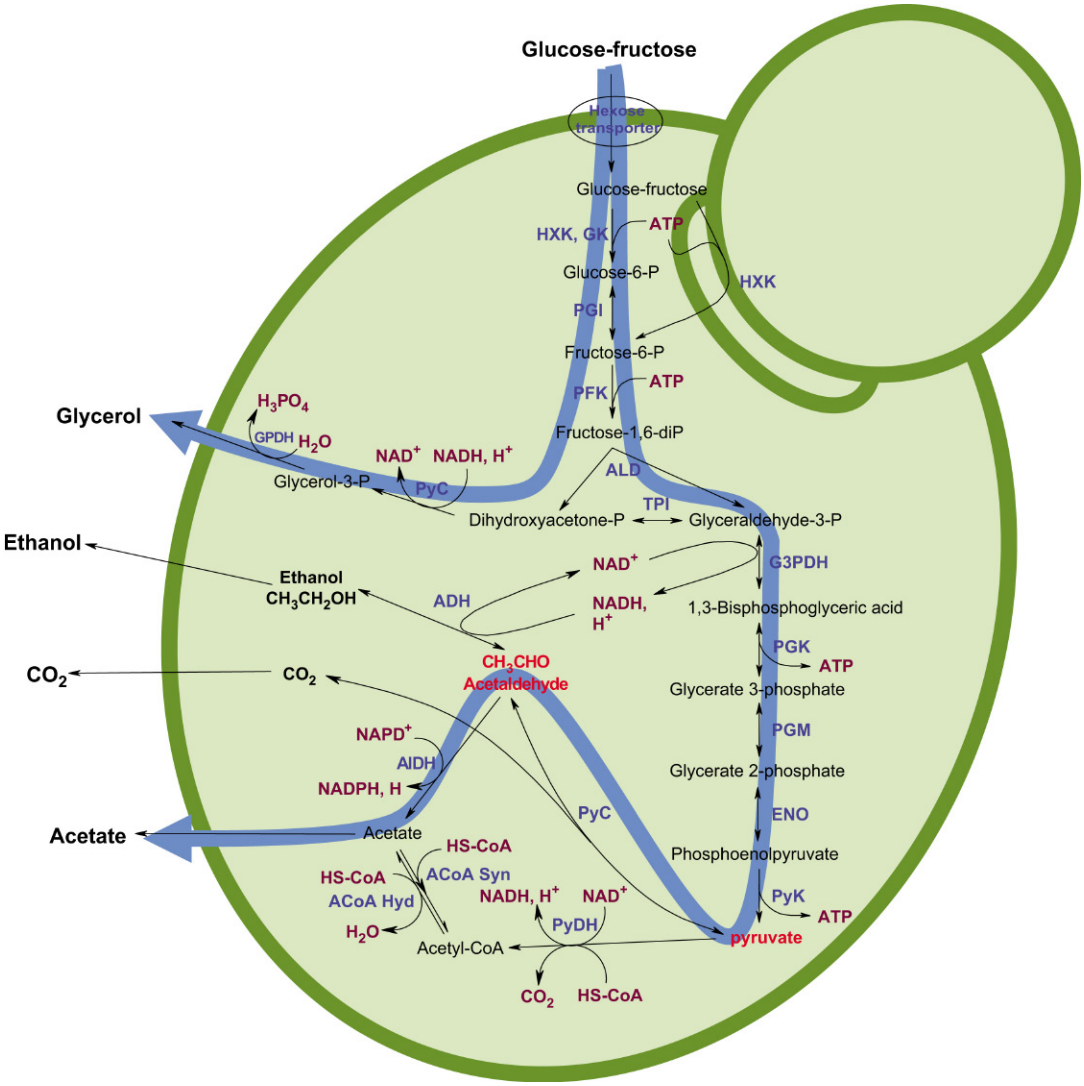


**Figure 10.8: Biochemistry of the High Production of Glycerol and Acetic Acid Under Hyperosmotic Conditions.**

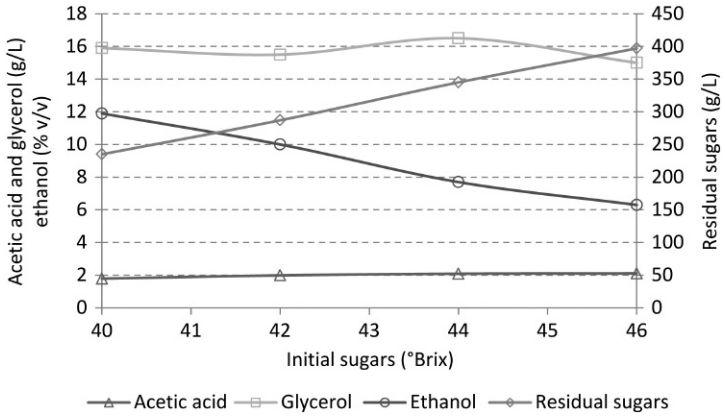


**Figure 10.9: Fermentative Production of Acetic Acid, Glycerol, Ethanol, and Residual Sugars Depending on the Initial Sugar Concentration.**

*Adapted from Pigeau, G.M., Bozza, E., Kaiser, K., Inglis, D.L., 2007. Concentration effect of Riesling ice wine juice on yeast performance and wine acidity. J. Appl. Microbiol. 103, 1691–1698.*

however, ice wine musts (38–40°Brix) are frequently around or below log 7. The lag phase is considerably longer and the fermentation rate is slower than for table wines.

Acetic acid, glycerol, and total acidity formation standardized to metabolized sugar strongly correlate to must concentration (Pigeau et al., 2007). The fermentation of 38°Brix must increases acetic acid and glycerol production 8.4- and 2.7-folds, respectively (Pigeau and Inglis, 2007). Fig. 10.10 shows value ranges of acetic acid and glycerol depending on initial sugar concentration in must.

Acetaldehyde concentration reaches values of four times higher than normal wines during the first week, but later the concentration is lower due perhaps to the high volatility of such molecules and its metabolization by aldehyde dehydrogenases. The expression of several mitochondrial aldehyde dehydrogenases has been observed and it can also affect the formation of glycerol-3-phosphate. Production of acetic acid means more than an 80% increase in total acidity (Pigeau et al., 2007). The content of ethyl acetate in ice wines is frequently higher than in traditional wines because of the levels of volatile acidity (Lukić et al., 2016).

Typical prolonged lag phases and sluggish fermentations of highly concentrated musts in ice wine production mean that it may take several months to obtain the suitable alcohol volume. The use of stepwise rehydration protocols increases ethanol concentration levels and improves yeast viability making the fermentation process safer and shorter (Fig. 10.11). The procedure includes several acclimatization steps from more diluted juice to higher concentrate media to adapt yeast metabolism and cell envelops to extreme osmotic conditions (Kontkanen et al., 2004). The use of warm temperatures makes the yeast membrane and cell

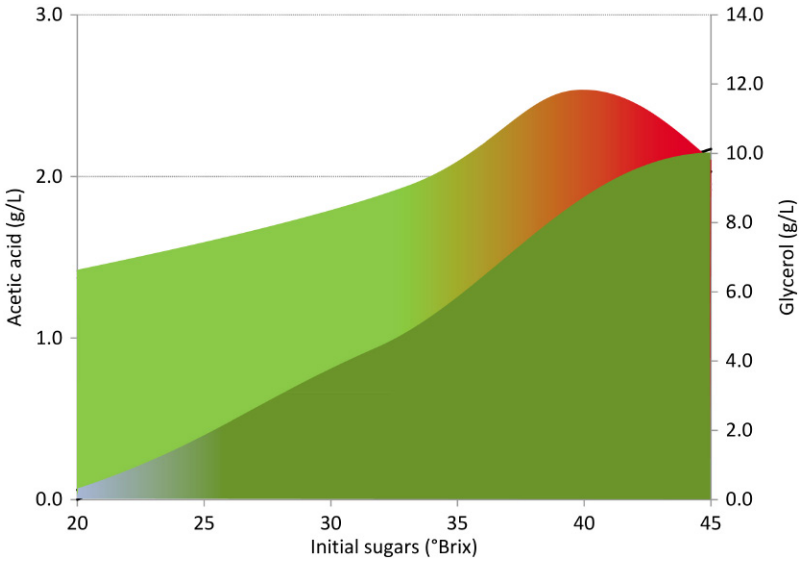


**Figure 10.10: Range of acetic acid (*grey line*) and glycerol (*black line*) in high sugar fermentations.**
*Adapted from Pigeau, G.M., Bozza, E., Kaiser, K., Inglis, D.L., 2007. Concentration effect of Riesling ice wine juice on yeast performance and wine acidity. J. Appl. Microbiol. 103, 1691–1698 and Inglis, D., 2012.* Torulaspora delbrueckii *and ice-wine fermentation: the start of a winning combination. XXIIIes Entretiens Scientifiques Lallemand. Monestier, France.*

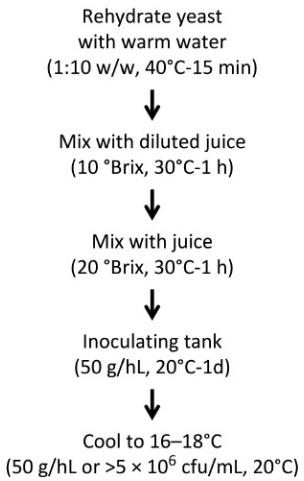


**Figure 10.11: Acclimatization Protocol for Yeast Starters to Ferment Ice Wines.**
*Adapted from Kontkanen, D., Inglis, D.L., Pickering, G.J., Reynolds, A., 2004. Effect of yeast inoculation rate, acclimatization, and nutrient addition on icewine fermentation. Am. J. Enol. Viticult. 55, 363–370.*

wall more flexible to pressure adaptation. Under these conditions, it is possible to get higher yeast counts and better-adapted cells to the fermentation of highly concentrated musts. When acclimatized yeasts are used at 0.2 g/L, the alcoholic volume increases slightly, however, when double inoculation dose (0.5 g/L) and stepwise acclimatization are used together, it is easier to reach 10% (v/v) of ethanol (Kontkanen et al., 2004).

To increase the quality of ice wine fermentation yeast, selection and breeding are powerful tools for obtaining strains or hybrids with improved performances that are better adapted to the osmotic stress of high concentrated musts. The hybridization of a robust *Saccharomyces cerevisiae* wine strain with *S. bayanus* with a strong fermentative power and low production of volatile acidity has been used to reduce its contents during ice wine fermentation (Bellon et al., 2015). The yeast strain also produces variations in the wine's aromatic profile but it is also strongly influenced by interactions between the yeast effect, vintage, and variety (Crandles et al., 2015).

The choice of yeast strain has also been found to significantly affect the accumulation of acetic acid, glycerol, reduced-sulfur odor, and color (Erasmus et al., 2004). Compared with using *S. cerevisiae*, spontaneous fermentation in Cabernet Franc ice wine is unique in its production of geranyl acetone and ethyl benzoate. Using *S. bayanus* in Cabernet Franc ice wine produces the highest concentrations of 15 compounds including 4 alcohols, 7 esters, furfural, hexanoic acid, TDN, β-damascenone (Kinga et al., 2015), and highest concentrations of 2-methyl-1-butanol, isoamyl acetate, and ethyl decanoate in Riesling ice wines (Crandles et al., 2015). This demonstrates that different yeast species produce unique compounds or higher concentrations of specific compounds relative to other treatments (Kinga et al., 2015).

The addition of nutrients, especially to increase yeast assimilable nitrogen, is normally carried out by dosing diammonium phosphate or yeast hydrolysates. Also, yeast fragments release unsaturated lipids and vitamins that help yeast to grow and to make fermentation, even under extreme conditions, easier. Some commercial yeast activators have been described because of the positive role it plays in ice wine fermentations or in problem musts or stuck fermentations: GO-FERM (Lallemand, Montreal, and Canada) is a rehydration nutrient containing pantothenate, biotin, magnesium, zinc, and manganese (Kontkanen et al., 2004).

Ice wines are frequently found in 375 mL bottles because of its high price. In bottling, refermentations and alterations can be controlled by the addition of sulfur dioxide and sorbate. In Canada, average ice wine values were 180 and 140 mg/L, respectively (Soleas and Pickering, 2007). The addition of $SO_2$ can be diminished by using lysozyme to control bacterial growth (Chen et al., 2015). In ice wines, sulfite content ranges 2–3 times the average values found in table wines because of the high amount of residual sugar, helping to avoid refermentation in the bottle by yeast, but also bacterial developments. Lysozyme, an enzyme able to hydrolyze the peptidoglycan layer of a bacteria cell wall, reduces the viability of Gram-positive bacteria. Its use is allowed in oenology to control or delay malolactic

fermentation and bacterial alterations. So, the use of lysozyme, together with $SO_2$ and sorbate, produce synergistic effects that largely help to control bacterial developments at lower $SO_2$ values (Chen et al., 2015).

## 7 *Use of Non*-Saccharomyces

The use of non-*Saccharomyces* yeasts is a trend in current oenology (Morata and Suárez-Lepe, 2016; Suárez-Lepe and Morata, 2012), with some species, such as *Torulaspora delbrueckii,* which behave as an osmotolerant, becoming an interesting alternative to *S. cerevisiae*. *T. delbrueckii* (Fig. 10.12A–B) is a low producer of acetic acid and ethyl acetate, when it is used in ice wine fermentation whose volatile acidity is near 0.4 g/L, reaching a final alcoholic degree of 9% (v/v). When the same yeast is used in sequential fermentation with *S. cerevisiae*, it is possible to obtain 10% (v/v) of ethanol, which is more suitable for ice wine production, and volatile acidity remains below 0.6 g/L (Inglis, 2012). Moreover, the control of osmolality in *T. delbrueckii* can be managed by the production of glycerol and arabitol (Lucca et al., 2002), perhaps facilitating a better adaptation to hyperosmotic substrates. The production of some interesting aroma compounds, such as 2-phenylethyl acetate (rose petals), 3-ethoxy propanol (solvent and blackcurrant), and diacetyl (buttery and nutty) can be enhanced with many *T. delbrueckii* strains, helping to improve the sensory profile (Loira et al., 2014) (Table 10.2).

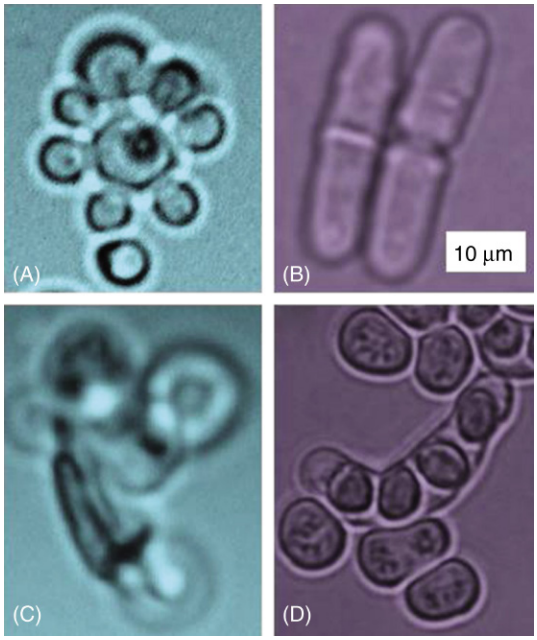


**Figure 10.12: Optical Microscopy of *T. delbrueckii*.**
(A) Budding cell, (B) conjugation and *Schizosaccharomyces pombe,* (C) reproduction by bipartition, and (D) lineal sporulation with four asci.

**Table 10.2: Some non-*Saccharomyces* yeasts used or with potential applications in ice wine production.**

| Species | Brand | Producer | Nitrogen Requirements | Alcohol Tolerance [% (v/v)] | Volatile Acidity | Sugar Tolerance |
|---|---|---|---|---|---|---|
| *T. delbrueckii* | Level2 TD | Lallemand | Moderate | 10 | Low | High |
| | Viniflora PRELUDE | CHR Hansen | Moderate | 9 | Low | High |
| *L. (Kluyveromyces) thermotolerans* | Viniflora CONCERTO | CHR Hansen | Moderate | 10 | Low | Medium |
| *Schizosaccharomyces pombe* | ProMalic | Proenol | Very low | 13 | High | High |

http://www.enolviz.com/modulos/pdfs/productos/levaduras/28/699118_PRELUDE.nsac.pdf; http://www.lallemandwine.com/products/catalogue/product-detail/?range=9&id=54; http://www.proenol.com/files/editorials/ProMalic_FT204-09_PT.pdf?u=.

*Schizosaccharomyces pombe* (Fig. 10.12C–D) reduces malic acid levels by maloalcoholic fermentation (Suárez-Lepe et al., 2012), and could prove interesting in achieving a better acidity balance in some ice wines. Malic acid degradation is very efficient in *S. pombe* because of the existence of a specific malic acid carrier **mae1p**, and also because malic enzyme is cytosolic nonmitochondrial like in *S. cerevisiae* (Saayman and Viljoen-Bloom, 2006).

Many *S. pombe* strains can reach more than 13% (v/v) of ethanol in table wines, so can be used as single inoculum to make complete fermentations in ice wines. The only problem is the high production of acetic acid, frequently near 1 g/L in table wines (Benito et al., 2012), but this has never been studied in highly concentrated musts. Also, acetate production can be controlled by means of mixed or sequential fermentations with *S. cerevisiae* (Loira et al., 2015). The peculiar metabolism of *S. pombe* increases the amount of pyruvate during fermentation, affecting the formation of stable pyranoanthocyanins, such as vitisin A and vitisin A derivatives. These pigments behave as very stable pigments in enological conditions and can improve color and color persistence of red ice wines (Morata et al., 2012). Furthermore, nitrogen requirements of *S. pombe* are very low and it has been proposed as a biotechnology to reduce biogenic amines due to low yeast assimilable nitrogen needs and the metabolization of malic acid by maloalcoholic fermentation reducing the development of lactic acid bacteria (Benito et al., 2012).

*Lachancea* (*Kluyveromyces*) *thermotolerans* has been described as a low acetate producer (Comitini et al., 2011) and is able to produce +L-lactic acid (Gobbi et al., 2013). Moreover, *L. thermotolerans* is able to ferment until it reaches 10% (v/v) of ethanol, a property that makes it really interesting in ice wine production. Additionally, it has been reported that an increase in both glycerol and 2-phenylethanol levels is associated with *S. cerevisiae* (Gobbi et al., 2013).

Non-*Saccharomyces* offer new tools to control volatile acidity in ice wines and at the same time they are able to increase aromatic complexity and there is a possibility of other improvements in biological ageing and stability with some species.

## 8 Ageing of Ice Wines

### 8.1 Ageing on Lees

Ageing on lees (AOL) is a biological ageing technique consisting of keeping wines in contact with lees (Fig. 10.13) for several months/years to increase mouth feel by the release of yeast polysaccharides and mannoproteins and to improve aromatic complexity by some yeast metabolites or because of the formation of new aromatic compounds. Lees are the death cells that have fermented the wine. The AOL of ice wines can improve structure, increasing the integration and smoothing of acidity. Moreover, it is possible to obtain a more complex sensory profile by the formation of new aromatic compounds with notes of baked goods and yeast.

AOL starts with the autolysis process of yeast-by-yeast enzymes that starts to depolymerize cell envelops. In this process, not only cell wall constituents but also cytosolic contents are released in wines. Yeast cell walls are formed by chitin, glucans, and mannoproteins. The cell wall is 30% of the yeast's dry weight, polysaccharides represent approximately 85%, and proteins 15% (Nguyen et al., 1998). Yeast cell wall polysaccharides increase mouthfeel, reduce astringency and bitterness, improving wine softness and density.

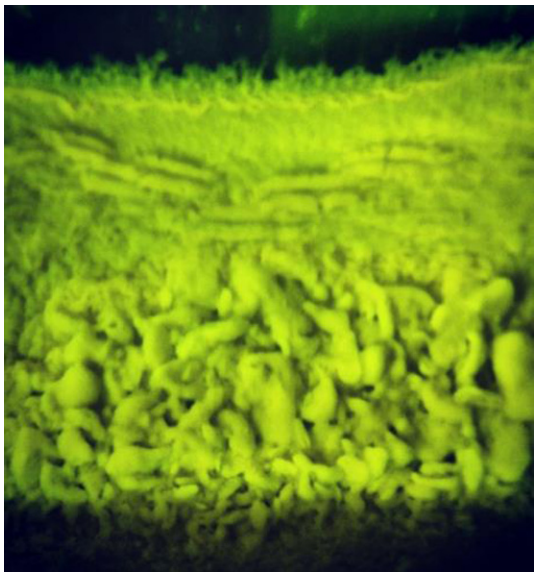

**Figure 10.13: Yeast Lees Sediments During Bottle Ageing on Lees (AOL).**

Moreover, AOL is reductive protecting fruitiness and it probably helps to preserve variety aromas in ice wines. Yeast cell walls contain glutathione, a tripeptide with strong antioxidant properties located mainly in its thiol groups. Glutathione is currently used by many wine-makers to control oxidations and minimize levels of $SO_2$, which are higher in sweet wines than in dry ones.

Yeast autolysis is a very slow process but is also strain dependent, so selection of suitable strains can help speed it up. The production of pure yeast biomasses of a single yeast culture selected for its specific positive properties for AOL improves the microbiological application of this technique (Suárez-Lepe and Morata, 2006). Moreover, cocktails of several yeast species/strains can be used for AOL processes to increase the amount of polysaccharide released and to increase aromatic complexity.

The use of non-*Saccharomyces* yeast has been recently described as a tool to improve AOL processes (Kulkarni et al., 2015; Suárez-Lepe et al., 2012), speeding it up, and reducing the time needed for autolysis. When osmophilic yeasts with specific configurations of double-layer cell walls, such as *S. pombe* or *S. ludwigii* are used, the possibilities presents to increase polysaccharide contents in a shorter time is a technical advantage (Palomero et al., 2009). At the same time, it is possible to improve mouthfeel given the large average size of their polysaccharides compared to *S. cerevisiae*.

The use of AOL in ice wines can help to improve the aromatic complexity and flavor balance and, at the same time, protect the aroma compounds from oxidation. Moreover, AOL can soften the acidity making a better-balanced wine. AOL can be applied in a barrel or steel tank. Several biotechnologies can be used, from the simple use of lees from fermentation to the production of pure lees of a specific yeast strain or even the use of non-*Saccharomyces* yeasts.

## 8.2 Sparkling Ice Wines

First sparkling ice wine was made in Canada in 1998, by Inniskillin Company using the Charmat Method in stainless steel tanks. At sensory level, the integration of $CO_2$ bubbles reduces sweetness and helps to improve integration and freshness. To make sparkling wines, there are main technologies for traditional bottle fermentation and Charmat process.

In the first technique, the most traditional one, partial fermentation of the sugar must be done in the bottle reaching the desired $CO_2$ pressure, after fermentation the typical AOL stage improves mouthfeel and aromatic complexity. As AOL take a long time to develop yeast autolysis and promote the formation of aromatic compounds, a period longer than 9 months is frequently recommended to reach suitable expression. After fermentation and AOL, it is necessary to remove lees to obtain a clean and clear product (disgorging). Dosing, capping, riddling, disgorging, and corking in the usually small quantities of 375 mL bottles is labor intensive. However, a lengthy AOL time produces a better integration of $CO_2$ with fine

bubbles, a creamy texture, and a complex AOL flavor. A second possibility is the Charmat Method in which fermentation is carried out on a bigger scale in steel pressure tanks, where the sparkling wine is settled and later transferred to bottles under isobaric conditions. The Charmat Method is easier and has lower costs compared with traditional bottle fermentation. In both techniques, yeasts undergo a very difficult fermentation process because of the high osmotic and $CO_2$ pressures.

### 8.3 Oak Aged Ice Wines

Also available in the market are ice wines aged in French and American oak barrels for a variable time (4–9 months) for added complexity. Barrel ageing also means an evolution in color, with it being typical for these white ice wines to turn into golden or honey tones. The great natural acidity is softened by barrel aging, but at the same time, the high acidity of ice wines protects them during the long ageing period and helps to maintain structure and balance. The complex aroma evolves from fruity notes to reach greater complexity and depth and also improves with a creamy vanilla flavor. Simultaneous use of lees and oak aging together with agitation (*Bâtonnage*) will result in a smooth buttery wine with toffee characteristics. During ageing, temperatures must remain below 15°C and there should be high humidity levels. Bottled ice wine should have a long life if kept at a suitable temperature in the absence of light and vibrations.

## 9 Analytical Controls

Due to its relatively high price, the authenticity of ice wine is becoming a real issue in wine production. Ice wines are produced according to strict production standards and regulations. According to German law, Eiswein is classified under Qualitätswein mit Prädikat (QmP). Frozen grapes for ice wine production must be harvested at the minimum sugar concentration level and should be between 110 and 128° Oechsle (26–30°Brix). The minimal alcohol by volume content is 5.5% and the maximum quantity of total $SO_2$ allowed is 400 mg/L. In Canada, ice wine is regulated by the VQA. A VQA regulation states that frozen grapes for ice wine production must be harvested at the minimal level of sugar must be 35°Brix equivalent to 153.5° Oechsle. According to the VQA, ice wines in Canada must have at least 7% alcohol, a concentration of acetic acid not above 2.1 g/L and residual sugars must be 125 g/L (100 g/L in British Colombia). Austrian law dictates that Eiswein must have 25° Klosterneuburger Mostwaage (KMW). Under Luxembourg legislation, Vin de Glace must have 120° Oechsle (28.1°Brix) (Mencarelli and Tonutti, 2013). According to the National Standard of the People's Republic of China, the alcohol content should be between 9% and 14% by volume and residual sugar must be above 125 g/L in 冰酒.

Analytic measurements are a necessary approach in ice wine production for product monitoring and quality control. OIV recognized and published many analysis methods, and some countries consider that the methods of analysis shall prevail as reference methods

for the determination of the analytical composition of the wine in the context of control operations. Many vine-growing countries have introduced its definitions and methods into their own regulations (OIV, 2015). In fact, the fast and unambiguous identification of potential markers, suitable to qualitatively differentiate the crops from various areas and farming systems, is a crucial requirement in trade and regulations. Conclusive analysis is a matter of highly reliable and objective instrumentation (Setkova et al., 2007).

## *9.1 GC Applications*

Today, Gas Chromatography (GC) and GC-MS with specific sample preparation are widely used for the detection and quantification of the organic molecules and also organometallic species in ice wine, especially for the analysis of both volatile and semivolatile compounds. Setkova et al. (2007) utilized a headspace solid-phase microextraction-gas chromatographic-time-of-flight mass spectrometric (SPME–GC–TOF-MS) method for the analysis of volatile and semivolatile components of ice wine from Canada and the Czech Republic. The entire method for ice wine analysis did not exceed 20 min per sample (Table 10.3). An odor assessment of 25 Canadian and German ice wines was carried out using a GC olfactory detector system (SGE International Pty.

**Table 10.3: Optimized conditions of the analytical method for the determination of volatile and semivolatile components of the ice wine aroma.**

| SPME | |
|---|---|
| Ice wine sample volume | 3 mL in 10 mL headspace vial |
| Salting out | 1 g NaCl |
| Sample incubation conditions | 45°C, 5 min |
| SPME fiber and mode | DVB/CAR/PDMS 50/30 µm; headspace |
| Sample extraction conditions | 45°C, 5 min |
| Sample agitation speed (incubation/extraction) | 500 rpm/500 rpm |
| Fiber desorption conditions | 260°C, 2 min |
| GC–TOF-MS | |
| GC column | RTX-5 (10 m × 0.18 mm I.D., 0.2 µm) |
| Injection mode | Split less |
| GC oven program | 40°C (30 s); 50°C/min to 275°C (30 s) |
| Carrier gas, mode, and flow | Helium at constant flow, 1.5 mL/min |
| Transfer line temperature | 275°C |
| GC run time | 5.7 min (last wine component eluted at 4.45 min) |
| Mass analyzer | High-speed time-of-flight |
| Ionization type/energy | EI/70 eV |
| Ion source temperature | 200°C |
| Detector voltage | 1700 V |
| Mass fragments collected | 35–450 *m/z* |
| Data acquisition rate | 50 spectra/s |

SPME, Solid-phase microextraction.

Ltd, Ringwood, Australia). The end of the GC column was connected to a variable outlet splitter set to deliver flow to the mass selective detector and the olfactory detector port at a ratio of 30:70. This aroma port was fitted to vacant detector housing, and the tubing passing through the oven wall was heated with oven temperature air by means of an air venturi (Cliff et al., 2002). Other researchers have been analyzing ice wine volatiles using a GC equipped with a Carbowax column (Cliff and Pickering, 2006; Nurgel et al., 2004). GC-MS has also been used to determine amino acid. Ice wine stored for 24 years contained 0.9% D-Proline, 6.4% D-Glx, 3.0% D-Asparagine, and 1.5% D-Alanine determined by GC-MS-MS (Ali et al., 2010).

## 9.2 LC Applications

High performance liquid chromatography (HPLC) methods are widely used for the analysis of many nonvolatile compounds in ice wine. The technique is suitable for sugar determination and fast detection glucose and fructose in ice wine. Two types of sugars in six types of ice wine were determined by HPLC with evaporative light-scattering detector. The samples were analyzed on a $NH_2$ chromatographic column (5 μm × 4.6 mm × 25 cm). The flowing phase was acetonitrile:$H_2O$ = 80:20 and the flow was 0.8 mL/min. The temperature in column and detector was 40°C and the injection volume was 20 μL (Wang et al., 2014). HPLC also has been used for the determination of phenolic compounds in ice wine. Li et al. (2016) determined phenolic compounds in two types of ice wine using the HPLC-MS-MS method. For anthocyanins, the mobile phase was composed of (A) 6% (v/v) acetonitrile containing 2% (v/v) formic acid, and (B) 54% (v/v) acetonitrile with 2% (v/v) formic acid. The gradient elution was as follows: 10% B for 1 min, from 10% to 25% B for 17 min, isocratic 25% B for 2 min, from 25% to 40% for 10 min, from 40% to 70% for 5 min, and from 70% to 100% for 5 min, with a flow rate of 1.0 mL/min. The injection volume was 30 μL and the detection wavelength was 525 nm. The column temperature was 50°C. The MS conditions were as follows: electrospray ionization, positive ion model; nebulizer, 35 psi; dry gas flow, 10 L/min; dry gas temperature, 325°C; scan, 100–1000 *m/z*. For the nonanthocyanin phenolic compounds, the mobile phase was comprised of (A) 10% (v/v) acetic acid, and (B) 90% (v/v) acetonitrile containing 10% (v/v) acetic acid. The elution gradient ranged from 5% to 8% B for 5 min, from 8% to 12% B for 2 min, from 12% to 18% for 5 min, from 18% to 22% for 5 min, from 22% to 35% for 2 min, from 35% to 100% B for 2 min, 100% B for 4 min, and from 100% to 5% B for 2 min with a flow rate of 1.0 mL/min. The injection volume was 2 μL and the detection wavelength was 280 nm. The column temperature was 25°C. The MS conditions were as follows: electrospray ionization, negative ion model; nebulizer, 35 psi; dry gas flow, 10 L/min; dry gas temperature, 325°C; scan, 100–1000 *m/z*. Also, glucose, fructose, and malic acid could be monitored by LC equipped with a UV detector set to 210 nm and a refractive index detector.

## 9.3 FT-MIR Applications

Using Fourier transform-middle infrared spectrometry (FT-MIR), it is possible to analyze wine and must within a 90 s timeframe simultaneously on a significant number of important enological parameters, such as alcohol content, relative density, extract, sugar-free extract, refraction, conductivity, glycerol, total phenols, reducing sugar, fructose, glucose, sucrose, total acid, pH value, volatile acid, total $SO_2$ and tartaric acid, malic acid, lactic acid, and citric acid. Sample preparation is usually easy, fast, and cheap, which includes decarbonation and cleaning by centrifugation or filtration. FT-MIR is a secondary analytical method that first needs to calibrate the instrument against the chemical reference methods for the different components (Table 10.4). Once the system has been cleaned and zeroed, it is a good practice to perform the standardization procedure before running calibration samples. Some companies have applications to transfer calibrations developed on the unit to be transferred to another standardized instrument (Patz et al., 2004).

**Table 10.4: Concentration range in wines, average, and correlation ($R^2$).**

| Parameter | Range | Average | $R^{2a}$ |
|---|---|---|---|
| Alcohol (vol.%) | 7.4–14.0 | 11.4 | 0.982 |
| Alcohol (g/L) | 58.7–110.7 | 90 | 0.975 |
| Relative density (20/20) | 0.9908–1.0940 | 1.0021 | 0.999 |
| Extract (g/L) | 19.8–238.1 | 42.7 | 0.999 |
| Sugar-free extract (g/L) | 14.7–55.6 | 22.2 | 0.859 |
| Conductivity (µS/cm) | 1150–3230 | 1879 | 0.948 |
| Glycerol (g/L) | 5.20–27.80 | 7.85 | 0.983 |
| Total phenol (mg/L) | 134–2260 | 570 | 0.959 |
| TEAC (mmol/L) | 2.5–30.9 | 7.1 | 0.920 |
| Sugars | | | |
| Fructose (g/L) | 0.0–165.7 | 14.9 | 0.998 |
| Glucose (g/L) | 0.2–63.5 | 6.5 | 0.996 |
| Sugar before inversion (g/L) | 1.5–220.8 | 23.1 | 0.998 |
| Sugar after inversion (g/L) | 1.5–234.7 | 23.8 | 0.998 |
| Acidity and organic acids | | | |
| Total acid (g/L) | 3.72–14.10 | 6.14 | 0.973 |
| pH | 2.49–3.99 | 3.37 | 0.834 |
| Volatile acid (g/L) | 0.14–1.41 | 0.44 | 0.768 |
| Tartaric acid (g/L) | 0.8–3.3 | 2 | 0.423 |
| Malic acid (g/L) | 0.0–6.6 | 2.3 | 0.811 |
| Citric acid (g/L) | 0.0–2.3 | 0.32 | 0.487 |
| $SO_2$ | | | |
| Total $SO_2$ (mg/L) (Tanner–Brunner) | 32–588 | 120 | 0.703 |
| Total $SO_2$ (mg/L) (photometry) | 7–415 | 86 | 0.843 |
| Free $SO_2$ (mg/L) | 0–58 | 20 | 0.120 |

[a]Averaged from validation with two independent data sets (A and B).

### 9.4 Texture

As texture analysis is a fast and low-cost analytical technique, it can also be favorably applied in oenology as a routine tool for monitoring wine grape quality. Among the different mechanical parameters measurable, berry skin thickness and hardness are indices that reflect anthocyanin extractability and dehydration kinetics with adequate reliability (Rolle et al., 2012). Universal testing machines are those currently used in texture tests applied in grape studies and provide precise measures of force, time, distance, and deformation (Rolle et al., 2010). In grapes for ice wines, this technique can report information on frozen grape extractability.

## 10 Sensory Quality

### 10.1 Visual Appearance and Color

Appearance is very important for a wine. Sight is the first of our senses to be used in wine tasting. The eyes introduce a wine, providing an initial reference point, such as clarity, transparency, sediment, brilliance, color, bubbles, and fluidity. Meanwhile, sight can also affect the sensitivity of smell and taste. A glass of quality ice wine should be clear, bright, transparent, shiny, and reflective. Depending on its depth of color, a limpid red ice wine might not be transparent, but transparency is necessary for white ice wine.

Clarity is closely related to taste. A cloudy wine with particles in suspension will directly and adversely affect one's taste bud sensory qualities. The wine will be masked by a screen of impurities and the flavor will be distorted. A cloudy wine never tastes right; it is rough and lacking in harmony. Furthermore, haze is always a sign of spoilage for consumers. An appropriate level of wine clarity implies the level of visible impurities under analysis does not exceed the threshold of what is considered acceptable, because there is no such thing as absolute limpidity in wine. A clear wine becoming hazy once again is quite natural, regardless of whether it's been cleared by natural settling, or by a clarification procedure. Some fragile clear wines can become cloudy when exposed to air, while others can be similarly affected by light, cold, heat, or microbiological changes, all of which can detract from the wine's clarity and quality. It is only a stable wine that can harmoniously develop all its qualities as it ages. As long as the wine remains unstable, it is susceptible to various changes in clarity, known as cases.

In general, a glass of ice wine will reduce fluidity due to a high viscosity, which is largely dependent on the sugar, ethanol, glycerol, and soluble polysaccharide content. This is discernible only at unusually high sugar (15 g/L fructose or 5 g/L glucose) and/or alcohol (10%–15%) content or when the glycerol content exceeds 25 g/L (Jackson, 2009), and ice wine just meets this condition in terms of sugars. At and above this level, viscosity could reduce the perceived intensity of astringency and sourness of wine (Smith and Noble, 1998). In addition, ice wine will form more rivulets than dry wine due to capillary action.

The color of a wine may provide some indication of a wine's body, its age, health, and maturity. Generally, color is related to characteristics, a certain color indicates a certain type of wine. A white ice wine with a yellowish-golden color makes us recall a cluster of overripe grapes with yellow skins and a sweet fruity taste. Such a special golden color probably results from the joint effects of juice concentration, caftaric acid oxidation, and the release of catechins on freezing (Jackson, 2008).

## 10.2 Aroma Profile

Ice wine has distinctive aromatic characteristics, varying from peach, pear, fig, green apple, raisin, dried apricot, citrus, pineapple, litchi, mango, to violet, honey, and caramel, depending on its origin, grape variety, and vinification. Young ice wine has a lively fruity flavor, the sensory profile evolves into honey, caramel, and dried fruit notes upon aging. The complex flavor of ice wine may be a cause of the following: using aromatic white varieties, aromatics adequately accumulate during the slow maturity of the berry, high sugar content enriched yeast fermentation to produce more alcohol and esters, besides, being aged in barrel or aged on lees can show more complex or evolved aromas.

Canadian white ice wine is known for its honey, lemon, apple, and tropical fruits, such as mango, fig, pawpaw, pineapple, lychee, dried apricot, and smoky, cinnamon fragrance, balanced with the proper acidity levels. Its sensory profile evokes dried apple, jam, and caramel when aged in barrels, and finishes with the smell of oranges. Aroma profile of red ice wine is a mix of strawberry, cream, spice, and mild herbs (Kinga et al., 2015). Sparkling ice wine has a fragrance of nectarine, apricot, lemon, honey, and the bubbles are finer than sparkling Brut wine.

German Eiswein (Qualitätswein mit Prädikat) are ice wines of beerenauslese concentration, made from grapes collected and pressed while frozen to concentrate sugar, acidity and extract, it is a truly special wine with a singular concentration of fruity acidic freshness and sweetness. Some of the wines present nutty and oily notes. Riesling is, without doubt, Germany's most highly esteemed grape variety, and also the main variety for Eiswein. Riesling is relatively cold hardy so the fruits can mature slowly to form its elegant and delicate aroma, and maintain high acidity in the pulp (Zhan, 2010). Riesling Eiswein is truly special with a singular concentration of fruity acidic freshness and sweetness, full of green lemon, pear, apple, peach, cherry (jam), chamomile, honey, raisin, dried apricot, syrup, cinnamon, and caramel aromas. Some of them have nut and oily notes (Parker, 2012).

Austria Zweigel red ice wine has a rose appearance with a fresh, sweet but not cloying taste. White ice wine made from Scheurebe, Grüner Veltliner, Riesling, Gewürztraminer, and Welschriesling show freshness and sweetness, that is, well balanced with the high acidity, full body, similar with Germa *Eiswein*.

Beibinghong ice wine is deep purple in color, has a special fruity aroma of *V. amurensis*, and sweet, honey, roast caramel, and is typically full-bodied with a lasting finish. Sensory profiles of Vidal Blanc ice wine in China depend on the region, and in the Jilin province is full-body,

sweet, balanced, refreshing, and has a lasting finish, while on the contrary, in the Liaoning province it is tart, watery and light (Table 10.5).

Luxembourg Riesling ice wines show a typical golden-yellow color. They have complex and concentrated aromas of dried flowers, lemon grass, and pineapple. Later, you are left with a fresh and powerful mouthfeel—large and generous mineral freshness with citrus hints in a perfect balance.

Sensory profiles of some typical varieties used in the elaboration of ice wines share common aspects but also remarkable specificities. Vidal Blanc has an intense character that is enhanced by the freezing process, resulting in an ice wine with a typical flavor profile that is greatly appreciated by the market. It is typical to find honey and peach aromas, as well as pineapple, apricot, and butterscotch in ice wines made with the Vidal grape. Gewürztraminer ice wines show terpene, floral, pungent, and ripe fruit aroma series (Lukić et al., 2016). Riesling ice wine provides a medium-bodied taste full of sweet apricot, peach, apple, citrus, and grapefruit and the aromas of honey and sweet grape. Fruit aroma is well balanced with a suitable and stimulating acidity. Cabernet Franc produces a wine with a bright red ruby tone and the powerful aroma of fresh strawberries. Sensory features and pairing of main ice wine varieties are as:

- Riesling (*V. vinifera*) is Germany's leading grape variety, a white grape known for its characteristic "transparency" in flavor. Aroma varies from sweet apricot, peach, apple, citrus, grapefruit, floral to mineral substance. The color will change to deep golden yellow through aging, accompanied by a more complex flavor of ripe fruit and honey. Its fine aroma is well balanced with a suitable and stimulating acidity. Riesling Eiswein can be paired with fish, chicken, shell fish, and soft cheese (Zhan, 2010).
- Vidal Blanc is an ideal variety for ice wine making. Matured fruit can form a mix fruity of orange, pineapple, grapefruit, apricot, and honey. When aged in barrel, it looks like a golden liquid with a complex aroma and delicate mouthfeel, is full bodied with a long finish. "Vidal Blanc" ice wine can be paired with fried goose liver, fruit, complex flavored cheese, ice cream, chocolate, etc. (Zhan, 2010).
- Gewürztraminer (*V. vinifera*), a white grape, has high sugar content and a relative low acidity level. It is famous for its high density complex aroma, varying from tropical fruit, such as banana, mango, lemon, litchi, peach to rose, honeysuckle, peony, violet, geranium, locust, verbena, gingersnap, lilac, cinnamon, even musk (Zhan, 2010). "Gewürztraminer" ice wines show terpene, floral, pungent, and ripe fruit aroma series (Lukić et al., 2016). It can be paired with meat sauces, cheese, foie gras, and venison, goes especially well with Asian food like Sichuan cuisine, Indian Curries, Vietnamese, and Thai cuisines (Zhan, 2010).
- Sylvaner (*V. vinifera*, Traminer × Osterreichisch-Weiβ), a white grape, has a unique aroma like "Riesling" but relative low acidity. With the fragrance of soil, basil, cotton, fern, ripe fruit, pale in color, "Sylvaner" ice wine can be paired with seafood, and asparagus (Zhan, 2010).

**Table 10.5: Sensory properties of ice wine produced by several companies in China.**[a]

| Region | Province | Company | Ice Wine Style | Varieties | Aroma | Color | Mouthfeel |
|---|---|---|---|---|---|---|---|
| Northeast | Jilin | Tonghua Wine Industry Co., Ltd | White | Vidal blanc | Floral, fruity, tropical fruit, honey | Yellow–golden | Sweet, vinous, balance, refresh, long finish |
| | | | Red | Beibinghong | Dried fruit, honey, dried grape | Ruby-red | Sweet, balance, refresh, long finish |
| | | Qingshanyuan Wine Industry Co., Ltd | Red | Beibinghong | Fine | – | Vinous |
| | | Xuelan Wine Industry Co., Ltd | Red | Beibinghong | – | – | – |
| | | | White | Vidal blanc | – | – | – |
| | | Tonghua Zilong Wine Industry Co., Ltd | White | – | Apricot, pineapple, honey, tropical fruit | – | Sweet, fat, balance, long finish |
| | | | Red | Shuanghong | Honey, peach, pineapple, black berry, fruity | Ruby-red | – |
| | | | Red | Beibinghong | Grape, fruity, nut | Garnet | Soft, balance, bouquet |
| | Liaoning | Sun Valley Ice Wine Industry Co., Ltd | White | Riesling | Floral, fruity, delicate, fine, complex | Yellow–golden | – |
| | | | White | Vidal blanc | Pineapple, mango, apricot, peach, honey, sweet melon | Yellow–golden | Tart, watery, light |
| | | | White | Pinot blanc | Complex, fine, long finish | Yellow–golden | Refresh, sweet, balance |
| | | Wunv Mountain Milan Liquor Company | Red | Beibinghong | – | – | – |
| | | | White | Vidal blanc | – | – | – |
| | | Zhangyu Golden Ice Valley Wine Industry Co., Ltd | White | Vidal blanc | Fruity, pineapple, honey, tropical fruit | Yellow–golden | Sweet, fine, delicate, full-body, long finish |
| Northwest | Xinjiang | Yizhu Wine Industry Co., Ltd | Red | – | – | – | – |
| | | | White | Vidal blanc | | | |
| | Gansu | Qilian Wine Industry Co., Ltd | White | Italian Riesling Semillon | Fruit, honey, apple, fruity, complex, fine, harmony | Pale yellow–golden | Mellow, fat, bouquet, typical |
| | | | Red | Merlot | – | Ruby-red | Sweet, vinous, long finish |
| | Ningxia | Hangsheng Xixia King Liquor Company | White | Riesling | Lemon, rose | Yellow–golden | Sweet, vinous, special |
| | | | White | Gewürztraminer | | | |
| | | Florian Wine Co., Ltd | White | Chardonnay | Fruity | Straw-yellow | Sweet, mellow |
| | | | Red | Pinot nior | Fruity | Ruby-red | Sweet, mellow |

–, Not mentioned.

[a]Data from the company's website.

- Cabernet Franc (*V. vinifera*), a red grape variety, produces a wine with a bright red ruby tone and the powerful aroma of fresh strawberries. It's frequently used in Canada for ice wine production but Merlot, Pinot noir, and even Cabernet Sauvignon can also be used. The Niagara-on-the-Lake region was the first to produce Syrah ice wine in 2004 and Sangiovese in 2007.

Ice wines show a complex aroma formed by terpenes, furans, acetals, esters, etc. (Lukić et al., 2016). When aromatic profiles by stir bar sorptive extraction-gas chromatography-olfactometry-mass spectrometry (SBSE-GC-O-MS) of both table and ice wines made from Riesling and Vidal blanc varieties, higher concentrations of more aroma compounds were observed in the second ones (Bowen and Reynolds, 2012). The highest odor activity was observed in: β-damascenone, 1-octen-3-ol, ethyl octanoate, *cis*-rose oxide, and ethyl hexanoate; these molecules were above threshold in Riesling and Vidal ice wines. Also in ice wines of these varieties, Crandles et al. (2015) measured odor activity values higher than unit in: Linalool, *cis*-rose oxide, 1,1,6-trimethyl-1,2-dihydronaphthalene, β-damascenone, β-ionone, ethyl cinnamate, *p*-vinyl guaiacol, and decanal.

Ice wine is quite refreshing, despite the high amount of residual sugars, due to high acidity levels (normally >10 g/L). Many people describe it as a delicious dessert in a glass, and it is quite common for a small 375 mL bottle to be enjoyed by 6–8 people.

Ice wine usually has a medium to full body, with a prolonged finish. Its aroma is frequently evocative of peach, pear, dried apricot, honey, citrus, figs, caramel, green apple, raisins, and a hint of violet on both the nose and palate, etc., depending on the variety (Fig. 10.14).

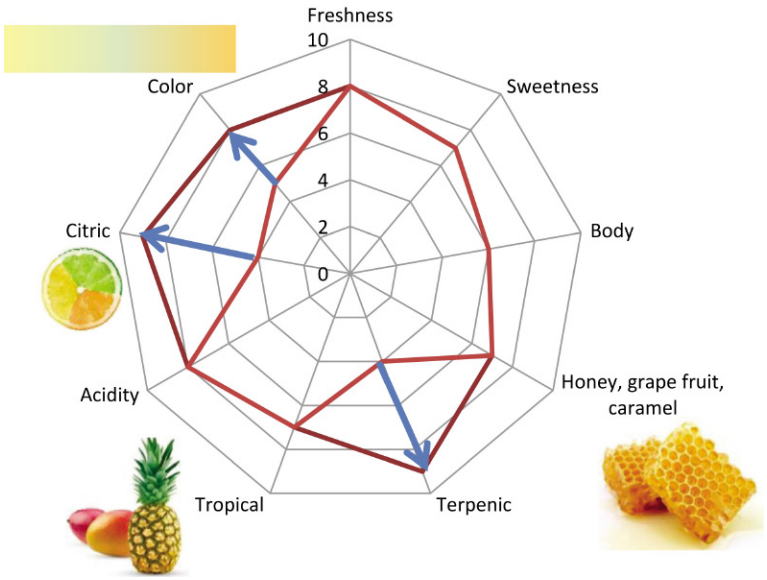


**Figure 10.14: Sensory Profile of Typical White Ice Wines.**
Intensity of some descriptors is variable according to grape variety.

The perfume of tropical and exotic fruits, such as pineapple, lychee, or mango is quite common, especially with white varietals. Moreover, its sensory profile evolves according to harvest times, with more complex late harvest ice wines being more frequent. On the other hand, nonconventional ice wines: sparkling, aged in barrel, aged on lees, can show more complex or evolved aromas according to the specificities of the ageing process. Barrel ageing increase notes like butterscotch and brown sugar and it further improves body and achieves a more rounded mouth feel.

## 11 Conclusions

Ice wines are produced in only a few special regions worldwide, ideal climatic conditions are necessary for good grape maturation together with a requirement for frozen grapes at harvest times. Furthermore, although ice wines are produced on small scale compared with traditional still wines, they are in a high price bracket and they enhance the prestige of wine-making regions able to create these wines. As ice wine making is a hard process, new biotechnological improvements can help oenologists to improve fermentation quality and safety. Lot of improvements and trends are being incorporated to ice wine technology: the use of nontraditional varieties in ice wine technology allowing new sensory profiles or better resistance against the cold, pests, or diseases, new ageing technologies, such as sparkling ice wines, barrel-aged ice wines, and so on, open new possibilities to develop very interesting products. Finally, ice wine is a magic synthesis of extreme climate conditions, human tradition, and knowhow, and extremely adverse fermentations that makes a really beautiful wine.

## Acknowledgments

We would like to express our gratitude to Chen Yangxiong, enologist of Gansu Qilian Winery Co. Ltd., for kindly providing us the photos used in Figs. 10.2, 10.3, 10.4, and 10.5.

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CHAPTER 11

# Metagenomics of Traditional Beverages

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## 1 Introduction

Fermented beverages have been considered as basic foods in many countries, because the fermentation process is an old technique to conserve, produce, or transform foods and beverages. Along history, these beverages have been elaborated mainly in towns of Africa, Asia, East Europe, Latin America, and several Pacific islands. These beverages are consumed in religious ceremonies, agricultural festivities, and social events and are also used in traditional medicine. Fermented beverages are characterized because of use of a great diversity of raw materials for their preparation, the elaboration process, and the microorganisms involved during the fermentation. These foods are consumed as soups, porridge, gruel, and beverages (Lappe-Oliveras et al., 2008).

Some of the cereals used during the preparation of fermented foods and beverages include maize, sorghum, wheat, rice, millet, and among others (Guyot, 2012). A lot of maize fermented products have been consumed in Latin America for many centuries (Elizaquível et al., 2015). Also, vegetables, fruits, milk, sap of plants, and plant leaves have been used to prepare fermented foods and beverages (Cocolin et al., 2013).

Preparation form of fermented beverages varies among countries, even among different regions of the same country, and these processing steps confer different biochemical properties, sensory properties, and nutritional properties of each product. The sensory properties, such as taste, flavor, smell, texture, and color of fermented beverages play a key role on the quality and acceptance of these beverages. Most of traditional beverages are produced by traditional processes and at small-scale levels.

Most of food fermentation is spontaneous, however involves specific microorganisms that depend of raw material, processing conditions, and environmental conditions as well as temperature and pH. A variety of microorganisms are presents in these foods; usually the nonstandardized starter cultures formed for some mesophilic aerobic bacteria, lactic acid bacteria (LAB) aerobic bacteria, and yeasts are used. LAB mainly preserves food and

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00011-6

prevents growth of pathogenic microorganisms and spores because of the acidification and production of bacteriocins. These relevant microorganisms play a key role during the spontaneous fermentation (Escalante-Minakata et al., 2012).

Some microorganisms are not detected by dependent cultures methods, because they cannot grow in traditional culture media, under standard conditions; the 99% of the microorganisms are considered uncultivable. Therefore, metagenomics based methods have been using (Cocolin et al., 2011; Sarethy et al., 2014) to study the microorganisms present during traditional fermentations. These methods can be applied in foods research and may allow the characterization of all microbiota in a product, besides ensuring the quality and safety of food (Yeung, 2012). The aim of this chapter is to highlight and discuss the current techniques used to identify nonculture microorganisms in traditional fermented beverages by metagenomics, and its approach in the characterization, storage, and security of these beverages to understand beverage ecosystems.

## 2 Metagenomics

Metagenomics allows gain of information about communities less explored, because it might obtain all genome sequences of different microorganisms that form a microbial population by extraction and analysis of total DNA, without growing these microorganisms in synthetic culture. Through metagenomics, studies have been obtaining valuable information about genes that encode for enzymes, biocatalysts, and biosynthetic pathways than using traditional techniques of molecular biology (Wilson and Piel, 2013). Metagenomics is a useful tool to meet the high biodiversity of the environmental samples and the evolution of communities and the interaction among the members of a specific community (van Hijum et al., 2013).

The metagenome may follow three main pathways (Fig. 11.1): (1) PCR amplification of the *16S* and *18S rRNA* genes to estimate the microbial diversity; (2) digestion and cloning into expression vectors; and (3) direct sequencing of the sample (Hernández-León et al., 2010).

### 2.1 General Aspects of the Culture-Independent Molecular Methods

Diversity, evolution, and dynamics of microbial populations in different natural ecosystems have been determined by culture-independent molecular methods (Table 11.1). These techniques were based on the isolation of total DNA/RNA of microbial population in an ecological niche and subsequent amplification of metabolic genes or marker genes; these genes must be present in the microbial population and must have conserved regions. Culture-independent methods use primers encoding the *16S rRNA* gene (V3 and V4 regions) and primers for different phylogenetic markers, such as *26S rRNA* gene (D1/D2 regions), *Tu* elongation factor, *RecA* protein, and RNA polymerase β subunity (*rpo β*), and others genes of interest (Cocolin et al., 2013).

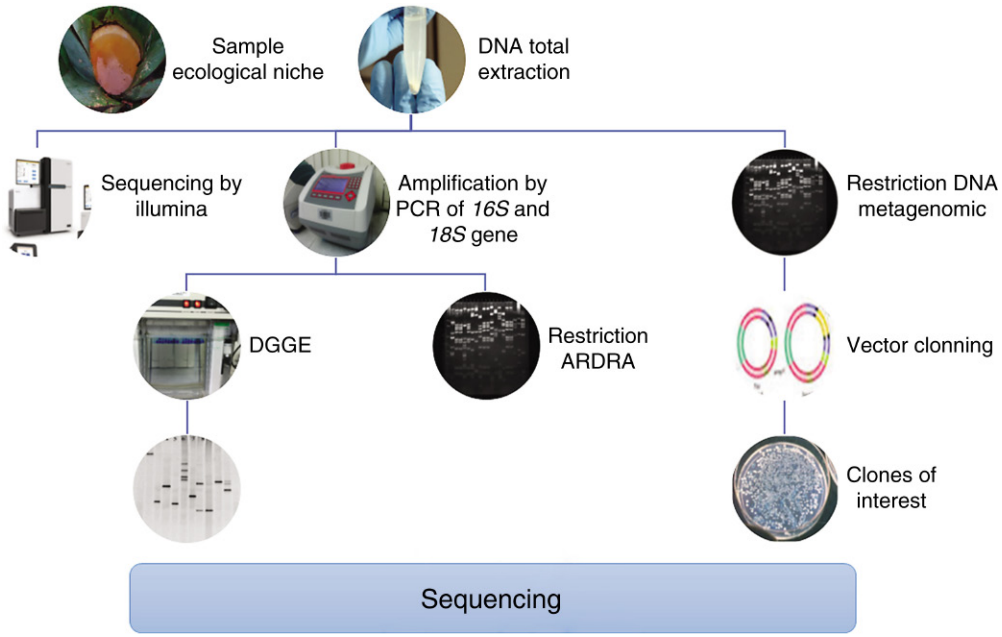


**Figure 11.1: General Pathways of the Metagenome.**

In microbial ecology of foods, some culture-independent molecular techniques have been used, such as length heterogeneity polymerase chain reaction (LH-PCR), amplified fragment length polymorphism (AFLP), single stranded conformational polymorphism (SSCP), ribosomal intergenic spacer analysis (RISA), restriction analysis of internal transcribed spacer (ITS), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis (DGGE/TGGE), and the next-generation sequencing (NGS) by HiSeq Illumina System and pyrosequencing.

### *2.1.1 SSCP*

It is based on the mobility of single strands of DNA. Detects sequence variations between different amplified DNA fragments and need low temperatures to avoid denaturing electrophoresis (Cocolin et al., 2013).

### *2.1.2 T-RFLP*

It is used to study variations in genes from different organisms and to characterize the dynamic changes over time in an ecological niche. It is based on the digestion by restricting endonuclease of PCR products labeled with fluorescence. Profiles represent the obtained specie; however, it can generate complex patterns (Cocolin et al., 2013).

**Table 11.1: General aspects of the culture-independent molecular methods.**

| Methods | Taxonomic Resolutions | Genes | Primers | Applications | References |
|---|---|---|---|---|---|
| PCR-DGGE/ PCR-TGGE | Genus and species | *16S rRNA* (V3 and V6 regions), *26S rRNA (D1/D2* regions) | Using a clamp in primer, formed for G and C (40 pb) | Community fingerprinting, microbial population dynamics, and mutation analysis | Giraffa and Carmianati (2008); Cocolin et al. (2013) |
| SSCP | Genus and species | *16S rRNA* | Fluorescent primers | Community fingerprinting, microbial population dynamics, and mutations analysis | Cocolin et al. (2013) |
| T-RFLP | Genus, species, and strains | *16S rRNA* and *18S rRNA* | Fluorescent primers | Community fingerprinting and microbial population dynamics | Cocolin et al. (2013) |
| LH-PCR | Genus and species | *16S rRNA* | Fluorescent forward primers | Community fingerprinting and microbial population dynamics | Bottari et al. (2010) |
| PCR-ARDRA | Genus and species | *16S rRNA* | — | Community fingerprinting and microbial population dynamics | van Hijum et al. (2013) |
| RIS/ITS-PCR | Genus and species | *16S* and *23S rRNA; 18s* and *28S rRNA* | Fluorescent forward primers | Community fingerprinting and microbial population dynamics | Justé et al. (2008) |
| RAPD | Genus and species | *26S rRNA (D1/D2 dominio)* | Fluorescent primers of 10 bp | Community fingerprinting and microbial population dynamics | Stephan (1996) |
| AFLP | Genus, species, and strain | Randomly fragments DNA | Fluorescent primers | Community fingerprinting and microbial population dynamics | Esteve-Zarzoso et al. (2010) |
| Illumina system | Genus and species | 16S rRNA (V3 and V4 regions) | Multiple primers | Community fingerprinting, microbial population dynamics, and gene quantification | Liu et al. (2012); Quail et al. (2012); Koboldt et al. (2013) |

AFLP, Amplified fragment length polymorphism; ARDRA, amplified ribosomal DNA restriction analysis; DGGE, denaturing gradient gel electrophoresis; ITS, internal transcribed spacer; LH-PCR, length heterogeneity polymerase chain reaction; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RISA, ribosomal intergenic spacer analysis; SSCP, single stranded conformational polymorphism; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism.

### 2.1.3 LH-PCR

It is used to distinguish between different organisms based on natural variations in the length of the 16S rDNA gene sequences (Bottari et al., 2010).

### 2.1.4 PCR-ARDRA

ARDRA may produce fingerprint in bacteria. The PCR products are labeled with fluorescence and digested with restriction enzymes. It generates band patterns of the predominant species in a microbial population, though ARDRA have shown reproducible and congruent result of LAB species (Sarethy et al., 2014; van Hijum et al., 2013).

### 2.1.5 RIS/ITS-PCR

It is based on the amplification of intergenic regions, separated into different size, allowing amplification of intergenic spacer (IGS) microorganisms' sample. A pattern of bands considered community fingerprinting is obtained (Justé et al., 2008).

### 2.1.6 RAPD

This method amplified DNA regions arbitrarily using random oligonucleotide primers. Low temperatures of annealing are used and generated patterns of bands. It is used for microorganisms typing, useful in genetic mapping, in the study of relationship, and might distinguish different microorganisms simultaneously (Sarethy et al., 2014; Stephan, 1996).

### 2.1.7 AFLP

Amplifies two genomic regions and amplicons are separated under denaturing conditions. It is based on: (1) DNA restriction with two different enzymes and ligation of adapters; (2) amplification of restriction fragments; and (3) separation of fragments in denaturing polyacrylamide gels. However, this technique does not differentiates dominant homozygotes from heterozygotes (Esteve-Zarzoso et al., 2010).

### 2.1.8 PCR-DGGE/PCR-TGGE

DGGE and TGGE allow obtaining the community fingerprint by PCR amplification of the *16S rRN*A and *26S rRNA* genes, followed by separation of DNA fragments. These techniques are based on decrease of electrophoretic mobility of PCR products, and double strand DNA molecules in polyacrylamide gels containing a denaturant linear gradient (containing urea as denaturing agent) or a linear temperature gradient. A strategy to amplify a double strand DNA is using a primer with a G-C-rich (40 bp) clamp. This clamp maintains both strands covalently attached at the ends. DNA molecules with different sequences migrate to different positions in the gel, because they have different resolution. The largest DNA fragments and with more content of guanine and cytosine are retained at the end of the gel of polyacrylamide, and therefore the smaller fragments are retained at the beginning or near

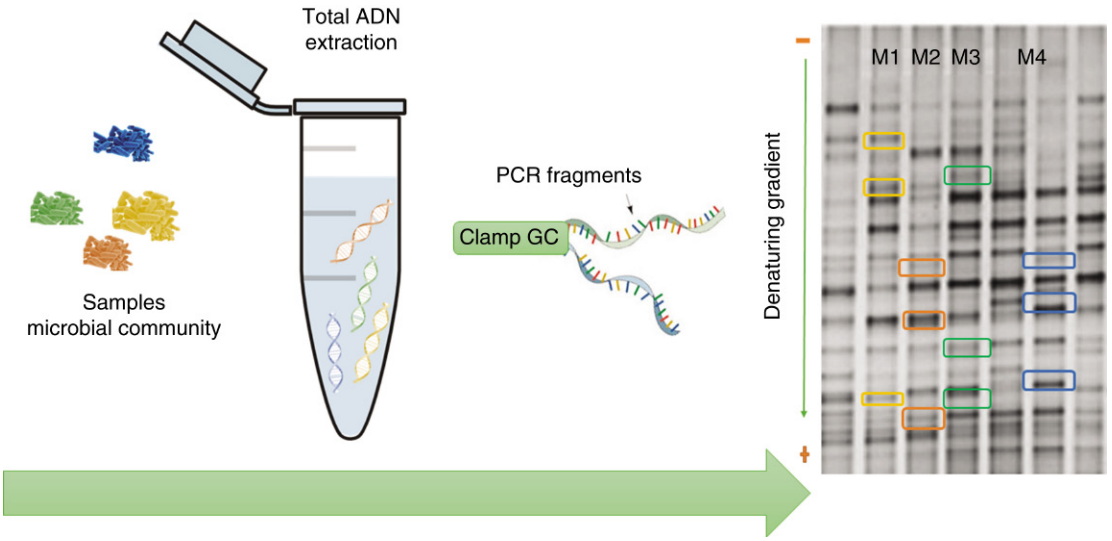


**Figure 11.2: Diagram Process of DGGE.**

this site (Fig. 11.2) (Cocolin et al., 2011, 2013; Giraffa and Carmianati, 2008; van Hijum et al., 2013). Different foods have been analyzed by DGGE, some of them are: fermented wines, meat, and dairy products (Cocolin et al., 2013).

DGGE is one of the most used techniques to identify microorganisms during fermentation processes, food spoilage, and food security. However, NGS presents an advantage over the DGGE; NGS might be analyzing large amount of sequences in a short time using technologies, such as Illumina and Roche 454 pyrosequencing, also NGS allows the detection of microorganisms that are not detected by DGGE, and does not require cloning of microorganisms (Cocolin et al., 2013; Sarethy et al., 2014).

### *2.1.9 Illumina system*

Illumina sequencing by chemical synthesis technology is based on next-generation sequencing. V3 and V4 region of the *16S rRNA* gene may be amplified and sequenced by this technique; also other regions, such as D1/D2 of *26S rRNA* gene may be sequenced. It has been used to study defined regions of the genome. HiSeq system provides amplification, sequencing, and analysis of data quickly (3 at 8 h), which is effective and the cheapest sequencing process because it features the biggest output and lowest cost (Liu et al., 2012; Quail et al., 2012). In this technique, a mix of multiple primers are combined with the genomic DNA of interest to amplify by PCR specific DNA regions, to which specific adapters are linked to the ends. A library is generated for sequencing; each sequence represents a single DNA product (Koboldt et al., 2013), and thousands of samples simultaneously can be generated with multiplexing primers and adapters (Liu et al., 2012). This technology has been used successfully to detect pathogens in humans, bacteria,

mutation detection, large genome sequencing, and other uses with 98% accuracy (Frey et al., 2014; Ross et al., 2013), and can also generates millions of sequences at a fraction of cost of the Sanger sequencing or Roche 454 pyrosequencing, from the sample of a microbial community (Bartram et al., 2011).

#### *2.1.10 454 Pyrosequencing*

Roche 454 pyrosequencing is a rapid method for studying the microbial ecology of various environments, it can be also used for the analysis of microorganism sequences, mutation detection, and large genome, but other sequences can be analyzed, too. Roche 445 was the first commercially successful NGS and its advantage is the speed as it needs only 10 h for sequencing. The 454 pyrosequencing is one high throughput sequencing system that can read several hundred base pairs with 99.5% of accuracy and quality (Glenn 2011; Liu et al., 2012). These technologies are very interesting because microorganisms that are not dominant in the community of niche ecology can be detected by pyrosequencing (Leite et al., 2012).

There is scarce information about microbial studies of fermented foods through culture-media independent molecular methods (Humblot and Guyot, 2008). These molecular methods may lead to the identification of microorganism formerly not detected in these foods and help in the characterization of the genetic diversity of microbial communities involved during fermentation of traditional foods and beverage (Kergourlay et al., 2015; Lappe-Oliveras et al., 2008). The use of techniques based on metagenomic to study beverages microbial communities is relatively new, however, the use of this technique in foods has become affordable for food industry (Kergourlay et al., 2015).

## 3 Traditional Fermented Beverages

Traditional beverages have been produced, since long time ago, from fermentation process of different raw materials and consumed during religious ceremonies and rituals or as medical treatments (Lappe-Oliveras et al., 2008). Therefore, they are representative of various regions and communities of a country (Table 11.2).

Through history, diet culture of different human groups has been characterized by use of specific raw materials for elaboration of their traditional fermented beverages. The millet, sorghum, and maize have been used for porridges in Africa and Latin America, fruits are used to produce wine in Europe, America, and Australia and rice is employed to produce alcoholic beverages in China and Japan (Tamang, 2010).

Preparation of traditional beverages occurs through a process of spontaneous fermentation. This fermentation is affected by the microbiota contained in the raw material and by the environmental factors that can or cannot benefit the fermentation process and the final product (Navarrete-Bolaños, 2012). Fermentation occurs during four principal food processes: (1) alcoholic fermentation, in which yeast and bacteria, such as

**Table 11.2: Culture-independent methods applied to traditional fermented beverage.**

| Countries | Beverages | Raw Materials | Reported Microorganisms | Methods | Genes | References |
|---|---|---|---|---|---|---|
| Mexico (SLP) | Mezcal | *Agave salmiana* | *Saccharomyces cerevisiae, Kluyveromyces marxianus, Pichia kluyveri, Zygosaccharomyces bailii, Clavispora lusitaniae, Torulaspora delbrueckii, Candida ethanolica,* and *Saccharomyces exiguus* | PCR-DGGE | 26S rRNA | Verdugo Valdez et al. (2011) |
| Mexico | Mezcal | *Agave salmiana* | *Pediococcus parvulus, Lactobacillus brevis, Lactobacillus composti, Lactobacillus parabuchneri,* and *Lactobacillus plantarum* | PCR-DGGE | 16S rRNA | Narváez-Zapata et al. (2010) |
| Mexico | Pulque | Aguamiel (maguey sap) | *Lactobacillus acidophilus, Leuconostoc mesenteroides, Gluconobacter oxydans,* and *Hafnia alvei* | ARDRA | 16S ARNr | Escalante et al. (2004) |
| Mexico (Morelos) | Pulque | Aguamiel (maguey sap) | *Acinetobacter orientalis, Zymomonas mobilis, Kluyver ascorbata, Acinetobacter radioresistens, Lactobacillus* sp., *L. acidophillus, L. hilgardii, L. paracollinoides, L. sanfranciscenci, Lactococcus* sp., *Leuconostoc kimchi, Leuconostoc citreum, Leuconsotoc gasicomitatum,* and *Leuconostoc mesenteroides* | ARDRA | 16S ARNr | Escalante et al. (2008) |
| Mexico, Guatemala | Pozol | Maize | *Lactobacillus casei, L. delbrueckii, L. fermentum, L. plantarum, Bifidobacterium mínimum, Sphingomonas* sp., *Streptococcus bovis,* and *Enterococcus saccharolyticus* | PCR-DGGE | 16S ADNr | Ben Omar and Ampe (2000) |
| Mexico | Taberna | Sap of palm | *Zymomonas mobilis, Fructobacillus* spp., *Pantoea agglomerans, Lactobacillus nagelii, L. sucicola*, and *Acetobacter pasteurianus* | Clone libraries | 16S ADNr | Alcántara-Hernández et al. (2010) |
| USA | Kava | Rhizome and roots of kava (*Piper methysticum*) | *Weissella soli, Lactobacillus* spp., and *Lactococcus lactis* | PCR-DGGE | 16S rRNA | Dong et al. (2011) |
| Brazil | Cauim | Cassava, rice, peanuts, cotton seeds, and maize | *Lactobacillus plantarum, L. fermentum, L. paracasei, L. brevis, P. guilliermondii, K. lactis, Candida* sp., *R. toruloides,* and *Saccharomyces cerevisiae* | PCR-DGGE | 16S and 26S rRNA | Lacerda Ramos et al. (2010) |
| Brazil | Caxiri | Cassava and sweet potato | *Paenibacillus* sp., *Bacillus subtilis, Lactobacillus fermentum, Saccharomyces cerevisiae, Pichia membranifaciens,* and *Rhodotorula mucilaginosa* | PCR-DGGE ARDRA | 16S and 26S rRNA | Santos et al. (2012) |
| Brazil | Chicha | Rice | *Propionibacterium* sp., *Bifidobacterium* sp., *Leuconostoc lactis, Lactobacillus casei, Enterobacter* sp., *Escherichia coli, Enterococcus lactis, Klebsiella pneumoniae, Streptomyces* sp., *Bacillus subtilis,* and *Enterococcus durans* | PCR-DGGE | 16S rRNA | Puerari et al. (2015) |
| South Africa | Fruit juice | Apple, grape, pear, mango, and aloe vera | *Alicyclobacillus acidoterrestris* and *Alicyclobacillus pomorum* | PCR-DGGE | 16S rRNA | Duvenage et al. (2007) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Cameroon | Palm wine | Sap of palm | LAB, *Saccharomyces cerevisiae, S. ludwigii, Zygosaccharomyces baili, Hanseniaspora uvarum, Candida parapsilopsis, Candida fermentati,* and *Pichia fermentans* | PCR-DGGE | 26S rRNA | Stringini et al. (2009) |
| Turquía | Kefir | Kefir grains | Lactobacillaceae, Leuconostoccaceae, Enterococcaceae, and Streptococcaceae family | rRNA | 16S rRNA | Nalbantoglu et al. (2014) |
| Spain | Tempranillo wine | | *Oenococcus oeni, Gluconobacter oxydans, Asaia siamensis, Enterobacter* sp., and *Serratia* sp. | PCR-DGGE | 16S rRNA | Ruiz et al. (2010) |
| France | Sauternes wine | Grape juice | *Saccharomyces cerevisiae, Candida stellata, Hanseniaspora uvarum,* and *Botrytis cinérea* | PCR-DGGE | 26S rRNA | Divol and Lonvaud-Funel (2005) |
| Greece | Wines affected by Botrytis | Grape juice | *Metschnikowia pulcherrima, Zygosaccharomyces bailii, Issatchenkiav* spp., and *Botrytis cinerea* | PCR-DGGE | 26S rRNA | Nisiotou et al. (2007) |
| Greece | Wines affected by Botrytis | Grape juice | *Klebsiella oxytoca, Citrobacter freundii, Enterobacter* spp., *Erwinia* sp., *Pantoea dispersa,* and *Tatumella ptyseos* | PCR-DGGE | 26S rRNA | Nisiotou et al. (2011) |
| Italy | Erbaluce wine | Grape juice | *Candida zemplinina, Metschnikowia fructicola, Hanseniaspora uvarum,* and *Saccharomyces cerevisiae* | PCR-DGGE | 26S rRNA | Rantsiou et al. (2013) |
| Italy | Picolit wine | Grape juice | *Saccharomyces cerevisiae, Saccharomyces cerevisiae, Aureobasidium pullulans,* and *Candida zemplinina* | PCR-DGGE | 26S rRNA | Urso et al. (2008) |
| Italy | Wine | Grape must | *Acetobacter* sp., *Gluconobacter* sp., *Gluconoacetobacter, Fructobacillus* sp. *Lactobacillus* sp., and *Tatumella* sp. | Sequencing GS FLX | 16S rRNA | Campanaro et al. (2014) |
| Korea | Takju | Rice | *Lactobacillus paracasei, Lactobacillus plantarum, Leuconostoc pseudomesenteroides, Lactobacillus harbinensis,* and *Lactobacillus parabuchneri* | PCR-DGGE | 16S rRNA | Kim et al. (2010) |
| Taiwan | Millet | Rice | *Acinetobacter* sp., *Bacillus* spp., *Enterobacter* sp., *Lactococcus garvieae, Lactococcus lactis, Pedicoccus pentosaceus,* and *Pedicoccus stilesii* | PCR-DGGE | 16S rRNA | Chao et al. (2013) |
| China | Kefir | Kéfir grain | *Lactococcus* sp., *Lactobacillus* sp., *Acetobacter* sp., *Leuconostoc* sp., *Shewanella* sp., *Pseudomonas* sp., *Streptococcus* sp., *Acinectobacter* sp., *Pelomonas* sp., *Weissella* sp., and *Dysgonomonas* sp. | Sequencing Illumina HiSeq 2000 | 16S rRNA | Gao et al. (2013) |
| China | Kefir | Kefir grain | *Lactococcus* sp., *Lactobacillus* sp., *Acetobacter* sp., *Leuconostoc* sp., *Shewanella* sp., *Pseudomonas* sp., *Streptococcus* sp., *Acinectobacter* sp., *Pelomonas* sp., *Weissella* sp., and *Dysgonomonas* sp. | Sequencing Illumina HiSeq 2000 | 16S rRNA | Gao et al. (2013) |
| China | Kombucha | Black tea | *Gluconobacter* sp., *Acetobacter* sp., *Zygosaccharomyces* sp., and *Lactobacillus* sp. | NGS 454 Genome Sequencer FLX Titanium System | 16S rRNA V4-V5 and ITS-rDNA | Marsh et al. (2014) |

*Zymomonas mobilis* are involved; (2) lactic fermentation in which some LAB, such as *Lactobacillus acidophilus* are involved (3) acetic fermentation in which some bacteria, such as *Acetobacter* sp. are involved; and (4) alkali fermentation (Blandino et al., 2003). During the fermentation process of beverages, different compounds are originated, such as organic acids and amino acids which affect flavor, esters, and higher alcohol types that affect aroma of each beverage. The fermented alcoholic beverages in the world are produced from honey, plants, malting, fruits, and some cereals; and can classify as distilled and nondistilled alcoholic beverages (Tamang, 2010).

## 3.1 Microorganisms Involved in Traditional Fermented Beverages

During the fermentation process, microorganisms involved are already present in the raw material, added as starter cultures or present in the tools used during the process. Different microorganisms grow and develop during the different periods of the fermentation, until they are inhibited by the intolerable conditions because of formation of secondary metabolites, and so other microorganisms take over. The microorganisms modified the raw material by biochemical transformation them into more readily digestible compounds with nutritional properties and beneficial to health of consumers. Other compounds, such as alcohols, depending of fermentation process and step, are produced too. Most fermented beverages depend on the fermentation process by LAB, some species of *Bacillus* spp. and yeast (Trevanich et al., 2016). The abundance and diversity of these microorganisms change during the fermentation process (van Hijum et al., 2013); some of the most common microorganisms are mentioned later.

### 3.1.1 Bacillus *spp.*

*Bacillus* spp. are Gram positive bacteria, endospore forming, and are present in raw materials, such as grains and cereals. *Bacillus* proteases break down proteins in peptides and amino acids and provide products with higher nutritional value. The most of *Bacillus* spp. are safe microorganisms and are classified as GRAS. Representative of this kind of microorganisms are *B. subtilis*, *B. laterosporus*, *B. pumilus*, *B. brevis*, *B. macerans*, *B. licheniformis, B. polymyxa*, and *B. coagulans* (Trevanich et al., 2016).

### 3.1.2 *Lactic acid bacteria*

LAB include a group of rods and cocci, Gram positive, and has no spore, produces lactic acid, lactate, $CO_2$, and ethanol. LAB are related with the development of desirable organoleptic characteristic. In addition, LAB produce exopolysaccharides, different enzymes, and bacteriocins (Trevanich et al., 2016). This group of microorganisms includes homofermentatives bacterias, such as *Pediococcus, Streptococcus, Lactococcus,* and some *Lactobacilli* and heteroferentatives bacteria as *Weisella* and *Leuconostoc* and some *Lactobacilli* (Blandino et al., 2003).

### 3.1.3 Yeast

Yeast are involved in the fermentation of traditional beverages and they play a role on the production of volatile and nonvolatile compounds that give special characteristics to the beverage, such as aroma and flavor (Navarrete-Bolaños, 2012). The most common fermenting yeast are species of *Saccharomyces*, other genera included are *Pichia* spp., *Saccharomycopsis* spp., *Endomycopsis* spp., *Candida* spp., *Hansenula* spp., *Rhodotorula* spp., *Kluyveromyces marxianus,* and *Torulopsis* spp. (Trevanich et al., 2016).

The prevalence of microorganisms in the beverage is conditioned by adaptation to nutrient availability, such as amino acids, fatty acids, vitamins, proteins, and other compounds, presents in the ecosystem, therefore the type and quality of nutritional of raw material and the process technology play a key role in the microbial consortia (Elizaquível et al., 2015).

Preparation of many traditional fermented beverages remains today as a house of art. Some fermented beverages use a starter culture to produce the beverage, these starter cultures may be naturally prepared by (1) balls of rice or wheat with molds, amylolitic yeast, and LAB; (2) a combination of fungus, such as *Aspergillus oryzae* and *A. sojae*; and (3) cake of wheat flour moistened with its microbial flora (Tamang, 2010).

Characterization of cultured and noncultured microbiota present in a fermented beverage is essential for understanding the process of preparing and reproducing the typical organoleptic characteristics, such as odor, color, and taste of the beverage, because of the presence of microorganisms essential for the process. Therefore, this characterization allows setting the parameters for quality control during the process, also represents the first stage in the process of industrialization and scaling of these products (Escalante et al., 2008).

## 3.2 Application of Culture-Independent Molecular Techniques in Traditional Fermented Beverages

Culture-independent methods offer the opportunity to obtain information on the microbial communities, which are less explored. This information allows identifying some unexpected microorganisms that could be present in traditional beverages characterized only by conventional molecular techniques. Also metagenomic methods are important to follow the evolution of these communities during the fermentation process (Kergourlay et al., 2015). Metagenomic methods that have been used to amplify *16S rRNA* gene regions to identify bacteria are DGGE in beverages, such as pozol, mezcal, and chicha (Ben Omar and Ampe, 2000; Narváez-Zapata et al., 2010; Puerari et al., 2015; Verdugo Valdez et al., 2011), and some methods by NGS, such as technology Illumina in beverages of kefir and the pyrosequencing (454 Roche) in beverages as kombucha (Gao et al., 2013; Marsh et al., 2014). In Table 11.2, it is showed that culture-independent methods applied to traditional fermented beverage for the identification of different microorganisms.

During the elaboration processes of fermented beverages and wines, microorganisms that have not been identified by dependent culture methods can take part and they may give special characteristics to the beverage. An example is the identification of yeast, such as *Candida stellata* during the process of production of sweet wine using DGGE (Cocolin et al., 2001) and the identification of non-*Saccharomyces* yeasts in beverages with more complex organoleptic profiles (Cocolin et al., 2013).

### 3.2.1 Fermented beverages from Mexico

In Mexico, there are different typical traditional fermented beverages elaborated in different towns and regions, which represent part of the site and people identity. In Mexico, traditional beverages can be grouped generally in distilled and nondistilled beverages. Some distilled beverages are: tequila, mezcal, bacanora, raicilla, and sotol, while some nondistilled beverages are: pulque, pozol, tuba, tesguino, tepache, and tavern, among others (Alanís and González, 2011; De León-Rodríguez et al., 2008; García et al., 2010; Lappe-Oliveras et al., 2008). Only few beverages have been characterized by metagenomic, such as mezcal, pulque, and pozol (Ben Omar and Ampe, 2000; Escalante et al., 2008; Narváez-Zapata et al., 2010; Verdugo Valdez et al., 2011).

#### 3.2.1.1 Mezcal

Mezcal is a traditional beverage from Mexico, elaborated from alcoholic fermentation of pineapple of several Agave species, such as *Agave angustiofolia, A. esperrima, A. weberi, A. potatorum,* and *A. salmiana*. The taste and aroma of mezcal depend on cooking process, pineapple fermentation and distillation of the beverage. Recently, it was reported different studies of the LAB and yeast communities during Mezcal fermentation from *Agave salmiana* in Tamaulipas and San Luis Potosi, Mexico. Microbial diversity of LAB was characterized using DGGE technique, and analyzing the *16S rRNA* gene sequences were found *Pediococcus parvulus, Lactobacillus brevis, Lactobacillus composti, Lactobacillus parabuchneri, Lactobacillus plantarum,* and some species of *Weissella* and *Bacillus* (Narváez-Zapata et al., 2010). While yeast population during *A. salmiana* fermentation was analyzed using two different methods, DGGE (D1 region of 26S rRNA gene) and RFLP (5.8S-ITS region and D1/Ds dominion of 26S rRNA gene); this characterization allowed to report the presence of *Saccharomyces cerevisiae, Torulaspora delbrueckii*, and *Kluyveromyces marxianus* (Verdugo Valdez et al., 2011). The presence of particular populations of microorganisms during fermentation gives the flavor and aroma of the beverage in each location and therefore the quality.

#### 3.2.1.2 Aguamiel

The maguey or Agave is one of the most important plants used in Mexico, since prehispanic periods to produced beverages and it is abundant in the semidesert zones. The species used to produce beverages are *Agave salmiana, A. mapisaga, A. atrovirens, A. Americana*, and *A. ferox* and are known as "magueyes pulqueros" (Ortiz-Basurto et al., 2008). The

aguamiel is *Agave* sap obtained from maguey in adult stage (8–10 years), which is a liquid with a sweet, pleasant taste, characteristic odor, and colorless to slightly herbaceous light yellow; in most cases, the aguamiel is collected by rustic and traditional procedures; and it is consumed as a refreshing typical beverage in locations where maguey is cultivated. This beverage has a sweet and pleasant flavor, it is colorless and with a characteristic herbaceous scent. It is composed of sugars, such as glucose, sucrose and fructose, fructans, gums, amino acid, proteins, and minerals, the amount of these components varies according to Agave specie and development conditions of maguey (Ortiz-Basurto et al., 2008); and has a native microbiota mainly formed by LAB, yeasts, and some exopolysaccharides-producing bacteria. To obtain the aguamiel, it is necessary to maguey castration before flowering, which consists of being cut the tenderest leaves of the plant center (meyolote) removing the flower stalk, thus preventing that plant juices are consumed during development of quiote (inflorescence) and flowers. Subsequently, let the stem or mezontete achieve its development and sap has its maximum sugar content, over a period of 6–12 months, with the aim that the maguey aguamiel can produce enough good quality. When the maguey reached the necessary aging spots on the leaves appear, and it is the time of perforation to open the mezontete (pineapple) in order that the sap begins to flow. Then clean or scrape the mouth of the bowl (mezontete) to open the pores and vessels through which the aguamiel flows, the ideal time is when the bowl acquires a color between red and dark yellow. Initial production of aguamiel is low; after 2–3 weeks reaches peak production of up to 3 L in each rasp and held from 3 to 6 months; then aguamiel production drops drastically (Alanís and González, 2011; Ortiz-Basurto et al., 2008). Recollection of aguamiel is done daily, 3 times a day (morning, noon, and night), using a jug, after aguamiel collection, maguey is scraped back to cavity to open the pores again and let flow the sap to subsequent collection at the end, empty bowl is covered with fleshy leaves and a stone to prevent animals falling (Fig. 11.3). Aguamiel is transported in tubs for marketing; this procedure is rudimentary and unhygienic.

The aguamiel microbiota begins the process of fermentation, which can last from 12 to 48 h at 25°C, reducing sugar content of 7%–14% to 0.5%–2%, increasing the ethanol content and the formation of exopolysaccharides, increasing viscosity and thus obtaining an alcoholic, white, strong smelling, and viscous drink called pulque. They have reported several studies to characterize microbial diversity of aguamiel and pulque using traditional methods of culture-dependent identification; using this technique, it was found that microbial diversity is composed by *S. cerevisiae, Kluyveromyces*, LAB, such as *Lactobacillus acidophillus, Leuconostoc mesenteroides*, and others microorganisms (Escalante et al., 2008).

The characterization of complex microbiota present in aguamiel has not been well defined despite the various studies that have been conducted. Not dependent methods of molecular identification can lead to identification of microbial groups previously undetected in these products, expanding knowledge of the genetic diversity of microbial communities involved in the fermentation of this traditional beverage (Lappe-Oliveras et al., 2008).


**Figure 11.3: Production and Recollection of Aguamiel in Maguey Pulquero (*Agave atrovirens*).** (A) Maguey on production of Aguamiel, (B) recollection of Aguamiel, (C) Aguamiel in mezontete, and (D) Aguamiel obtained from maguey in adult stage.

The span of natural fermentation of Aguamiel sap varies, for commercialization in most cases a traditional heat treatment is applied for preservation. Aguamiel is the raw material to elaborate other products such as agave honey, fructo-syrups, natural sweeteners and pulque bread elaboration, also because of its content of agave fructans, this beverage has prebiotic properties, by this reason this product can be considered as a functional beverage. The chemical characterization of aguamiel is very important to ensure nutritional quality of this beverage (Muñiz-Márquez et al., 2015). However, the boiled product life is short of almost 7 days, because fermentation process of this product is very quickly. The metagenomic studies can help to understand the microbial ecology in this beverage and can set the guidelines to preserve this beverage for longer time.

#### 3.2.1.3 Pulque

The pulque is a traditional Mexican beverage, produced by fermentation of agave sap called aguamiel; this alcoholic product is obtained after 24–48 h of fermentation and it is not distilled. Aguamiel is rich in fructose, glucose, sucrose and polyfructans, and is extracted from several Agave species, such as *A. atrovirens* and *A. salmiana.* The pulque is a whitish liquid, slime, alcoholic, foamy, odor and with characteristic taste (Valadez-Blanco et al., 2012). The fermentation process of aguamiel to pulque is not controlled. However, in order to accelerate this process a portion of pulque previously fermented call seed is added to aguamiel (Ortiz-Basurto et al., 2008). Fermentation is generally conducted under poor aseptic conditions, which involved the indigenous microbiota of maguey and the microbiota associated with the collection, transport and handling of aguamiel, therefore, characterization of the microbiota during fermentation of aguamiel through metagenomics, may help to control the process and industrialization. During pulque elaboration, a viscous and alcoholic fermentation takes place; so fresh pulque consumption is recommended, because after 36 h acetic and putrina fermentation begins. The pulque viscous consistence depends on exopolysaccharides production by *Leuconostoc mesenteroides* and other microorganisms. Several studies have been carried out to characterize microbial diversity of pulque using traditional methods of identification based on culture mediums. More recent studies using ARDRA revealed different microorganisms, which are actively involved during pulque fermentation and confer organoleptic characteristics to the beverage. Some identified microorganisms by ARDRA were: *Acinetobacter orientalis, Zymomonas mobilis, Kluyverumyces ascorbata, Acinetobacter radioresistens, Lactobacillus* sp.*, L. acidophillus, L. hilgardii, L. paracollinoides, L. sanfranciscenci, Lactococcus* sp., *Leuconostoc kimchi, Leuconostoc citreum, Leuconsotoc gasicomitatum*, and *Leuconostoc mesenteroides* (Escalante et al., 2008). All microorganisms and their proportion in the fermentation process of pulque must be defined to control the conditions of fermentation.

#### 3.2.1.4 Pozol

Pozol is one of the first fermented foods in which the microbiota was analyzed by culture-independent methods. Pozol is a traditional food made from fermented maize; prepared in towns of Mexico and Guatemala with white nixtamalized maize kernels. First, maize is ground and mixed with water and the obtained dough is wrapped with banana leaves. This dough is fermented at room temperature for 7 days. Then, dough is suspended in water and dinking as a traditional beverage. From the spontaneous fermentation of this beverage, yeasts, fungi and bacteria have been identified using metagenomics techniques. Bacterial species identified by DGGE (V3 region of *16S rRNA* gene) are: *Lactobacillus casei, L. delbrueckii, L. fermentum, L. plantarum, Bifidobacterium mínimum, Sphingomonas* sp.*, Streptococcus bovis*, and *Enterococcus saccharolyticus* (Ben Omar and Ampe, 2000).

#### 3.2.1.5 Taberna

Taberna is a sweet and effervescent traditional alcoholic beverage from the southern part of Mexico and other areas of Central America; it is produced by natural fermentation of coyol palm sap (*Acrocomia aculeate*) and is very similar to wine palm produced in Africa. The bacterial diversity of taberna was characterized by the sequencing of bacterial 16S rDNA libraries from metagenomic DNA and were identified by the following microorganisms: *Zymomonas mobilis, Fructobacillus* spp., *Pantoea agglomerans, Lactobacillus nagelii, L. sucicola*, and *Acetobacter pasteurianus; Z. mobilis* was found in most of the sequenced clones during the fermentation process with a largest proportion in the microbial community as well as *Lactobacillus nagelii* (Alcántara-Hernández et al., 2010).

Some beverages until now have been studied by culture-dependent methods, such as distilled beverages: bacanora and sotol, and nondistilled beverages, for example, tuba, tesgüino, colonche, axokot, and tepache. The sotol is a traditional Mexican alcoholic beverage with designation of origin for the Mexican states of Durango, Coahuila, and Chihuahua. The liquor is obtained by cooking sotol pineapple (*Dasylirion* spp.), then the raw material is fermented and the product is distilled; the process is long and has no quality control (Buenrostro-Figueroa et al., 2012). Others common beverages are: tepache, which is prepared with pineapple pulp and maize, apple or orange can be added; and tuba, which is prepared with the spontaneous fermentation of coconut palm sap, this beverage is similar to taberna, palm wine and others beverages consumed in Africa but the main difference is the palm specie used for their elaboration. The microbiota in tepache and tuba has been studied because of their production of bacteriocins (De la Fuente-Salcido et al., 2015). However, the study that the total microbiota involved in each fermentation process of these traditional beverages can be characterized by techniques based on metagenomics and can be helpful to understand the microbial ecology better in each beverage.

### *3.2.2 Fermented beverages from Africa*

Africa has rich variety of traditional fermented food and beverages, which may contain probiotics that can have a positive effect on human health. Palm wines, and traditional beers, such as: Mahewu, Togwa, Teji, Burukut, Pito, Sherbote, and Mwenge are traditional fermented beverages consumed by people of Africa (Franz et al., 2014). However, some beverages, such as Teji, an alcoholic fermented beverage from honey and whose flavor depends on the region where it is elaborated. In Ethiopia and Pito, a light brown sweet beverage from Niger, produced with maize or sorghum (Tamang, 2010), only have been studied by identification methods based on culture of microorganisms.

#### 3.2.2.1 Palm wine

Palm wine is an alcoholic beverage produced from the fermentation of sap of different palm species. Palm wine is a sweet, milky, effervescent, and alcoholic beverage. The

Palm wine is composed by amino acids, proteins, vitamins, and sugars. This traditional beverage is elaborated in different regions, such as Ghana, Cameroon, and West Africa regions; but in India three types of palm wine called *sendi, tari,* and *nareli* are produced, too (Tamang, 2010). Palm wine microbiota has been analyzed by culture and nonculture methods to identify the microorganisms present during fermentation; the main yeasts identified were: *S. cerevisiae, S. ludwigii, Zygosaccharomyces baili, Hanseniaspora uvarum, Candida parapsilopsis, Candida fermentati,* and *Pichia fermentans;* but some LABs were identified, too (Stringini et al., 2009).

### *3.2.3 Fermented beverages from Eastern Europe*

#### 3.2.3.1 Kefir

Kefir is a beverage produced by the fermentation of milk or water with kefir grains, sucrose, and fruits. This beverage called kefir or water kefir is very common in different counties of Eastern Europe. Beneficial properties, such as antimicrobial, antitumor, antimutagenic, and antioxidant by consumption of this beverage have been suggested. In addition, it has been mentioned for improving digestion and human health. The microbial diversity of kefir and starter grains in Ireland was evaluated using high-throughput parallel sequencing. In this study, *Lactococcus* spp. was found as the dominant family, but the microbiota is not uniform (Dobson et al., 2011). Some metagenomics analysis of this beverage has reported presence of microbial species of the *Lactobacillaceae, Leuconostoccaceae, Enterococcaceae*, and *Streptococcaceae* families (Nalbantoglu et al., 2014). In other sample of kefir from Brazil and analyzed by DGGE and pyrosequencing, it was found that the community was dominated by yeasts, such as *Kazachstania, Kluyveromyces* and *Saccharomyces*, and by both techniques, *Lactobacillus kefiranofaciens* was identified*;* however, the technology of pyrosequencing allowed the communities to be identified better in kefir than DGGE, because minor bacterial groups in the communities may not be detected using DGGE (Leite et al., 2012).

The microbiota in water kefir from Germany was identified, too. The technique ARDRA was used to study the bacterial microbiota and it was identified that species belonging to *Lactobacillaceae* and *Bifidobacteriaceae* families were found to be the most abundant microorganisms in this beverage (Gulitz et al., 2013). In other sample of water kefir, it was found that the bacteria strains from *Zymomonas* and *Lactobacillus* families were dominant and along with the yeasts *Hanseniaspora* and Dekkera, too (Marsh et al., 2013a,b). The microbiota of kefir varies depending on the regions where they are elaborated, because of conditions of the fermentation process and raw material employed, these factors may affect sensory properties of each product in each county or town.

### *3.2.4 Fermented beverages from Korea*

#### 3.2.4.1 Takju or Makgeolli

Takju is a traditional alcoholic beverage from Korea, it is fermented with grains of rice and nuruk. The nuruk is a starter culture from flour and semolina. In this fermentation, some

fungal species are involved, such as *Rhizopus* sp., *Mucor* sp., and *Aspergillus* sp.; yeasts, such as *Sacharomyces cerevisiae* and bacterial species, such as *Bacillus subtilis* and LAB have been reported, too. The wine reaches to 15% alcohol and traditionally is produced using a fermentation process which is not completely controlled; the microbiota present in this beverage was investigated by culture-independent methods, such as DGGE, analyzing the *16S rRNA* gene, results of this study indicated the presence of *Lactobacillus paracasei, L. plantarum, L. parabuchneri*, and *Leuconostoc pseudomesenteroides* in the beverage (Kim et al., 2010). Using 454 pyrosequencing and ITS, the dynamics of microbial communities during the fermentation to laboratory scale of makgeolli was studied (Jung et al., 2012). The analysis of the regions V1 and V3 of *16S rRNA* gene allowed the identification of *Acetobacter, Bacillus, Streptomyces*, and *Saccharopolyspora* in starter culture samples, these microorganisms disappeared after fermentation. *Enterococcus, Lactobacillus, Leuconostoc, Pediococcus, Weissella*, and *Lactococcus* were present throughout the fermentation process. The yeasts present during the fermentation process were *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*, while in some stages, *Aspergillus fumigates, A. oryzae, Rhizopus microspores*, and *Kazachstania barnettii* were observed (Jung et al., 2012). In this study, the largest proportion of yeasts corresponded to *S. cerevisiae* was found and this yeast increased the ethanol content.

### *3.2.5 Fermented beverages from China*

#### 3.2.5.1 Kombucha

Kombucha is a black tea beverage that was originated from China and is called tea fungus and Haipao, too. This tea is fermented by a symbiosis of bacteria and yeast embedded with the cellulosic matrix; kombucha contains metabolites that promote the health of consumer, also contains ethanol, acid acetic, and lactic. Culture-independent analyses of the microbiota present in this beverage using high-throughput sequencing, revealed the presence during the fermentation of *Gluconobacter, Acetobacter, Lactobacillus*, and *Zygosaccharomyces* species (Marsh et al., 2014).

#### 3.2.5.2 Maotai liquor

Maotai liquor is a beverage from China that involves a complex fermentation system. In the beginning, sorghum is mix with different type of Daqu (white, yellow, and black) and water for 11 days and then is stored in pool to allow anaerobic fermentation under noncontrolled conditions. This liquor has unique flavor and a complex of aromas, which is based on the microbiota involved during the process. The microorganisms that participates throughout fermentation were analyzed by DGGE, also the microbiota was studied in samples of sorghum and Daqu. Authors identified *Weissella cibara*, *Bacillus oleronius*, and *Lactobacillus fermentum* in most of the samples, while that Hanseniaspora varoom and *Monascus ruber* were only appeared in Daqu samples, and *Zygosaccharomyces kombuchaensis*, *Penicillium decumbens, Cryptococcus gastricus,* and *Saccharomyces* sp. were appeared in sorghum samples (Wang et al., 2016).

#### 3.2.5.3 Fen liquor

Fen liquor is a traditional fermented beverage that is produced by the fermentation process of broomcorn, in which fermentation starters are used and involves numerous steps. The starter is formed by barley, wheat, and peas, and involved six stages of fermentation during 1 month; and the entire fermentation process, it is formed by 12 stages with duration of 6 months. The diversity of bacterial and fungal populations that are involved in each stage of entire fermentation process was studied by high-throughput pyrosequencing technique. The bacterial communities were grouped in the *Lactobacillaceae* family, the most important bacteria during the fermentation process in this beverage, and *Bacillaceae* family. The fungal diversity was assigned to *Pichia kudriavzevii* of the *Saccharomycetaceae* family, which were the most abundant, but yeasts from *Saccharomycopsidaceae* and *Trichocomaceae* families were observed, too (Li et al., 2013).

#### 3.2.5.4 Chinese yellow rice wine

Chinese yellow rice wine or Shaoxing rice wine is a traditional fermented alcoholic beverage from China. It is similar to brewing and the microorganisms involved in this process are responsible of aroma, flavor, and color. During the production process of this beverage, Chinese starter culture and wheat Qu (Chinese koji) are used. The microbiota of rice wine was identified by DGGE and some microorganisms were found, such as: *Lactobacillus, Staphylococcus, Saccharopolyspora, Buttiauxella,* uncultured microorganisms, and some microorganisms belonging to the *Enterobacteriaceae* family (Luan et al., 2013). In addition to microorganisms found by DGGE, *Enterobacter*, *Pantoea, Pseudomonas, Propionibacterium, Acinetobacter, Burkholderia, Streptococcus, Weisella, Leuconostoc*, and *Lactococcus* were found in other study of microbiota of rice wine. However, *Enterobacter* and *Pantoea* were related with most of reads and these bacteria can cause opportunistic infections in some hosts (Fang et al., 2015). The microbial community in Shaoxing rice wine was characterized by Illumina-based sequencing using Illumina Hiseq2000, for the purpose of understanding better, the microbial ecology during the fermentation process of this beverage. In this study, from 21 to 23 million reads were generated by Illumina-based sequencing and B*acillus* sp., *Staphylococcus* sp., *Sacharopolyspora* sp., *Streptomyces* sp., *Mycobacterium* sp., *Rhodococcus* sp., *Frankia* sp. and other were identified in the 5 days, while those identified at 30 days, were *Lactobacillus* sp., *Pantoea* sp., *Arthrobacter* sp., *Kocuria* sp., and others, too. However, some unculturable microorganisms were not identified (Xie et al., 2013).

#### 3.2.5.5 Puer tea

Puer tea is a postfermented tea from China and is a beverage with brown to red color and fragrant. This beverage involves a complex of microorganisms that participate in its fermentation and produce secondary metabolites, such as polyphenols and volatile compounds that are related with the taste of puer tea. Using DGGE, it was possible to identify

the puer tea microbiota and was found that *Aspergillus niger* and *Blastobotrys adeninivorans* were some of the predominant microorganisms (Abe et al., 2008). The microbial community that is taking part during the fermentation of puer tea was studied by pyrosequencing, too. In this case, authors found that the bacteria are those microorganisms, more present in the process of fermentation (70%), following by yeast (16%), and the other microorganisms unassigned (13%). The most abundant yeasts were *Yarrowia* and *Saccharomyces* and the *Aspergillus* fungi (Lyu et al., 2013).

### *3.2.6 Fermented beverages from Taiwan*

#### 3.2.6.1 Taiwanese millet alcoholic beverage

A Taiwanese alcoholic beverage produced from millet is not a distilled traditional beverage. It is obtained from the fermentation of glutinous rice and is used in ceremonies of the Taiwanese tribes. To improve the quality of controlled drinking and preparing starter cultures, Chao et al. (2013) analyzed the microbiota involved in the initial fermentation by DGGE, identifying the following bacteria: *Acinetobacter* sp., *Bacillus* spp., *Enterobacter* sp., *Lactococcus garvieae, Lactococcus lactis, Pedicoccus pentosaceus*, and *Pedicoccus stilesii.*

### *3.2.7 Fermented beverages from Argentina*

#### 3.2.7.1 Chicha

Chicha is a traditional alcoholic beverage produced by fermentation of some varieties of maize in Northwestern Argentina. Chicha is a clear, yellow, effervescent, and alcoholic beverage, also called maize beer. The fermentation process includes saliva as starter culture and the microbiota of chicha changes in the different locations, depending on maize varieties and local traditions, but the LAB and yeast are the dominant populations. The populations of LAB in chicha were evaluated in two locations of Argentina, Maimará (M), and Tumbaya (T), by pyrosequencing. In both locations *Enterococcus, Lactococcus, Streptococcus, Weissella, Leuconostoc*, and *Lactobacillus* were identified; only in Chicha M *Pediococcus* was identified (Elizaquível et al., 2015).

In addition to the identification and selection of the microbiota that is actively involved in the fermentation of a beverage, there are critical variables that must be controlled in order to improve the process. These variables are: temperature, acidity, pH, agitation, and aeration. These variables will control the growth of biomass and production of metabolites necessary for each fermentation process (Navarrete-Bolaños, 2012). Metagenomics can be used as a tool in the manufacturing process and may allowed understanding the changes related to taste and aspect of foods affected by microorganisms (Kergourlay et al., 2015).

The new metagenomic methods include sequencing and identify species of heterogeneous taxon associated to foodstuffs, such as the "All-Food-Seq" approach used by Ripp et al. (2014). In this case, characterization of dominant microbial communities facilitates understanding the population dynamics during fermentation process of beverages (Kergourlay et al., 2015).

## 4 Potential Health Benefits of Traditional Fermented Beverages

The traditional fermented beverages may contain several functional active compounds that are generated during the fermentation process of each beverage, such as enzymes, vitamins, amino acid, soluble fiber, antioxidants, lactic acid, and other products; some of these bioactive substances may confer health benefits to consumers, such as such as reduction of allergies, antimicrobial activity, and promote the cardiovascular health (Trevanich et al., 2016).

One of these beverages is the puer tea that provided health benefits to consumer because it contains compounds, such as polyphenols and terpenoids. The puer tea is associated with the reduction of cholesterol level, regulation of blood pressure, and prevention of cardiovascular diseases (Lyu et al., 2013). Other beverage based on tea is kombucha or tea fungus, which contains bioactive peptides and secondary metabolites that promote human health benefits, such as antidiabetic and anticarcinogenic (Aloulou et al., 2012; Jayabalan et al., 2011; Marsh et al., 2014).

The probiotic microorganisms, such as *Lactobacillus*, which are present in many traditional beverages and promote gastrointestinal health and reduction of cancer risk in the consumers (Trevanich et al., 2016). Consumption of fermented food, such as beverages with probiotics have been positive effects on human health and socioeconomy of African people, preventing diarrheal diseases because it causes the intestinal microbial balance. The mechanisms under which probiotics act and the health properties that confer to health consumer are: alleviation of lactose intolerance symptoms, interference or antagonism versus pathogen, and others (Franz et al., 2014). However, the probiotic potential and effectiveness of each isolate LAB that it will add to a food in controlled conditions, it must be evaluated by some tests, such as survival under conditions simulating the human gastrointestinal tract, adherence to the intestinal mucosa, adhesion to mucin, antimicrobial activity, antibiotic resistance, hemolytic activity, etc. (Castro-Rodríguez et al., 2015). In addition, the yeasts present in beverage contribute to increase the nutritional value because these microorganisms contain high-value proteins, micronutrients, and vitamins (Trevanich et al., 2016).

In some beverages, bacteriocins, peptides with antimicrobial activity against pathogenic and spoilage bacteria were identified, which currently are increasingly used as antibiotics. Bacteriocins are produce by LAB versus others bacterial. One of these beverages are the kefir and Irish kefir. In these beverages, the production of Lacticin 3147 by *Lactococcus* spp. was found. This lantibiotic is a class I bacteriocin and has been shown to inhibit pathogens, such as *Clostriduym difficile*, *Staphylococcus aureus*, and *Enterococcus sp* (Dobson et al., 2011). The Mexican Tuba and Tepache are fermented beverages that contain bacteria, such as *Lactococcus lactis,* which produces peptides with antimicrobial activity as nisin and *Enterococcus faecium* that synthesizing enterocin. These bacteriocins have antimicrobial activity versus strains, such as *L. moncytogenes, S. aureus, K. pneumoniae,*

*E. coli, Salmonella* spp., and *S. typhimurium* (De la Fuente-Salcido et al., 2015). However, the composition of bioactive substances and their health benefits to consumer in each beverage are scattered (Trevanich et al., 2016).

## 5 Conclusions

Traditional fermented beverages are important part of population diet around the world. Genetic analyses of the microbiota present in the fermentation of traditional beverages, using techniques based on metagenomics, promote the extensive characterization in each ecology niche. This characterization can be exploited to establish the microorganisms that are actively involved in this process, giving particular organoleptic characteristics to each beverage. The quality of beverages depends on flavor and aroma conferred by microbial communities in each beverage. Also, the identification of the microorganisms is needed for monitoring the hygiene, quality, and safety of the beverages. Additionally, this type of analyses opens the opportunity to scale and industrialize the fermentation processes for elaboration of these traditional beverages, knowing with certainty, the essential microbiota for each fermented beverage. In addition, these techniques may have employed for characterization during storage and safety of fermented traditional beverages.

## Acknowledgments

SLVM thanks to the Autonomous University of Coahuila (UAdeC) and the National Council of Science and Technology (CONACYT) for the financial support to study a PhD in Food Science and Technology in the UAdeC.

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CHAPTER 12

# Process Engineering Applying Supercritical Technology for Obtaining Functional and Therapeutic Products

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## 1 Introduction

The application of supercritical technology as an effective extraction process for obtaining bioactive compounds from vegetable matrices was consolidated in the previous years, as much in aspects of operation as process costs. In special, the characteristic of supercritical carbon dioxide as a solvent with adjustable physicochemical properties in function of pressure and temperature makes it a selective solvent for the extraction of chemical compounds with different applications. Moreover, their critical properties are achieved at low temperature conditions, which enable obtaining thermolabile compounds, which are widely used in various applications in pharmaceutical, cosmetic, and food products.

The extracts obtained from the supercritical carbon dioxide have unique features when compared to those obtained by conventional procedures, such as extraction with organic solvents. Supercritical extracts are safe for human consumption because they are solvent free and their functional activities preserved, as carbon dioxide is an inert fluid, while extracts produced by other techniques show residual content of organic solvents and often have their chemical properties changed as a result of the extraction process. In addition, supercritical technology is an environmental friendly technology.

These extracts can be used in the formulating and processing of novel products and are potentials to innovation on the life cycle of several products available worldwide. Supercritical extracts meet the global demand for products that promote healthiness and well-being, products with natural additives and ingredients; sensoriality and pleasure, products with strong sensory appeal through the extraction and application of flavor compounds;

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00012-8

reliability and quality, safe production process; sustainability and ethics, low environmental impact products.

In this context, the aim of this chapter was to present the supercritical technology as a potential extraction process for obtaining bioactive extracts with functional and therapeutic properties, as well as the potentialities of such compounds in formulating novel products were overviewed along the chapter.

## 2 Supercritical Fluid Technology Applied to Extractions: A Realistic Overview of Parameters and Operating Conditions

Supercritical fluid technology applied to the extraction of functional and therapeutic products is a subject of study in several research centers around the world. This technology has gained notoriety in the decaffeination of coffee at the ending of 20th century and has been recently used in several countries at industrial level, especially for the recovery of natural products (Eggers and Pilz, 2011; Herrero et al., 2010; Melo et al., 2014c; Mendiola et al., 2013; Oliveira et al., 2012; Pereira and Meireles, 2010). Although less used than essential oils, supercritical fluid extraction (SFE) has been reported for recovering edible oils as well (Balvardi et al., 2015; Jokić et al., 2010; Martínez and Aguiar, 2014). Industrially, sesame oil has been extracted by SFE by UMAX Co. (Korea), in a plant designed by NATEX Prozesstechnologie GesmbH (Austria). The plant consists of two extraction vessels of 3.8 $m^3$, supporting pressure up to 55 MPa. Furthermore, pharmaceuticals and nutraceuticals compounds have been extracted and purified using supercritical fluids by PHASEX Co. (USA) at a 2 × 320 L extraction plant. Likewise, FLAVEX Naturextrakte (Germany), one of the pioneers in SFE, produces supercritical botanical extracts for distribution worldwide, concentrating specifically on food supplements, cosmetics, and perfumery.

The increasing number of organizations applying supercritical fluid technology is a consequence of the recent trend of manufacturing products through a sustainable and renewable way. In the extraction field, the use of "green" solvents as $CO_2$ and water is becoming increasingly popular across several segments of industry, especially the nutraceuticals, polymers, and pharmaceuticals ones. The food supplements sector has also the participation of supercritical technology, which stable, sterile, and solvent-free products are marketed. The wide spectrum of active ingredients offers new possibilities in creative product development, such as food flavors, nanoemulsions, nanocarriers, and edible coatings. The formulations are especially concentrated on quality and health functionalities. Then, the SFE has all the features to be applied to the development of innovative products.

$CO_2$ is the most used solvent in SFE of natural products. $CO_2$ is nontoxic, cheap, renewable, and nonflammable substance. Under ambient temperature and pressure (approximately 25°C and 0.1 MPa), it is released from the extracts, after extraction, leaving no solvent traces in the extracts. Critical point of $CO_2$ is relatively easy to achieve ($T$ = 31°C and 7.4 MPa)

compared to other solvents, which may be used for this process and at this temperature thermolabile compounds might be stable. In addition, under supercritical conditions, $CO_2$ presents nonpolar characteristics, which allows extracting oils and natural flavorings at mild temperatures, thus preserving their thermosensitive compounds. $CO_2$ can also be recirculated in the process, preserving the environment, and diminishing the variable cost. When considering the whole SFE process, other features are seen. SFE increases the generation of viable waste streams and reduces the generation of hazardous waste streams (several times it is null). This occurs because the only waste accumulated at the end of the process is the dry exhausted solid matrix, which can be reused for many purposes, including animal feed, special and modified starches and phenolic compounds productions, and/or lignocellulosic biomass for energy generation or incorporated into the soil.

To overcome the drawbacks of conventional extraction methods, some patents have been developed. One example is the SFE of alkaloids with $CO_2$ and cosolvent after crushing caper alkaloid raw materials (Liu and Wan, 2011). Another example is the extraction of annatto (*Bixa orellana*) seed oil and defatted annatto seeds by SFE with $CO_2$ at mild condition of temperature (Albuquerque and Meireles, 2012). Likewise, a patent has been assigned for providing an SFE process for extracting oil from kenaf (*Hibiscus cannabinus* L.) seeds, wherein the SFE process uses a supercritical fluid and it is conducted from 20 MPa to 60 MPa with temperature ranging from 40 to 80°C (Ismail et al., 2014). The application of SFE to recover natural products of high added value is commonly preferred, as the high commercialization prices of such extracts allow diluting the high investment cost associated with SFE implantation, as well as the patents, several systematic studies have been performed to optimize SFE conditions. The main operating parameters influencing the extraction yields from solid matrices have been assessed as temperature and pressure (Melo et al., 2014a; Zabot et al., 2012), solvent flow rate and time (Moraes et al., 2015; Zabot et al., 2014c), average size of particles (Santos et al., 2015), use of cosolvents (Takeuchi et al., 2010), and bed height to internal diameter ratios (Zabot et al., 2014a). However, in some situations, the behavior of the extractions overtime is not clear when some of the operating parameters are changed. Therefore, a realistic overview of processes is provided in the following subsections, attempting to explain the pros and cons of taking some operational decisions along the runs.

### 2.1 Temperature Distribution Through Pressurized Beds

The success of establishing the supercritical technology in industrial scale depends on having enough and useful information about the parameters that affect transport phenomena in the processes. One of the parameters is the temperature, which is a fundamental operating parameter that directly influences the yields and composition of extracts. Overall, it is known that increasing temperature favors mass transfer and solubility of compounds. However, little is known about the real temperature distribution through pressurized beds during the extractions.

Most of the studies dealing with extraction of target compounds in cylindrical vessels commonly consider homogeneous beds with constant temperature (uniform distribution) during the whole process, even though variations might exist. However, this approach is not as real as the theory suggests. Controlling temperature during SFE process is a hard and systematic task, because temperature depends on the preheating of the solvent (or mixture of solvents), the volume/size of the bed, the geometry of the extraction vessel (bed height to internal diameter ratio), and the mode/efficiency that the bed is heated. Then, gradients of temperature in pressurized beds are commonly present in the process, which are more remarkable as the scale increases. According to some reports, the gradients of temperature can affect the fluid dynamics of the mixture solvent + solute and its phase equilibrium (Brunner, 1994; Pronyk and Mazza, 2009). Consequently, global yields and extraction kinetics can be influenced as well.

Considering that temperature heterogeneity occurs, learning about temperature distribution through extraction beds during SFE process can contribute to validate further studies performed in different bed geometries and scales. Up to now, the systematic determination of temperature distribution in this segment has been rarely reported. The lack of studies is an important gap that needs to be further studied, especially on pressurized systems. The need of studies mapping temperature distribution in the beds is an opportunity to create strategies that avoid local overheating or local portions of low temperature. The importance of this study is highlighted because thermosensitive compounds may be degraded at moderate to elevated temperatures; additionally, hydrolysis and isomerization reactions can also occur, and starch-rich matrices can undergo gelatinization reactions.

As aforementioned, temperature is typically considered as constant and homogenous through pressurized beds, especially because the fluid flow can distribute efficiently and uniformly the heat. For example, a temperature equal to 60°C is set, and then all the boundary layers are set to be 60°C for maintaining the target temperature: feeding of solvent equal to 60°C and external surface of the extraction vessel equal to 60°C. However, as preliminary findings obtained by our research group (data not published), temperature can change according to the extraction process. Then, temperature in the boundary layers should be different. A typical behavior of temperature distribution for preheating, pressurizing, static time, and dynamic periods during SFE is shown (Fig. 12.1). As target temperature equal to 60°C, temperatures at the center of the bed (radial axis) and at $r/R$ equal to 0.5 (at the middle of radius) have been assessed.

Some typical inferences can be done about the behavior of temperature in beds (solid matrices) submitted to SFE:

1. Preheating by conduction (region in black, Fig. 12.1) can take a long time with no substantial increase of temperature at the center of the bed. As the scheme shows, variations of temperatures upon radial direction might exist and could be significantly different. This occurs because the beds are typically filled with solid materials and

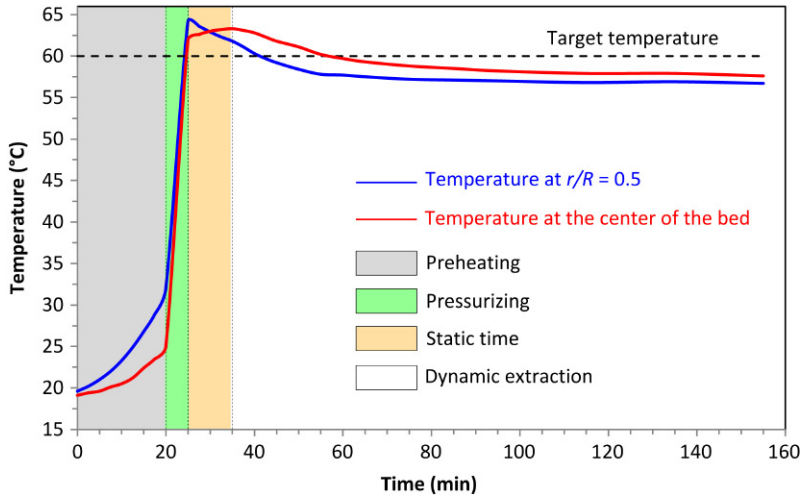



**Figure 12.1: Typical Behavior of Temperature During Supercritical Fluid Extraction (SFE) of Bioactive Compounds From Vegetal Raw materials (Solid) Inside an Extraction Vessel of 1 L Heated by an External Band Heater.**

*r* is the radial position of the bed; *R* is the radius of the bed; measurements were done at the *h*/*H* equal to 0.5 (middle), where *h* is the axial position of the bed and *H* is the height of the bed.

present void fractions (porosity) filled by air, thus diminishing the efficiency of heat transfer by conduction. Therefore, except specific conditions, the preheating period can be usually ignored.

2. Pressurizing step (region in dark gray, Fig. 12.1) provides a significant increase of temperature. The fast increase of temperature can be attributed to the effective transport properties of supercritical $CO_2$, especially the heat transfer coefficient. When preheated $CO_2$ fills the void fractions, it transfers its energy to the solid particles and the thermal energy from the internal wall of the vessel to the bulk of the bed. Curiously, after thoroughly evaluating all the findings, another thermal gain seemed to contribute with the increase of temperature in the process: the increase of molecular collisions during pressurization period. If the thermal gain during pressurizing step is not being properly considered, bed overheating might occur. Furthermore, controlling the thermal gain after pressurization is important, because the right control favors reducing or ignoring the preheating period. Consequently, it is possible to enhance the productivity of an industrial plant operating in continuous mode by diminishing the whole time of each batch. The control can be done by changing the inlet temperature of $CO_2$ (or other solvent) or by changing the temperature of the fixed bed (solid particles). In almost all the times, the first option is preferable. Therefore, the inlet temperature of solvent is a key parameter for reaching the target temperature after pressurization;
3. Static time (region in light gray, Fig. 12.1) is the period established to have equilibrium of pressure and temperature in the pressurized bed. Pressure usually reaches its

equilibrium in a few seconds. Then, for determining the suitable static time, the time should be left running up to the differences among all the temperatures and the target temperature remain smaller than 1°C (typically). As observed in the schematic drawing (Fig. 12.1), the static time should have been higher than 30 min. However, it influences the productivity: the larger the static time, the lower the productivity. Therefore, it is recommendable to associate the static time with pressurizing step, thus achieving the target temperature at shorter periods;

4. Dynamic extraction (region in white, Fig. 12.1) is the period in which the total extract starts to be exhausted from the bed, that is, the extract starts to be collected. The extent of this period depends on the characteristics of raw materials. Typically, this period is long when triacylglycerols and fatty acids are extracted, while it is short when volatile oils are extracted. Regarding to the temperature behavior, the contact of the heated vessel with the environment can promote a dissipation of heat through the boundaries, even though they are insulated. Consequently, the temperatures in different positions of the bed can be reduced. Therefore, one alternative for maintaining the temperatures closer to the target one is increasing the inlet temperature of solvent during the dynamic extraction period.

Measurement of temperature in SFE processes should be rather taken in several positions through pressurized beds. Overall, when controlling the temperature only by accounting it at positions as the band heater controller, the jacket of the extraction vessel, the solvent inlet or the solvent + extract outlet, a strong difference between the real value (average temperature of the bed) and the measured value might occur. To facilitate interpreting the temperature variations along the runs and then taking some operational decisions, an illustration was created (Fig. 12.2).

### 2.2 Bed Height to Internal Diameter Ratio and Criteria for Scale up SFE Process

In the supercritical technology field, researches are performed to increase the total extraction yield by changing process conditions (i.e., pressure, temperature). However, there is a need for further discriminations about the influence of other variables on the yields, as the bed height to internal diameter ratio ($H_B/D_B$). Such parameter plays an important role on SFE kinetics. The success of scale up is linked to the reproduction of kinetic extraction curves using different $H_B/D_B$ ratios. As reported for the decaffeination of coffee beans with a particle size of approximately 7 mm, large $H_B/D_B$ ratios should be used ($H_B/D_B$ of approximately 9). However, for smaller particles in the range of 0.4–0.8 mm that tend to swell, the $H_B/D_B$ ratio should be only 3 (Laurent et al., 2001). In addition, this parameter is important for plant design and mathematical modeling.

Different $H_B/D_B$ ratios influence the fluid dynamics and the temperature distribution in the beds. In long vessels (high $H_B/D_B$ ratios), axial dispersion might be significant, whereas in short vessels (short $H_B/D_B$ ratios), radial effects can cause heterogeneous distributions of temperature and mass (Brunner, 1994; Valle et al., 2004; Zabot et al., 2015). Other restriction

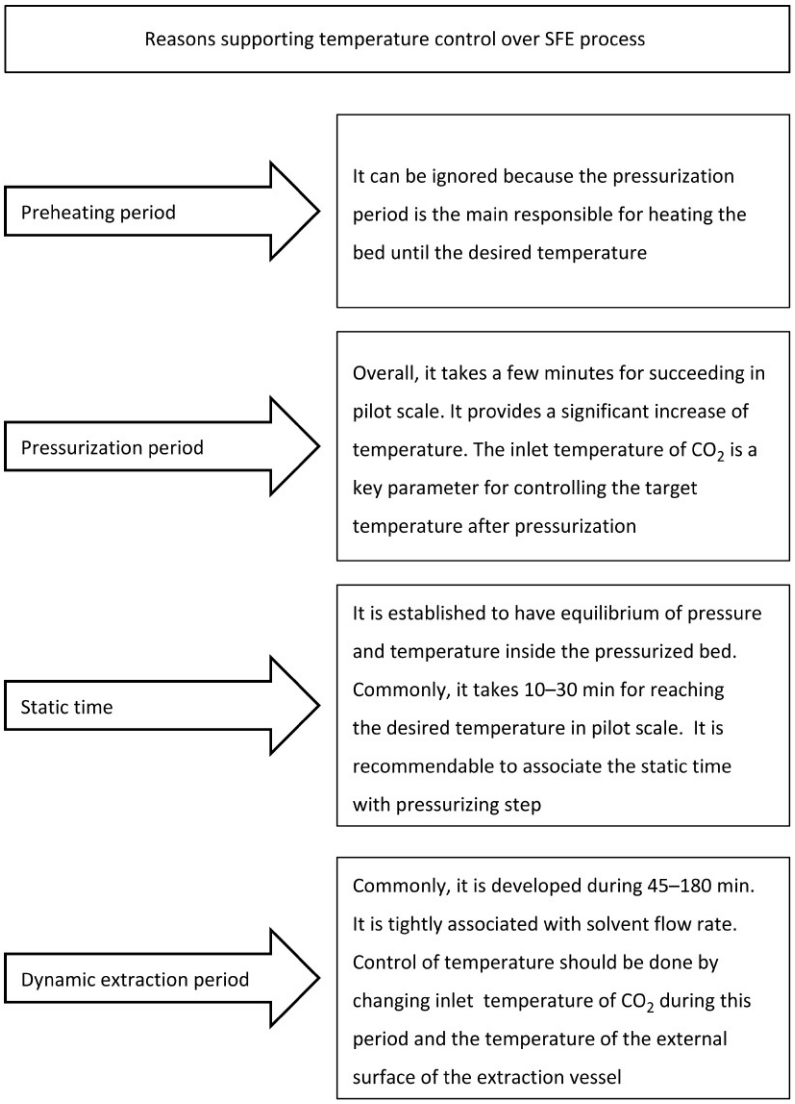



**Figure 12.2: Characteristics of Periods During SFE with Respect to Temperature Control.**

is the bed compaction, which typically increases as the $H_B/D_B$ ratio increase. Therefore, designing a vessel with suitable $H_B/D_B$ ratio is very important, as the increase in the bed diameter is accompanied by the increase in the thickness of the wall of the vessel and thus increase in the cost of investment. Then, vessels with beds of $H_B/D_B$ ratios commonly ranging from 5 to 7 seems to be more suitable for SFE applications (Meireles, 2003).

Zabot et al. (2014c) evaluated the influence of $H_B/D_B$ ratio on the kinetics of clove oil extraction. The authors studied $H_B/D_B$ ratios equal to 2.7 and 7.1 for vessels of equal volume (1 L), whereas all the other main parameters were maintained constant: pressure,

average temperature, bed porosity, diameter of solid particles, and bed density. The only parameter that changed with the $H_B/D_B$ ratio was the solvent interstitial velocity, while the solvent mass to feed mass ratio for a fixed time was maintained constant as well. According to the findings, no differences on extractions yields and extract compositions were observed. Otherwise, Zabot et al. (2014a) evaluated the influence of $H_B/D_B$ ratio on the kinetics of rosemary compounds extraction by developing the same systematic study as that aforementioned one. According to the findings about overall extraction curves and kinetic parameters, the bed with lower $H_B/D_B$ ratio ($H_B/D_B$ equal to 2.7) was more favorable for obtaining rosemary extract. The kinetics of extraction of oxygenated monoterpenes (i.e., 1,8-cineole and camphor) and phenolic diterpenes (i.e., carnosic acid) were also different for both bed ratios. These behaviors suggest that the bed geometry presents a remarkable influence on the mass transport properties in supercritical fluid, depending on the material.

To maintain the same behavior of the extraction curves in different beds with the objective to propose the scale up, there are relationships among process parameters that must be followed. These relationships are referred as criteria for scale up SFE process (Table 12.1). Thus, understanding which parameters should be held constant and which information should be input for process development is a challenge for attaining similar responses in different $H_B/D_B$ ratios and scales. It is important to point out that the same criterion can be suitable for a specific vegetal source and unsuitable for another vegetal source. The likely reason for the differences is associated with the composition of each raw material and also with the composition of the bulk extract, which rather changes as different botanical parts are used.

As seen (Table 12.1), two criteria have been reported to obtain clove oil by SFE. The first one [Eq. (12.1)] maintains the solvent mass to feed mass ($S/F$) ratio constant for a fixed extraction time. For instance, if 100 g of clove is loaded in the bed 1 and 500 g of supercritical $CO_2$ flows through this bed during 60 min, a total of 1000 g of supercritical $CO_2$ should flow through the bed 2 during 60 min if 200 g of clove is loaded in this second bed. For obtaining clove oil rich in terpenes, this criterion [Eq. (12.1)] has been suitable, because similar kinetic extraction yields have been reported for different $H_B/D_B$ ratios (Fig. 12.3). Otherwise, the second one (Eq. 12.2) maintains the solvent superficial velocity constant through a fixed bed. However, this criterion has been not considered valid for scale up because it does not match the solvent flow rate with the feed mass loaded into the extraction vessel. In this case, for beds with an elevated $H_B/D_B$ ratio, $CO_2$ might be saturated with extractable substances before leaving the extraction vessel. As it is known, the saturation condition should be avoided in SFE process. For obtaining clove oil rich in terpenes, this second criterion (Eq. 12.2) has been unsuitable, because an expressive difference on kinetic extraction yields have been reported for different $H_B/D_B$ ratios

**Table 12.1: Remarks about the criteria used for geometry shift and scale up in SFE of bioactive compounds.**

| Vegetal Sources | Criteria | Remarks | References |
|---|---|---|---|
| Clove buds (*Eugenia caryophyllus*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Suitable*: Maintaining an equal *S*/*F* ratio and an equal extraction time when using different $H_B/D_B$ ratios provided similar kinetic extraction yields | Zabot et al. (2014c) |
| Rosemary leaves (*Rosmarinus officinalis*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Unsuitable*: Different behaviors of the extraction curves and different behaviors of extract composition for each extraction bed have been reported | Zabot et al. (2014a) |
| Ginger rhizomes (*Zingiber officinale*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Suitable*: The extraction curves were similar when a 17-fold scale up was performed | Prado (2010) |
| Sugarcane bagasse (*Saccharum* spp.) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Unsuitable*: The yields of octacosanol-rich extracts were larger in $H_B/D_B$ equal to 5.9 than in $H_B/D_B$ equal to 2.3 | Prado (2010) |
| Grape seeds (*Vitis vinifera*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Suitable*: The criterion was successful in reproducing kinetics parameters in different scales and $H_B/D_B$ ratios | Prado et al. (2012) |
| Peach seeds (*Prunus persica*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Unsuitable*: Different extraction yields have been reported during the kinetic curves | Sánchez-Vicente et al. (2009) |
| Black sage leaves (*Cordia verbenacea*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Suitable*: Similar extractions curves have been reported for some different $H_B/D_B$ ratios | Quispe-Condori et al. (2008) |
| Annatto seeds (*Bixa orellana*) | $\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall t_1 = t_2\right)$ | *Suitable*: Similar extractions yields and kinetic parameters have been reported for two different $H_B/D_B$ ratios | Moraes et al. (2015) |
| Macela inflorescences (*Achyrocline satureioides*) | $\frac{Q_2}{Q_1} = \left(\frac{F_2}{F_1}\right)^2 \cdot \frac{H_{B1}}{H_{B2}} \cdot \frac{D_{B1}}{D_{B2}}$ | *Unsuitable*: Differences in the kinetic parameters between the $H_B/D_B$ ratios were presented | Takeuchi (2009) |
| Red pepper fruit (*Capsicum frutescens*) | $\frac{Q_2}{F_2} = \frac{Q_1}{F_1}$ | *Unsuitable*: Differences for the convective mass transfer coefficient ($k_{YA}$) values between laboratorial and pilot scales were presented | Silva (2013) |
| Fennel seeds (*Foeniculum vulgare*) | $\frac{Q_2}{Q_1} = \left(\frac{F_2}{F_1}\right)^2 \cdot \frac{H_{B1}}{H_{B2}} \cdot \frac{D_{B1}}{D_{B2}}$ | *Suitable*: Similar extraction yields were provided after varying the $H_B/D_B$ ratios | Moura et al. (2005) |
| Clove buds (*Eugenia caryophyllus*) | $\frac{Q_2}{A_2} = \frac{Q_1}{A_1}$ | *Unsuitable*: Maintaining the solvent velocity constant influenced the mass flow rate | Martinez et al. (2007) |

A, Crosssectional area of the bed; $D_B$, bed diameter; *F*, feed mass; $H_B$, bed height; *Q*, solvent mass flow rate; *S*, solvent mass; *t*, time.

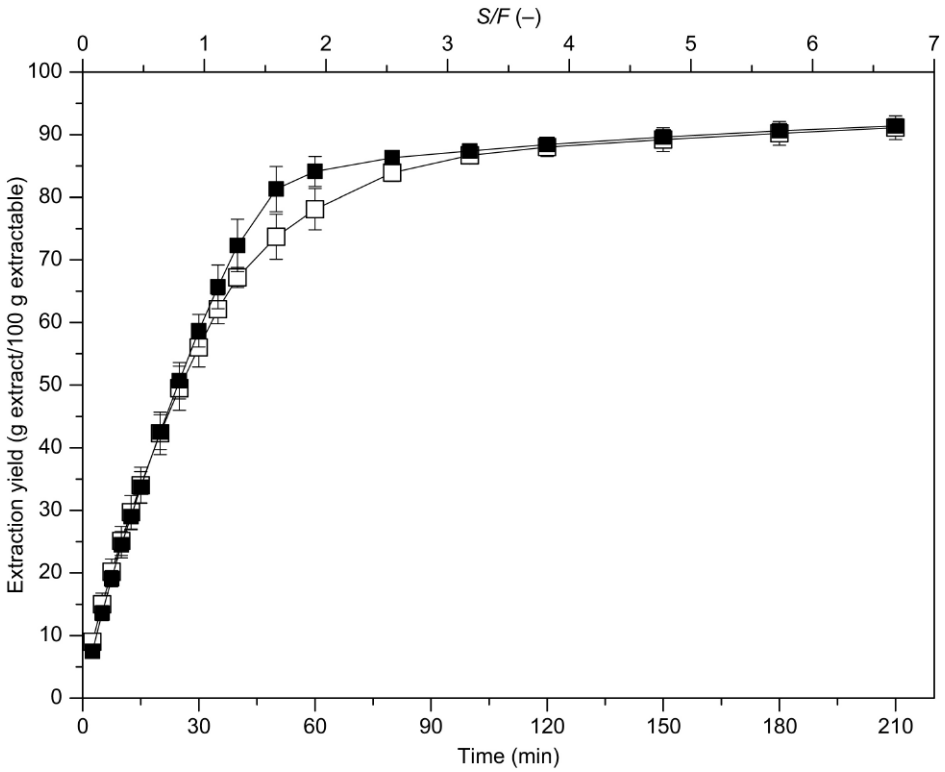



**Figure 12.3: Extraction Curves of Clove Oil Obtained by Supercritical Technology in Beds of $H_B/D_B$ Ratio Equal to 7.1 (□) and $H_B/D_B$ Ratio to 2.7 (■) Using the Criterion Described in Eq. (12.2).**
*Adapted from: Zabot, G.L., Moraes, M.N., Petenate, A.J., Meireles, M.A.A., 2014c. Influence of the bed geometry on the kinetics of the extraction of clove bud oil with supercritical $CO_2$. J. Supercrit. Fluids 93, 56–66, with permission from Elsevier.*

(Fig. 12.4). For the same corresponding value of solvent velocity, the solvent mass used in the bed of $H_B/D_B$ ratio equal to 7.1 was lower than that used in the bed of $H_B/D_B$ ratio equal to 2.7 at a given process time and equal feed mass for both beds.

$$\frac{S_2}{F_2} = \frac{S_1}{F_1}\left(\forall\, t_1 = t_2\right) \tag{12.1}$$

where, $S$ is the solvent mass, $F$ is the feed mass, and $t$ is the time.

$$\frac{Q_2}{A_2} = \frac{Q_1}{A_1} \tag{12.2}$$

where, $Q$ is the solvent mass flow rate and $A$ is the bed crosssectional area.

Another form of evaluating the extraction yields of extracts from a specific raw material using beds of different $H_B/D_B$ ratios rather than that qualitative evaluation (Figs. 12.3 and 12.4) is

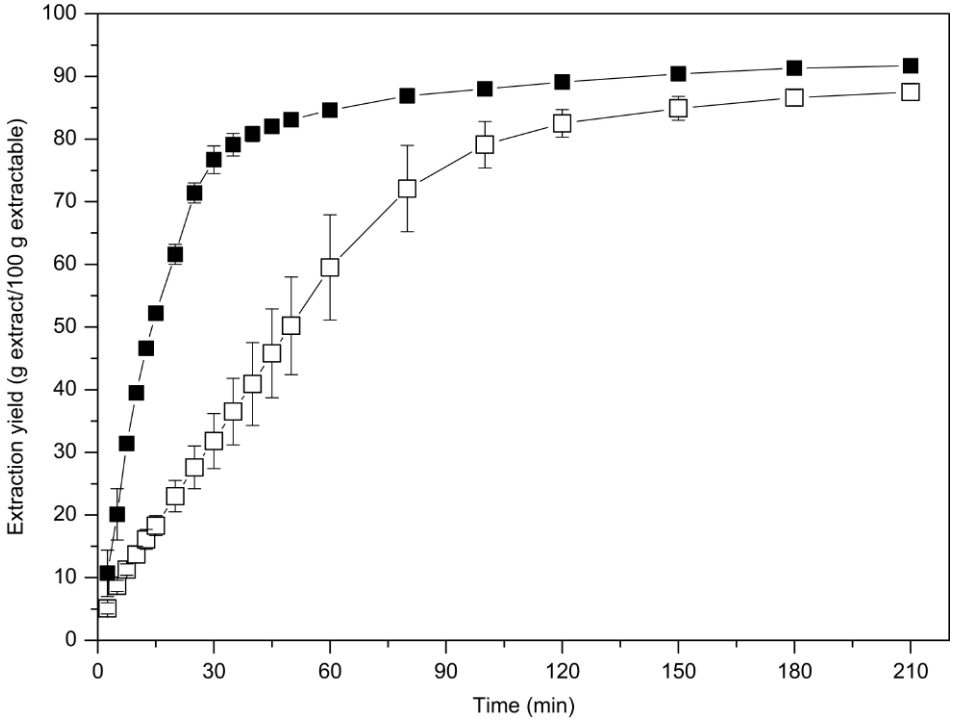



**Figure 12.4: Extraction Curves of Clove Oil Obtained by Supercritical Technology in Beds of $H_B/D_B$ Ratio Equal to 7.1 (□) and $H_B/D_B$ Ratio to 2.7 (■) Using the Criterion Described in Eq. (12.2).**
*Adapted from Zabot, G.L., Moraes, M.N., Petenate, A.J., Meireles, M.A.A., 2014. Influence of the bed geometry on the kinetics of the extraction of clove bud oil with supercritical $CO_2$. J. Supercrit. Fluids 93, 56–66, with permission from Elsevier.*

providing a quantitative description. Such description provides information about the kinetic parameters fitted to the curves. One example done with clove oil (Zabot et al., 2014c) is presented (Table 12.2).

It is evident that the kinetic parameters ($t_{CER}$, $R_{CER}$, $M_{CER}$, and $Y_{CER}$) are similar between the beds with different $H_B/D_B$ ratios when using the first criterion (Eq. 12.1). However, differences on the values of the kinetic parameters are presented between the beds with different $H_B/D_B$ ratios when using the second criterion [Eq. (12.2)]. Therefore, further systematic studies are needed for providing the right criterion for bed geometry shift and scale up with other raw materials containing therapeutic compounds, especially when different botanical parts are used, such as seeds, flowers, leaves, stems, fruits, roots, rhizomes, and so on.

### *2.3 Choosing the Ideal $CO_2$ Flow Rate*

As previously cited, $CO_2$ is the mostly used solvent in SFE of bioactive compounds. Then, this subsection is going to discuss about its flow rate on fixed beds, especially when relative

**Table 12.2: Kinetic parameters fitted to extraction curves of clove oil obtained by SFE in two different extraction beds of 1 L (Zabot et al., 2014c).**

| Criteria | $H_B/D_B$ | $t_{CER}$ (min) | $R_{CER}$ (g extract/ 100 g extractable) | $M_{CER} \times 10^4$ (kg/min) | $Y_{CER}$ (g extract/g $CO_2$) |
|---|---|---|---|---|---|
| $\frac{S_2}{F_2} = \frac{S_1}{F_1}(\forall t_1 = t_2)$ | 2.7 | 45 ± 2 | 83 ± 1 | 22.5 ± 1.3 | 0.13 ± 0.01 |
| | 7.1 | 45 ± 1 | 78 ± 2 | 21.0 ± 0.8 | 0.12 ± 0.01 |
| $\frac{Q_2}{A_2} = \frac{Q_1}{A_1}$ | 2.7 | 27 ± 1 | 76 ± 2 | 32.6 ± 0.1 | 0.17 ± 0.01 |
| | 7.1 | 82 ± 13 | 75 ± 1 | 10.6 ± 1.8 | 0.09 ± 0.01 |

$t_{CER}$, Period of constant extraction rate; $R_{CER}$, extraction yield at the constant extraction rate period; $M_{CER}$, mass transfer rate at the constant extraction rate period; $Y_{CER}$, mass ratio of solute in the fluid phase at the extractor vessel outlet at the constant extraction rate period.
In Figs. 12.3 and 12.4 and in Table 12.2, the term "extractable" means the amount of total extract obtained at the same condition of pressure and temperature, but with *S/F* ratio high enough (excessive amount of solvent), guaranteeing the depletion of extract from the raw material in these conditions.

high or low mass flow rates are selected. The behaviors can be extended to other solvents as well, with light variations on the magnitude for each solvent.

In general, the yields of the extracts rich in phytochemicals (especially the kinetic extraction yields) depend on the solvent flow rate. The $CO_2$ flow rate is associated with the velocity of the supercritical $CO_2$ flowing through the loaded material inside the extraction vessel. Two situations can be considered:

### 2.3.1 High $CO_2$ flow rate

When the solvent flow rate is sufficiently high, and consequently the superficial velocity is sufficiently high, the contact time between $CO_2$ and solid interface is reduced, and the extractions cannot show high efficiency. The restraint can be therefore attributed to solubility and/or to intraparticle diffusion. In such case, $CO_2$ is misused, and that unnecessary spent in excess increase utilities costs, mainly the energy cost related to pumping. Therefore, another trouble is associated with an operating aspect: pumping. High $CO_2$ flow rate requires pumps with specific characteristics, especially to support pumping large amount of $CO_2$ at high pressures. In addition, the pumps should be equipped with a cooler system to maintain the $CO_2$ at liquid phase, which is commonly reached under some negative degrees Celsius. If the SFE process is performed at industrial scale (extraction vessels with a few hundred liters or a few cubic meters), then several high capacity pumps should operate in parallel mode to provide the elevated flows. Consequently, the total cost of the operation is increased.

### 2.3.2 Low $CO_2$ flow rate

When the solvent flow rate is sufficiently low and consequently the superficial velocity is sufficiently low, the penetration of the $CO_2$ into the vegetal matrix is deeper and prolonged,

generally being a time-restrictor for the SFE process. Besides, the $CO_2$ can be saturated on solutes before leaving the extraction vessel. Thus, after the saturation, the $CO_2$ does not extract any solute. The film resistance and/or the accumulation in the bulk can predominate over intraparticle diffusion and solubility issues. Consequently, the mass transfer rates and kinetic extraction yields are affected. Experimentally, this was the behavior observed on the extraction of clove oil when using a low solvent flow rate (4.2 g/min) for a fixed bed of 1 L (570 g of clove) of $H_B/D_B$ ratio equal to 7.1 (Fig. 12.4, white squares). When the $CO_2$ flow rate is too low, longer process times are needed and the productivities are then reduced.

After extracting phytosterol-rich oil from 25 g of dried Kalahari melon seeds loaded in a 500 mL extraction vessel, Nyam et al. (2011) observed the oil recovery increased from 67 to 76 wt.% when the pressurized $CO_2$ flow rate was increased from 10 to 15 mL/min, respectively. This occurred because increasing the flow rate reduced the external mass transfer resistance, leading to a higher oil extraction. However, further increase in supercritical $CO_2$ flow rate (20 mL/min) decreased the oil recovery (69 wt.%). The reason of this behavior is the presence of the diffusion-controlled period, whereas increasing the flow rate could not increase the rate of diffusion. Contrarily, increasing the flow rate from 15 to 20 mL/min negatively influenced the yields as the axial dispersion could have hampered the uniform distribution of the solute on the supercritical $CO_2$. Similar behavior was reported by Rodrigues et al. (2002) for the extraction of volatile oil from 80 g of ginger rhizomes (1.2 g $CO_2$/min was the optimum) and 120 g of eucalyptus leaves (0.9 g $CO_2$/min was the optimum). According to Casas et al. (2008), it is not advisable to work with a $CO_2$ flow rate above 25 g/min in the extraction of bioactive compounds from 190 g of sunflower leaves loaded in a 2 L extraction vessel because the increased solvent consumption is not compensated by increases in the yields. Then, these mentioned data are elucidative examples of a rational selection of ideal flow rate values.

Therefore, it is important to balance the technical/operational features with the scientific features to choose the ideal $CO_2$ flow rate. When fulfilling the SFE process, the operator should consider the capacities installed of the plant to select operating flow rates under 60%–90% of the maximum solvent flow rate supported. This selection avoids the $CO_2$ saturation with solute. The more unsaturated is the $CO_2$, the more is its ability to extract the functional compounds. However, the extraction process is limited by the solubility of the solute in supercritical $CO_2$ or by the occurrence of immediate withdrawal of volatile oils as the process started running. Then, any additional mass flow rate increase can be unnecessary. If the plant is being designed and has been not installed yet, the engineers should consider on the project the suitable solvent flow rates that reduce mass transfer resistance and allow the maximum extraction yield during a smaller time. For specific vegetal sources, the engineers can consult some scientific studies whereas the extraction yields have been optimized by varying the $CO_2$ flow rate. As examples, the extractions of phytosterol-enriched oil from Kalahari melon seeds (Nyam et al., 2011), xanthones from mangosteen pericarp (Zarena et al., 2012), essential oil

from spearmint leaves (Ansari and Goodarznia, 2012) and essential oil (camphor, 1-8 cineole, camphene, borneol, myrcene, and α-pinene) from sage leaves and flowers (Langa et al., 2009) have been optimized. For a wide range of other vegetal sources, Melo et al. (2014b) provided a compilation of publications comprising SFE of nutraceutical compounds from 2000 to 2013 and their respective features, which include the solvent flow rate. Other influences associated with solvent flow rate are the axial dispersion, unsteady or nonstable dynamic flow, nonuniform distribution of the solvent viscosity, and nonideal flow patterns (Brunner, 1994). Whenever possible, $CO_2$ flow rate should be measured on mass basis. If volumetric flow rate is provided, temperature and pressure on which the flow is measured should accompany the flow value.

### 2.4 Other Main Operating Parameters for SFE Process

As known, SFE is traditionally performed at high pressure, commonly between 8 and 50 MPa (Zabot and Meireles, 2016). The pressure influences straightforwardly the density and the diffusivity of the solvent, which in most cases $CO_2$ is the solvent. Density of $CO_2$ can be tuned by changing the system pressure or temperature (or both), mainly in the region close to the critical point. In this region, the distinction between gas and liquid phases disappears, and the $CO_2$ is continuously interchanging between gas-like/liquid-like properties. Within the region around the critical point the properties are highly sensitive to changes in temperature and pressure. Thus, the selective extraction of different nutraceutical compounds can be achieved by changing the system pressure as well. The increase in pressure raises the solvation power of the solvent due to the increase in the density. Nonpolar and low-polarity compounds can be extracted at moderate pressures (<15 MPa), as the case of volatile oils (Zabot et al., 2012).

Total extract and ar-turmerone were obtained by SFE from turmeric rhizomes (Carvalho et al., 2015). As reported for the pressure range studied (10, 15, 20, 25, 30, and 35 MPa), the isotherms (40, 50, and 60°C) presented cross one another in the region near to 20 MPa. Then, 20 MPa was found to be the suitable pressure for obtaining approximately 8 g extract/100 g of turmeric rhizomes. Likewise, 15 MPa was found to be the suitable pressure for obtaining approximately 15 g oil/100 g of clove buds (Maschke et al., 2007). When extracting caffeine from Robusta coffee husks, 30 MPa was the best pressure, the highest one among those studied (10, 15, 20, 25, and 30 MPa). For 100°C, the extraction yield of such alkaloid was approximately 13-fold higher when using 30 MPa than 10 MPa (Tello et al., 2011). However, the influence of pressure upon solubility of compounds is tightly associated with temperature. Then, the pair pressure–temperature should be evaluated at the same time to make the proper selection of this binomial condition.

Supercritical $CO_2$ is a suitable solvent for nonpolar prevailing compounds, such as terpenes, tocopherols and tocotrienols, sterols, carotenoids, fatty acids, and among others. On the other

hand, the efficacy of supercritical $CO_2$ diminishes for polar prevailing compounds. In such case, cosolvents (commonly ethanol, isopropanol and water) are used to favor extracting these target compounds. For example, the highest yields of sunflower extract were obtained with mixing 5 wt.% water to the $CO_2$. However, the percentage of water present in the solvent system has an influence on the bioactivity of the extract. An increase in the percentage of cosolvent proved detrimental to the extraction process in terms of the bioactivity of the extracts (Casas et al., 2008).

Anthocyanins and other phenolics were extracted from blackberry bagasse after mixing 5 wt.% ethanol to the $CO_2$ or 5 wt.% water to the $CO_2$. The use of water as cosolvent resulted in a significant increase of anthocyanins content (cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, cyanidin 3-O-(6″-malonyl-glucoside), and cyanidin 3-O-(6″-dioxalyl-glucoside)). The polar nature of water and ethanol and the acidification of the solvent mixture favored the solubilization of the anthocyanins. The addition of water and ethanol as cosolvents in SFE increased the global yield, as well as the contents of phenolics, anthocyanins, and the antioxidant activity of the extracts (Reátegui et al., 2014). Ethanol was also suitable for extracting quercetin-rich extract (Zabot and Meireles, 2016). Therefore, in order to maintain SFE as a green technology, the cosolvent must be carefully chosen when needed, being ethanol and water the most recommendable. $CO_2$ + ethanol + water may in some cases be a suitable choice as well.

An important aspect of SFE studies with $CO_2$ plus cosolvent is that the solvents are loaded in the beds in the following two different forms (Melo et al., 2014b):

1. The more conventional procedure comprises mixing the $CO_2$ with the cosolvent in a fixed proportion along time, which requires an additional pump and independent feed line for each fluid;
2. The alternative procedure is through the impregnation of the vegetal matrix with the cosolvent at the onset of the experimental run, followed by the extraction with pure $CO_2$.

Even though there are no works targeting a comparative study of the two procedures, it is hypothesized that the conventional procedure allows better controlling the percentage of cosolvent in the system. Regarding to the drying stage, the cosolvent should be removed from the collected extract by an additional unit operation. Although water is inexpensive, the unit operation of forced-air drying or freeze-drying leads to an additional cost. Likewise, ethanol should be removed from the extract by evaporation or distillation. Then, the dryer stage can be optimized to evaluate if the use of the modifier is profitable or not, that is, if the increase in the extracts yields and quality (composition and bioactivity) are a rentable advantage against the energy and utilities additional expenditures. But, it is important to point out that if the whole aqueous extract can be used for further purposes (aqueous food supplements, aqueous additives, etc.), the potential inclusion of water on SFE of phytochemicals from vegetal sources rich in polyphenols is advisable.

Therefore, the supercritical technology is a promising technology competing with other extraction techniques that shows advantages to be applied on food, chemical, and pharmaceutical industries. Other related applications of this breakthrough technology are covered on the next sections.

## 3 Products Obtained by SFE and Their Functional Properties

The extractions with SFE are already consolidated and proved that are able to provide natural products of high quality and economically feasible to market. However, there is a lack of studies involving applications of the products obtained via SFE in food products. To start the product development process is necessary to know the functional properties and physicochemical characteristics of the compounds involved. Therefore, the following topics of this section meant to show the main vegetable matrices studied by LASEFI (Laboratory of Supercritical Technology: Extraction, Fractionation, and Identification of Vegetal Extracts) research group, as this is a group of globally recognized research on supercritical technology area (Zabot et al., 2014b), and the possible applications of the bioactive extracts in some foods in order to promote innovations in the processing of food products.

### 3.1 *Clove Bud (*Eugenia caryophyllus*)*

*Eugenia caryophyllus* is one spices known worldwide for its medicinal and culinary qualities (Deladino et al., 2008). The extract of this plant obtained via SFE is rich in volatile oils that are used in medicine as an antiviral agent, antifungal, antitumor, analgesic, antiseptic, and also in the treatment of asthma, allergic diseases, and periodontal treatments. In the food industry, it is used as flavoring and antimicrobial; this extract can also be used in the agricultural industry as an insecticide (Acosta, 2009; Chen et al., 2006; Noubigh et al., 2013; Waterhouse, 2001).

Main compounds present in clove oil are eugenol, β-caryophyllene, α-humulene, and eugenol acetate. Among these eugenol is the major compound of clove essential oil (Deladino et al., 2008; Noubigh et al., 2013). Chemical structures of these compounds are shown in Fig. 12.5.

Eugenol is a compound of great industrial interest because of its antioxidant and antimicrobial properties (Deladino et al., 2008), and also due their antiseptic, antiinflammatory, and analgesic characteristics (Maschke et al., 2007). The physicochemical characteristics of eugenol are listed later (Brand-Williams et al., 1995):

- Molecular formula: $C_{10}H_{12}O_2$;
- Molecular weight (g/mol): 164.20;
- Solubility: water, alcohol, chloroform, ether;
- Melting point (°C): −9.2;
- Boiling point (°C): 225.

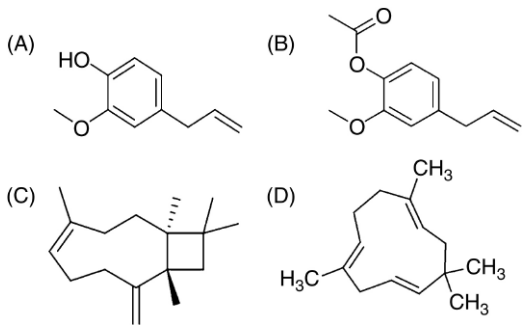



**Figure 12.5: Chemical Structures of the Main Compounds Present in Clove Bud.**
(A) Eugenol; (B) eugenol acetate; (C) β-caryophyllene; (D) α-humulene. *Obtained from: Deladino, L., Anbinder, P.S., Navarro, A.S., Martino, M.N., 2008. Encapsulation of natural antioxidants extracted from* Ilex paraguariensis. *Carbohydr. Polym. 71, 126–134, with permission from Elsevier.*

The use of clove essential oil and eugenol as a food additive are *generally recognized as safe* (GRAS) by the Food and Drug Administration (FDA) (Benzie and Strain, 1996). Therefore, in the past decades, many industries have used these natural additives to replace synthetics due to their antimicrobial and antioxidant potent activity able to increase the shelf life and product quality (Bos and van Vliet, 2001).

Microorganisms easily contaminate food products during processing, storage, and transport. Such contamination may lead to loss of quality, quantity, and composition of the nutrients, thus reducing the market value of these products. To reduce contamination, various essential oils from higher plants have been used not only as condiments and seasonings, but also as preservatives (Bos and van Vliet, 2001).

Some studies showed the possibility of using eugenol how a substitute of the chemical additives used for preserving meats and fishes (Wilde, 2000). Several studies reported antimicrobial action of eugenol. Mahmood and Al-Koofee (2013) showed that the eugenol has action against Gram-positive and Gram-negative microorganisms. Fasolin et al. (2014) demonstrated the antimicrobial action of eugenol on apples. Benichou et al. (2001) reported that eugenol can effectively eliminate free radicals in soybean oil during the storage period.

However, the positive effect of the addition of essential oils in foods is limited by the quantity of essential oil added. In general, to get an effective antimicrobial action, it is necessary to add elevated concentrations of essential oil in the foods, and this can lead to obtaining products with strong flavor and low sensory acceptance (Dickinson et al., 2003; Ushikubo and Cunha, 2014). In this context, essential oils appear as an interesting ingredient in biodegradable food packaging, especially due to its natural origin and their functional properties (antioxidant/antimicrobial), allowing obtaining active materials in order to extend the shelf life and add value to the product (Dickinson et al., 2003). Packaging based on edible films with antimicrobial coatings have provided an innovation in the concept of active

biodegradable packaging, because they prevent microbial growth through direct contact of the packaging material with the food surface (Kobayashi et al., 2005).

Therefore, products with great potential for application of clove essential oil are packaging for meats, chocolates, and dairy products due to the high lipid content contained in these products; in fruits, juices, jams, and sweets that have high water activity and are therefore, susceptible to develop microorganisms. In this sense, the use of eugenol as an antimicrobial agent adds quality to food products, by maintaining their sensory properties, increase their shelf life, and human health benefits.

### 3.2 *Jabuticaba (*Myrciaria cauliflora*)*

Jabuticaba (*Myrciaria cauliflora*) is a plant native from Brazil, its fruits are of pleasant taste, dark color, and represent a rich source of phenolic compounds, including flavonoids, anthocyanins, tannins, and phenolic acids (Di Mattia et al., 2010). These bioactive compounds present in jabuticaba fruits act beneficially on the human organism. Several authors point out that jabuticaba has antioxidant activity, antiinflammatory, antimicrobial, anticancer, antimutagenic; besides aid in the treatment or prevention of lung diseases, cardiovascular diseases, and diabetes (Losada-Barreiro et al., 2012; Mahdavee Khazaei et al., 2014; Sato and Cunha, 2011).

The jabuticaba peel is a waste from the processing of juices, liquors, wines, jellies and candy, and also by the direct consumption of fresh fruits (Betz et al., 2012). This waste is rich in anthocyanins (Asnaashari et al., 2014; Uekusa et al., 2008), natural compounds considered as potential replacements for synthetic dyes used in food products (Tonon et al., 2012). These compounds are present particularly in fruits with dark color and are responsible for the red, purple, and blue coloring (Sato and Cunha, 2011).

Although they are a rich source of nutrients and functional compounds, the wastes from jabuticaba are still discarded by industry and consumers. The extraction technology with supercritical fluids allows adding value to jabuticaba peel waste through the recovery of anthocyanins present in this part of the fruit, producing an extract with high antioxidant activity (Betz et al., 2012). These extracts, rich in natural pigments with functional activity, can be preserved by encapsulation in polymeric matrices (Asnaashari et al., 2014; Santos et al., 2013).

Anthocyanins identified in jabuticaba peel were: cyanidin-3-glycoside; delphinidin-3-glycoside; peonidin; peonidin-3-glycoside (Lin et al., 1995; Santos and Meireles, 2010; Sato and Cunha, 2011). Fig. 12.6 presents the chemical structures of these compounds.

Antioxidants and colorants are present in most food products; however, very few occurrences are found of these compounds obtained from natural sources. Therefore, the addition of

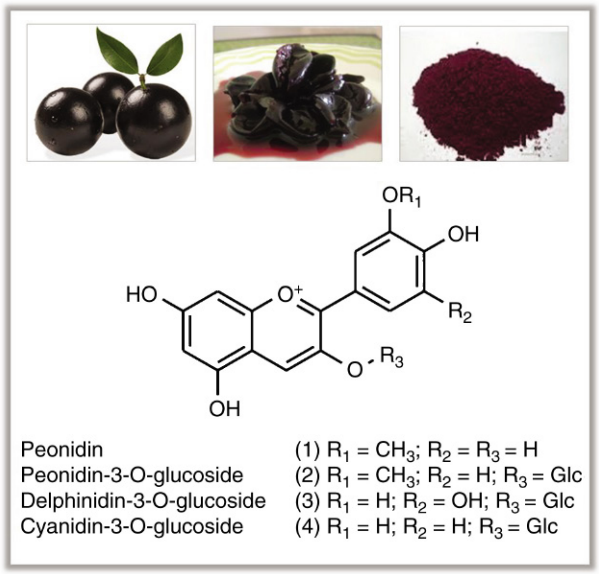



**Figure 12.6: Chemical Structure of Anthocyanins Present in Jabuticaba Peel.**
*Adapted from: Sato, A.C.K., Cunha, R.L., 2011. Influence of dispersing media and particle characteristics on rheological behavior of noncolloidal suspensions. J. Disper. Sci. Technol. 33, 437–446, with permission from Elsevier.*

pigments extracted from jabuticaba peel in food products, can meet both the industrial demand for natural resources as the needs of moderns consumers, because in addition to maintain the sensory characteristics, they increase the nutritional value of products and promote beneficial to human health.

Extracts obtained by SFE from jabuticaba processing wastes were incorporated by Di Mattia et al. (2010) in probiotic Petit Suisse cheese. The results obtained by these authors were satisfactory, because the product developed had good sensory acceptance.

We can think on the application of jabuticaba extracts in candies and chocolates, as they add sensoriality due to coloration, these products are consumed by a very diverse audience, from children to adults. The application of jabuticaba extracts in products aimed for people with diabetes is also interesting, because the compounds found in the extract also can help in the treatment of this disease. The application of jabuticaba extracts in products aimed for people with diabetes is interesting, because the compounds found in the extracts also can help in the treatment of this disease. Probiotic yogurts are already accepted and appreciated by a selected group of people concerned about the wellbeing, so the incorporation of these extracts in dairy products would increase the diversity of flavors and functional properties expected in this market niche. Doughs and cereals are other products with potential for the application of jabuticaba extracts, because there are available on the market for sale doughs with different colors obtained from natural dyes.

### 3.3 *Brazilian ginseng (*Pfaffia glomerata*)*

*Pfaffia glomerata* or Brazilian ginseng is popularly known for its many medicinal properties (Bitencourt et al., 2014). This plant has analgesic and antiinflammatory effects, acts as an antimicrobial agent and inhibits in vitro tumor cell growth (Fang and Bhandari, 2010; Debien et al., 2015). Recently, the scientific community, as well as the industries of food, beverages, and cosmetics showed great interest in the products extracted from *Pfaffia glomerata* due to the presence of compounds with functional activity (Quideau and Feldman, 1996).

Debien et al. (2015) studied the extraction with supercritical $CO_2$ from *Pfaffia glomerata* using ethanol as cosolvent. The researchers found that the extract was able to reduce the surface tension due to the presence of saponin, a biosurfactant known for excellent activity in reducing the surface tension of aqueous solutions. Saponins are active constituents of Brazilian ginseng. They have glycosides in their structure with an aglycone as a fundamental core linked to different types of sugar units (Rakić et al., 2006; Debien et al., 2015). The aglycones present in saponins are classified triterpene or steroid (Hsieh et al., 2007). The aglycone can have different functional groups (—OH, —COOH, —$CH_3$) leading to a variety of saponins. The number of saponins can be further increased due to different compositions of sugar chains, number, and type of branching replacement of sugars linked to aglycones (Uozaki et al., 2007). Fig. 12.7 illustrates the structure of aglycone present in the saponins.

The presence of hydrophobic and hydrophilic domains in the saponin molecules are responsible for the ability of this compound to reduce surface tension and control microbial spoilage in foods (Rakić et al., 2006). This property of saponins makes it a surfactant, which is characterized by being an amphiphilic compound capable of altering the surface and interfacial properties of the system and thus lead the formation of emulsions (de Mejia et al., 2006). Therefore, surfactants are part of an important class of chemical compounds with applications in various segments of the food industry because of its ability to increase

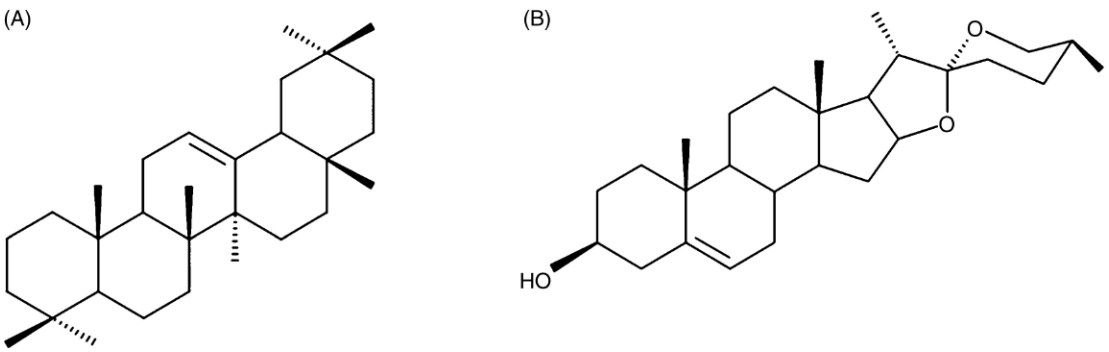



**Figure 12.7: Structure of Aglycone Present in Saponins.**
(A) Triterpenic; (B) steroidal.

the solubility or stability of immiscible mixtures besides acting in foaming (Manach et al., 2004).

The use of saponins extracted from the Brazilian ginseng is not yet exploited industrially, but some studies showed the effectiveness of using saponins for the formation and stabilization of emulsions. For example, the Quillaja saponin is a naturally occurring surfactant extracted from a tree native to semiarid zones of Chile, the surfactant is already available for commercial use and is widely used by the food industry (Yang et al., 2013).

Munin and Edwards-Lévy (2011) compared the performance of modified starch and Quillaja saponin on the formation of orange oil nanoemulsions incorporated with ester gum in the oil phase. At the end of this study, the authors concluded that the production of nanoemulsions with Quillaja saponin showed better performance compared to the nanoemulsions obtained with modified starch. They noted that Quillaja saponin transmitted high interfacial load and low interfacial tension, which promoted the formation and stabilization of the orange oil nanoemulsions. Yang et al. (2013) obtained excellent results regarding ability of saponin to stabilize emulsions. These authors also found that Quillaja saponin was highly surfactant and had similar interfacial properties to Tween 80, a synthetic nonionic surfactant widely used in the food industry.

Rakić et al. (2006) compared the use of commercial saponins with the saponins extracted from Brazilian ginseng in the formation of annatto seed oil emulsions. The authors developed a research work with the purpose of showing the performance of the saponin extracted from Brazilian ginseng on the emulsions formation of bioactive compounds by different emulsification processes, such as ultrasonication and mechanical stirring by means rotor-stator, in an attempt to establish the use of a biosurfactant for applications in pharmaceutical, cosmetic, and food products. The results showed that saponins obtained from Brazilian ginseng were able to produce emulsions with smaller droplet size of the dispersed phase, and therefore resulted in more kinetically stable emulsions to phase separation compared to other treatments evaluated.

Given the earlier mentioned, it is possible to see the benefits that the use of extracted saponins from natural sources using clean technologies can bring to different productive sectors, especially of food products, thus increasing the shelf life of products, sensory quality, and nutritional value of different types of processed foods. In the baking industry the use of saponins extracted from Brazilian ginseng, for example, could be used as an emulsifier where their presence would act on the improving the structure of the shell of breads, aeration of cake batter, and inhibiting the retrogradation of starch. In addition, they could be used as stabilizing emulsions of the margarine and mayonnaise industries. The interactions between the proteins these products with surfactants can ensure stability, quality, and longer shelf life of the emulsions formed. Saponins may also be employed as surfactants for maintaining foams in dairy beverages, ice creams, juices, soft drinks, and beers.

## 3.4 *Rosemary* (Rosmarinus officinalis)

Rosemary (*Rosmarinus officinalis* L.) is a perennial herb belonging to the family *Lamiaceae*, typical of the Mediterranean region, it has been widely used for years in medicine and food preparation, due to their different biological properties (Hernández et al., 2016). These biological activities are mainly due to the presence of volatile and phenolic compounds (Ribeiro-Santos et al., 2015).

*Rosmarinus officinalis* is known in medicine for antimicrobial, antimutagenic, antidiabetic, antidepressive, and chemopreventive activities (Okoh et al., 2010; Vicente et al., 2013). In the food industry, the rosemary essential oil has many applications due to its antimicrobial activity, antimycotic, and antioxidant (Hernández et al., 2016). When compared with others antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, rosemary volatile oil has a higher thermal resistance (Leal et al., 2003), which makes its attractive use in products that are subjected to high temperatures. Rosemary extracts have a volatile fraction (essential oil), consisting of 1.8-cineol, camphor, α-pinene, and camphene (Ahmed et al., 2016; Carvalho et al., 2005) and a nonvolatile fraction containing phenolic compounds, such as rosmarinic acid, carnosic acid, carnosol, and rosmanol (Ibanez et al., 2003; Silva et al., 2016b).

The rosemary extract may be directly added to foods or incorporated into packagings (Ribeiro-Santos et al., 2015). The rosemary extract content allowed in foods depends on the type of product and is expressed as the sum of main antioxidant compounds present in this extract, carnosol, and carnosic acid (Zabot et al., 2014a). Fig. 12.8 presents the rosemary herb and your essential oil obtained by the SFE.

Studies on extraction of bioactive compounds from rosemary showed that rosmarinic, carnosol, and carnosic acids are the compounds found in larger quantities in rosemary extracts (Ribeiro-Santos et al., 2015). Table 12.3 shows the physicochemical characteristics of the main chemical compounds found in rosemary extracts.

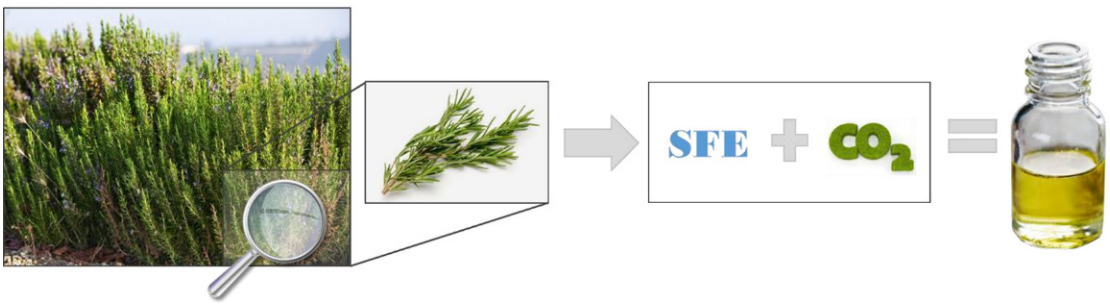
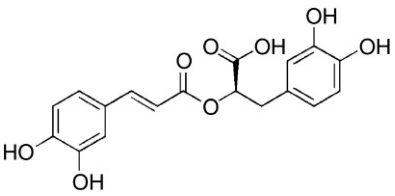
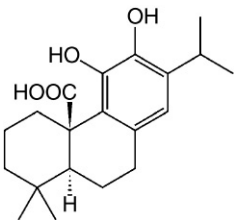
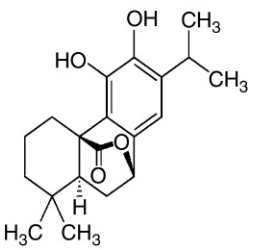



**Figure 12.8: Rosemary Herb and the Essential Oil Obtained by SFE.**

**Table 12.3: Physicochemical characteristics of the main compounds obtained in rosemary extracts (ChemicalBook, 2016a,b,c).**

| Physicochemical Characteristics | Rosmarinic Acid | Carnosic Acid | Carnosol Acid |
|---|---|---|---|
| Structural formula |  |  |  |
| Molecular formula | $C_{18}H_{16}O_8$ | $C_{20}H_{28}O_4$ | $C_{20}H_{26}O_4$ |
| Molecular weight (g/mol) | 360.31 | 332.43 | 330.42 |
| Solubility | Water, ethanol, formaldehyde, dimethyl sulfoxide | Water, dimethyl sulfoxide | Dimethyl sulfoxide, ethanol, methanol |
| Melting point (°C) | 171–175 | 185–190 | 210–220 |
| Boiling point (°C) | 694.7 | 499–503 | 524.8 |

There are studies that proved the effectiveness of rosemary extracts to preserve and transmit flavor to many food products (Rocio Teruel et al., 2015). The antioxidants present in these extracts are able to prevent lipid oxidation reactions avoiding the rapid deterioration of products rich in fats. These reactions are undesirable in food products, because they contribute to the loss of the sensory characteristics (taste, aroma, texture, and appearance), and promote the destruction of fat-soluble vitamins (Yang et al., 2016). Therefore, the use of this extract is a great alternative to extend the shelf life of a range of products with high lipid content.

Due to the features mentioned earlier, the rosemary extracts can be applied on meat products, such as hams, sausages, and salami to increase the shelf life of these products through inhibition of the oxidative processes, besides providing desirable aromas and flavors. Another interesting application of these extracts, both the quality viewpoint of the final product, as well as to human health is the incorporation of rosemary extracts into edible vegetable oils, as these oils are used in the preparation and frying of many food products.

### *3.5 Annatto Seeds (*Bixa orellana *L.)*

Annatto is a fruit native to the tropics of South America that belongs to the family *B. orellana* (Rodrigues et al., 2014). The extraction of the bioactive compounds from annatto seeds using

**Figure 12.9: Annatto Tree and Annatto Seeds.**

supercritical technology has been extensively studied because of their functional activities by the food and pharmaceutical industries (Moraes et al., 2015; Shahid Ul et al., 2016; Taham et al., 2015). Fig. 12.9 illustrates the annatto tree and annatto seeds.

Among the products obtained by means the extraction process with supercritical carbon dioxide from annatto seeds, geranylgeraniol and δ-tocotrienol are main bioactive compounds (Silva et al., 2015). Tocotrienols together with tocopherols are antioxidants popularly known as vitamin E (Knecht et al., 2015). They occur naturally in eight isoforms of α-, β-, γ-, and δ-tocopherols and tocotrienols (Fig. 12.10). Antioxidant activity of this vitamin is associated with breaking of soluble chains in natural oils, to prevent oxidation of lipids in food products and biological systems (Zou and Akoh, 2015). Vitamin E is considered essential, as the human body is unable to synthesize it naturally. Therefore, its consumption happens through fortified foods or through vitamin supplements (Sen et al., 2006). During industrialization,

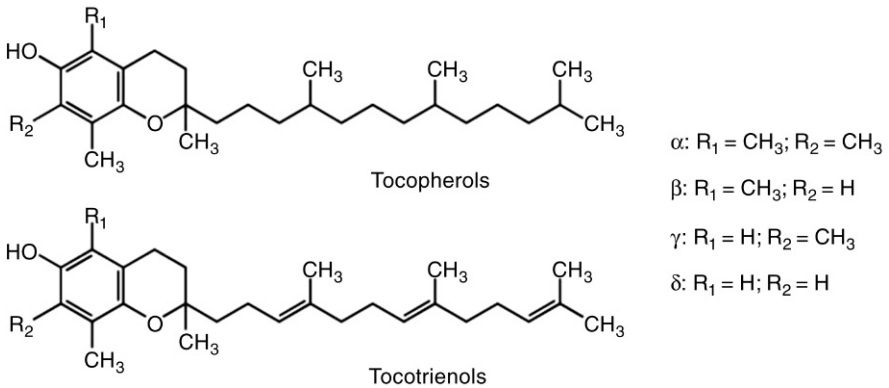



**Figure 12.10: Chemical Structure of Tocotrienols and Tocopherols.**

*Obtained from: Knecht, K., Sandfuchs, K., Kulling, S.E., Bunzel, D., 2015. Tocopherol and tocotrienol analysis in raw and cooked vegetables: a validated method with emphasis on sample preparation. Food Chem. 169, 20–27, with permission from Elsevier.*

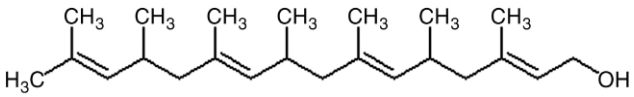



**Figure 12.11: Molecular Structure of Geranylgeraniol.**

foods undergo some processes, such as heating, cooling, and dehydration. These processes can lead to the loss or reduction of vitamin E in fresh foods (Bramley et al., 2000). Due to this, the addition of these compounds in food products becomes necessary through supplementation from natural or synthetic sources.

In addition to acting as an antioxidant, tocotrienols are highlighted in the pharmaceutical and medical fields due their properties as anticancer, neuroprotective, and controlling serum cholesterol levels (Karmowski et al., 2015; Zou and Akoh, 2015). These compounds can be identified in palm oil, rice bran oil, grape seed oil, maize, wheat germ, hazelnut, olive, rye, oats, barley, and monocotyledonous plants seeds, such as annatto (Aggarwal et al., 2010). Recently, the oil obtained from annatto seeds by supercritical extraction was recognized as the main natural source of δ-tocotrienol, a promising antioxidant and chemotherapeutic agent with the potential angiogenic inhibitor (Moraes et al., 2015). The antioxidant activity of this compound is associated with its ability to give phenolic hydrogens to free radicals and thereby prevent oxidation of lipids. Thus, the use of δ-tocotrienol is of interest to the food industry, as they can inhibit lipid oxidation, a reaction that negatively influences the flavor and decreases the quality and safety of food products (Zou and Akoh, 2015).

Annatto seeds also stand out as a source of geranylgeraniol, the main terpenic constituent of the seeds, representing 1% of dry seeds (Jondiko and Pattenden, 1989). Fig. 12.11 shows the chemical structure of this compound. Geranylgeraniol can be used as nutritional supplement in foods and beverages (Moraes et al., 2015) and is also the target of the pharmaceutical industry because of its antiparasitic action against *Trypanosoma cruzi* (Menna-Barreto et al., 2008), therapeutic action against leishmaniasis (Monzote et al., 2014), and mainly for its anticancer action (Marcuzzi et al., 2012), besides being an important intermediate in the synthesis of coenzyme Q10, vitamin K, and tocotrienols (Tan and Foley, 2002).

If these compounds present in annatto can contribute to the human health, why not incorporate them into food products? The enrichment of foods with these compounds may bring technological innovations for processing foods. However, studies involving this type of application in food is still a major challenge for science because of the instability and fragility of bioactive compounds that can be easily degraded when exposed to external factors, such as oxidation, heat, and light (Asbahani et al., 2015). An alternative to stabilize such unstable compounds is to employ the encapsulation technology aiming to protect and preserve the bioactive compounds from external factors while maintaining the biological activity (Ray et al., 2016). Silva et al. (2016a) studied encapsulation of annatto seed oil by emulsification

with biopolymers and obtained a good geranylgeraniol retention using gum acacia and inulin as encapsulating matrices. Annatto seed oil microparticles preserved the chemical characteristics of geranylgeraniol and kept oil antioxidant capacity of annatto seeds. These results show the real possibility of applying geranylgeraniol in functional food products.

The annatto seed oil can be used by the dairy industry for the production of many types of cheese, besides being used in the manufacture of butter, giving to these products several yellow shades (Sathiya Mala et al., 2015). The addition of the extract in food products can inhibit oxidative processes in foods with high levels of lipids. Santana et al. (2013) evaluated the effectiveness of incorporating annatto as an antioxidant additive in biodegradable films based on chitosan. The authors verified that developed packaging was feasible.

## 4 Conclusions and Future Trends

This chapter presented the potential applications of extracts obtained via SFE on the formulation and production of several products, mainly focusing on the food industry products. These extracts are obtained by a clean technology from natural sources and, therefore provide several benefits to human health due their functional and therapeutic properties. They meet the global demand for healthy products promoting wellbeing and sensoriality. In this sense, supercritical extracts can act as an agent of innovation in the life cycle of products with already established market. However, all applications mentioned or suggested in this chapter must be accompanied by a chemical, technological, and economic study to keep the quality, competitiveness, and especially to ensure the safety of consumers. The next steps are to stimulate practical researches for application of these extracts, evaluating the sensory characteristics and functional properties of the product; consumer acceptance; economic viability of production on an industrial scale; and product safety to the consumer.

## Acknowledgments

Authors are grateful to CNPq (470916/2012-5) and FAPESP (2012/10685-8 and 2015/13299-0) for the financial support. Eric Keven Silva thanks FAPESP (2015/22226-6) for the postdoctoral assistantship. Giovani L. Zabot thanks Federal University of Santa Maria (043038/2016; 041501/2015). Gislaine C. Nogueira thanks CNPq (166060/2015-1) for the postdoctoral assistantship. M. Angela A. Meireles thanks CNPq (302423/2015-0) for the productivity grant.

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CHAPTER 13

# Sugar Beet Pulp as a Source of Valuable Biotechnological Products

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## 1 Introduction

Sugar beet was already being grown as a garden vegetable over 2000 years ago. The plant was probably selected from various *Beta* species, which grew around the shores of the Mediterranean, and it was widely used for various culinary purposes. It was not until the 17th century, however, that beet was first grown on a field scale, and only then as fodder for cattle. In Germany in 1747, saccharose was obtained from beets by the chemist Andreas Margraff, and the first sugar beet factory was built in Silesia in 1802. By 1818, world beet sugar production had overtaken that of cane sugar. Growing sugar beet (*Beta vulgaris*) for sucrose production found further success in the United States from around 1870. The United States now provides around 11% of the world's supply (Finkenstadt, 2013), and sugar beet provides about 20% of the global demand for sugar. The European Union is also a global leader in sugar production, and beet sugar accounts for almost all sugar production in Europe. In other markets, sugar beet plays a more marginal role. Sugar beet and sugarcane dominate their respective domestic markets, mainly due to preferential international trade and agriculture agreements. For example, in China sugar beet accounted only for around 6% of total sugar output in 2006. Currently, sugar beet is grown in some 50 countries. The top world sugar beet producing countries in the world are France, the United States of America, Russia, Germany, Ukraine, Turkey, Poland, China, Belgium, Egypt, the Netherlands, and Iran (Rogers, 2011).

### 1.1 Sugar Beet

Sugar is obtained as sucrose from only two crops, beet and cane. Sugar from beet is exactly the same as cane sugar. Sugar is used as a major component in a wide range of foods and drinks, for its sweetening, energy giving, and preservative properties. Despite concerns over

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00013-X

its health effects and competition from other sugars (cereal isoglucose, maize fructose, and artificial sweeteners), the demand for sugar continues to rise. In the last 60 years, world consumption of sugar from cane and beet has increased fivefold.

Sugar beets consist of around 75% water, 18% sugar, and approximately 5% cell walls. Through centuries of selective breeding, the sugar concentration in beets has increased by around one-third. Resistance and/or tolerance to pests and diseases have also increased. Plant breeding continues to improve yields and the chemical properties of the root, which helps to increase the amount of white sugar extracted at processing factories (Draycott, 2006). However, genetic modification of beet has not been approved by the European Union and it is illegal to grow genetically modified beets for commercial purposes (*British Sugar* http://www.britishsugar.co.uk/sugarbeet.aspx).

### 1.2 Sugar Beet Processing

Root quality is the most important parameter affecting beet processing. The aim of processing is to produce pure sugar from the purchased sugar beet at the least cost. The efficiency of processing can vary appreciably, depending on the factory equipment and how it is operated, but in case of the beet, it is the greatest manufacturing cost. Producers in some countries commence their operations by harvesting and storing the beet in the field. These are the primary stages of processing. In subsequent stages, the beets are flumed, washed, and sliced into thin slices called cossettes. Once these have been diffused with hot water in a process of counter-current extraction, the resulting dark, opaque raw juice is purified using lime/$CO_2$ (carbonatation), yielding a clear second carbonatation filtrate. During carbonatation, soda may be added if the juice alkalinity is too low, or acid if it is too high. The filtrate, which contains around 12%–17% dissolved solids, may or may not be sulfitated at this point, to inhibit discoloring reactions which sometimes occur in the process that follows. This process involves evaporating the "thin" juice at high temperature to yield a "thick" juice containing over 60% of dissolved solids. The thick juice is crystallized under vacuum, yielding two or more crops of sugar. The second and any subsequent crops of crystals are generally too impure to be sold, so they are redissolved, mostly in evaporated thick juice. This gives the thick juice higher purity and it can, in turn, yield a very high purity (>99.9% sucrose) first sugar crop, which is then dried and sold (Draycott, 2006). The syrup separated from the last crop of sugar, from which no more sugar can be economically crystallized, is called molasses. This is one of two valuable coproducts that result from the processing of sugar beet roots, along with sugar beet pulp (SBP), the insoluble beet tissue that remains from counter-current raw juice extraction (Fig. 13.1). These products can be used combinedly or separately, dried or processed in different ways, including as animal feed or as fermentation substrates (Van der Poel et al., 1998). Utilization of coproducts reduces waste and adds value to the crop (Finkenstadt, 2013).

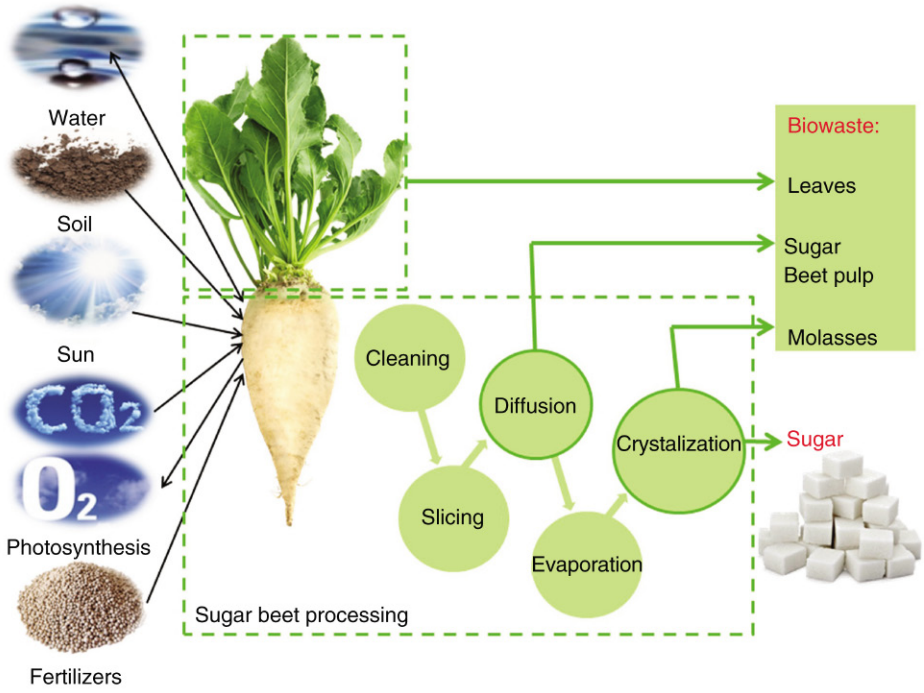


**Figure 13.1: Sugar Beet Processing.**

### *1.3 Sugar Beet Tops*

Sugar beet tops may comprise of sugar beet leaves only or a mixture of the leaves and crowns, depending on the harvesting machinery used. The tops weigh roughly the same as the roots, depending on the variety, time of harvest, and growing season. Sugar beet leaves normally contain 12%–14% dry matter (d.m.), and leaves and crowns contain 16%–18% d.m. They are very palatable and high in proteins, vitamin A, and carbohydrates. They can therefore provide inexpensive fodder for a wide range of farm livestock. They can be grazed in the field, as is common practice for feeding sheep or cattle, or they can be harvested for transportation. They may be consumed fresh or wilted, or preserved in silos for use in winter (Draycott, 2006). To minimize soil contamination, special equipment is required for direct collection at topping. The energy content of 10 kg beet top silage is equivalent to that of 1.5 kg barley. Beet top silage may also be used in combination with other feeds (Harland et al., 2006). In the Netherlands, studies on the possibility of using plant waste as a potential substitute for environmentally unsustainable proteins, such as meat, dairy, and soy showed that a vegan gluten-free plant-based protein extracted from the pressed green juice of sugar beet leaves was as versatile as chicken egg.

## 1.4 Sugar Beet Pulp

Around 20 million tons of SBP is produced in Europe each year. SBPs differ only in terms of dry mass content. *Wet pulp* usually contains 6%–12% d.m., *pressed pulp* 18%–30% d.m., and *dried SBP* 87%–92% d.m. The low dry matter content of wet pulp means that it is somewhat fluid in nature, and therefore difficult to handle and store. It can be fed fresh to ruminant livestock and pigs, in which case its major attributes are its energy and digestible fiber contents. If wet pulp is to be ensiled, no silage additive is necessary because the high sugar content ensures good fermentation. Pressed pulp is produced by all beet sugar facilities worldwide and is a very popular feed for livestock. Like wet pulp, its major attributes are its energy and digestible fiber contents. Pressed pulp, if used fresh, should be consumed within 5–7 days, as mold will grow on any surfaces exposed to air, causing spoilage. This spoilage occurs more rapidly under warm, humid conditions, and in such cases immediate ensilage is therefore recommended. When dried, SBP is very stable, and can be either used directly or stored for up to a year without any adverse effect on its feed value or fermentation possibilities. It is normally processed as pellets, which are convenient to use. Referred to as "shreds" the unpelleted form provides a stable feed for customers close to beet sugar factories. The density of shreds is one-third that of pellets, so transportation over long distances may be costly. *Dried molassed pulp* or *molassed pressed pulp* are manufactured in several European countries and the United States of America. These products are produced by mixing pressed pulp with warm beet molasses in the beet sugar factory. The resulting molassed pressed pulp can be sold directly for use on farms, or may be dried in shredded form and pelletized into sugar beet pellets 6–8 mm in diameter (Harland et al., 2006). SBP contains carbohydrates, proteins, and fiber. The main carbohydrates are sucrose (10% d.m.) and polysaccharides, such as cellulose (22%–40% d.m.). The main hemicelluloses are galactan and araban (24%–32%) and pectin (24%–32%) (Table 13.1).

**Table 13.1: Fermentable sugars in sugar beet pulp (Finkenstadt, 2013).**

| Component | Dry Matter (%) |
|---|---|
| Glucose | 68 |
| Arabinose | 22 |
| Uronic acids | 18 |
| Galactose | 5 |
| Rhamnose | 2 |
| Xylose | 2 |
| Mannose | 1 |
| Saccharose (residual) | 4 |
| Ferulic acid | 0.5 |
| Acetic acid | 1.6 |
| Methanol | 0.4 |
| Protein | 8 |

Since it is not mature, the fiber in sugar beet root crops is not extensively lignified. In addition to carbohydrates, it also contains lignins (3%–4% d.m.), associated ferulic acid ester (0.8% d.m.), and acetic acid (3.9 % d.m.). Generally, it comprises approximately one-third pectin, one-third hemicellulose, and one-third cellulose. SBP may be used as a palatable, fibrous food ingredient in food for human consumption. The inclusion of sugar beet fiber in the diet of healthy volunteers resulted in significant physiological changes, such as reductions in the levels of both postprandial plasma glucose and blood cholesterol (Morgan et al., 1988).

The high pectin content in beet pulp makes it a source of available energy for microbial protein synthesis in ruminants. Sugar beet fiber is highly digestible, with digestibility of almost 90% for both ruminants and pigs. This makes SBP an important part of feeding regimes, increasing the fiber content of the diet without reducing the energy density. Its combination of high energy and fiber makes SBP unique among feed ingredients (Harland et al., 2006). However, the direct use of this coproduct as a feed component decreases significantly during summer, due to the availability of green fodder. The drying process is also quite energy intensive and not economically profitable, while reusing dried SBP requires rehydration. Our previous studies have shown that hydration of dried SBP requires large amounts of water: around 8 $m^3$ per 1 ton of raw material.

As well as for producing dietary fiber, arabinose, rhamnose, galactose, and ferulic acid, SBP may also be used as a substrate in various biotechnological processes: ethanol or lactic acid (LA) fermentation coupled with various catalytic processes for obtaining valuable bioproducts and preparations, or for production of biogas and single cell protein (SCP). The composition of SBP and sugar beet leaves suggests that they could be used to produce a number of value-added products. Apart from their use as foodstuffs and animal feed, sugars can be processed for industrial purposes using biorefining technology.

## 2 Sugar Factories as Biorefineries

The development and large-scale implementation of clean, effective, and renewable technologies for the production of energy and valuable chemicals, including biofuels and biocomponents, is an area of key concern for governments, scientists, and industry (Preisig and Wittgens, 2012). Requirements for the addition of biocomponents to the pool of conventional fuels and the reduction of greenhouse gas emissions have driven the implementation of novel technologies for the production of advanced biofuels, which make optimal use of the energy contained in biomass, as well as the development of effective methods of $CO_2$ sequestration (EPA, 2010; Harvey and Bharucha, 2016; Kousoulidou and Lonza, 2016). A number of raw materials have been given double credit for their use as biofuels. The modern approach taken by biorefineries provides a package of interrelated technologies that make up an integrated system for the production of liquid biofuels,

gas, feed, and valuable chemicals (Kalta et al., 2016; Lopez-Díaz et al., 2016; Moncada et al., 2016). The combination of technologies is designed to enable nonwaste processing and manufacturing (de Jong and Jungmeier, 2015).

The sources of biomass that can be used to produce energy and valuable chemicals are many and varied. They range from field crops to wastes from agriculture, the agrifood industry, households, and the municipal sector (Singh et al., 2014). Biomass is produced in special plantations of fast-growing trees (willow, Platanus, poplar, eucalyptus), sugarcane, rapeseed, sunflowers, and selected grass species (Aravanopoulos, 2010; Nissim et al., 2013; Santos et al., 2016; Sixto et al., 2015). It can also be derived from forestry wood waste, wood and pulp waste from the paper industry, agricultural waste (manure, biogas), and municipal waste (sewage sludge, household waste, and waste paper). A significant proportion of this biomass can be used in the power industry as biofuel. Biomass can be used for energy production by direct combustion of solid biofuels (e.g., wood, straw, and sewage sludge), or processed into liquid fuels (e.g., esters of rapeseed oil and alcohol), or gas (e.g., agricultural biogas, biogas, and gas from sewage treatment plants and waste dumps) (Ben-Iwo et al., 2016; de Jong and Jungmeier, 2015; Melero et al., 2011). Biomass can be converted into energy carriers using physical, chemical, or biochemical methods.

Sugar beets are grown on a large agricultural scale, and so are readily available in large quantities. Sugar beet is a multifunctional material, which can be used in biorefineries for the total production of bioethanol (second generation biofuels), energy, heat, feed, and other valuable chemicals and biomaterials. However, generally it is used to produce sugar, during which waste is generated in the form of SBP and beet leaves. Drying, pelletizing, and transportation are energy-intensive processes and therefore the profits from SBP-derived animal feed depend mainly on the economics of feed industries. Dehydrating and pelletizing SBP can add 30%–40% to the total cost of sugar beet processing. Many factories therefore make marginal use of SBP (Zheng et al., 2012a,b).

There is clear potential for finding new, economically viable ways of using waste biomass from sugar production as a raw material. Research conducted at Lodz University of Technology (TUL) in the years 2009–2016 ("Sugar beet pulp biomass as a raw material for new fermentation media" PBS1/B8/3/2012 and "Processing of waste biomass in the associated biological and chemical processes grant" BIOSTRATEG2/296369/5/NCBR/2016 funded by the Polish National Centre for Research and Development) has proven the production of bioethanol from various final and semifinished products to be technologically feasible (Dziugan et al., 2013). Fig. 13.2 shows the elaborated concept for utilization of sugar beet waste biomass.

### 2.1 Sugar Beet Pulp as a Substrate for Biorefineries

Worldwide, in 2011 alone, 271.6 million tons of sugar beets were produced (with Europe accounting for the production of 195.6 million tons). After saccharose extraction, this resulted

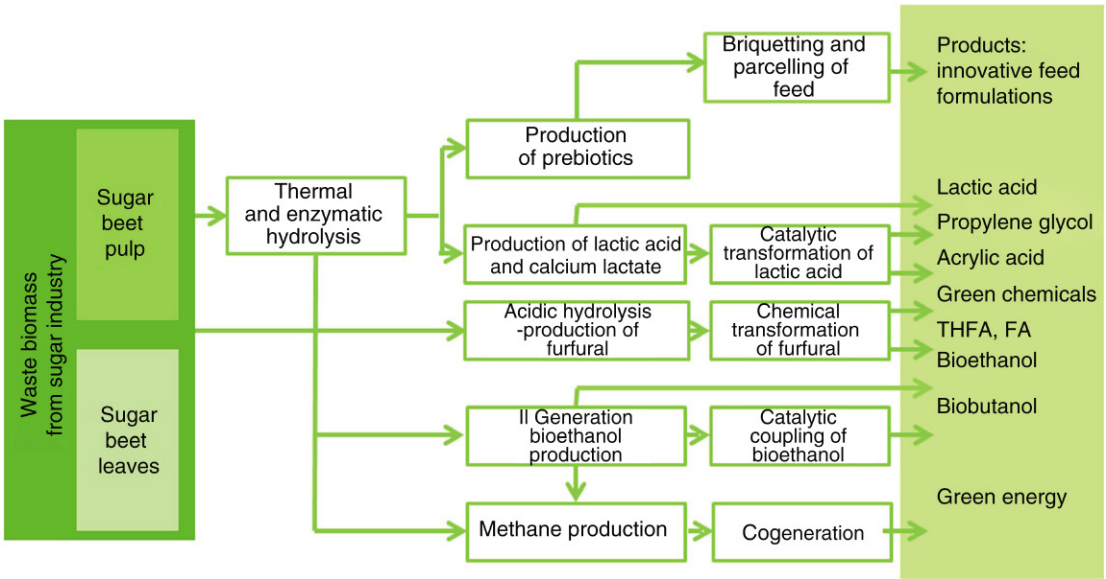


**Figure 13.2: Concept for Conversion of Waste Biomass From the Sugar Industry Into Valuable Products.**

in the production of approximately 68 million tons of wet SBP or 17 million tons of dried biomass (Kracher et al., 2014). Its major constituents are polysaccharides consisting of 22–24 wt.% cellulose, 30 wt.% hemicelluloses, and 15–25 wt.% pectin, with small amounts of fat, protein, ash, and lignin, at 1.4 wt.%, 10.3 wt.%, 3.7 wt.%, and 5.9 wt.%, respectively. Heteropolysaccharides are composed mainly of xylose, glucose, mannose, galactose, and arabinose (Olmos and Hansen, 2012; Wang et al., 2015). Sugar beet pectin (polymeric galacturonic acid backbone with intermittent blocks of alternating monomers, unbranched galactan, and highly branched arabinan side chains) consists of glucose with arabinose and galacturonic acid (GA) and smaller amounts of galactose and rhamnose (Hamley-Bennett et al., 2016; Micard et al., 1996; Ward et al., 2015).

Waste materials have attracted much attention as alternative carbon sources, which could reduce the cost of fermentative processes (Zhang et al., 2014). SBP biomass, consisting of 60%–70% carbohydrates in dry matter, has the potential to replace pure sugars in various biotechnological processes (Nguyen et al., 2013). The release of hydrolysis products (glucose from cellulose, together with xylose, glucose, mannose, galactose, and arabinose from hemicelluloses and pectin) is a promising step toward valorizing this by-product. Arabinose and GA are value-added products, which can be utilized as intermediate raw materials in the pharmaceutical industry as well as in organic synthesis and for vitamin C production (Bellido et al., 2015).

Lignocellulose biomass (straw, leaves, stems, wood waste, etc., often left lying in fields) can be decomposed into monosaccharides, which may be used as substrates for biotechnological

processes (Singh et al., 2014). Delignification is often a necessary step in plant biomass bioconversion, since the cellulose and hemicellulose fibers, which provide the structural framework of plant cell walls are protected from depolymerizing enzymes by strongly crosslinked lignin coatings (Singh et al., 2014; Sindhu et al., 2016). A variety of pretreatment methods have been developed to loosen the structure of lignocellulose through the partial removal of lignin (Pang et al., 2016; Yiin et al., 2016). Simultaneous partial depolymerization of hemicelluloses, decreasing cellulose crystallinity, and increasing porosity also reduce the recalcitrance of lignocellulosic biomass to enzymatic hydrolysis (Hendriks and Zeeman, 2009; Kotarska et al., 2015; Yu et al., 2011). The use of low-lignin SBP is therefore advantageous.

Our work has shown that the optimal model is one in which all biotechnological processes are located at the sugar factory (PBS1/B8/3/2012). At location, all by-products from sugar production, such as molasses or SBP, can be further processed. The possibility of using SBP for the production of ethanol and other valuable compounds becomes extremely attractive from the economic standpoint. SBP after hydrolysis can also be used to produce yeast biomass and other valuable products from yeast, as well in more sophisticated, innovative technologies, such as the production of propylene glycol.

### 2.1.1 Biomass pretreatment

The limited efficiency of enzymatic SBP hydrolysis results from the structure of the biomass, which impedes access by hydrolytic agents to specific substrates. To achieve rapid and complete hydrolysis, biological, thermal, or acid/base pretreatments are used in combination with enzyme preparations with high cellulolytic activities (Kracher et al., 2014). Biological ensilage enables storage and decomposition of biomass for use by biorefining industries. Compared with raw SBP, which is characterized by up to 71% enzymatic digestibility, ensiled SBP has higher enzymatic digestibility, in the range 74%–95% (Zhang et al., 2011). In a study by Zheng et al. (2012a), the reducing sugar yield from ensiled SBP under enzymatic hydrolysis was greater than that from raw SBP, but required washing with water. The cost of the necessary concentrations of enzymes may, however, be prohibitive. Chemical pretreatments, such as ammonia pressurization/depressurization (APD) or acid-base pretreatment, can be employed prior to hydrolysis to reduce the number of enzymes needed to achieve significant fermentable sugar yields (Zheng et al., 2012b). To increase the concentration of monomeric sugars in the hydrolysates, an effective pretreatment should render cellulose more degradable while avoiding the formation of degradation products and minimizing sugar loss (Bellido et al., 2015). Moreover, pretreatments should be strong enough to disrupt the cellulose-hemicellulose network and the cellulose-lignin network generally. On the other hand, the more severe the pretreatment, the more biomass is degraded into inhibitory compounds (e.g., furfural or hydroxymethylfurfural) and nonfermentable products (Kühnel et al., 2011).

When SBP-hemicellulose is suitable for subsequent biological processes, the pretreatment method chosen must be able to remove the lignin without causing disintegration of the hemicellulose. After calcium hydroxide treatment, the SBP structure usually appears more amorphous (with indistinguishable fibers) (Dredge et al., 2011). The structure of SBP can be modified by APD, when the biomass is exploded in a reactor vessel by sudden evaporation of ammonia. APD has been found to significantly increase the efficiency of hydrolysis of the cellulose fraction, but by addition of hemicellulose- and pectin-degrading enzymes, the hydrolysis of treated pulp was no better than with the untreated control (Foster et al., 2001).

Dilute acid pretreatment promotes disruption of the polymeric structure, improving enzymatic hydrolysis and the fermentation yield of SBP. Scanning electron microscopy (SEM) images evidently demonstrate the change in surface morphology of feedstock after acid hydrolysis (Yücel and Aksu, 2015). In research conducted by Zheng et al. (2013), acid concentration and temperature were found to have a significant effect on hemicellulose removal, while solid loading had a noticeable effect on cellulose solubilization. When SBP was pretreated at 170°C, arabinose and GA yields in the supernatant fraction decreased, while the levels of the other sugars remained constant. Decomposition levels of 40% and 90%, respectively, were noted for these compounds, suggesting that arabinose and GA are both heat-labile. In studies by Zheng and Bellido, the optimum pretreatment conditions for solid loading 6%, were found to be: $T = 120°C$, $C_{acid} = 0.66\%$, which resulted in a hydrolysis yield of 93% and a total reducing sugar yield of 63% (Bellido et al., 2015; Zheng et al., 2013).

The most common method of raw material pretreatment, however, is steam with pressure. The process of thermal hydrolysis can be conducted in either acid or alkaline environments, with factors including time, temperature, pressure, and the amount of dry mass influencing the effectiveness of cellulose decomposition (Singh et al., 2015). In the case of bioconversion of SBP, the costs of thermal processing could be reduced, due to its chemical composition and relatively low lignin content (Zheng et al. 2012b). One of the most promising and environmentally-friendly methods of pretreatment involves either steam explosion (SE) or liquid hot water (LHW) (Behera et al., 2014). Hydrothermal pretreatment enables partial lignin removal and can result in a discernible increase in the dimension of pores in the coherent structure of plant biomass (Kumar et al., 2009; Mosier et al., 2005; Singh et al., 2014). It also causes a partial breakdown of hemicelluloses, which are converted (mainly) to oligomers and simple sugars. The principle of hydrothermal pretreatment is based on autoionization of water at elevated temperatures (130–220°C) and pressure, leading to the release of protons ($H^+$), and a significant decrease in pH (Mosier, 2013). Hot water treatment has been shown to be an effective pretreatment for SBP, resulting in the solubilization of 40%–60% of the total biomass. The principal advantages of hot water treatment are that it requires the use of significantly lower amounts of reagents, costs less than chemical or enzymatic treatments and produces only small amounts of degradation products that could inhibit subsequent fermentation when at higher concentrations (Zieminski et al., 2014).

A small number of studies have been performed at low (1% and 8%) dry matter concentrations (Zhang et al., 2011), with some requiring a milling step to reduce the particle size before treatment (Kühnel et al., 2011). These treatments can solubilize all the hemicellulose, up to 22% of the cellulose and 60% of the lignin (Hamley-Bennett et al., 2016). The resulting hydrolysates are characterized by high concentrations of monosaccharides, which increases the efficiency of further enzymatic digestion. The type and quantity of products generated by degradation of nonstarch polysaccharides were strongly affected by temperature. The weak thermal stability of hemicelluloses, such as xylan and glucomannan degrading at temperatures over 180°C caused the appearance of low concentrations of xylose and mannose. At temperatures of 120–150°C, arabinose, galactose, and uronic acids were the predominant components of solids remaining after LHW processing. Raising the processing temperature (to 200°C) caused the arabinose content in the insoluble fractions to drop from 66% to 2%. The same tendency was observed for rhamnose, galactose, and uronic acids, while the amounts of xylose, mannose, fucose, and glucose remained constant (Zieminski et al., 2014). It has also been found that a combination of mild hydrothermal pretreatment at 140°C with subsequent enzymatic digestion allows fast and efficient hydrolysis of SBP. Within 24 h, more than 90% of all cellulose was hydrolyzed. In contrast to chemical pretreatments, this mild pretreatment with enzymatic digestion did not result in a loss of biomass or in the production of any undesirable compounds (Kühnel et al., 2011).

In the SE method, biomass particles are heated using high-pressure saturated steam for a short period of time and the pressure is swiftly reduced to terminate the reactions, causing the biomass to undergo an explosive decompression (Singh et al., 2014). Typical conditions are: temperature 160–260°C, pressure 0.7–4.83 MPa, and time ranging from several seconds to a few minutes. During steam pretreatment, parts of the hemicellulose hydrolyze and form acids, which are thought to catalyze the further hydrolysis of the hemicellulose and make cellulose more accessible to hydrolytic enzymes. LHW pretreatment is similar to SE, but uses water in the liquid state at elevated temperatures instead of steam. It results in hemicellulose hydrolysis and the removal of lignin. During this process, lower temperatures (optimally between 160 and190°C) are used, avoiding the formation of fermentation inhibitors, which occurs at higher temperatures, and no washing or neutralization of the slurry is necessary (Behera et al., 2014; Kumar et al., 2009; Mosier et al., 2005; Singh et al., 2014). The low cost of the solvent is a further advantage for large scale applications.

The low lignin content of SBP means that high temperature treatments are not required to solubilize the pectin fraction. Thermal treatment of SBP has a strong physical effect on lignocellulosic substrates, due to the sudden release of pressure during explosive decompression and the fraying of polymer fibers (Deutcher, 2008). Monosaccharides are released from cellulose and hemicelluloses, and high amounts of free glucose have been detected following this procedure (Rezic et al., 2013). Hamley-Bennett et al. (2016) used steam pretreatment on native SBP and evaluated the effects of time and pressure on the

extraction of selected pectins. Over 24 min, the process with steam pretreatment provided the highest solubilization of pectin oligomers at comparatively low pressure (5 Bar), leaving the cellulose blocks almost intact. The steam pretreatment of biomass pulp is included in the concept of a sugar beet biorefinery, as it could make use of waste stream from the existing operation, avoiding the need for such processes as grinding or dilution with water.

### *2.1.2 Saccharification*

A popular way of pretreating plant biomass is enzymatic hydrolysis (Badiei et al., 2014; Hemsworth et al., 2015; Mood et al., 2013). SBP contains three types of carbohydrate: cellulose, hemicellulose, and pectin. Generally, treatment of SBP with a combination of cellulase and pectinase shows much higher saccharification efficiency compared with cellulase alone (Zheng et al., 2012b). The addition of pectinase has the strongest effect on galactose, resulting in the release of 91.3% of total galactose from SBP. Likewise, cellulose degradation into glucose monomers and the release of arabinose from hemicellulose increases as a result of the addition of pectinase (Rezic et al., 2013). For the purposes of ethanol fermentation, pectinase is most commonly used together with cellulase/β-glucosidase to break the SBP polymers into GA and monosaccharides (Zheng et al., 2012b).

Many studies use commercial enzyme mixtures for the saccharification of SBP: Celluclast 1.5 L, β-glucosidase (Novozymes 188), and Pectinex Ultra SPL; Celluclast lo5L FG, Novozym 431, and Viscozyme L; Celustar XL, and Agropect (Foster et al., 2001; Zheng et al., 2012b; Zieminski and Kowalska, 2015). Micard et al. (1997) report using commercial cellulases and pectinase complexes (Multieffect XL, Celluclast, Novozym 342, SP 584, Cellulase, and Cellulyve Tr 300G+ AN 6000) in different combinations. Cellulose hydrolysis was found to be more complete when pectinases and cellulases were added sequentially. The degree of hydrolysis was increased to 41.6% by applying pectinolytic mixture SP 584, followed by treatment with Celluclast. The use of other enzymes resulted in yields of 0.1% and 19.3% for individual sugars (Kühnel et al., 2011; Micard et al., 1997; Rezic et al., 2013). In research conducted by Lodz University of Technology and the Krajowa Spolka Cukrowa S.A. (Polish Sugar Company), a mixture of Viscozyme and Ultraflo Max (Novozymes) was used to obtain SBP hydrolyzates in the Dobrzelin Sugar Factory (Poland). The components of the hydrolyzate obtained on a semiindustrial scale (3 $m^3$ bioreactor) are presented in Table 13.2.

Much work over recent decades has focused on the degradation of SBP, which has been shown to provide good yields of solubilized carbohydrates. However, in these studies, high levels of enzymes or chemicals have been required (Micard et al., 1996). No data are available for the degradation of SBP in commercially reasonable time spans and with economically viable enzyme levels (Kühnel et al., 2011). Therefore, research has been directed at finding new, less expensive, crude multienzyme preparations. Kracher et al. (2014) identified high-levels of lytic enzymes, degrading cellulose, pectins, and hemicelluloses in the plant pathogenic fungus *Sclerotium rolfsii* grown on SBP. *S. rolfsii* cultured on an

**Table 13.2: The main saccharide components in sugar beet pulp pectin (SPB) hydrolyzate obtained following Viscozyme and Ultraflo Max treatment.**

| Sample | Glucose | Fructose | Mannose | Raffinose | Galacturonic Acid | Xylose | Arabinose | Galactose |
|---|---|---|---|---|---|---|---|---|
| | c (g/L) | c (g/L) | c (g/L) | c (g/L) | c (g/L) | c (g/L) | c (g/L) | c (g/L) |
| Trial 1 | 12.90 | 4.730 | 0.342 | 23.21 | 4.208 | 0.702 | 11.85 | 14.22 |
| Trial 2 | 8.828 | 7.403 | 1.819 | 21.60 | 4.423 | 0.631 | 11.54 | 13.84 |

inexpensive SBP medium adapts its own exoenzyme system to produce more cellulolytic enzymes. When grown on SBP, the white-rot fungus *Trametes multicolor* produces fewer hemicellulases, but more of the oxidoreductases which are also required to degrade the substrate. Cellulases, hemicellulases, and some oxidoreductases are produced by saprotroph *Neurospora crassa* grown on SBP. Analysis of postfermentation media indicates *S. rolfsii* to be an efficient degrader of hemicellulose. However, the profile of the conversion products shows that degradation of cellulose was limited. Supplementing *S. rolfsii* with a *Trichoderma reesei* postfermentation medium dramatically increases cellulose degradation. Even without pretreatment, very low enzyme doses might enable efficient hydrolysis of sugar beet pulp pectin (SPB) pectin, hemicelluloses, and cellulose (Kracher et al., 2014).

### 2.1.3 Fermentation

Sugars released during enzymatic hydrolysis can be converted into bioproducts (ethanol, butanol, or organic acids) using microorganisms. However, there are several technical barriers to the use of lignocellulose as a substrate in fermentation processes. Polymeric compounds broken down into fermentative sugars form a mixture of hexoses and pentoses. The main problem is the small number of microorganisms, which are able efficiently and simultaneously to convert hexoses and pentoses into ethanol. Most commercial producer strains (both bacteria and conventional ethanol-fermenting yeasts) cannot assimilate pentose. For instance, *Saccharomyces cerevisiae* is unable to metabolize either arabinose or GA into ethanol (Zheng et al., 2012b). Sugars formed by the hydrolysis of lignocellulosic beet pulp may also contain xylose from hemicellulose. There is therefore a need to find unconventional strains to improve fermentation efficiency. *Pichia stipitis* yeast, known to produce ethanol in synthetic growth media with xylose, is able to metabolize media containing acid hydrolysate of SBP (Yücel and Aksu, 2015). The ethanol yield after fermentations of SBP hydrolyzates carried out at TUL was between 10% and 43% of the theoretical value, with a degree of total sugar attenuation between 10% and 58%. The yeast *S. cerevisiae* Ethanol Red (Lessafre) showed high efficiency for glucose fermentation. Fermentation of other sugars present in the medium (galactose, mannose, arabinose, and xylose) is possible after inoculation with *P. stipitis* or *Pachysolen tannophilus* (Berłowska et al., 2016; Pielech-Przybylska et al., 2014).

Based only on the US annual production of SBP (1.6 million tons) and the ethanol yields presented in the literature (from 0.2 to 0.4 g ethanol/g biomass), SBP could provide yearly

between 400,000 and 800,000 $m^3$ of ethanol. A potential drawback might be the conflict between using SBP for liquid fuel generation to produce animal feed (Zhbeng et al., 2012a). We recommend the use of SBP as a supplementary substrate for distilleries working on sugar factory-based feedstocks.

Despite intense pretreatment and high enzyme loads, the effectiveness of hydrolysis is usually only 40%–80%. The main goals therefore are to reduce the energy required in the process by optimizing enzymatic treatment and to increase fermentative conversion of the main carbohydrates into valuable products (Kracher et al., 2014). Zheng et al. (2012b) suggest that enzyme activity loss is primarily caused by end-product inhibition. Generally, the fermentation process can be performed either separately or simultaneously alongside enzymatic hydrolysis, in separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) (Ohgren et al., 2007). SSF may be one way to achieve high biofuel yield from SBP, since SSF would help maintain relatively low end-product concentrations, leading to low end-product inhibition. These enzymes could work synergistically with fermenting microorganisms to convert the carbohydrate components of SBP into biofuels (Zheng et al., 2012a; Zheng et al., 2012b). In SHF, pretreated biomass is degraded to glucose and afterward fermented in separate units. The major advantage of this solution is the possibility to conduct cellulose hydrolysis and fermentation under the optimum conditions for each process. However, the main drawback is the inhibition of cellulase activity by the sugars released, mainly cellobiose and glucose, and the risk of microbial contamination due to the length of time the process requires (Paschos et al., 2015; Saha et al., 2011). In SSF, hydrolysis and fermentation are conducted in the same time, in the same reactor. This method gives a higher reported product yield, by eliminating product inhibition, and the risk of contamination is much lower. Furthermore, there are fewer vessels used compared to SHF, resulting in lower costs. The drawback of SSF is the difference between the optimum conditions for enzymatic hydrolysis and those for fermentation (Liu et al., 2014).

Zheng et al. (2012a) reports that the modified ethanologenic bacterial strains *Escherichia coli* KO11, *Klebsiella oxytoca* P2, and *Erwinia chrysanthemi* EC 16 are suitable for efficient conversion of SBP carbohydrates into ethanol in SSF processes. *E. coli* KO11 was the most active in fermenting arabinose and GA and yielded the highest ethanol concentration (25.5 g/L), followed by *K. oxytoca* P2 and *E. chrysanthemi* EC 16. Rorick et al. (2009) used *E. coli* KO11 and *S. cerevisiae* (Type II -YSC2) in both parallel and serial fermentation processes to convert SBP into ethanol. They found that, of the two, *E. coli* KO11 was able to achieve a much higher ethanol yield (Rorick et al., 2009; Zheng et al., 2013).

Research conducted at TUL has shown that SBP is also a promising feedstock for LA synthesis (Berlowska et al., 2015). Simultaneous utilization of sugars derived from lignocelluloses has been reported for only a few LAB strains (Datta and Henry, 2006; Gallezot, 2012; Zhang et al., 2008). Xylose, galactose, arabinose, and fructose derived from

**Table 13.3: Productivity of lactic acid bacteria in SHF and SSF processes.**

| LAB Strains | Productivity of Lactic Acid (g/L) | |
|---|---|---|
| | SHF | SSF |
| *Lactobacillus delbrueckii* PCM 490 | 11.96 ± 0.19 | 29.16 ±0.14 |
| *Lactococcus lactis* PCM 2379 | 13.44 ± 1.75 | 25.45 ± 0.41 |
| *Lactobacillus plantarum* HII | 12.60 ± 0.37 | 30.11 ± 1.15 |
| *Lactobacillus acidophilus* PCM 2510 | 12.48 ± 0.65 | 25.18 ± 0.95 |

lignocellulose are less effective carbon sources for LA fermentation processes than glucose (Vennestrøm et al., 2011). The presence of glucose often prevents the use of secondary carbon sources (Aidelberg et al., 2014). LA fermentation of sugar beet hydrolyzates was therefore tested in SHF and SSF modes (Table 13.3). Rapid and complete consumption of the released carbohydrates was observed for SSF processes. Simultaneous release and utilization of carbohydrates is also advantageous in this case because of carbon catabolite repression. With these LAB strains, glucose represses the consumption of other less-favorable sugars, such as xylose and arabinose (Abdel-Rahman et al., 2015).

Waste from the agricultural industry, such as straw, SBP, sugar beet silage (Vazifehkhoran et al., 2016), beet leaves, and tree bark, all of which contain lignocellulosic complex in their structures, is also commonly used for methane fermentation. In our concept, the liquid fraction derived from hydrolysis would be subjected to alcoholic fermentation, while other uses should be found for the remaining solid fraction. Recent studies have indicated that, when added to sewage sludge, SBP lixiviation has huge potential as a renewable energy source. The organic fraction of the sewage sludge and beet pulp lixiviation can be converted into methane and carbon dioxide by anaerobic codigestion (Montañés et al., 2015). Anaerobic codigestion of sewage sludge and lixiviated SPB increased biogas productivity and organic matter removal, as well as lowering solids retention times (Montañés et al., 2013). In research by Zieminski et al. (2014), the temperature of LHW treatment clearly affected the chemical composition of the SBP hydrolyzates and the yield from methane synthesis. This fermentation process also led to the sharpest decrease in the COD (chemical oxygen demand) of the hydrolysate, which was significantly lower than for untreated SBP.

At TUL, SBP residues were successfully codigested in a mixture with sewage sludge. This solution can increase the efficiency of digestion chambers in wastewater treatment plants. Digestate analysis did not indicate any destabilization of the biogas production process, via either volatile fatty acids or ammonia. The highest biogas yields were achieved with SBP residues, both when treated as a monosubstrate and when mixed with municipal sewage sludge (1:1 by weight). The highest level of methane production was observed from codigestion conducted with sewage sludge and 35% residues (Borowski and Kucner, 2015). Simultaneous anaerobic digestion of mixed substrates (codigestion) is an effective option for improving the economic viability of biogas production (Mata-Alvarez et al., 2000).

The possible positive synergies from anaerobic digestion of SPB residues from enzymatic hydrolysis mixed with poultry manure were also evaluated at TUL. Due to an unfavorable C/N balance and the high concentration of LA, the monodigestion of SBP residues required pH adjustment to achieve satisfactory biogas production and stable digestion operation. The addition of poultry manure to SBP residue improved the nutrient balance, and stabilized the pH (Borowski et al., 2016). With SBP hydrolyzate cofermented with stillage (3:1), biogas productivity of 750 mL/g VS was achieved. Biogas yields from separately-fermented SBP and stillage were 13% and 28.6% lower, respectively. Enzymatic pretreatment of SBP hydrolyzate before methane fermentation, causing partial degradation of the component polysaccharides, can increase biogas production considerably (Zieminski and Kowalska, 2015).

Stillage is produced in large volumes during industrial alcohol production. Due to its high level of COD coefficient, stillage is an unused waste product and currently represents a significant economic and ecological issue. Subjecting stillage from distilleries to methane fermentation can help reduce its COD coefficient level and the amount of organic substances in the fermentation base (Cesaro and Belgiorno, 2015). Methane fermentation also eliminates the negative environmental impact of leaving waste lying on fields, where it emits gases including mercaptans, ammonia, and hydrogen sulfide (López et al., 2012). The biogas generated from methane fermentation (a so-called second-generation fuel) can be used in the production of heat and electricity, which may in turn be used to power other technologies that require thermal and electric energy, such as the alcohol fermentation process. Almost all organic substances that are biodegraded during methane fermentation are transformed into biogas (Herbes et al., 2016).

## 3 Conversion of Biomass and Bioproducts into Valuable Chemicals

### 3.1 Acidic Hydrolysis of Sugar Beet Pulp and Sugar Beet Leaves

Acidic hydrolysis is generally the best known and most commonly applied method of chemical hydrolysis for the fermentation of lignocellulosic biomass. The effectiveness of this method is based on the dissolution of hemicellulose and partial hydrolysis of cellulose, but using concentrated acid solutions can lead to issues with serious economic consequences (e.g., corrosion and the need for recovery after hydrolysis), as well as to the formation of toxic inhibitors which reduce the effectiveness of hydrolysis and fermentation. For these reasons, in the research at TUL, we investigated the influence of dilute inorganic (sulfuric, hydrochloric, nitric) acids on the efficiency of enzymatic hydrolysis. Dilute acid pretreatment resulted in the removal and then recovery of most hemicelluloses in the form of dissolved sugars, enabling higher glucose productivity.

Acidic hydrolysis of waste biomass from a variety of sources including SBP, straw, bagasse, corn cobs, oats, and wheat bran can be used to produce furfural. Furfural is widely used as an important renewable building block for products with huge market potential, such as solvents

(tetrahydrofuran), plastics (polyamides, etc.), resins, furfuryl alcohol, and fuel additives (e.g., methyl-tetrahydrofuran, methylfuran) (Rao et al., 1999; Win, 2005; Xing et al., 2011; Zeitsch, 2000). Furfural is also receiving attention as a possible bio-based alternative for use in the production of a wide range of products, from antacids and fertilizers to bioplastics and biofuels (Gruter, 2009; Gruter and Dautzenberg, 2007). Furfural and its derivatives have been used to make jet and diesel fuel-range alkanes (Huber et al., 2005, 2006; Xing et al., 2010) and as a gasoline blendstock (Lange et al., 2012).

The first furfural processes, such as the Quaker Oats process, were batch processes (Hoydonckx et al., 2002; Karinen et al., 2011; Zeitsch, 2000). Currently, most furfural is produced by continuous processes, such as the Westpro-modified Huaxia Technology used in China (Mamman et al., 2008; Win, 2005). A promising technology for improving the energy efficiency and yields is the Supra Yield process (Zeitsch, 2004), which is used for furfural production in Australia. Today, over 85% of furfural is produced in three countries, with China leading the market followed by South Africa and the Dominican Republic (FMI, 2016). Global furfural production stands at around 300,000 tons per year (Mamman et al., 2008).

The Institute of General and Ecological Chemistry at Lodz University of Technology has been developing a method for the acidic hydrolysis of SBP and sugar beet leaves, using $H_2SO_4$ acid (Fig. 13.3), as well as conducting research into the influence of the mode of waste biomass preservation (freezing, ensiling, drying) and length of storage on the yield of furfural. All tested methods of fixation provided high productivity of furfural, comparable with the values for fresh material (Table 13.4.). Long-term storage of ensiled waste biomass did not result in the loss of furfural productivity (Table 13.5). This confirms the usefulness of these methods for the commercial production of furfural.

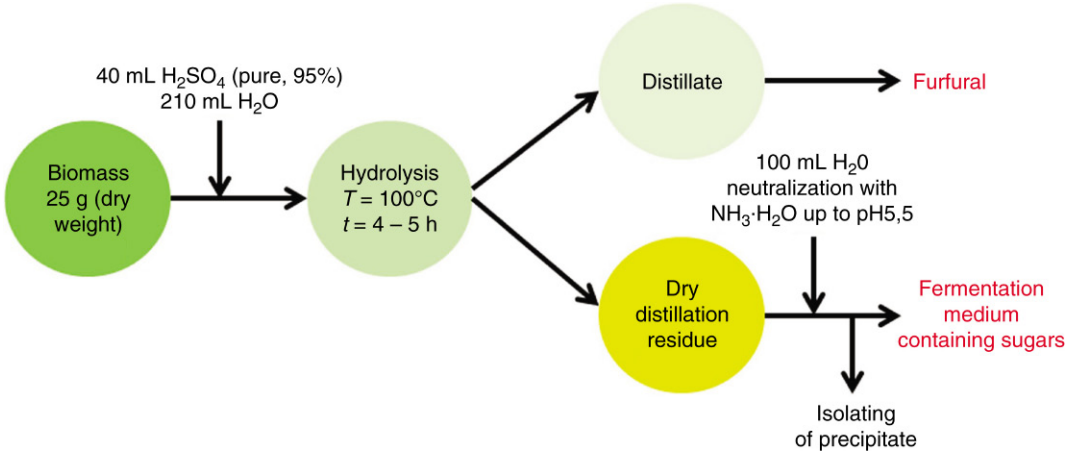


**Figure 13.3: Scheme for Acidic Hydrolysis of Waste Biomass.**

**Table 13.4: Yield of furfural obtained by acidic hydrolysis of waste biomass from the sugar industry.**

| Biomass | | Yield of Furfural (% m/m of d.m.) | | | | Dry Matter (%) |
|---|---|---|---|---|---|---|
| | | Sample 1 | Sample 2 | Sample 3 | $\bar{x}$ | |
| Sugar beet pulp | Fresh | 4.87 | 5.30 | 4.75 | 4.97 | 21.12 |
| | Ensiled | 4.37 | 4.84 | 4.76 | 4.66 | 19.34 |
| | Dried | 4.82 | 4.77 | 4.77 | 4.79 | 92.90 |
| | Frozen | 4.65 | 4.60 | — | 4.63 | 20.02 |
| Sugar beet leaves | Fresh | 1.77 | 0.91 | 1.54 | 1.41 | 19.69 |
| | Ensiled | 1.31 | 1.43 | 1.23 | 1.32 | 46.35 |
| | Dried | 1.95 | 2.17 | 1.76 | 1.96 | 96.20 |
| | Frozen | 2.16 | 1.90 | 2.07 | 2.04 | 18.58 |

**Table 13.5: Yield of furfural obtained by acidic hydrolysis of ensiled waste biomass from the sugar industry.**

| Date of Sampling | | Yield of Furfural (% m/m of d.m.) | | | | Dry Matter (%) |
|---|---|---|---|---|---|---|
| | | Sample 1 | Sample 2 | Sample 3 | $\bar{x}$ | |
| Ensiled sugar beet pulp | 06.12.2015 | 4.37 | 4.84 | 4.76 | 4.66 | 19.34 |
| | 10.01.2016 | 4.60 | 4.07 | 4.41 | 4.36 | 18.27 |
| | 21.02.2016 | 4.00 | 4.36 | 5.32 | 4.56 | 14.81 |
| | 3.04.2016 | 4.65 | 4.80 | 4.75 | 4.73 | 16.51 |
| | 08.05.2016 | 5.98 | 4.84 | 5.20 | 5.34 | 17.03 |
| Ensiled sugar beet leaves | 06.12.2015 | 1.31 | 1.43 | 1.23 | 1.32 | 46.35 |
| | 10.01.2016 | 1.21 | 1.23 | 1.23 | 1.22 | 45.05 |
| | 21.02.2016 | 1.16 | 0.92 | 1.43 | 1.17 | 44.65 |
| | 3.04.2016 | 1.29 | 1.20 | 1.05 | 1.18 | 48.34 |

### *3.1.1 Furfural as a platform chemical*

Biomass is considered as the only sustainable source of energy and organic carbon for the industry and has the potential to displace petroleum in the production of chemicals and liquid transportation fuels (Serrano-Ruiz et al., 2011). In particular, furfural, which is one of the main products of acidic hydrolysis of biomass (Fig. 13.4.), is often cited as a chemical platform compound for the production of chemicals and fuels (Bakhmutsky et al., 2012; Cai et al., 2014). Furfural is already widely used in the chemical industry. Furfural contains a C=O group which undergoes reactions, such as reduction, oxidation, acylation, acetylation, aldolization, and Knoevenagel condensation, decarboxylation, and Grignard reactions, as well as the double bonds of the furan ring (C=C—C=C) which can undergo hydrogenation, oxidation, alkylation, halogenation, ring opening, and nitration (Yan et al., 2014).

Our investigations have been chiefly concerned with furfural reduction. Hydrogenation yields valuable products, including furfuryl alcohol (FA), tetrahydrofurfuryl alcohol (THFA),

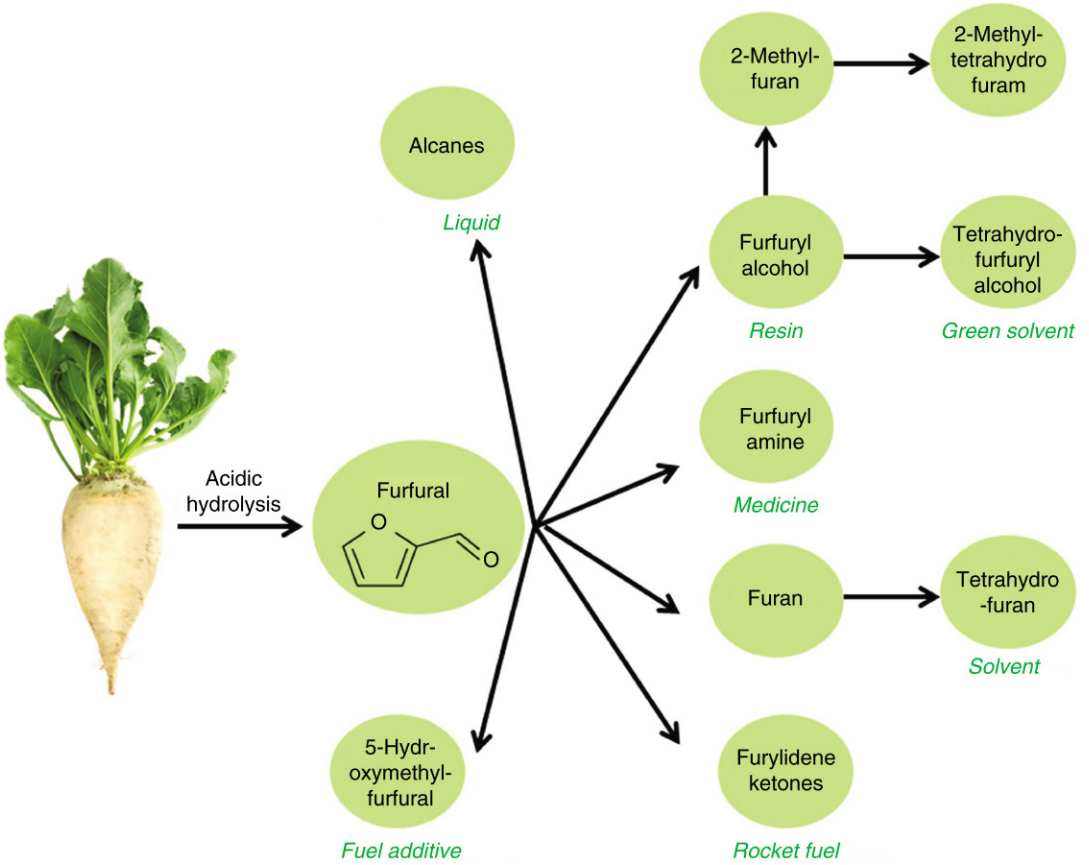


**Figure 13.4: Furfural as a Chemical Platform Compound to Produce Chemicals and Fuels.**

methyl furan (MF), and methyl tetrahydrofuran (MTHF) (Fig. 13.4.), through the reduction of furfural with hydrogen gas over catalysts based on Cu (Bankmann et al., 1997; Rao et al., 1997; Seo and Chon, 1981), Ni (Lee and Chen, 1999; Luo et al., 2001; Wojcik, 1948), on noble metals, such as Ru and Rh, or on Pt complex compounds (Burk et al., 1994). These systems are often promoted using Na, Ca, Co, La, or Ni (Reddy et al., 2007). In our investigations, the process of hydrogenation of furfural in water solution under high pressure hydrogen was performed over supported palladium, platinum, rhodium, and nickel catalysts promoted with other metals (Au, Bi, Cu, In, etc.) (Lesiak et al., 2014; Modelska et al., 2015). The choice of which metal is used as a promoter of 5% $Pd/SiO_2$ systems is very important for determining their selectivity. Higher selectivities to desired products were observed in the cases of each of the bimetallic systems used (5% Pd-1%$M/SiO_2$, M = Tl, Fe, In, Bi, Te, Au). FA was the main product of the reaction using Pd-$Tl/SiO_2$, Pd-$Fe/SiO_2$, and Pd-$In/SiO_2$. Other metallic promoters, such as Bi, Te, and Au, incorporated into a $Pd/SiO_2$ system, resulted in increased selectivity toward THFA and condensation products. Because of the different

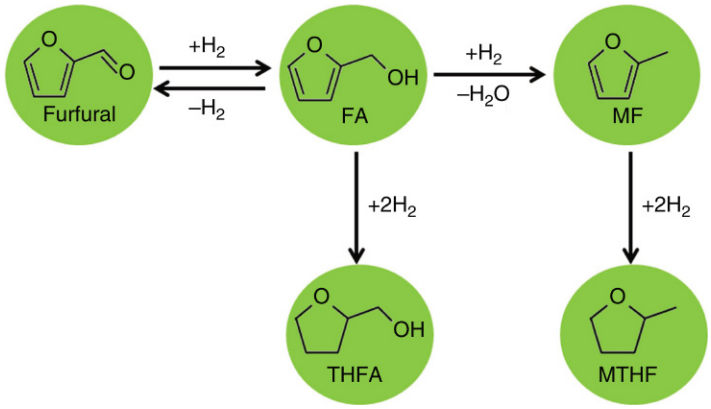


**Figure 13.5: Hydrogenation of Furfural.**
*FA*, Furfuryl alcohol; *MF*, methyl furan, *MTHF*, methyl tetrahydrofuran; *THFA*, tetrahydrofurfuryl alcohol, and furanic components to biofuels.

selectivities observed for bimetallic catalysts, based on Pd, their physicochemical properties were further studied. Activation of the bimetallic catalysts in $H_2$ atmosphere at 300°C caused the formation of intermetallic compounds or alloys. The presence of these intermetallic systems was detected using FTIRS-CO, XRD, SEM-EDS, SIMS-ToF, and TPR-$H_2$ (Witonska et al., 2013). The effect on the performance of palladium-based catalysts caused by the presence of metals depends on the interactions between the two, and on the chemical states of both the Pd and the cometal. Understanding the nature of this interaction is important for the creation of new and stable Pd-based catalysts with the required catalytic properties for the reduction of furfural.

Selective reduction can also give partially hydrogenated products, such as 2,5-dihydroxymethylfuran (Zhu et al., 2015), and fully hydrogenated materials, such as 2,5-bis (hydroxymethyl)tetrahydrofuran (Nakagawa et al., 2013) (Fig. 13.5). Each of these materials can serve as alcohol components for the production of new polyesters, and their combination with 2,5-furandicarboxylic acid (FDCA) leads to a new family of completely biomass-derived products. The use of FDCA as a PET/PBT analog has great potential in a high volume, high-value chemical market.

## 3.2 Valorizing Products of Biosynthesis

### 3.2.1 Biobutanol production from sugar beet pulp

Butanol is an important chemical feedstock, which is used mainly as a solvent or a substrate in the production of polymer materials (e.g., butyl acrylate, butyl metacrylate). It is also of interest as a liquid-fuel additive (Gautam and Martin, 2000). This alcohol is fabricated via chemical pathways based on oxosynthesis (hydroformylation of propene) (Bahrmann et al., 1980) (Cornils and Kuntz, 1995; Lenarda et al., 1996; Wiebus and Cornils, 1996;

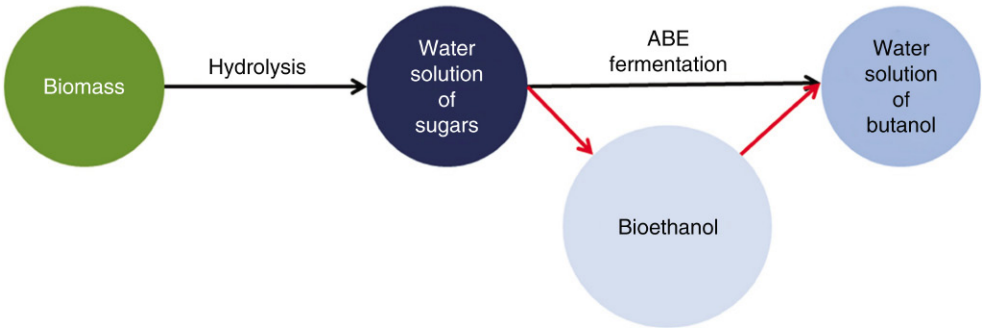


**Figure 13.6: Scheme for Biobutanol Production From Biomass.**

Wilkinson et al., 1981), Reppe synthesis (Bochman et al., 1999) or crotonaldehyde synthesis (Lee et al., 2008), followed by hydrogenation. These processes are performed over metal catalysts using petrochemical materials at high pressure, making them expensive and energy-intensive, especially for producing butanol for use as an additive in liquid fuels.

Industrial processes being considered to produce biobutanol from agricultural biowaste include fermentation using *Clostridia* strains (Jurgens et al., 2012; Kumar et al., 2012) and catalytic coupling of bioethanol produced through classical fermentation (Dziugan et al., 2013, 2015; Ghaziaskar et al., 2013). Unfortunately, these biological routes proceed not to biobutanol but to a mixture of acetone-butanol-ethanol (ABE). Both of these methods require biomass hydrolysis as an initial step (Fig. 13.6). Autohydrolysis of SBP at pH 4 has been established as the best form of pretreatment, improving overall yields of sugars released by enzymatic hydrolysis, and acetone and butanol yields in the fermentation process. Simultaneous consumption of both hexoses and pentoses has been observed from waste biomass using this method for *C. beijerinckii* DSM 6422. A mixture of butanol and acetone was obtained with up to 0.4 g solvents/g sugars (Bellido et al., 2015). However, ABE fermentation has drawbacks, such as the high cost of the fermentation substrate, significant inhibition by butanol, and the low butanol concentration in the product (Kaminski et al., 2011). These limitations result in low butanol productivity, cofabrication of additional by-products, high downstream processing costs for butanol recovery, the formation of mixed solvents, and relatively low butanol content in the crude product. Attempts have been made to develop more economical and sustainable processes for bio-based butanol production, by modifying the metabolic pathways of native *Clostridia* microorganisms (Duerre, 2011; Jang et al., 2012; Tashiro et al., 2013). Relatively little attention has been given to optimize the distillation process (Wang and Li, 2012). A process consisting of liquid–liquid extraction (LLE) followed by stream-stripping distillation may be economically viable (Sanchez-Ramirez et al., 2015). Biobutanol can also be obtained using alternative product recovery techniques, such as ionic liquids, gas stripping, or adsorption (Visioli et al., 2014). For example, ionic liquids, such as 1-hexyl-3-methylimidazolium hexafluorophosphate and

1-hexyl-3-methylimidazolium bis(trifluoromethyl-sulfonyl)imide enable efficient recovery of *n*-butanol from aqueous solutions when the volumes of the liquids are approximately equal (Kubiczek and Kamiński, 2013). Such processes could possibly be employed on an industrial scale, improving the economic viability of biobutanol production processes.

Another way to produce biobutanol is through ethanol fermentation of biomass hydrolysates and catalytic coupling of bioethanol. Based on the results of Ghaziaskar et al. (2013), who noticed high conversion rates of absolute ethanol to butanol on alumina-supported Ni systems, and of Riittonen et al. (2012), we decided to test the activity of 8%–20% $Ni/Al_2O_3$ catalysts, using as substrates rectified alcohol (96% ethanol) and raw spirit (83% ethanol). The use of raw spirit and rectified spirit (crude distillery products) could substantially reduce the costs of butanol synthesis, as it would eliminate the necessity of transporting bioethanol from the distillery to the plant. Dziugan et al. (2015, 2014) employed a continuous-flow process for the catalytic condensation of distillates to butanol on an industrial scale. Using supported nickel systems, a butanol yield of almost 13% was achieved after a single pass of rectified ethanol or raw spirit at 330°C under 120 atm through the catalyst bed. The butanol yield in the reaction mixture was improved (to 31.4% (v/v) by distilling the reaction mixture and recycling the low-boiling fraction into the catalytic reactor. The efficiency of the process of ethanol coupling can be improved again using different catalytic systems in a two-zone reactor. This approach has the potential to be extremely cost effective, since even a relatively small reactor would be sufficient for large-scale production of car fuel additives. The main advantage of these technological solutions is that they do not require the purification and dehydration of rectified spirit. The distillates employed are produced in commercial distilleries and can be used directly in on-line processes of butanol manufacture on an industrial scale.

### *3.2.2 Lactic acid as a platform chemical*

LA can be obtained easily by fermentation of saccharide feedstock (i.e., SBP) and is an extremely useful building block for the synthesis of chemicals, such as acrylic acid, pyruvic acid, 2,3-pentanedione, LA esters, and propylene glycol (Fig. 13.7) (Datta and Henry, 2006; Dusselier et al., 2013). Catalysis is one of the key issues in this platform approach to the commercial transformation of LA into a range of useful chemicals.

#### 3.2.2.1 Production of propylene glycol from lactic acid

TUL researchers, in cooperation with KSC, have investigated a method of propylene glycol synthesis via catalytic hydrogenation of LA obtained through the fermentation of hydrolyzates obtained from SBP. Reduction of LA to propylene glycol was conducted over ruthenium catalysts supported on various carriers, such as $SiO_2$, $Al_2O_3$, and C, under mild temperature conditions and $H_2$ pressure (70–150°C, 3–5 MPa) (Berlowska et al., 2015; Binczarski et al., 2016). This process of propylene glycol production is of interest to industry.

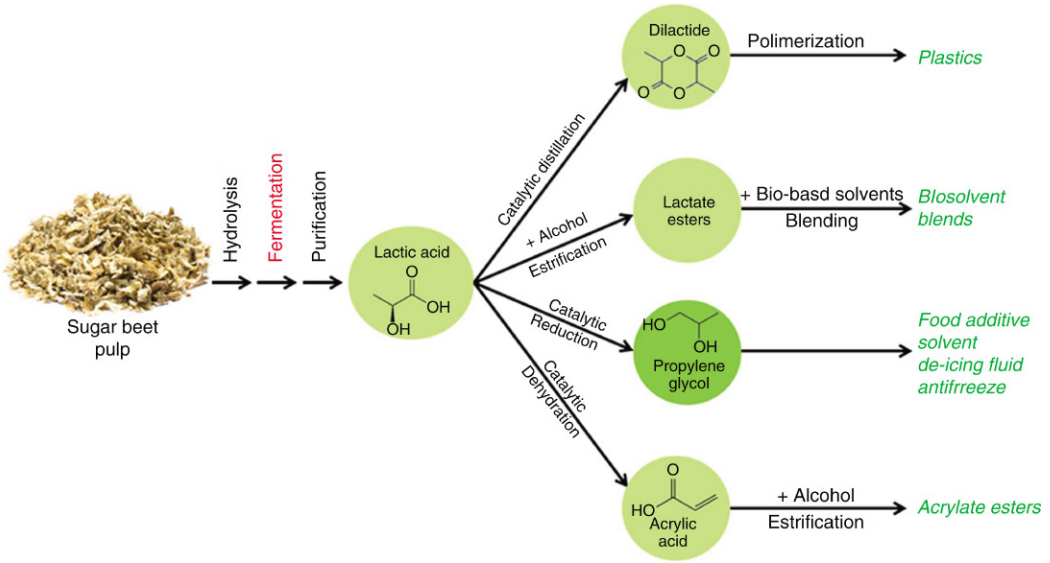


**Figure 13.7: Potential Lactic Acid-Based Products and Uses.**

However, its disadvantage is that it is material intensive (requiring 1.25 kg LA/1.00 kg PG). It is, therefore necessary to reduce costs and increase efficiency. The use of less pure LA as a feedstock is one possibility. In the conventional biological method of pure LA production, the separation and purification stages account for up to 50% of production costs (Corma et al., 2007). Moreover, the sulfide-containing amino acids present in postfermentation broths irreversibly poison the ruthenium catalysts, whereas nonsulfurous amino acids only partially and reversibly poison the catalysts (Zhang et al., 2008). For this reason, it is necessary to develop a new method of treatment for postfermentation broth containing calcium lactate, which would be less expensive than the conventional method of industrial LA production. We decided on the use of active carbon for the purification of acidified to pH 2–3 postfermentation broth, and on the catalytic reduction of diluted water solution of LA over Ru-based supported catalyst. The yield of propylene glycol obtained in this way is satisfactory and the water solutions of propylene glycol obtained can be used in concentrated form as a component in antifreeze.

## 4 Sugar Beet Pulp as a Substrate for Complete Feed Formulation

Fiber products from SBP are generally recognized as safe (GRAS) and relatively simple to produce. Beet fiber contains around 67% carbohydrates, such as cellulose (19%), hemicelluloses (28%), and pectin (18%) and 8% protein (by weight). For human digestion, the dietary fiber availability is above 20% (Finkenstadt, 2013). SBP contains large amounts of pectin which is located, along with other polysaccharides, in the cell walls or extracellular

matrices of the sugar beet root. The main building blocks of SBP-pectin are three structural polymers: homogalacturonan (HG), rhamnogalacturonans I (RG-I), and II (RG-II). HG is built of a backbone of GA units combined by an α-(1,4) linkage that is variably esterified with methanol ($O_6$ carboxyl esters) and acetic acid ($O_2$ and $O_3$ hydroxyl esters). RGI is the second most abundant polymer in pectins, and is made up of chains with alternating units of GA and rhamnose. Linear β-(1,4)-linked galactan and highly branched arabinan, composed of α-(1,5)-linked backbones with α-(1,2) and/or α-(1,3) arabinofuranosyl substitutions, are side chains of RG-I. RG-II is a polysaccharide made up of GA, rhamnose, galactose, and unusual neutral sugars (Ralet et al., 2005; Ridley et al., 2001; Levigne et al., 2002). Commercial pectins are obtained by acid extraction and are used as gelling agents, stabilizers, and emulsifiers in the food and pharmaceutical industries. In comparison to other pectins, sugar beet pectin has a higher proportion of neutral sugar side chains, and higher contents of higher acetic acid, phenolic esters, or protein, to which they are bound in covalent linkages. The gel-forming ability of pectin from sugar beet is very poor in comparison to other types of pectin obtained from other sources. However, sugar beet-derived pectin is reported to show ability of emulsification due to its relatively hydrophobic nature. The higher protein content in pectin from sugar beet means that it has potential for widespread applications in biomaterial science, food, and pharmaceuticals (Cooke et al., 2010; Marry et al., 2000; Rahman et al., 2013).

### 4.1 Protein-Enriched Biomass

The advantages of using microorganisms for SCP production compared with conventional sources of protein (soybeans or meat) are well known. Microorganisms share such features as relatively fast growth and high protein content, allowing for rapid biomass production. The production process can be continuous and independent of environmental conditions. Different types of microbes, such as bacteria, fungi, molds, algae, and yeasts can be used as sources of SCP. Algal single-cell protein has limitations, such as the need for warm temperatures. Fungi, especially yeasts, are particularly suitable for SCP production, as they can be easily propagated using inexpensive raw materials and readily harvested due to their larger cell sizes and flocculation abilities. Various microorganisms, including fungi (*Penicillium, Aspergillus,* etc.), algae (*Chlorella, Spirulina, Laminaria, Rhodymenia*, etc.), bacteria (*Cellulomonas, Lactobacillus, Alcaligenes*, etc.), and yeasts (*Saccharomyces, Candida, Kluyveromyces, Pichia,* and *Torulopsis*) are already used worldwide for food production, as SCP or as components of starter cultures in traditional food production. Of the yeast species, *S. cerevisiae, Kluyveromyces* spp. and *Candida utilis* are fully recognized as safe for human consumption (Bekatorou et al., 2006).

Patelski et al. (2015) demonstrated the possibility of converting SBP into single-cell protein (SCP) using five yeast strains, frequently described as "fodder yeasts," which are used in the preparation of animal fodder: *Trichosporon cutaneum, Candida tropicalis, P. stipitis, Candida*

*guilliermondii*, and *S. cerevisiae*. In studies on the bioconversion of SBP to SCP by four *Candida* spp. strains (*utilis, tropicalis, parapsilosis,* and *solani*), a maximum protein content of 43.4% was achieved in 48 h. *C. utilis* and *C. tropicalis* performed better than the other yeast strains. For *C. tropicalis*, the maximum bioconversion efficiency (46%) was reached in batch flask fermentations. The bioconversion of beet pulp under controlled conditions using *C. tropicalis* and a 51 fermenter, gave 29% and 48% product recoveries and protein levels of 39% and 25%, respectively, in two- and one-stage processes. The one-stage process (simultaneous saccharification and fermentation) was also run in a larger volume and gave 50% product recovery with 29% protein content (Nigam and Vogel, 1991; Singh et al., 2014). In studies conducted by Ghanem (1992), a satisfactory SCP yield was achieved from a mixed culture of *T. reesei* and *Kluyveromyces marxianus* grown on SPB. In accordance with both FAO guidelines and the profile of soy bean oil, the protein synthesized in this way contained all the essential amino acids.

Patelski et al. (2015) studied the possibility of producing yeast biomass after enzymatic hydrolysis of SBP. Preparations of Viscozyme and Ultraflo Max were used for hydrolysis, and from 250 kg of wet beet pulp 375 L of hydrolysate were obtained. *T. cutaneum*, *C. tropicalis, P. stipitis*, *C. guilliermondii,* and *S. cerevisiae* were grown on SPB hydrolysate after enrichment with sources of mineral compounds (N, P, K, Mg). The highest biomass yield from shake cultures was observed for *C. tropicalis*, with a protein content of 52.3% of dry mass.

### 4.2 Prebiotics

An economically important aspect of SBP management is the valorization of its nutritional content as animal feed. From January 1, 2006, the European Union introduced in all member countries, a total ban on the use of antibiotic growth promoters in animal feed [Regulation (EC) No 1831/2003]. Instead, alternative nutritional amendments in the form of probiotics, prebiotics, enzymes, herbs, and minerals should be used to strengthen and stabilize the natural resistance of beneficial microflora in the digestive systems of animals. Feed additives should be selected based on their ability to stimulate more efficient production of farmed animals, to provide nutrition, and ensure food security. Scientists and nutritionists have conducted several studies confirming the positive effects of the use of pro- and prebiotics, especially for young animals. The health benefits of prebiotic oligosaccharides contribute to increase the efficiency of livestock production by improving animal health (reducing the incidence of diarrhea), heightening resistance to stress, reducing the number of falls, increasing body weight, and improving the use of feed nutrients (Cho et al., 2011; Gaggìa et al., 2010; Hajati and Rezaei, 2010). The use of SBP to produce prebiotics may offer a new and economically viable application. The market for new-generation animal feeds is still wide open.

Probiotics confer a health benefit on the host by maintaining the normal intestinal microbiota, preventing the growth of pathogenic microorganisms, promoting digestion and intake of feed, and stimulating the immune system (UN FAO, 2006a,b). Prebiotics, on the other hand, are unviable nutrients that have beneficial effects on the health of the host due to the modulation of intestinal microbiota (FAO, 2007). They differ from other fermentable carbohydrates in that they interact with selective microorganisms. Organisms associated with health benefits are stimulated, shifting the composition of the intestinal microbiota toward beneficial organisms and selectively promoting the growth of probiotics. A synbiotic is a product which contains both prebiotics and probiotics and in which the prebiotic compound selectively favors the probiotic compound. Synbiotics benefit the host by improving the implantation and survival of live microbial dietary supplements in the gastrointestinal tract, activating the metabolism, and selectively stimulating the proliferation of health-promoting bacteria (Gibson and Roberfroid, 1995). Nondigestible oligosaccharides (NDOs) are the best-known prebiotics. Many oligosaccharides with prebiotic properties (e.g., fructooligosaccharides and galactooligosaccharides) are commercially available, but there is increasing interest in the development of new prebiotics, with added functionalities. Some prebiotics can be obtained by extraction from plants or fruits. However, most are industrially synthesized by chemical or enzymatic methods. SBP, a low-value and very common waste from the sugar industry, could compete with other materials for use in the production of prebiotic oligosaccharides, especially pectin-derived oligosaccharides (POS), which are promising candidates for new-generation prebiotics.

SBP is a generally unexploited source of cell wall polysaccharides, such as pectin, which can be converted into higher value products. Pectin isolated from SPB differs considerably from that isolated from fruit cell walls, in terms of its structure and physicochemical properties, because of the different composition and functional adaptations of the beet root as an underground storage organ. Compared to fruit pectin, pectin isolated from SBP is characterized by lower GA content, lower molecular weight, lower degree of methyl esterification, and a higher degree of acetyl esterification (Fishman and Jen, 1986). These features contribute to the weak gelling properties. However, the composition of SBP suggests that it could be a suitable starting material for obtaining a variety of biologically active NDOs, such as oligogalacturonides (OGaU), arabino oligosaccharides (AOS), and galacto oligosaccharides (GaOS).

Several methods are available to obtain POS from SPB. The breakdown of pectic polymers into soluble hydrolysis products can be achieved by treatment with hot, compressed water (hydrothermal or autohydrolysis treatments). Alternatively, the samples can be subjected to hydrothermal processing under nonisothermal conditions. Experiments conducted in a temperatures range of 160–175°C yielded comparatively high concentrations of both oligogalacturonides and arabino oligosaccharides. POS can be also produced by

enzymatic hydrolysis. Different enzymatic preparations have been tested for this purpose. Enzymatic hydrolysis of SPB pectin with a combination of endopolygalacturonase and pectinmethylesterase yielded three pectic oligosaccharides. These were composed of a mixture of polymers of different molecular weights and with different GA contents (Combo et al., 2013). Arabinohydrolases have also been used in the enzymatic hydrolysis of SBP arabinan, producing neutral branched arabinooligosaccharides (Westphal et al., 2010). Colquhoun et al. (1994) determined the structure of feruloylated oligosaccharides obtained by enzymic degradation or mild acid hydrolysis of SBP. Feruloylated oligosaccharides were identified as being derived from pectic neutral side-chains containing arabinose or galactose residues. In the feruloylated arabinose oligosaccharides, feruloyl groups were linked to 0–2 of l,-Araf residues.

POS are known to exhibit different biological activities, such as immunomodulation (Matsumoto et al., 2008) and anticancer activities (Manderson et al., 2005; Olano-Martin et al., 2002). Moreover, POS have been reported to have prebiotic potential, since they selectively increase the populations of beneficial bacteria in the gastrointestinal tract. Vigsnaes et al. (2011) studied the potential prebiotic properties of AOS released from rhamnogalacturonan I during sequential acid extraction of pectin from SBP. Al-Tamimi et al. (2006) determined the fermentation profiles of arabinooligosaccharides, following varying degrees of polymerization, by human gut bacteria. Bifidobacteria were stimulated to different extents, depending on the molecular weight of the POS.

Our research is ongoing into the possibilities for producing prebiotics from SBP through enzymatic hydrolysis combined with fermentation (SSF) and the use of probiotic bacteria, or for producing synbiotic animal feed with yeast. If this research proves fruitful, SSF could contribute not only to increasing the efficiency of enzymatic hydrolysis by limiting the effect of product inhibition, but could also enrich the final product in terms of nitrogen content and probiotic activity. Rational use of SBP could further contribute to reducing the environmental impact of industrial sugar production and to increasing the pool of materials available for use as supplements in animal feed.

## 5 Conclusions

Sugar beet is an important crop in almost all European countries. This green carbohydrate-rich material is currently used principally in foodstuffs and as animal feed, but new opportunities are available for a wide range of potential applications in the chemicals industry and material science. Using biorefinery technologies, biomass carbohydrates can now be released for industrial applications, as well as for animal feed and foodstuffs. There is clearly both a need and an opportunity to use SBP in sustainable, economically viable, and ethical ways. The complete utilization of sugar beet should be examined in the context of a bio-based economy. We hope that this chapter has provided readers with an insight into the environmental, economic, and social value of SBP.

## Acknowledgments

The authors would like to acknowledge the contribution of the Applied Research Programme of the National Centre for Research and Development—Project "Sugar beet pulp biomass as a raw material for new fermentation media" PBS1/B8/3/2012 and Biostrateg Programme—Project "Processing of waste biomass in the associated biological and chemical processes" BIOSTRATEG2/296369/5/NCBR/2016.

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CHAPTER 14

# Biofilms in Food Industry: Mitigation Using Bacteriophage

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## 1 Introduction

Bacteriophages play a role in biocontrol of microbes in fresh foods, without compromising the viability of other normal flora or food quality. However, there is little published material on biofilms–bacteriophage interactions (Laxmi and Bhat, 2015). Phages can infect and grow within their specific hosts even if they are antibiotic resistant. Host specificity is commonly observed at a strain level, species level, or, more rarely, at genus level. This specificity led to their application in directed targeting of dangerous bacteria (Hagens and Loessner, 2010). Lytic phages with their ability to adhere to bacteria and integrate into their cellular machinery, utilize host resources to reproduce, with the destruction of the bacterial cell causing phage release. Virulent (strictly lytic) phages are therefore the clear choice for food safety applications (Mahony et al., 2011).

Biofilm formation being an important source of contamination is a significant problem in the food industry, since it causes food spoilage or transmission of diseases (Di Bonaventura et al., 2008). The safety of bacteriophages in preserving food products is assured because they are nontoxic and ubiquitous in foods (Bruttin and Brüssow, 2005). Much of the phage preparations used in foods are reported to be safe for use as preservatives and GRAS (Gerner-Smidt et al., 1993).

Among pathogenic bacteria, *Pseudomonas aeruginosa,* with multiple virulence factors, is an important human and plant pathogen, responsible for various infections (Deep et al., 2011). The notable ability of *P. aeruginosa* to form biofilms in many environments renders antibiotic treatments incompetent and therefore promotes chronic infectious diseases through contamination in many industries (Bjarnsholt, 2013; Hoiby et al., 2010). Confronting the confinements of common antibiotics (Rasamiravaka et al., 2015), there is an expanding requirement for the disclosure and the advancement of antimicrobial agents that present novel or unexplored properties to efficiently control and manage bacterial diseases that spread in the food and processing industry.

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00014-1

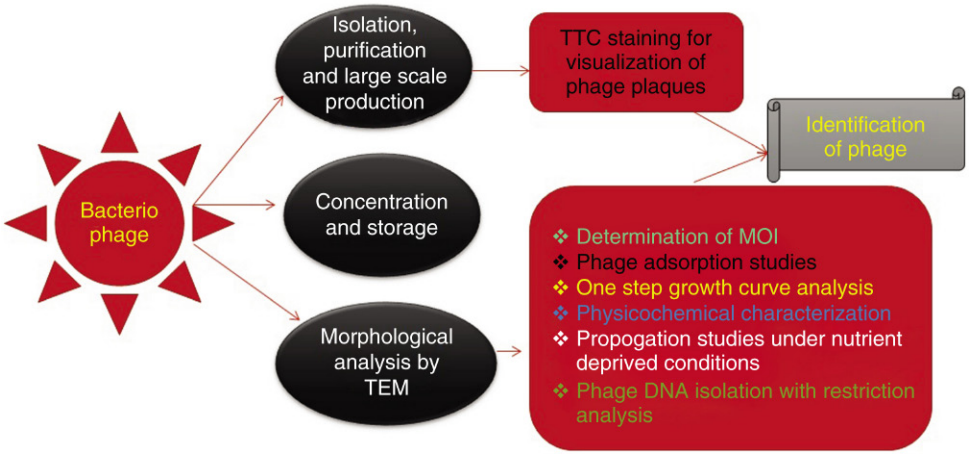


**Figure 14.1: The Illustration for the Steps in Identification of Bacteriophage Used in the Study.**

There are numerous studies with promising outcomes on phage utilization to control biofilm development by *Pseudomonas sp.* (Knezevic et al., 2011; Pires et al., 2011). The absence of bacterial resistance against enzymes, such as lysins, is considered a major advantage in using phage lysins and other enzymes of phage origin (Fischetti, 2010); as the bacteria would have to modify the structure of its cell wall to avoid enzymatic action.

This chapter provides evidence of the biocontrol ability of the phage ΦPAP-1 and its proteins against biofilms formed by host *P. aeruginosa* strain BTRY1. Toward this, various sections deal with the isolation, purification, and characterization of the bacteriophage and its host, (Fig. 14.1) followed by host range studies, extraction of phage proteins and their antibiofilm activity thereby proving its ability as biocontrol agent (Fig. 14.2).

## 2 Methodologies Adopted

### 2.1 Host Culture

The host, *P. aeruginosa* BTRY1 was isolated from milk sample obtained from local markets at Kochi, Kerala, India. The isolate was confirmed to be *P. aeruginosa* by the amplification of the 16S rRNA gene (1.5 kb) from the genomic DNA. The sequence of the isolate was retrieved with genbank accession number KF460558 (Laxmi and Bhat, 2014).

### 2.2 Bacteriophage Isolation

The milk sample was centrifuged at 4000*g* (Sigma, 3K30, Germany) for 10 min at 4°C, filtered through 0.22 µM membrane filter (Millipore, USA) to make it bacteria-free and then the filtrate was screened for bacteriophage by double-agar overlay method (Adams, 1959;

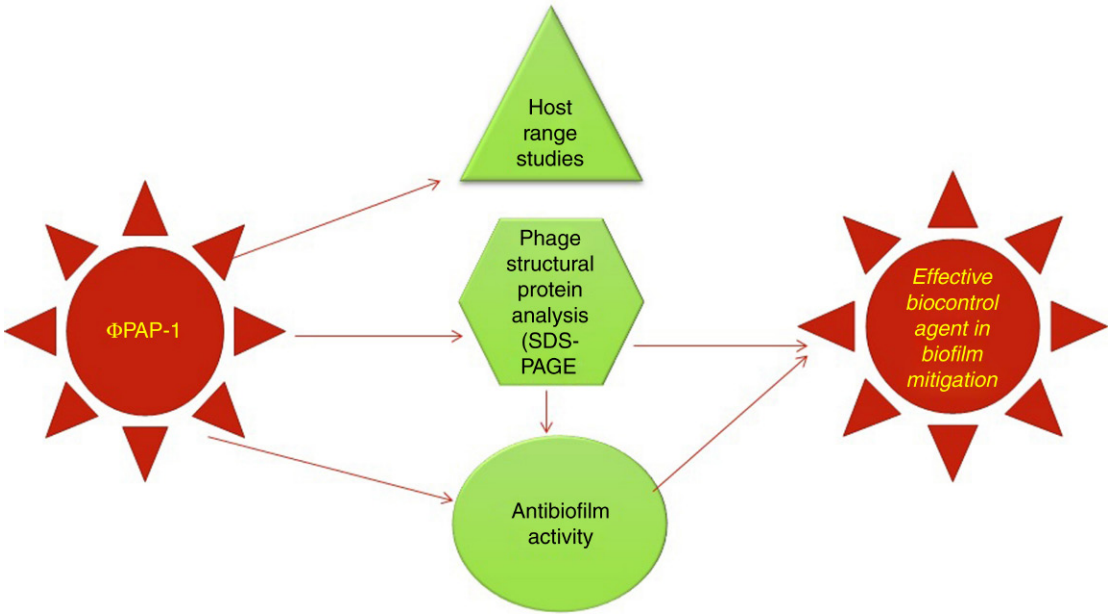


**Figure 14.2: Different Methods Proving Bacteriophage as a Biocontrol Agent.**

Anderson et al., 2011). Tetrazolium staining revamps phage plaque visibility against the backdrop of bacterial growth. Each plaque appears as a sharp, clear area against the intense red background produced by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to an insoluble formazan by the bacterial cells (Pattee, 1966). The petri plates with plaques were then flooded with 10 mL of trypticase soy broth (HiMedia) containing 0.1% of TTC (HiMedia, Mumbai). After incubation for 20–30 min at 37°C, the broth was poured off and the plaques were observed.

### *2.3 Phage Purification and Large Scale Production*

Purification, large scale production, and concentration of phage lysate were as per Sambrook et al. (2000). Aimed at purification, a single plaque picked with a sterile tooth pick was introduced into 3 mL of log phase host culture. Incubated at 37°C in an environmental shaker (Orbitek, Scigenics, India) for 16 h at 120 rpm, then centrifuged at 10,000*g* followed by filtration through 0.22 µM membrane (Millipore, USA). The lysate was used for double agar overlay. This procedure was repeated 5–6 times until even sized plaques were obtained. For mass or large-scale production, plates with uniform plaques were overlaid with 10 mL of SM buffer, incubated overnight with gentle rocking and the phage suspension recovered and pooled. Chloroform was added at a final concentration of 5% (v/v), assorted well using a vortex mixer, and incubated at room temperature for 15 min. The cell debris was discarded by centrifugation at 5000*g* for 10 min. The supernatant subsequently was transferred to a sterile

polypropylene tube, added chloroform at 0.3% (v/v) concentration, and stored at 4°C until use. Plaque forming unit (PFU) of $1 \times 10^{10}$/mL was produced following this method.

### 2.4 Phage Concentration and Storage

Phage was concentrated using polyethylene glycol (PEG) 6000 (Sambrook et al., 2000) with modifications (Li and Zhang, 2014). Concisely, 1% (v/v) overnight culture of the host bacteria was transferred to 200 mL nutrient broth (Himedia, Mumbai, India), and incubated at 37°C for 3.5 h in an environmental shaker at 100 rpm (Orbitek,Scigenics, India). Phage was added at a multiplicity of infection (MOI) of 0.2 and incubation continued for 12–16 h at 37°C with 100 rpm. This broth was centrifuged at 10,000*g* for 20 min (Sigma, 3K30, Germany), supernatant filtered through 0.22 µM membrane filter (Millipore, USA). RNAase (Bangalore Genei) and DNAase I (Bangalore) were added at a final concentration of 1 µg/mL each, and incubated at room temperature for 30–35 min. Solid NaCl was added at a final concentration of 1 M and then dissolved by stirring. The mixture was kept in ice for 1 h, trailed by centrifugation at 11,000*g* for 10 min at 4°C. Solid PEG 6000 (SRL, India) was added to the supernatant at a final concentration of 10% (w/v), dissolved by gentle stirring on a magnetic stirrer at room temperature. This was then kept in ice overnight and centrifuged at 11,000*g* for 10 min at 4°C. The supernatant was cast-off completely, while the pellet was resuspended in 5 mL of phosphate buffered saline (PBS). PEG and cell debris were removed from the phage suspension by the addition of an equal volume of chloroform, vortexing for 30 s, followed by centrifugation at 3000*g* for 15 min at 4°C. The aqueous phase containing the phage particles were recovered and stored at −20°C.

### 2.5 Maintenance and Storage of Phages

Phage lysate for long term storage was maintained employing two methods, namely storage at 4°C as such and as glycerol stock (Jeena and Bhat, 2012).

### 2.6 Characterization of Phages

#### 2.6.1 Morphological analysis

Transmission electron microscopy (TEM) was used for the morphological analysis. A drop of high titer phage sample was spotted on a carbon-coated TEM grid, permitted to settle for 2–3 min and excess of sample removed by blotting. A drop of 2% phosphotungstic acid hydrate (Sigma-Aldrich) used for negative staining (Bock et al., 2007; Quintarelli et al., 1971) was placed on the spot, allowed to react for 2–3 min and drained off by poignant a blotting paper strip to the edge of the grid. The grids were dried for 15 min, examined and photographed using a transmission electron microscope (Model Jeol/JEM 2100 2000X) operated at 200 kV at Sophisticated Test and Instrumentation Centre, Kalamassery, Kerala. Phage morphology was observed from the micrographs.

### 2.6.2 Optimal multiplicity of infection

MOI is the ratio of phage particles to host bacteria, calculated by dividing the number of phage added (volume in mL × PFU/mL) by the number of bacteria added (volume in mL × colony forming units/mL). MOI giving maximum yield was determined as optimal MOI (Li and Zhang, 2014).

### 2.6.3 Phage adsorption studies

The first step in the growth of bacteriophage is a process called adsorption, which is the attachment to susceptible bacteria. Adsorption studies were carried out as per Augustine et al. (2013a). Log phase of host culture was infected by optimal phage MOI and incubated at 37°C. After 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min time intervals following infection aliquots of 5 mL were sampled. All were immediately filtered through 0.22 μM membrane filter (Millipore, USA) and the phage titer was determined using double agar overlay method after appropriate dilutions. All plating's were done in triplicates and appropriate controls were maintained. The percentage of phage adsorption was calculated as follows: [(control titer − residual titer)/control titer] × 100%. The phage titer observed at time zero was considered as the control.

### 2.6.4 One-step growth curve

One step growth curve was executed and the graph plotted with log of PFU against time was used to calculate the latent period, the rise, and the burst size of the phage (Capra et al., 2006). Mid log phase culture of the host (200 mL) was harvested by centrifugation at 9000 × *g* for 10 min and resuspended in one-fifth of the initial volume (40 mL) of prewarmed nutrient broth. The phage suspension at the optimal MOI was added, allowed to adsorb for 15 min at 37°C, harvested by centrifugation at 10,000*g* (Sigma, 3K30, Germany) for 5 min and resuspended in 200 mL nutrient broth. This was incubated at 37°C. Samples were taken at 10 min intervals (up to 2 h) and immediately tittered by the double agar overlay method. Assays were carried out in triplicates and proper controls were maintained. The graph was plotted with log of PFU/mL against time. The latent period, the rise period, and the burst size of the phage were estimated from the one step growth curve.

### 2.6.5 Influence of physical and chemical parameters on phage viability/infectivity

The influence of temperature on phage dissemination was studied at temperatures ranging from 50 to 100°C (Lu et al., 2003). To study the impact of NaCl on phage viability, solutions of NaCl with wavering concentrations, such as 0.1, 0.25, 0.5, 0.75, 1, 2, and 3 M were prepared in deionized water and incubated for 30 min at 37°C. Effect of pH on phage infectivity was assessed by incubating the phages in suitable buffers of different pH, ranging from 2 to 11. Impact of sugars, such as sucrose, dextrose, galactose, fructose, maltose, mannitol, mannose, lactose, rhamnose, ribose, and xylose on phage viability was

also studied (Capra et al., 2006) with little modifications. Sugars were added to a final concentration of 500 mM to each phage sample. All samples after incubation were tested using double agar overlay plate method to conclude the number of surviving PFU. The results were compared with control titer in case of effect of sugars and then expressed as a percentage of phage inactivation. All the experiments were performed in triplicates and plotted with ±SD.

#### 2.6.6 Influence of physical and chemical parameters on phage adsorption

The adsorption of phages on the host *P. aeruginosa* BTRY1 was determined at temperatures of 0, 10, 20, 30, 37, 40, 45, and 50°C. Impact of various concentrations of NaCl (0.1, 0.25, 0.5, 0.75, and 1 M) on adsorption was also investigated. Adsorption rate of ΦPAP-1 was determined at the pH values ranging from 2 to 11 (Son et al., 2010). The influence of calcium ions (0, 1, 10, 20, and 30 mM concentrations) on phage propagation was determined (Lu et al., 2003). In all experiments, the supernatant obtained after centrifugation was assayed using double agar overlay method for unabsorbed free phages and the counts were compared with control titer. The results were stated in percentages of adsorption. All the experiments were conducted in triplicates and plotted with ± SD.

#### 2.6.7 Effect of optimized physicochemical parameters on phage propagation

The collective effect of all the parameters optimized was studied (Augustine et al., 2013a). Phage lysate was added at its optimum MOI to mid log phase host cells grown in nutrient broth (pH adjusted to 8 with optimal NaCl and $CaCl_2$ concentrations). The incubation temperature was set at optimum. One step growth curve experiment was repeated as described in Section 2.6.4. Aliquots were sampled at 10 min intervals, mixed with mid log phage host cells grown in nutrient broth followed by incubation for 30 min and were immediately tittered by double agar overlay method. All plating's were done in triplicates. Proper controls were maintained. The graph was plotted with log of PFU/mL against time. The latent period, the rise period, and the burst size of the phage were calculated.

### 2.7 Bacteriophage genome analysis

Phage DNA extraction was as per Sambrook et al. (2000) and Li and Zhang (2014) with modifications. Concisely, 1 mL of the concentrated phage suspension was incubated at 56°C with proteinase K (50 µg/mL) and sodium dodecyl sulfate (SDS) (0.5%) for 1 h. After incubation, the digestion mix was extracted once with an equal volume of phenol, once with 50:50 phenol and chloroform, and finally with equal volume of chloroform. The DNA was then precipitated with double the volume of ethanol in presence of sodium acetate and dissolved in Tris EDTA (TE) buffer (pH 7.6). Finally, the DNA was run on 1% agarose gel, stained with ethidium bromide, and visualized in UV light. The image of the gel was captured using gel documentation system (Syngene, United Kingdom).

The restriction pattern of phage DNA was studied using *Bam* HI. (Fermentas, USA). Enzyme digestions were performed as mentioned by the manufacturer. Each 20 µL digestion solution containing 1 µg of bacteriophage DNA and 1 U of the restriction enzyme in reaction buffer was incubated for 1 h at reaction temperature. Restricted fragments were separated by agarose (1.0%–1.2%) gel electrophoresis, stained with ethidium bromide, and visualized in UV light. The image of gel was captured using gel documentation system (Syngene, UK) (Augustine et al., 2013a).

## 2.8 Phage Host Range Determination

The host range of the phage ΦPAP-1 was assessed on the basis of their ability to form plaques on respective test strains by spot test assay (Jensen et al., 2015). Two phages were tested against 20 strong biofilm producers (Laxmi and Bhat, 2014) namely *Bacillus altitudinis strains (BTMW1*, *BTMG1*, *BTCW2*, *BTMW3*, *BTPW1*, and *BTTP1), Bacillus pumilus strains (BTMY2, BTMW2, BTMY4,* and *BTCP1), P. aeruginosa (BTRY1), Brevibacterium casei (BTDF1), Staphylococcus warneri (BTDF2), Micrococcus luteus strains (BTDF3, BTFF1), Micrococcus sp (BTDP2), Bacillus niacini (BTDP3), Bacillus sp (BTSD1), Bacillus licheniformis (BTSD2)*, and *Geobacillus stearothermophilus (BTFF2).*Host range studies was also conducted using NCIM cultures including *P. aeruginosa (2863), Salmonella Typhimurium (2501), Escherichia coli (2343),Klebsiella pneumoniae (2957), Proteus vulgaris (2027), Clostridium perfringens (2677), Staphylococcus aureus (2127), Bacillus cereus (2155), Bacillus pumilus (2189), Bacillus circulans (2107)*, and various isolates from the culture collection of Microbial Genetics laboratory, CUSAT that include *Vibrio diabolicus (TVMS3), Vibrio alginolyticus (KK16), Vibrio harveyi (KKS4), Vibrio parahemolyticus (KK10), Salmonella enteritidis (S37)*, and *S. enteritidis (S49).* Phage lysate was added at their exponential phase, incubated for 1 h and plated using the double agar overlay method. All platings were performed in triplicates. The plates were incubated at 37°C and were observed for plaques.

## 2.9 Propagation of Phage Under Nutrient Depleted States of the Host Cell

Phages recommended for use as biocontrol agents have an added advantage, when they have the ability to infect host under stationary as well as various nutrient deprived conditions. Thus the ability of the phage to infect host under different nutrient deprived conditions was studied.

### 2.9.1 Preparation of log- and stationary-phase, starved- and nutrient-depleted cultures

The host cells grown as overnight culture at 37°C were used for the stationary-phase cell infection experiments. A fresh 6 h culture was taken for the exponential-phase host cell infection experiments. The starved host cells were made by resuspending cell pellet obtained after centrifugation of a 6-h-old culture, in an equal volume of physiological saline, and incubating it for 24 h at 37°C (Kadavy et al., 2000). Nutrient depleted cultures were prepared by growing bacteria to log-phase in nutrient broth, and the cells harvested by centrifugation

were resuspended in appropriate starvation suspension media, like minimal medium without carbon, minimal medium without phosphate, and minimal medium without ammonium chloride (nitrogen source) (Nystrom et al., 1992). In all cases except for stationary-phase, the $O.D_{600}$ of cultures was adjusted to 0.5 ($1 \times 10^5$ colony forming units CFU/mL) using respective medium prior to the addition of the phage. Phage was introduced into each of these cultures at a very low MOI of 0.1, incubated at 37°C for 24 h and plated (double agar overlay method) to observe the efficacy of phage multiplication under various nutrient deprived conditions. All platings were in triplicates with suitable controls.

Statistical evaluations were done by ANOVA, followed by Newman–Keuls Test and Tukey Test using StatsDirect statistical software (version 2.8.0, Cheshire, United Kingdom) computer program.

## 2.10 Phage Structural Protein Analysis

### 2.10.1 Nonreductive sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel electrophoresis (PAGE) of extracted phage proteins was performed under nonreducing conditions for evaluating the nature of the phage capsid protein using vertical slab electrophoresis (Genei, Bangalore, India) by the method of Laemmli (1970) as adopted by Sambrook et al. (2000). The protein marker from New England Biolabs, United Kingdom was kept as standard and molecular weight was determined using Image J software (Image J 1.49v/Java 1.6.0_24, 64-bit).The gel plates were cleaned and assembled. Resolving gel (16%) of 5 mL was prepared by mixing 2.65 mL of acrylamide: *bis*-acrylamide (30:0.8), 1.25 mL of resolving gel buffer stock, 100 µL of 10% SDS and 1.05 mL of water followed by 100 µL of ammonium persulfate solution (10%), and *N,N,N',N'*-tetramethylethylenediamine (TEMED) (10 µL). The mixture was immediately poured into the cast and a layer of water was added over the gel and allowed to polymerize for at least an hour. Water layer was poured following polymerization. The stacking gel (4%) of 2.5 mL was prepared by combining 0.425 mL of 30:0.8 acrylamide: *bis*-acrylamide solution, 0.625 mL of stacking gel buffer stock, 25 µL 10% SDS, and 1.425 mL of distilled water, followed by 25 µL of ammonium persulfate, and 2.5 µL of TEMED. The stacking gel was then poured into the gel assembly, above the resolving gel and the comb immediately inserted. Gel was allowed to polymerize for 30 min, placed in the electrophoresis apparatus and upper and lower reservoirs filled with reservoir buffer and was prerun for 1 h at 80V (Jeena and Bhat, 2012).

### 2.10.2 Sample preparation

Phage sample was prepared by mixing 15 µL of 1X sample buffer for nonreductive SDS-PAGE with concentrated phage lysate. This sample and 5 µL low molecular weight marker (New England Biolabs, UK) was loaded on to the gel and run at 80 V. The current was increased to 100 V, when the dye front entered the resolving gel. The run was stopped when

the dye front reached 1 cm from the lower end of the plate, the gel was removed and stained (Jeena and Bhat, 2012).

#### *2.10.3 Silver staining*

The gel was fixed for 30 min in fixing solution 1, followed by incubation in fixing solution 2 for 15 min. This gel was washed 5 times in water, each for 5 min duration. Sensitized the gel in freshly prepared sensitizer for 1 min and washed in water twice for 2 min each. The gel was then incubated in staining solution for 25 min at 4°C, washed twice, each for 1 min duration, and then incubated in developing solution until the bands appear. To prevent over staining, the gel was treated for 10 min in sodium EDTA, washed in water twice for 2 min each. The image of gel was captured using gel documentation system (BIORAD, USA) (Jeena and Bhat, 2012).

### *2.11 Antibiofilm Activity of Whole ΦPAP-1 and Proteins Extracted from ΦPAP-1*

The antibiofilm activity of whole phage and proteins (100 µg/mL each) extracted from ΦPAP-1 was tested against nine strong biofilm producing strains (Laxmi and Bhat, 2014) using the standard microtiter plate assay as a semiquantitative method (Rode et al., 2007). Briefly, 220 µL of tryptone soy broth (TSB) was added to the wells of 96-well microtiter plate, followed by 20 µL each of the bacterial culture ($OD_{600}$ = 1), in triplicates for each test organism and incubated aerobically for 24 h at 37°C. 10 µL of whole phage and phage proteins were added to respective wells and incubated for 24 h at 37°C. The contents of the plates were poured off; washed thrice with phosphate buffer (0.01 M, pH 7.2), and attached bacteria were fixed with methanol. After 15 min, plates were decanted, air dried, and stained with 1% crystal violet for 5 min and excess stain rinsed under running tap water and airdried. The dye bound to adherent cells was extracted with 33% (v/v) glacial acetic acid, and measured at 570 nm using a UV-VIS spectrophotometer (Schimadzu, Japan). TSB served as negative control. Positive control for the assay is those particular wells containing only the test microorganisms. All tests were repeated thrice independently and statistically analyzed (Christensen et al., 1988; Stepanović et al., 2000).

Finally, the percentage reduction in biofilm formation was calculated as: percentage in biofilm reduction = (OD of Control − OD of Test/OD of Control) × 100 statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program (Laxmi and Bhat, 2015).

## *3 Observations and Findings*

### *3.1 Bacteriophage Isolation, Purification, Concentration, and Storage*

A lytic phage producing large, clear plaques was obtained on the lawn of the host strain *P. aeruginosa* (BTRY1) from milk sample and named as ΦPAP-1. The tetrazolium stained plate showing plaques by phage ΦPAP-1 on bacterial lawn of host is as presented in Fig. 14.3.

**Figure 14.3: Tetrazolium Plates Showing Plaques Formed by Phage on Bacterial Lawn (ΦPAP-1) on *Pseudomonas aeruginosa* BTRY1.**

ΦPAP-1 was concentrated up to $1 \times 10^{10}$ PFU/mL using PEG precipitation and the phage concentrate was used for all further studies. Glycerol stocks of ΦPAP-1 were maintained at −80°C.

## *3.2 Characterization of Phage*

### *3.2.1 Morphological analysis*

TEM is routinely used in the morphological characterization of phage. TEM elucidated morphology has pronounced significance as it forms the basis for the classification of

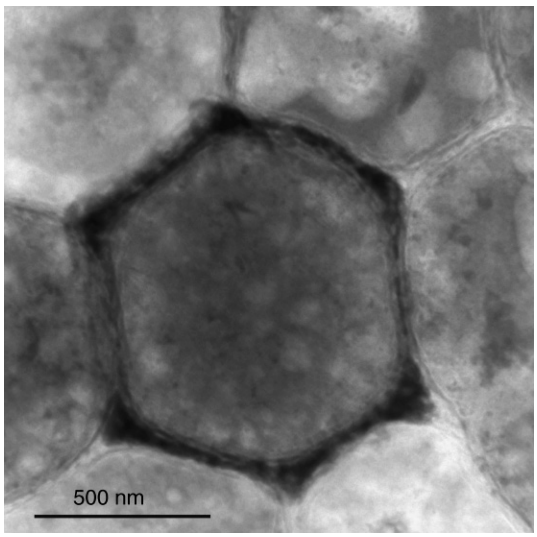


**Figure 14.4: Transmission Electron Micrograph Image of ΦPAP-1 Stained With 1% Phosphotungstic Acid Hydrate (Bar Represents 500 nm).**

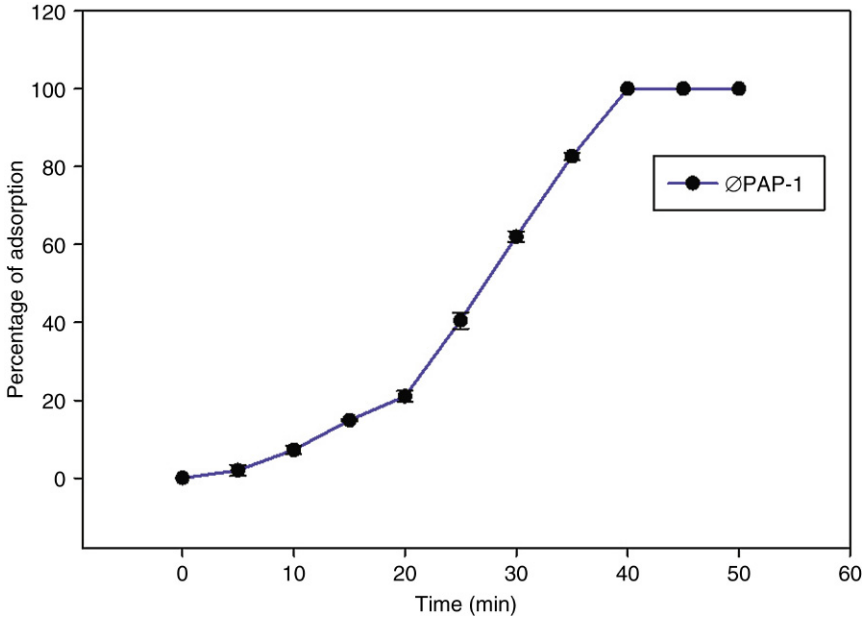


**Figure 14.5: Adsorption Curve of ΦPAP-1.**

bacteriophages (Fig. 14.4). The electron micrograph of ΦPAP-1 clearly showed bacteriophage with a hexagonal head of size 264.47 ± 0.91 nm without any tail. The phage sizes were determined from the average of three independent measurements (mean ± standard deviation). ΦPAP-1 was thus observed to be nonenveloped with icosahedral head and with no head-tail structure, which is a typical of family *Tectiviridae.*

### *3.2.2 Determination of optimal multiplicity of infection*

The optimal MOI of PAP-1 was five phages per bacterium with *P. aeruginosa*s strain BTRY1 as host.

### *3.2.3 Phage adsorption studies*

For ΦPAP-1 adsorption nearing 100% was achieved after 40 min of exposure to the host bacteria (Fig. 14.5).

### *3.2.4 One step growth curve*

The one step growth curve helped in understanding the growth kinetic parameters like latent period, rise period, and the burst size of the bacteriophage ΦPAP-1 and is given in Fig. 14.6. These experiments were performed at 37°C, with an MOI of five for ΦPAP-1. The growth curve revealed that the latent period was about 30 min while rise period was 60 min. The calculated burst size was 60 phages per bacterium.

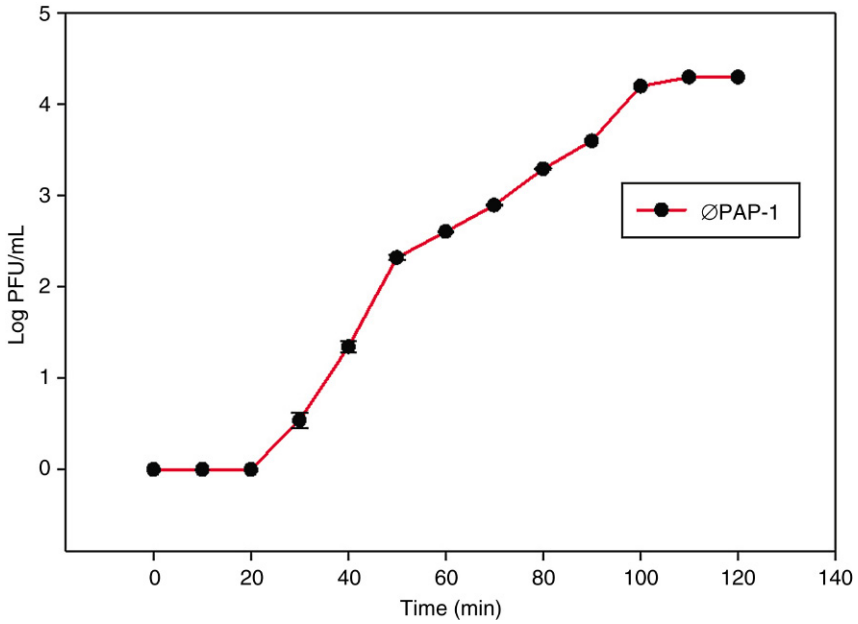


**Figure 14.6: One Step Growth Curve of ΦPAP-1.**

### *3.2.5 Influence of physical and chemical parameters on phage viability*

The influence of temperature variation on viability of phage ΦPAP-1 is as depicted in Fig. 14.7A. Sufficient viability was noted at 50 and 60°C. Viability was drastically reduced to a few PFU/mL at 70°C, while exposure to temperatures above 70°C even for a few seconds was fatal as there were no survivors at this temperature. Optimal concentrations of NaCl for ΦPAP-1 survival was 0.1 M NaCl. The phage showed higher viability even at 0.75 M, while higher concentrations of NaCl caused a decline in the viability of ΦPAP-1 as observed from Fig. 14.7B.The optimum pH for ΦPAP-1 viability was at pH 8, but it showed survival at the range of pH 6–11 [Fig. 14.7C].The influence of 11 different sugars on viability of ΦPAP-1 is depicted in the Fig. 14.7D. Sugars, such as fructose, lactose, and maltose caused drastic inactivation of ΦPAP-1 by as much as 80%. Dextrose, rhamnose, sucrose, and ribose caused 63% inactivation while galactose and xylose affected 66% and 72% inactivation, respectively. Mannitol caused the least inactivation at 18% compared to control.

### *3.2.6 Influence of physical and chemical parameters on phage adsorption*

The effect of temperatures on the adsorption of ΦPAP-1 is as represented in Fig. 14.8A. Maximum adsorption was observed at 30°C. At temperatures above 45°C, ΦPAP-1 could not adsorb well resulting in low survival. The influence of sodium chloride on adsorption by ΦPAP-1 is as represented in Fig. 14.8B. 0.25 M concentrations of NaCl favored 75%

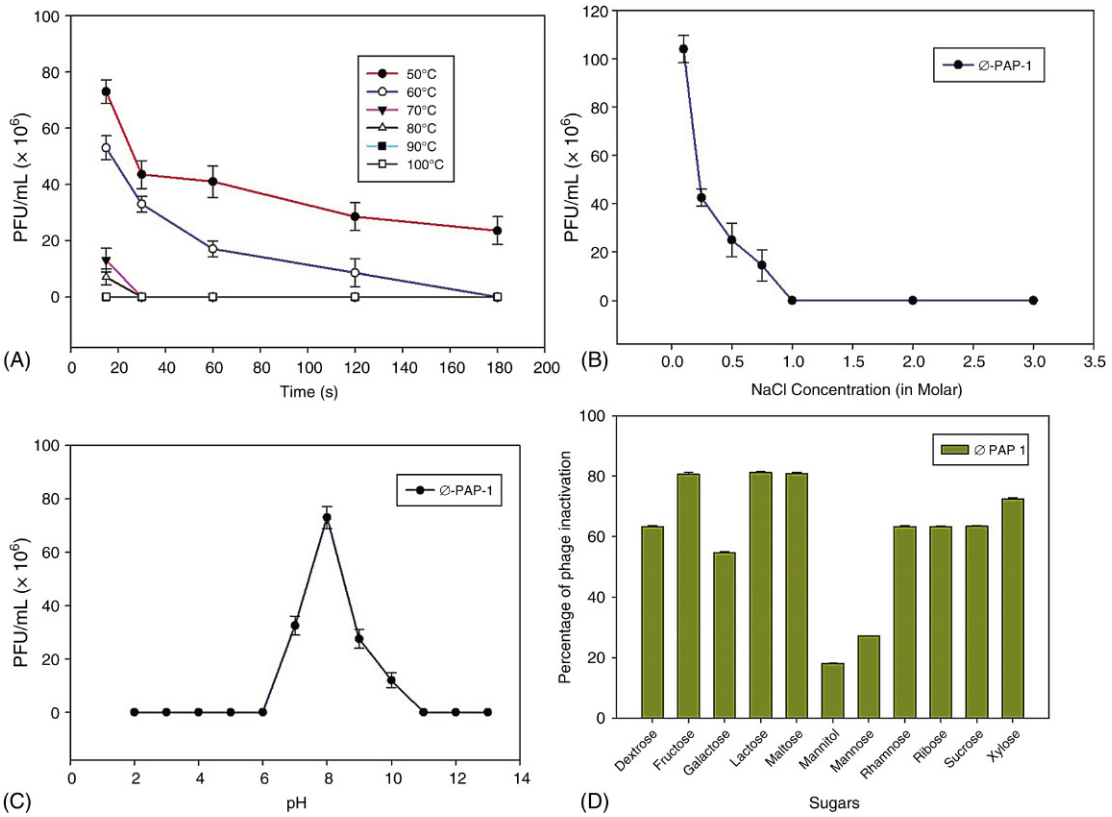


**Figure 14.7: Effect of Physicochemical Parameters on Viability of ΦPAP-1.**
(A) Temperature versus ΦPAP-1 viability. (B) NaCl versus ΦPAP-1 viability. (C) pH versus ΦPAP-1 viability. (D) Sugars versus ΦPAP-1 viability.

adsorption. Maximum adsorption of 94% was at 0.5 M sodium chloride followed by 91% at 0.75 M. Adsorption continued to occur at higher concentration of NaCl, that is, 1 and 2 M (85% and 70%, respectively). An adsorption of 30 % was found even at 3 M NaCl. Thus this phage proved to adsorb even at high concentration of NaCl, albeit at lower rates. The influence of pH on the adsorption was depicted in Fig. 14.8C. Optimum pH was 9, giving a maximum adsorption of 96%. pH ≤ 7 was detrimental with no observed adsorption. The propagation of phage under different concentrations of calcium chloride was as depicted as in Fig. 14.8D and optimum $CaCl_2$ concentration for phage adsorption was 10 mM.

### *3.2.7 Cumulative effect of optimized parameters on propagation of ΦPAP-1*

Under optimized conditions, the latent period was minimized to 20 min and the rise period was increased to 100 min. Burst size was raised from 60 to 73 phages per bacterial cell) (Fig. 14.9).

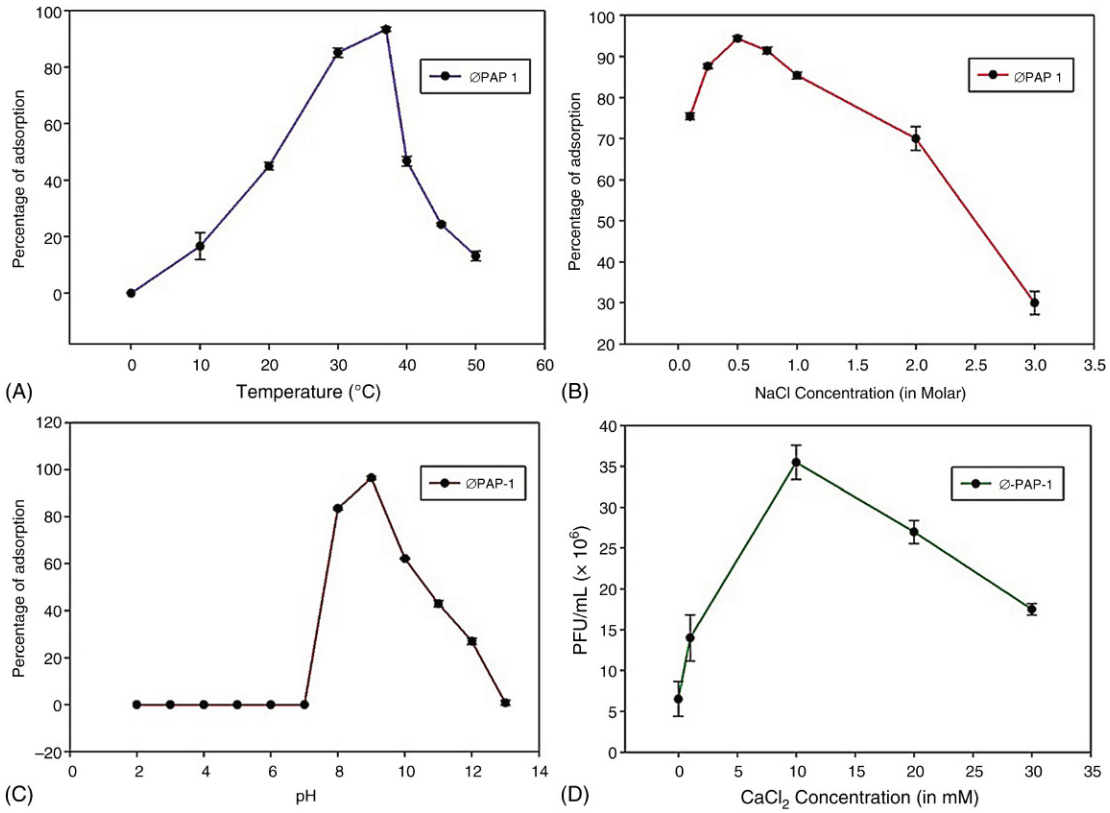


**Figure 14.8: Effect of Physicochemical Parameters on Adsorption of ΦPAP-1.**
(A) Temperature vs ΦPAP-1 adsorption. (B) NaCl vs ΦPAP-1 adsorption. (C) pH vs ΦPAP-1 adsorption. (D) $CaCl_2$ vs ΦPAP-1 adsorption.

### *3.3 Bacteriophage Genome Analysis*

The genomic DNA of ΦPAP-1 was isolated and was visualized by agarose gel electrophoresis as single band. The restriction pattern of the phage DNA revealed the susceptibility of phage genome to the restriction endonuclease *Bam* HI (Fig. 14.10).

The nature of the genome of ΦPAP-1 was identified as double stranded DNA on the basis of its sensitivity to digestion by restriction of endonuclease. The double-stranded nature of the phage DNA along with its morphological features place ΦPAP-1 in the family *Tectiviridae* whose order is still unassigned since they have different characteristics from the orders *Caudovirales* and *Ligamenvirales.*

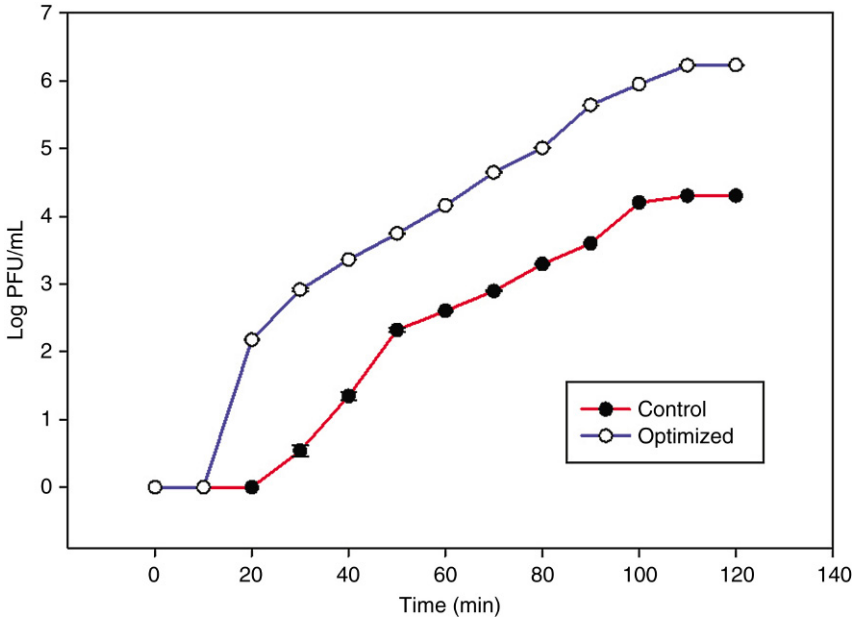


**Figure 14.9: Effect of Optimized Parameters on Propagation of ΦPAP-1.**

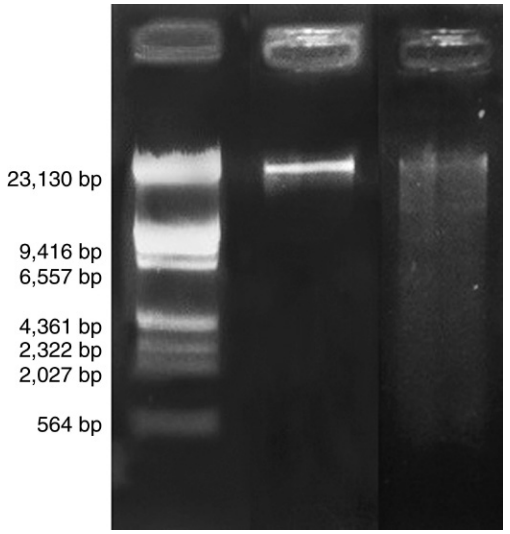


**Figure 14.10: Agarose Gel Electrophoresis of Phage DNA.**
Lane 1, Lambda DNA/*Hind* III Digest; lane 2, uncut ΦPAP-1 DNA; lane 3, *Bam*HI digest of ΦPAP-1 DNA.

### 3.4 Phage Host Range Determination

The host range of ΦPAP-1 was assessed on the basis of their ability to form plaques on different strains. The results are detailed in the table (Table 14.1). The phage was observed to be strain specific and formed plaques on lawns of the strains *P. aeruginosa* (BTRY1) and 2863 only. They did not infect the other organisms used in the study.

**Table 14.1: Host range studies of ΦPAP-1.**

| S. No. | Strain Name/NCIM Isolate Number | Identity | ΦPAP-1 |
|---|---|---|---|
| 1 | BTMW1 | *Bacillus altitudinis* | x |
| 2 | BTMY2 | *Bacillus pumilus* | x |
| 3 | BTMG1 | *B. altitudinis* | x |
| 4 | BTMW2 | *B. pumilus* | x |
| 5 | BTCW2 | *B. altitudinis* | x |
| 6 | BTMW3 | *B. altitudinis* | x |
| 7 | BTMY4 | *B. pumilus* | x |
| 8 | BTRY1 | *Pseudomonas aeruginosa* | ✓ |
| 9 | BTPW1 | *B. altitudinis* | x |
| 10 | BTCP1 | *B. pumilus* | x |
| 11 | BTTP1 | *B. altitudinis* | x |
| 12 | BTDF1 | *Brevibacterium casei* | x |
| 13 | BTDF2 | *Staphylococcus warneri* | x |
| 14 | BTDF3 | *Micrococcus luteus* | x |
| 15 | BTDP2 | *Micrococcus sp* | x |
| 16 | BTDP3 | *Bacillus niacini* | x |
| 17 | BTSD1 | *Bacillus sp* | x |
| 18 | BTSD2 | *Bacillus licheniformis* | x |
| 19 | BTFF1 | *Micrococcus luteus* | x |
| 20 | BTFF2 | *Geobacillus stearothermophilus* | x |
| 21 | 2863 | *P. aeruginosa* | ✓ |
| 22 | 2501 | *Salmonella typhimurium* | x |
| 23 | 2343 | *Escherichia coli* | x |
| 24 | 2957 | *Klebsiella pneumoniae* | x |
| 25 | 2027 | *Proteus vulgaris* | x |
| 26 | 2677 | *Clostridium perfringens* | x |
| 27 | 2127 | *Staphylococcus aureus* | x |
| 28 | 2155 | *Bacillus cereus* | x |
| 29 | 2189 | *B. pumilus* | x |
| 30 | 2107 | *Bacillus circulans* | x |
| 31 | TVMS3 | *Vibrio diabolicus* | x |
| 32 | KK16 | *Vibrio alginolyticus* | x |
| 33 | KKS4 | *Vibrio harveyi* | x |
| 34 | KK10 | *Vibrio parahemolyticus* | x |
| 35 | S37 | *Salmonella enteritidis* | x |
| 36 | S49 | *S. enteritidis* | x |

✓ denotes plaque formation; x denotes no plaque formation.

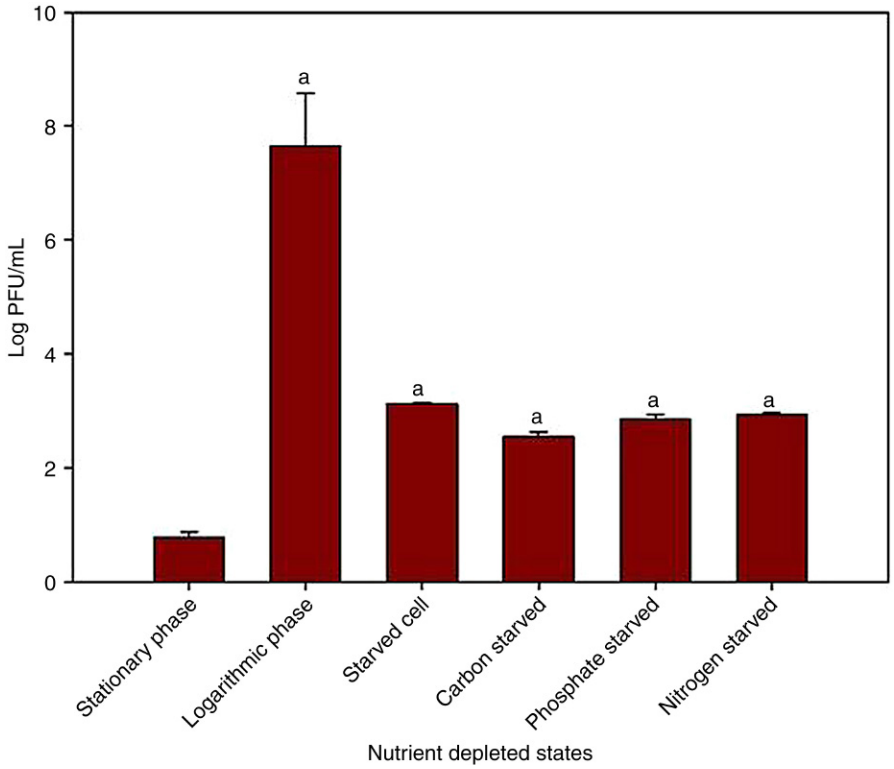


**Figure 14.11: ΦPAP-1 propagation in nutrient deprived conditions of the host cell BTRY1.**
*a* indicates $P < 0.0001$ when compared to stationary phase.

### *3.5 Propagation of Phage Under Nutrient Depleted States of the Host Cell*

The propagation ΦPAP-1 on host bacterial strain *P. aeruginosa* BTRY1 under various nutrient limited conditions exhibited significant outcome. ΦPAP-1 multiplication was maximum when the host was in the logarithmic phase ($\log_{10}$ 7.65 ± 0.9192[a] PFU/mL). But ΦPAP-1 also infected host under stationary phase, although in very few numbers ($\log_{10}$ 0.7650 ± 0.1061 PFU/mL) and was able to multiply even under multiple nutrient starved states, as evidenced by a significant level of increase in phage titer at the rate of $\log_{10}$ 3.12 ± 0.0283[a] PFU/mL. Successful propagation of ΦPAP-1 was also observed under carbon limiting conditions ($\log_{10}$ 2.48 ±0.0141[a] PFU/mL), nitrogen limiting conditions ($\log_{10}$2.925 ± 0.0707[a] PFU/mL), and phosphate limiting conditions ($\log_{10}$2.85 ± 0.0141[a] PFU/mL) as shown in Fig. 14.11.

### *3.6 Phage Structural Protein Analysis*

For ΦPAP-1 under nonreducing conditions, seven protein bands were observed, with the bands with molecular weights around 212,000; 191,750; 158,000; 91,372; 68,064; 36,410; and 18,298 Da, most prominently visualized on the gel (Fig. 14.12).

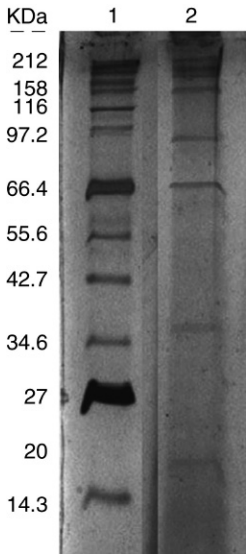


**Figure 14.12: SDS PAGE of ΦPAP-1 Under Nonreducing Conditions.**
Lane 1, marker; lane 2, ΦPAP-1.

### 3.7 Antibiofilm Activity of Whole ΦPAP-1 and Proteins Extracted from ΦPAP-1

ΦPAP-1 inhibited biofilm formation of *P. aeruginosa* (BTRY1) by 97%; 40% reduction in biofilm by *B. altitudinis* (BTMW1), 35% of *Bacillus sp* (BTSD1), while 30% reduction in biofilm formation was observed by *B. pumilus* (BTMY2*)* and *B. casei* (BTDF1). Thus although, it is host specific, the biofilm formation of *M. luteus* (BTDF3) *and G. staerothermophilus* (BTFF2) was also inhibited by 25%. On addition of ΦPAP-1, there was 25% biofilm inhibition in *S. warneri* (BTDF2) and 10% reduction *B. niacini* (BTDP3).

The reduction in biofilm formation was increased to 99, 44, 43, 34, 34, 29, 27, 24, and 18% in case of *P. aeruginosa (BTRY1), B. altitudinis* (BTMW1)*, Bacillus sp* (BTSD1), *B. pumilus* (BTMY2), *B. casei* (BTDF1), *M. luteus* (BTDF2), *G. staerothermophilus* (BTFF2), *S. warneri* (BTDF2), and *B. niacini* (BTDP3), respectively on addition of ΦPAP-1 proteins alone.

The comparison between the treatment with whole phage and phage proteins on the strong biofilm formers is depicted in Fig. 14.13.

## 4 Discussion

In the present study, ΦPAP-1, a lytic bacteriophage, was isolated from milk sample and as was its host, that is*, P. aeruginosa* (BTRY1). This is supported by the fact that bacteriophages being natural viral pathogens of bacteria coexist with their hosts, sharing the same ecological

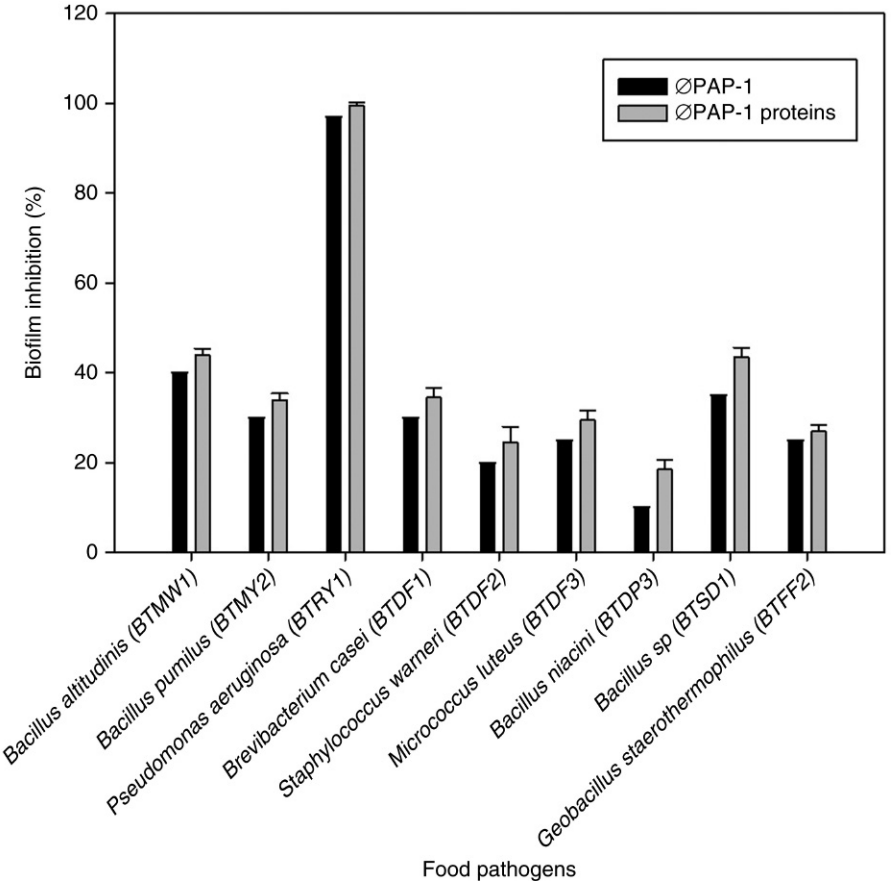


**Figure 14.13: Biofilm Inhibition (in Percent) by Test Pathogens on Treatment With Whole Phage and Proteins of ΦPAP-1.**

niches (Goyal, 1987; Heilmann et al., 2010). The phage obtained was isolated, purified, and concentrated using standard methods since these are prerequisites for structural and functional characterization of phages (Boulanger, 2009).

The morphological features of bacteriophage greatly aid in its classification (Ackermann, 2009). The TEM of ΦPAP-1 exhibited morphological traits, typical of family *Tectiviridae* according to ICTV (McGrath and van Sinderen, 2007). *G*enus Tectivirus is currently the only genus in the family *Tectiviridae* and the name is derived from Latin *tectus* (meaning "covered"). Gram-negative bacteria usually serve as natural hosts. There are currently four species reported in this genus including the type species *Enterobacteria phage PRD1* (Caldentey et al., 1994; Rydman and Bamford, 2003). Tectiviruses have no head-tail structure, but are capable of producing tail-like tubes. The virions of *Tectiviridae* species are nonenveloped, icosahedral, and displaying a pseudo T = 25 symmetry. The capsid has

two layers. The genome is a single molecule of linear double-stranded DNA 15 kb in length (San Martin et al., 2002). ΦPAP-1 has a hexagonal head 264.47 ± 0.91 nm and no tail. Other reported bacteriophages from family *Tectiviridae* with similar morphological dimensions as that of ΦPAP-1 are PR3, PR4, PR5, P722, and PRD-1 against different *Pseudomonas sp* (Fraenkel-Conrat, 2012; Knezevic et al., 2011).

Determination of optimal MOI is significant as many phages attaching to a single bacterial cell can cause cell lysis, even before the infection process can yield progeny (lysis from without). The optimal MOI for ΦPAP-1 was 5 phages/bacterium. Phage adsorption to the susceptible host is the second important factor affecting the booming phage-host interaction. Careful determination of the time taken by the phages to adsorb onto to the host cell is of supreme importance, as it may serve in later experiments for accurate characterization of the phage. Several reports on the lytic bacteriophages against *Pseudomonas sp* also showed the similar kinetic parameters like latent and rise periods along with the burst size as shown by ΦPAP-1 (Ceyssens et al., 2009; Di Lallo et al., 2014; Minor et al., 1996).

Best possible host and growth conditions must be carefully studied and selected for the production of each bacteriophage candidate for application as biocontrol agents (Sillankorva et al., 2010). In the present work, the influence of both physical and chemical parameters on phage viability/propagation and phage adsorption was studied. The parameters studied included temperature, pH, salinity, presence of calcium ions, and sugars. The effect of each factor and the knowledge about the phage growth dynamics in varying ecological conditions can be exploited during their future intended use as a therapeutic agent. Furthermore, it will also help in optimization of the large scale phage dissemination process in the laboratory conditions (Augustine et al., 2013a). The conditions for the phage viability was found to be 50°C, pH 8, and 0.1 M NaCl, while the sugars mannitol and mannose were favorable for the increased viability. The optimum temperature, pH, NaCl, and $CaCl_2$ concentrations for the phage adsorption was 37°C, 9, 0.5 M, and 10 mM for ΦPAP-1. Under optimized conditions, latent period decreased from 30 to 20 min, generation period increased from 60 to 100 min, and burst size increased from 60 to 73 phages/bacterium. Both burst size and the phage generation time are controlled by the phage latent period (Abedon et al., 2011; Augustine et al., 2013b) and in the present study, even though the latent period showed a dip, an overall increase in phage generation time was observed under optimized conditions, which ultimately resulted in the burst size increase.

Physiological state of the host, characterized by levels and activities of host cellular functions, plays a pivotal role in phage infection and propagation (You and Yin, 2002). Under various nutrient deprived conditions, ΦPAP-1 produced excellent results; a not so infrequent situation in the natural environment (Lenski, 1988). Restrictions in nutritional factors are known to limit the phage propagation (Miller and Day, 2008). There are only few reports on phages competent to infect their host under both nutrient-rich and nutrient-deprived conditions

(Augustine et al., 2013a; Chibani-Chennoufi et al., 2004). This characteristic of ΦPAP-1 is a distinctive quality required to be an effective, successful biocontrol agent.

The control strategies adopted, including preventive measures and chemical treatments are not unwavering in the eradication of the biofilm formation. There is a growing interest in the use of bacteriophages for the prevention and management of food borne bacterial infectious diseases, mainly due to the emergence of multiple antibiotic resistant (MAR) bacteria (Matsuzaki et al., 2005; Sulakvelidze et al., 2001).The lack of efficient bactericides pushed phage therapy as one of many promising approaches for the control of the biofilm producing bacteria. The ability of the phages to eradicate biofilms has been demonstrated for biofilms of various pathogens including *P. aeruginosa, B. cereus, K. pneumoniae, E. coli, Proteus mirabilis*, and *Staphylococcus Epidermidis* (Doolittle et al., 1995; Kudva et al., 1999).The main mechanism of action of phages on biofilm formation is the degradation of extra cellular polymeric substances or EPS. EPS probably may act as primary bacterial receptor for phage infection (Cornelissen et al., 2011). Application of bacteriophages in inhibiting mixed biofilms of *Pseudomonas fluorescens* and *Staphylcoccus lentus* has also been reported (Sillankorva et al., 2011). In that study, the biofilms were challenged with phage ΦBB-PF7A, specific for *P. fluorescens*, and showed that the phage readily reached the target host and caused a major population decrease. This phage was also capable of causing partial damage to the biofilms leading to the release of the nonsusceptible host (*S. lentus*) from the dual species biofilms. In the present study, ΦPAP-1, irrespective of its host and narrow host range was able to control biofilm formation by different food pathogens, making it a promising tool in the eradication of food industry biofilms.

Very recently, phages and phage-encoded proteins were proposed as natural food preservatives and antimicrobial agents to battle bacterial infections in humans, animals, or crops of agricultural importance (Drulis-Kawa et al., 2012; Glonti et al., 2010). In our study, the phage proteins were precipitated using acetone; and the crude protein extract along with the whole phages were assayed for their antibiofilm activity. The study revealed that the proteins alone could repress the bacterial adhesion to the polystyrene microtiter plates, which highlights the importance of phage proteins in the antibiofilm action of phages. Formation of a protected biofilm environment is one of the major causes of the increasing antibiotic resistance development. These certainties stress the need to create alternative antibacterial strategies, like phage therapy (Cornelissen et al., 2011).

The antibiofilm activity of phage endolysins is available, including reports on Φ11 endolysin (Sass and Bierbaum, 2007) and lysostaphin (Kokai-Kun et al., 2009) against staphylococcal biofilms. One governing difference between phages and lysins is that phages are natural while the endolysins are mostly purified from a recombinant expression system, thereby increasing the hurdles in the approval process for use in food safety. At present, there are

no approved enzybiotics (endolysins) for use in foods for human consumption. The specific use of peptidoglycan hydrolases has also been reviewed recently but the safety of its use in foods has not yet been proved (Callewaert et al., 2011; Garcia et al., 2010). Lately, potential of bacteriophage derived peptidases, $CHAP_K$, for the fast disruption of biofilms was reported against *staphylococci* where the purified protein completely eliminated *S. aureus* DPC5246 biofilms within 4 h. Furthermore, there was rapid degradation of *Streptococcus pyogenes* biofilms by PlyC, a bacteriophage-encoded endolysin (Shen et al., 2013). The role of engineered phages and coded proteins are also on their way to succeed in their aim to inhibit mixed biofilms (Lu and Collins, 2007; Pei and Lamas-Samanamud, 2014).

## 5 Conclusions

In food industries, biofilms are a source of strong contaminations causing food spoilage, and are possible causes of public health problems like outbreaks. Biofilms are challenging to eradicate due to their resistant phenotype, but mechanisms by which bacteria in biofilms attain resistance are still unknown. Furthermore, this resistance of biofilm-embedded bacteria to antimicrobial agents makes it necessary to search for agents to effectively kill them. Innovative strategies are therefore required to deal with biofilm-mediated food borne toxicities and infections. Scientists and clinicians alike are looking retrospectively to find a promising progressive treatment in the form of phage therapy.

In food industry, biofilms are highly impervious to chemical and physical treatments. Residues of disinfectants are also detrimental, hence it warrants prospecting for safe biofilm inhibitors for use in the food industry. Thus, this work focused on the biocontrol of bacterial biofilms. The phage proteins added an entirely new aspect to biofilm biocontrol and is worthy of more research. The production of phages in large scale would be cost effective and ecofriendly in nature and are microbiologically safe. These bioagents can be used as additives or as cleansers in the food processing environment. Future studies on the analysis and complete characterization of these phage proteins in detail as well as of other phages will add to the existing knowledge on the role of phage proteins in biofilm mitigation, including their mechanism of action. Hurdle technology which includes the combination of bioactive compounds along with bacteriophage preparations is a novel idea to resist food pathogens persisting in the food processing environments.

## Acknowledgments

The authors acknowledge Cochin University of Science and Technology, Kerala, India and Sophisticated Test and Instrumentation Centre, Kalamassery, Kerala for supporting the work with necessary facilities and this work was financially supported by the grant *Assured Opportunity for Research Careers—Innovation in Science Pursuit for Inspired Research* (AORC-INSPIRE) from Department of Science & Technology (DST) (DST-INSPIRE JRF-IF120536) to the first author.

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# Appendix

## Nutrient Medium

| Ingredients | g/L |
|---|---|
| Peptone | −5 g |
| Sodium chloride | −5 g |
| Beef extract | −1 g |
| Yeast extract | −2 g |

Suspended 13 g (Himedia, Mumbai, India) in 1000 mL distilled water. Mixed well, autoclaved at 15 lbs pressure (121°C) for 15 min and cooled to 50–55°C. When used as solid agar medium, 2.0% agar (w/v) was added to the medium. Final pH is 7.4 ± 0.2.

## Luria Bertani Broth

| Ingredients | g/L |
|---|---|
| Casein enzymic hydrolysate | −10 g |
| Yeast extract | −5 g |
| Sodium chloride | −10 g |

Suspended 25 g (Himedia) in 1000 mL distilled water. Heated to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Final pH is 7.5 ± 0.2.

## Minimal Media

| | |
|---|---|
| 5× minimal media | -20 mL |
| Distilled water | -80 mL |
| 1 M $MgSO_4$ | −0.2 mL |

Autoclaved and added:

| | |
|---|---|
| 20% glucose | −2 mL |
| 1 M $CaCl_2$ | −0.01 mL |

### *Minimal media (5× concentrate)*

| Ingredients | g/L |
|---|---|
| $Na_2HPO_4.7H_20$ | −6.4 g |
| $KH_2PO_4$ (dibasic) | −1.5 g |
| NaCl | −0.25 g |
| $NH_4Cl$ | −0.5 g |

Ingredients were dissolved in 80 mL distilled water. Adjusted the volume to 100 mL with distilled water and sterilized by autoclaving at 15 lbs for 20 min before use.

### *Physiological Saline*

| | |
|---|---|
| NaCl | −0.85 g |
| Distilled water | −100 mL |

Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min, cooled, and used.

### *0.01 M Phosphate Buffer (pH 7.5)*

| |
|---|
| Solution A: 0.2 M $NaH_2PO_4$ |
| Solution B: 0.2 M $Na_2HPO_4$ |

Mixed 16 mL of solution A with 84 mL of solution B and the volume was made up to 200 mL with distilled water. This was made up to 1 L to get 0.01 M buffer.

### *SM Buffer*

| **Ingredients** | **g/L** |
|---|---|
| NaCl | 5.8 g |
| $MgSO_4.7H_2O$ | 2.0 g |
| 1 M TrisHCl (pH 7.5) | 50 mL |
| 2% gelatin | 5.0 mL |

Ingredients were dissolved and were made up to 1 L with milli-Q water and autoclaved at 15 lbs for 20 min and stored at 4°C until use.

### *Phosphate Buffered Saline (PBS) (pH 7)*

| **Ingredients** | **g/L** |
|---|---|
| NaCl | 8.0 g |
| KCl | 0.2 g |
| $Na_2HPO_4$ | 1.44 g |
| $KH_2PO_4$ | 0.24 g |

Ingredients were dissolved in 800 mL of distilled water, pH adjusted to 7.00 with 1 N HCl. The volume was made up to 1 L with distilled water, autoclaved at 15 lbs for 20 min and stored at room temperature until use.

### *TE Buffer*

| | |
|---|---|
| 1 M Tris-Cl | −10 mL |
| 500 mM EDTA (pH 8.0) | −2 mL |

### Citrate Buffer (Hydrochloric Acid—Potassium Chloride Buffer (pH 2)

| |
|---|
| Solution A: 0.2 M KCl |
| Solution B: 0.2 M HCl |

Mixed 50 mL of solution A with 10.6 mL of solution B and made up to 200 mL with distilled water

### Citrate Buffer (pH 3–6)

| |
|---|
| Solution A: 0.1 M Citric acid |
| Solution B: 0.1 M Sodium citrate |

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to make up volume to 200 mLand then filter sterilized.

| Desired pH | Solution A (mL) | Solution B (mL) |
|---|---|---|
| 3 | 46.5 | 3.5 |
| 4 | 33.0 | 17 |
| 5 | 20.5 | 29.5 |
| 6 | 9.5 | 41.5 |

### Phosphate Buffer (pH 7)

Solution A: 0.2 M $NaH_2PO4$

Solution B: 0.2 M $Na_2HPO4$

Mixed 39 mL of solution A with 61 mL of solution B and the volume was made up to 200 mL with distilled water, followed by filter sterilization.

### Tris (Hydroxymethylamino Methane Buffer System (pH 8 and 9)

Solution A: 0.2 M Tris buffer

Solution B: 0.2 M HCl

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and filter sterilized.

| Desired pH | Solution A (mL) | Solution B (mL) |
|---|---|---|
| 8 | 50 | 26.8 |
| 9 | 50 | 5 |

### Carbonate–Bicarbonate Buffer (pH 10 and 11)

Solution A: 0.2 M $Na_2CO_3$

Solution B: 0.2 M $NaHCO_3$

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and filter sterilized.

| Desired pH | Solution A (mL) | Solution B (mL) |
|---|---|---|
| 10 | 27.5 | 22.5 |
| 10.7 | 45.0 | 5 |

### Sodium Hydroxide–Potassium Chloride Buffer (pH 12 and 13)

Solution A: 0.2 M KCl

Solution B: 0.2 M NaOH

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and then filter sterilized.

| Desired pH | Solution A (mL) | Solution B (mL) |
|---|---|---|
| 12 | 50 | 12 |
| 13 | 50 | 132 |

### Polyacrylamide Gel Electrophoresis

#### Stock Acrylamide Solution (30:0.8:1)

| | |
|---|---|
| Acrylamide (30%) | −60.0 g |
| Bis-acrylamide (0.8%) | −1.6 g |
| Distilled water | −200.0 mL |

Filtered through Whatman No. 1 filter paper and stored in amber colored bottle at 4°C.

#### Stacking gel buffer stock (0.5 M Tris-HCl, pH 6.8)

Tris buffer −6 g in 40 mL distilled water.

Titrated to pH 6.8 with 1M HCl (~ 48 mL) and made up to 100 mLwith distilled water. Filtered through Whatman filter paper No. 1 and stored at 4°C.

#### Resolving gel buffer stock (3 M Tris-HCl, pH 8.8)

Tris buffer −36.3 g

Titrated to pH 8.8 with 1 M HCl (~ 48 mL) and made up to 100 mL with distilled water. Filtered through Whatman No. 1 filter paper and stored at 4°C.

*Reservoir buffer for SDS-PAGE (pH 8.3)*

| | |
|---|---|
| Tris buffer | −3.0 g |
| Glycine | −14.4 g |
| SDS | −1.0 g |

Dissolved and made up to 1 L with distilled water. Prepared as 10× concentration and stored at 4°C.

*Sample buffer for Nonreductive SDS-PAGE (2×)*

| | |
|---|---|
| Tris-HCl (pH 6.8) | −0.0625 M |
| Glycerol (optional) | −10% (v/v) |
| SDS | −2% |
| Bromophenol blue | −0.01% |

*SDS (10%)*—1 g in 10 mL distilled water

*Sucrose (50%)*—5 g in 10 mL distilled water

Autoclaved at 121°C for 15 min and stored at 4°C until use.

*Stacking Gel (5%) (2.5 mL)*

| | |
|---|---|
| Acryl:Bis | –0.425 mL |
| Stacking gel buffer | –0.625 mL |
| 10% SDS | –25 µL |
| TEMED | –2.5 µL |
| 10% APS | −25 µL |
| Distilled water | –1.425 mL |

*Resolving Gel (12%) (5.0 mL)*

| | |
|---|---|
| Acryl :Bis | –2 mL |
| Resolving gel buffer | –1.25 mL |
| 10% SDS | –50 µL |
| TEMED | –15 µL |
| 10% APS | –37.5 µL |
| Distilled water | –1.66 mL |

*Protein Marker for SDS-PAGE*

Broad range molecular weight protein marker mix from New England BioLabs (UK) is a ready to load marker. The protein marker was mixed and 7 µL taken in a tube. Heated for 5 min at 100°C. After a quick microcentrifuge spin, loaded directly on to the gel. The composition of the marker mixed is as given below:

| **Components** | | **MW in Da** |
|---|---|---|
| Myosin | — | 212,000 |
| MBP-β-galactosidase | — | 158,194 |
| β-galactosidase | — | 116,351 |
| Phosphorylase b | — | 97,184 |
| Serum albumin | — | 66,409 |
| Glutamic dehydrogenase | — | 55,561 |
| MBP2 | — | 42,710 |
| Thioredoxin reductase | — | 34,622 |
| Triose phosphate isomerase | — | 26,972 |
| Trypsin inhibitor | — | 20,000 |
| Lysozyme | — | 14,313 |
| Aprotinin | — | 6,517 |
| Insulin A | — | 3,400 |
| B chain | — | 2,340 |

pH was 6.8 at 25°C and stored at −20°C.

*Silver staining*

Fixing solution 1—50 mL methanol and 5 mL acetic acid in 45 mL water

Fixing solution 2—50 mL methanol in 50 mL water

| | |
|---|---|
| Sensitizer | Sodium thiosulfate (20 mg/100 mL) |
| Staining solution (Mixed and prepared fresh before use) | Silver nitrate (200 mg/100 mL) |

*Developing solution (Mixed and prepared fresh before use)*

| | |
|---|---|
| Sodium carbonate (anhydrous) | −3 g/100 mL |
| Formaldehyde | −25 µl/100 mL |
| Sodium EDTA solution | −1.4 g/100 mL |

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CHAPTER 15

# Bioactive Properties and Biotechnological Production of Human Milk Oligosaccharides

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## 1 Structure of Human Milk Oligosaccharides (HMOs)

Human milk is considered the gold standard for nutrition of infants, providing essential nutrients, such as proteins, lipids, carbohydrates, and vitamins, but also bioactive components, such as enzymes, hormones, antibodies, growth factors, polyamines, nucleotides, and oligosaccharides that promote infant health and development (Bode, 2012). Human milk oligosaccharides (HMOs) can be present as free oligosaccharides, forming complex with proteins and lipids, and as glycosaminoglycans (GAGs) (Bode, 2012; Coppa et al., 2011; Newburg, 2009). In the past few years, much evidence suggests that some human milk glycans, especially HMOs, are involved in shaping a gut microbiota with health benefits for newborns. HMOs act as prebiotics, thus they are metabolic substrates of bacteria belonging to the genera *Bifidobacterium* and *Lactobacillus* (Becerra et al., 2015a; Bidart et al., 2014; Yu et al., 2013), which contain strains with demonstrated probiotic properties. There is also evidence suggesting that HMOs exhibit antiadhesive properties. They have identical structures that the glycans domains that serve as receptors for enteropathogens, including virus, bacteria, fungus, and protozoan parasites, and HMOs prevent their attachment by competitive inhibition (Bode, 2015). Epithelial and immune cell modulation has also been attributed to HMOs (He et al., 2016a,b).

### 1.1 Free Oligosaccharides

Human milk contains free oligosaccharides in concentrations ranging from about 12–13 g/L in colostrum and 22–24 g/L in mature milk, and constitutes the third largest solid component following lactose and lipids (Kunz et al., 2000; Thurl et al., 2010). HMOs are composed of the five monosaccharides D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc),

Advances in Biotechnology for Food Industry
http://dx.doi.org/10.1016/B978-0-12-811443-8.00015-3

L-fucose (Fuc), and sialic acid (Sia; *N*-acetylneuraminic acid). The chemical structures of more than 100 oligosaccharides have been characterized, which are variable in size, charge, sequence, and concentration (Kobata, 2010). In addition, not every woman synthesize the same set of oligosaccharides, as their synthesis is correlated with the secretor and Lewis blood group characteristics, which depend on the expression of certain glycosyltransferases (GTs). Despite their structural complexity, most HMOs contain lactose (Lac; Gal-β1-4Glc) at their reducing end, that is elongated by the addition of β-1-3-linked lacto-*N*-biose (LNB; Gal-β1-3GlcNAc; type-1 chain), and/or β1-3/6-linked *N*-acetyllactosamine (LacNAc; Gal-β1-4GlcNAc; type-2 chain) (Fig. 15.1). Extension with LNB seems to terminate the chain, whereas LacNAc can be further elongated by the addition of one of the two disaccharides. HMOs with β-1-6 bonds are branched oligosaccharides and they are named *iso*-HMO, while the linear structures with β-1-3 linkages are named *para*-HMO. Those three basic core disaccharides (lactose, LNB, and LacNAc) are often modified by L-Fuc with α-1-2, α1-3 or α1-4 linkage and/or Sia with α2-3 or α2-6 linkage (Fig. 15.1). Sia has a carboxyl group and therefore, sialylated HMOs contain one or more negative charges depending on the number of Sia added to the HMO backbone. The fucosylated HMOs constituted the majority; they ranged from 50% to 80%, while the sialylated ranged from 10% to 20% (Bode, 2012). HMOs can be simple trisaccharides as 2′-fucosyllactose (2′FL) and 3′-fucosyllactose (3′FL) or complex oligosaccharides with different degrees of

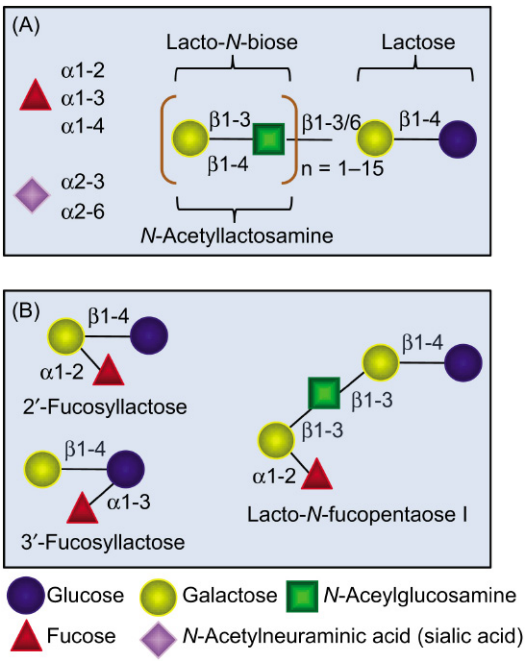


**Figure 15.1: Human Milk Oligosaccharides.**
(A) General structure. (B) Short-chain trisaccharides, for example, 2′-fucosyllactose and 3′-fucosyllactose; complex oligosaccharides, for example, lacto-*N*-fucopentaose I.

polymerization up to 20 monosaccharides. An important feature is that the HMOs containing the type-1 core are more abundant than those containing type-2. In fact, lacto-*N*-tetraose (LNT, Gal-β1-3GlcNAc-β1-3Gal-β1-4Glc) and its fucosylated derivatives lacto-*N*-fucopentaose I and lacto-*N*-difucohexaose I are among the most abundant HMOs (Thurl et al., 2010). In addition, LNB core is not present or is present in very low amounts in the milk of other mammals, included bovine milk (Urashima et al., 2013), which raises the question about the biological importance of the type-1 HMOs for both human health and human evolution.

### *1.2 Glycoproteins*

Proteins in milk are highly glycosylated and the glycan moieties (oligosaccharides) play an important role in infant health. *N*-glycans are linked to an asparagine residue, which is present in a conserved amino acid sequence (Asn-X-Ser/Thr, where X can be any amino acid except proline), through a GlcNAc. The *N*-glycan core contains two GlcNAc, the inner one linked to the asparagine can be fucosylated, and three mannose residues. This core can be extended by other monosaccharides, including Fuc and Sia, and it becomes a more complex and diverse structure. *N*-glycans have been classified in three types: high mannose, complex, and hybrid, based on the number of fucosylated and sialylated sites (Nwosu et al., 2012).

*O*-glycans usually contained an *N*-acetylgalactosamine (GalNAc) linked to a serine or threonine residue. Other carbohydrates can be added to this GalNAc and assembled into eight different core structures (Krasnova and Wong, 2016). In the type 1 sugar core, the GalNAc is extended with Gal, linked via a β1-3 bond, forming the disaccharide galacto-*N*-biose (GNB; Gal-β1-3GalNAc). GNB is also an important core structure in functional sugar chains, such as the T-antigen disaccharide and is also an essential part of the carbohydrate moieties of glycosphingolipids (Liu and Newburg, 2013; Moran et al., 2011).

More than 70% of human milk proteins are glycosylated, they range in size from 14–2000 kDa, and they include mucins, secretory immunoglobulin A, lactoferrin, lactadherin, bile salt-stimulated lipase, butyrophilin, leptin, adiponectin, lysozyme, α-lactalbumin, β-casein, and κ-casein. Human milk glycoproteins are present in both, skim milk fraction (contained about 60% whey and 40% casein) and in the milk fat globule membrane (MFGM) (Peterson et al., 2013). The biosynthesis pathway of free HMOs and glycans, bound to proteins, share the same GTs, and therefore they have similar structures. As described for HMOs, the glycan moieties of human milk glycoproteins have been involved in several biological effects protecting infants from disease. Attached glycans can act as binding epitopes for pathogens (Liu and Newburg, 2013; Peterson et al., 2013) and as prebiotic substrates promoting the growth of beneficial bacteria in the infant gastrointestinal tract. Regarding this, the genus *Bifidobacterium* can utilize mucin *O*-linked glycans by secretion of endo-α-*N*-acetylgalactosaminidase and 1,2-α-L-fucosidase (Ruas-Madiedo et al., 2008) and can cleave *N*-glycans by an endo-β-*N*-acetylgalactosaminidase (Le Parc et al., 2015).

### 1.3 Glycolipids

The major fraction, that is, about 98% of lipids present in human milk is triacylglycerides. While the remaining 2% is the glycolipids; these are constituted mainly by gangliosides, which are a group of Sia glycosphingolipids found exclusively in the MFGM. The gangliosides are composed by a hydrophobic ceramide lipid moiety linked to glycans (oligosaccharides) that can have one or more Sia (Ryan et al., 2013). Gangliosides are present in colostrum and mature milk at concentrations of about 9 mg/L (Bode et al., 2004a). The most abundant ganglioside on human colostrum is GD3 (α2-8NeuAcα2-3Galβ1-4Glcβ1-1Ceramide), while in mature milk is GM3 (NeuAcα2-3Galβ1-4Glcβ1-1Ceramide) (Lee et al., 2011a). Other gangliosides in minor concentrations include GM2 (GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Ceramide) and GM1 (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Ceramide) (Newburg and Chaturvedi, 1992).

The gangliosides present in the MFGM resemble the structure of the glycolipids that form part of epithelial cell membranes, and therefore they can act as decoys for pathogens and prevent the attachment of these to the epithelial cells of newborns, avoiding infections (Peterson et al., 2013). Regarding this, in vivo experiments have shown that bovine MFGM present in buttermilk probably prevents the adherence of the pathogen *Listeria monocytogenes* to the intestinal mucosa in rats (Sprong et al., 2012). Additionally, glycolipids from human milk can also adhere to toxins produced by pathogenic bacteria and to human viruses (Peterson et al., 2013).

### 1.4 Glycosaminoglycans (GAGs)

GAGs are linear heteropolysaccharides containing repeating nonsulfated or sulfated disaccharide units of hexosamine and uronic acid or galactose. GAGs have high molecular mass, strong anionic nature, and they are mainly complexed to proteins forming proteogycans (Jackson et al., 1991). All the GAGs are sulfated to different degrees with the exception of hyaluronic acid. The most abundant GAGs in human milk are chondroitin sulfate (about 55%) and heparan sulfate (about 40%) (Coppa et al., 2011). Hyaluronic acid is present in low concentrations that ranged from 500 ng/mL the first month after giving birth until 100 ng/mL at the end of the first year (Yuan et al., 2015). Recently, it has been shown that human milk GAGs are not present in the feces of breast-fed newborns, suggesting that they are catabolized by the gastrointestinal microbiota (Maccari et al., 2016). Like other glycans, human milk GAGs are also able to inhibit in vitro the adhesion of enteropathogenic bacteria as *Escherichia coli* serotype 0119 and *Salmonella fyris* to human epithelial cell lines (Coppa et al., 2016). Additionally, hyaluronic acid from human milk induces the antimicrobial peptide human β-defensin 2 and it has a role enhancing innate defense at the intestinal epithelium (Hill et al., 2013).

## 2 Biosynthesis: Genetic Determination

HMOs are the result of the extension of the milk sugar lactose by the action of several GTs, which transfer GlcNAc, Gal, Sia, or Fuc. For the latter, two genes are implicated; secretor (Se) gene encodes the α-1,2-fucosyltransferase (FUT2) and Lewis (Le) gene encoding the α-1,3/4-fucosyltransferase FUT3. Both are polymorphic with different alleles and as a consequence of the varying enzyme activities there is a measurable difference of specific HMOs and profiles (Blank et al., 2012). Thus, breast-fed infants naturally intake milk containing different types and amounts of oligosaccharides. Secretor women produce milk with high amounts of fucosylated HMOs, such as 2′-fucosyllactose and lacto-*N*-fucopentaose I. Nonsecretors women lack a functional FUT2 enzyme and their milk does not contain α-1,2-fucosylated HMOs. They represent about 15%–25% depending on their ethnic background (Castanys-Munoz et al., 2013). Le-negative women lacks specific α-1,4-fucosylated HMOs as lacto-*N*-fucopentaose II.

Depending on the expression of FUT2 and FUT3 enzymes, human milk can be assigned to one of the four women groups: Lewis-positive secretors (*Le*+ *Se*+), Lewis-positive nonsecretors (*Le*+ *Se*−), Lewis-negative secretors (*Le*−*Se*+), and Lewis-negative nonsecretors (*Le*− *Se*−). This classification, however, is too simple, as there is a wide range of enzyme expressions and activities, and as a consequence, a complex HMO profile throughout the population. Even Lewis-negative nonsecretors women that do not express FUT2 and FUT3 contain milk with fucosylated HMOs like 3′-fucosyllactose and lacto-*N*-fucopentaose III, suggesting that other FUT enzymes, such as FUT4-7 or FUT9 can be involved in HMOs fucosylation. Also, FUT1 may be involved in the presence of α-1,2-fucosylated HMOs in milk from nonsecretor women (Newburg et al., 2005).

Addition of Sia to HMOs backbone depends on the activity of several sialyltransferases. Unlike fucosylated HMOs, which can be absent in certain types of milk, a total deprivation of sialylated HMOs has not been found yet. HMOs sialylation differences among women are probably due to variations on the expression of sialyltransferases or other enzymes involved in the sialylation pathways (Bode, 2012).

## 3 Use of HMOs by Probiotic Bacteria: Prebiotics

### 3.1 Bifidobacteria

Bifidobacteria are typical inhabitants of the gastrointestinal tract of humans and other vertebrates. This bacterial genus has attracted great interest due to their implications in the health status of individuals, as many health-promoting activities have been reported for it (probiotics). Bifidobacteria constitute a considerable proportion of the intestinal microbiota of infants and it has been largely reported that their numbers are higher in breast-fed infants compared to that of formula-fed infants. However, some studies show that the bifidobacterial populations in both groups of infants reach similar numbers (Wang et al., 2015). Although

this discrepancies may be based on the bias introduced by the different molecular methods used to characterize the microbiota (qPCR, 16S rDNA amplification, and massive sequencing), the improvement in the quality and nutritional characteristics of modern infant formulas might account for it. Moreover, although the proportion of total bifidobacteria could be similar, the proportion of different species may differ between both groups. Thus, in breast-fed infants bifidobacterial species, such as *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), *Bifidobacterium bifidum, Bifidobacterium breve,* or *B. longum* subsp. *longum*) predominate, whereas other species which are typical of adults, intestinal microbiota (e.g., *Bifidobacterium adolescentis* or *Bifidobacterium catenulatum*) predominate in formula-fed infants (Haarman and Knol, 2005). Hence, some of the HMOs components determine the "bifidus factor" that was described for human milk. HMOs act as a class of prebiotic substances that promote growth of certain bifidobacterial species and it has been demonstrated that the differences in HMOs composition between different mothers determines the bacterial proportions in the intestinal microbiota of breast-fed infants (Lewis et al., 2015; Wang et al., 2015).

*Bifidobacterium* species are specially adapted to exploit HMOs as a carbohydrate fermentable source to support their growth. Their carry in their genomes complete enzymatic machineries for their catabolism, whose functions have been extensively reviewed (Fushinobu, 2010; Garrido et al., 2013). Strains of *B. infantis* and *B. bifidum* grow very efficiently in laboratory culture media supplemented with HMOs, which are difficult to degrade by other bacterial groups (Asakuma et al., 2011). HMOs are decorated with L-fucose residues, present in neutral HMO, and sialic acid (Sia) residues, present in acidic HMOs, that render their structures less attackable by the complex bacterial population in the colon. These structures are the target for different enzymatic activities present in bifidobacteria, such as sialidase activities that remove sialic acid. The sialidase SiaBb2 from *B. bifidum* belongs to the glycosyl hydrolase family 33 (GH33) and it is a surface-anchored enzyme that displays exo-α-sialidase activity and liberates sialic acid preferentially from α2,3 linkages. In accordance to this, its heterologous expression in *B. longum*, a species with a limited capacity to use HMOs, allows this species to utilize sialylated oligosaccharides (Kiyohara et al., 2011). Moreover, the HMOs gene cluster I of *B. infantis* (see later) carry putative sialidases with specificity toward α2,3 and α2,6 linkages that are induced after the growth on HMO (Sela et al., 2011). Other important activity is α-L-fucosidase, with members of the GH29 and GH95 families present in bifidobacteria. These two families differ in their mechanism of action and while GH29 members possess a double displacement mechanism where a conserved Asp acts as catalytic nucleophile, GH95 enzymes rely in an unique inverting mechanism where an Asn acts as a base catalyst and the acid catalyst is played by a Glu, as was first described for the AfcA enzyme from *B. bifidum* (Nagae et al., 2007). The two fucosidases from this species, AfcA (GH95) and AfcB (GH29), are extracellular and membrane-anchored enzymes with specificities for α1,2 and α1,3/4

linkages, respectively (Ashida et al., 2009), whereas *B. infantis* possesses five intracellular fucosidases of both families (1 GH95 and 4 GH29). Unlike AfcA, the GH95 fucosidase from *B. infantis* (encoded by Blon_2335) displays activity toward α1,2 and α1,3 linkages, being able to liberate L-fucose from 2′FL and 3′FL, while the second more active fucosidase (encoded by Blon_2336) is specific for α1,3/4 linkages. These enzymes are moderately induced by in vitro growth in the presence of HMOs (Sela et al., 2012). Despite the capacity of bifidobacteria to remove L-fucose and sialic acid from glycocomplexes and HMOs, the catabolism of these two compounds has not been deeply studied for this bacterial group and many strains are unable to use them (Asakuma et al., 2011). A gene cluster for the utilization of sialic acid (*nan–nag* genes) has been reported for *B. breve* strains and the construction of knockout mutants has evidenced its role in the growth of this species using sialic acid as a carbon source (Egan et al., 2014b). A similar gene cluster is also present in *B. infantis* ATCC15697, although these genes are absent for the rest of bifidobacterial genomes. Data on the growth of *B. breve* in coculture with *B. infantis* and with different sialyl-HMOs suggest that *B. breve* can feed on sialic acid released by sialidases from *B. bifidum* (Asakuma et al., 2011; Egan et al., 2014a). Finally, only four of the six enzymatic steps required for the oxidative catabolism of L-fucose and several putative L-fucose permeases are present in *B. infantis* ATCC15697, that are induced upon growth on fucosylated HMOs (Sela et al., 2012). Therefore, the exact route for L-fucose catabolism in bifidobacteria is still needs to be disclosed and many strains, such as *B. infantis* JCM1222, are not able to utilize this sugar (Asakuma et al., 2011).

HMOs deprived of sialic acid and fucose residues can be the target for different glycosidases. Most of these enzymes have been extensively studied in *B. bifidum* and *B. infantis*, while those of *B. longum* and *B. breve*, which are also prominent members of the infant's intestinal microbiota, are less known. Nevertheless, the potential to degrade HMOs in these last species is more limited and only moderate growth in HMOs and consumption of LNT have been reported for them (Asakuma et al., 2011). While *B. infantis* expresses a variety of transporters for taking up less complex HMOs and hydrolyze them intracellularly, *B. bifidum* expresses a repertory of extracellular enzymes able to hydrolyze HMOs externally. These two different strategies imply that a cooperative (or commensalist) association for the efficient use of HMOs may take place at the gastrointestinal tract, where some species probably take advantage of the glycosidic activities of other members of the microbiota (including bifidobacteria) that liberate shorter HMOs for cross-feeding. However, these two different specialized lifestyles may also reflect colonization of spatial and nutritional distinct niches within the gut (mucus layer and intestinal lumen), as occurs with other intestinal bacteria (Garrido et al., 2015).

The presence of an extracellular lacto-*N*-biosidase (LnBb, GH20 family) is a characteristic of *B. bifidum*, although such activity has also been described for *B. longum*, where an atypical lacto-*N*-biosidase that requires activation by a specific chaperone and is also

active on fucosylated and sialylated LNT has been described (Sakurama et al., 2013). This endoglycosidase releases LNB and lactose from LNT. The building block of type-2 HMOs (*N*-acetyllactosamine) and other type-2 HMOs, such as LN*n*T can be digested by the *B. bifidum* β-galactosidase BbgII (GH2) or the GH20 *N*-acetylhexosaminidases BbhI and BbhII, which have specificities toward β1,4 and β1,6 bonds, although lactose is a less preferred substrate for these enzymes (Goulas et al., 2009; Miwa et al., 2010). In *B. longum* and *B. breve* strains, the presence of different β-galactosidase activities that release galactose from LNT and LN*n*T have been described (Asakuma et al., 2011). Contrarily to *B. bifidum*, the glycosidase enzymes of *B. infantis* are intracellular. In this species, three GH42 β-glycosidases (Bga42A, Bga42B, and Bga42C) are present that act on different β1,3/4/6 galactosidic linkages present in HMO and other plant-derived oligosaccharides (Viborg et al., 2014).

LNB as well as GNB, a component of the *O*-glycosylation core, can be metabolized by the products of the LNB–GNB gene cluster, which in addition to *B. bifidum* and *B. infantis* is also present in *B. breve* and *B. longum* strains. The LNB–GNB cluster carries genes encoding an ABC transport system specific for the two disaccharides, an *N*-acetylhexosamine kinase (NahK) that phosphorylates the position 1 of GlcNAc and GalNAc and two enzymes of the Leloir pathway (GalT, a UDP-glucose hexose-1-P uridylyltransferase and GalE, a UDP-glucose 4-epimerase), responsible for channeling Gal-1-P, GlcNAc-1-P, and GalNAc-1-P to glycolysis and to the metabolism of aminosugars. A key enzyme for LNB and GNB hydrolysis is also encoded in LNB–GNB clusters: the LNB/GNB phosphorylase, a β-1,3-specific galactosyl-*N*-acetylhexosamine phosphorylase (GH112). This enzyme is characteristic in bifidobacteria and it catalyzes a phosphorolytic cleavage that produces *N*-acetylhexosamines and Gal-1-P. The LNB–GNB cluster is upregulated by growth on HMOs and proteome analysis confirmed the presence of its components when the bacteria grow with HMOs (Kim et al., 2013).

In addition to the LNB–GNB cluster, *B. infantis* strains carry a region in their chromosomes referred to as the HMOs cluster I that code for glycosidases (β-1,4-galactosidase, β1,3-*N*-acetylhexosaminidase, GH29 and GH95 α-L-fucosidases, GH33 sialidase ), an *N*-acetylhexosamine kinase active in both GlcNAc and GalNAc and carbohydrate transporters that are clearly devoted to the selective import and catabolism of HMOs constituents, and whose expression is induced by growth with HMOs (Garrido et al., 2015). Bifidobacteria are characterized by an overrepresentation of carbohydrate transporters of the ABC family in their genomes. The functions of some of these transporters have been characterized, showing that they are involved in the utilization of host glycans and, specifically, HMOs (Garrido et al., 2011). Members of this family possess membrane-bound extracellular solute-binding proteins that recognize the extracellular sugars and therefore have a lectin-like activity. The highly abundant family 1 solute-binding proteins from *B. infantis* are expressed in the presence of HMOs and increase the capacity of the

bifidobacterial cells to attach to epithelial surfaces (Chichlowski et al., 2012; Kavanaugh et al., 2013; Wickramasinghe et al., 2015), which contain glycoconjugates similar to that found in HMOs. This interaction produces an enhanced immunoregulatory performance and HMOs-grown *B. bifidum* and *B. infantis* strengthen the barrier function and display an increased production of antiinflammatory interleukin-10 and downregulation of proinflammatory TNF-α in a model of Caco-2 cells, compared to lactose-grown bacteria (Chichlowski et al., 2012). Furthermore, HMOs-grown *B. breve* and *B. infantis* downregulate the chemokines CXCL1, CXCL2, and CXCL3 in Caco-2 cells, which are involved in diseases characterized by inflammatory response and, in general, reduce inflammatory signaling (Wickramasinghe et al., 2015).

Bifidobacteria possess the capacity to release complex glycan structures from glycosylated proteins (*O*- and *N*-linked) that are present in mucins, mucosal glycoconjugates, and human milk proteins and use them for growth (Ruas-Madiedo et al., 2008). Endo-β-*N*-acetylglucosaminidases of GH18 or GH85 families are present in many pathogenic bacteria, where they are necessary to exploit the carbohydrates of the host mucosa, attacking the *N*,*N*′-diacetylchitobiose core characteristic of *N*-glycosylation. Similar enzymatic activities have been detected in isolates of *B. longum*, *B. Breve,* and *B. infantis* (GH18 and GH85) (Garrido et al., 2012). The EndoBI-1 enzyme has been characterized in *B. infantis*, showing its ability to deglycosylate *N*-glycoproteins, including human milk proteins, irrespective of its fucosylation or syalilation status. EndoBI-1 has a constitutive expression and incubation of *B. bifidum* with human milk proteins induces the expression of enzymes related to HMOs uptake and catabolism (Garrido et al., 2012), which demonstrates the cooperative action of this activity with the rest of the machinery for the utilization of carbohydrate resources from human milk. Mucin glycoproteins are characterized by their high *O*-glycosylation at Ser/Thr residues and an endo-α-*N*-acetylgalactosaminidase activity is required to cleave the α-GalNAc bond formed in these glycocomplexes. This activity has been described for some bacterial species, including bifidobacteria, where the enzyme EngBF (GH101) has been characterized in *B. longum* and *B. bifidum*. EngBF shows a high specificity for releasing *O*-glycans of the core-1 type that had been previously hydrolyzed by others glycosidases (e.g., Galβ1–3GalNAcα1-Ser/Thr) (Fujita et al., 2005). This activity would release GNB then can be readily taken up by the specific GNB ABC transporter and hydrolyzed by LNB/GNB phosphorylase (LNB/GNB cluster). Recently, a novel α-*N*-acetylgalactosaminidase with low identity to the EngBF family has been described in *B. bifidum* JCM1254 (NagBb), which has been ascribed to the new family GH129 (Kiyohara et al., 2012). This enzyme has the capacity to act on core-3 *O*-glycosylated peptides (e.g., GlcNAcβ1–3GalNAcα1-Ser/Thr) and displays both exo- and endoglycosidase activities, but functions primarily in the utilization of the Tn antigen (GalNAcα1-Ser). Moreover, it has an intracellular location, which suggests that this strain has the capacity to transport *O*-glycosylated peptides. EngBF and NagBb provide two different pathways that most probably cooperate in the degradation of different mucin

types. Also, as occurred for free HMO, cross-feeding in the utilization of glycosylated mucins have been reported between bifidobacteria species (Egan et al., 2014a).

### 3.2 Lactobacilli

Some species of *Lactobacillus*, the bacterial genus to which the biggest proportion of characterized probiotics belongs, are also constituents of the gastrointestinal tract. However, contrarily to bifidobacteria, lactobacilli are primarily present in the upper intestinal tract and they do not reach the high numbers found for bifidobacteria.

Genome inspection of several intestinal lactobacilli, such as *Lactobacillus gasseri*, *Lactobacillus johnsonii,* or *Lactobacillus acidophilus*, revealed that they are adapted to colonize the intestinal environment, although they probably rely on diet-derived carbohydrates for their growth and their capabilities to take advantage of HMOs or mucins are limited (Altermann et al., 2005; Azcarate-Peril et al., 2008; Pridmore et al., 2004). *L. gasseri* strains do not grow with mucin or HMOs as the sole carbon source and their genomes do not encode enzymes required for scavenging carbohydrates from these sources, such as α-fucosidases, α-*N*-acetylgalactosaminidases, β-*N*-acetylglucosaminidases, or sialidases (Azcarate-Peril et al., 2008). Despite this, *L. gasseri* has been described as a component of the human milk microbiota (Martin et al., 2003). The majority of the *L. gasseri* and *L. johnsonii* sugar transporters are specific for more simple mono- and disaccharides: glucose, fructose, cellobiose, lactose, sucrose, trehalose, and β-glucosides and belong to the phospho*enol*pyruvate: sugar phosphotransferase system (PTS; more than 20 systems) class. These species carry a PTS specific for GlcNAc, a constituent of HMOs and mucin. A homolog to this PTS from *Lactobacillus plantarum* is induced in vivo at the gastrointestinal tract in animal models (Golomb et al., 2016; Marco et al., 2007), reflecting the importance of this monosaccharide in the intestinal ecology. GlcNAc can be used by *L. gasseri* provided that it is released from mucin and/or HMOs by other members of the intestinal microbiota. *L. gasseri*, *L. johnsonii*, *Lactobacillus casei* and *L. acidophilus*, encode up to 20 glycosidases and phospho-glycosidases in their genomes, but the specificity of most of them is unknown. The enzymes encoding some catabolic steps of the sialic acid degradation pathway are also present in *L. gasseri*, although a complete catabolic route is lacking. Other lactobacilli that colonize mucosal surfaces or are adapted to develop in special environments, such as *Lactobacillus sakei*, *L. plantarum*, and *Lactobacillus salivarius* carry a *nan* cluster (genes *nanAEK*) that allows for the catabolism of *N*-acetylneuraminic acid (Neu5Ac) to GlcNAc-6-P (Almagro-Moreno and Boyd, 2009; Anba-Mondoloni et al., 2013). Accordingly, *nanA*, encoding *N*-acetylneuraminate lyase, was induced in *L. plantarum* in the small intestine of macaques (Golomb et al., 2016).

The generally limited capacity for HMOs or mucin utilization and the apparent lack of metabolic potential for HMOs catabolism encoded by the *Lactobacillus* genomes presents an

exception in the *L. casei/paracasei/rhamnosus* group. Members of this phylogenetically close group carry up to three genes encoding α-L-fucosidases from the GH29 family (Rodriguez-Diaz et al., 2011). Although some of these enzymes display in vitro activity on fucosylated HMOs, such as 2′FL or antigen H structures, these specific carbohydrates are not utilized by lactobacilli. By the contrary, the utilization of fucosyl-α1,3-GlcNAc (3FN) disaccharide, a component of HMOs, has been genetically characterized in *L. casei* [*alf* operon; (Rodriguez-Diaz et al., 2012a)]. The *alf* genes are induced by growth on 3FN via the GntR-class transcriptional repressor AlfR. Contrarily to the transport of most HMOs in bifidobacteria, this carbohydrate is taken up in lactobacilli by a PTS belonging to the mannose family (AlfEFG) by a process that does not involve phosphorylation of the incoming sugar. The disaccharide is then intracellularly split into L-fucose and GlcNAc by the α-L-fucosidase AlfB. The former is expelled from the cell, whereas the later enters the metabolism via the NAc-hexosamine catabolic pathway.

*L. casei* α-L-fucosidases act on short (di-) rather than long (tri, tetra-) oligosaccharides, which implies that this bacteria has the ability to take up previously hydrolyzed HMOs constituents. This is supported by the fact that their α-L-fucosidases lacks signal peptides for secretion and are therefore supposed to act intracellularly. In this sense lactobacilli follow similar strategies in the catabolism of HMOs to *B. infantis*, which also carry intracellular fucosidases. Although bifidobacterial strains from *B. breve* and *Bifidobacterium pseudocatenulatum* species are able to utilize 3FN, similar to lactobacilli, the L-fucose moiety is not catabolized (Becerra et al., 2015a). In fact, L-fucose utilization is not a common capability in lactobacilli and bifidobacteria. Although the genomes of bifidobacteria code for putative L-fucose transporters, as already discussed, the pathways for the catabolism of this sugar have not been elucidated for this group, which is in contrast to the idea that the presence of an L-fucose metabolism constitutes an advantage to develop at the gastrointestinal tract in both commensals and pathogenic bacteria. Thus, the apparent inability for L-fucose fermentation in lactobacilli and bifidobacteria is intriguing, due to the relative abundance of this sugar in HMOs and in the gastrointestinal niche. The faculty to use L-fucose in lactobacilli seems to be restricted to certain strains of *L. rhamnosus* isolated from intestinal environments, whereas this characteristic is absent from *L. rhamnosus* dairy isolates (Douillard et al., 2013). *L. rhamnosus* GG (LGG) carries a *fuc* operon which encodes a pathway for L-fucose catabolism equivalent to that present in *E. coli* (Becerra et al., 2015c). The *fuc* genes are induced by a DeoR-family transcriptional activator that most probably senses the presence of intracellular L-fucose metabolites, such as fuculose-1-phosphate. Similar to enterobacteria, in LGG L-fucose metabolism leads to formation of 1,2-propanediol as a final fermentation product.

The repertory of systems for HMOs and glycoconjugate utilization in the *L. casei/paracasei/rhamnosus* group is extended with the *gnb* operon, which is required for the use of the type-1 HMOs core LNB but also for the utilization of GNB, the core of type-1 *O*-glycosilation (Bidart et al., 2014). The *gnb* genes code for a PTS system of the mannose-class ($PTS^{Gnb}$) that takes up

the two disaccharides, as well as GalNAc, and phosphorylate them. Intracellular LNB and GNB are then hydrolyzed by a specific β-1,3-galactosidase of the GH35 family, GnbG. This enzyme is homolog to BgaG from pathogenic streptococci, although this latter enzyme is cell-wall anchored and only displays activity on LNB and LNT. The *L. casei* enzyme is very specific for β-1,3 linkages and in addition to LNB and GNB it is also active on LNT, although this tetrasaccharide is not fermented by *L. casei*, probably reflecting the lack of transport systems and/or a lacto-*N*-biosidase activity required for further metabolic steps. As transport through $PTS^{Gnb}$ is coupled to phosphorylation, the natural substrates of GnbG are probably LNB-phosphate and GNB-phosphate. According to the selectivity of phosphorylation of the PTS of the mannose family, LNB, GNB, and GalNAc are probably phosphorylated at position 6 in the galactose moiety. Galactose-6P and GalNAc-6P are routed though the tagatose-6-P pathway whereas the generated *N*-acetyl hexosamines (GlcNAc and GalNAc) need to be phosphorylated by yet uncharacterized kinases for being metabolized. The LNB and GNB catabolism in *L. casei* constitutes a novel pathway different to that present in bifidobacteria, which in this latter group consists of an ABC-type uptake system followed by intracellular hydrolysis catalyzed by a LNB/GNB phosphorylase. To date, LNB and GNB utilization in lactobacilli other than *L. casei* is only documented for *L. gasseri* and *L. johnsonii*, although GnbG homologs are not present in these species. Lactobacilli probably depend on other members of the microbiota which contribute with the required glycosidases to release GNB and LNB from complex glycoconjugates and HMOs (e.g., endo-α-*N*-acetylgalactosaminidase and lacto-*N*-biosidase from bifidobacteria releasing GNB and LNB, respectively). The only extracellular enzyme described so far for the utilization of HMOs in *L. casei* is the *N*-acetylglucosaminidase BnaG, an enzyme from the GH20 family which is active on β-1-3 linkages (Bidart et al., 2016). In the *L. casei* chromosome the *bnaG* gene is adjacent to the *gnb* genes but it is independently regulated. BnaG is a cell-wall anchored enzyme that releases GlcNAc from lacto-*N*-triose, a component of type-1 and 2 HMOs. The resulting lactose moiety is used by many lactic acid bacteria via a specific PTS that has been extensively studied due to its technological importance in the dairy industry. Recently, it has been shown that the PTS for lactose in *L. casei* [$PTS^{Lac}$, (Gosalbes et al., 1999)] is responsible for the efficient uptake of *N*-acetyllactosamine, which is hydrolyzed by the phospho-β-galactosidase LacG (GH1) into galactose-6-P and GlcNAc. Therefore, this species is adapted to the utilization of components derived from type-1 and type-2 HMOs. Important probiotic strains, such as LGG carry *lac* clusters homolog to that of *L. casei*, although in this strain the capacity to use lactose is limited due to the presence of mutations in *lacG* and in the regulatory gene *lacT* (Douillard et al., 2013).

All the exposed evidences show that the *L. casei/paracasei/rhamnosus* group is characterized by the capacity to use type-1 and 2 HMOs as well as structures from *O*-linked glycosylations. Although this group is probably not able to directly feed on glycoproteins and complex HMOs, it is able to use many structures derived from them. This reflects a particular adaptation that would increase their fitness for nutrient scavenging at the gastrointestinal tract of breast-fed infants.

## 4 Pathogen Antiadhesion

Many enteric pathogens use specific cell surface glycan structures of the intestinal epithelial cells as targets for binding, known as histo-blood group antigens (HBGAs). This pathogen-glycan binding is the first step in their pathogenesis. Human milk glycans (soluble or as parts of mucins, glycoproteins, or glycolipids) mimic host receptors structures interrupting the pathogen adherence process by acting as receptor analogs (Newburg, 2009), and they protect infants against intestinal diseases produced by viruses, bacteria, and their toxins, and by protozoan parasites. Evidences of the effectiveness of HMOs preventing intestinal infections have been found in vitro, in animal models, and in clinical follow-ups in humans.

### 4.1 Bacteria and Bacterial Toxins

The first evidences of the potential role of HMOs as antiadhesins was obtained using the oligosaccharide fraction of human colostrum and breast milk to block the adhesion of enteropathogenic *E. coli* to HEp 2 cells (Cravioto et al., 1991). HMOs have also been efficient to protect against bacterial toxins including the cholera toxin B subunit (CTB) and the stable toxin (ST) of *E. coli*. In 1995, Idota and collaborators showed that several human milk fractions were able to block the binding of CTB to its cellular receptor (the ganglioside GM1). The inhibition was more efficient (up to 80% inhibition) using the whey protein than the low molecular weight fraction (20% inhibition), while casein was not able to block the binding. Interestingly, the whey from infant formula milk was able to block the binding by 40%. Furthermore, the investigators found that the sialyllactose was able to block the fluid accumulation in rabbit intestinal loops prompted by the CTB (Idota et al., 1995). The same year it was found that the fucosyl-oligosaccharide fraction of human milk was able to inhibit the diarrhea produced by the ST in the suckling mouse model at the physiological concentration found in human milk (Newburg et al., 1995). Few years later, it was shown that the 3-sialyllactose (3′SL) was able to block the binding of *Helicobacter pylori* to human epithelial cells in vitro and to cure the symptoms produced in the rhesus monkey infection model (Mysore et al., 1999). Another oligosaccharide from human milk, the trisaccharide 2′FL blocked in vitro the binding of *Campylobacter jejuni* to the H (O) antigen expressed on the surface of mammalian cells and inhibited *Campylobacter* infection in vivo (Ruiz-Palacios et al., 2003). Oligosaccharides from human milk were also able to impair the adhesion of *L. monocytogenes* to Caco-2 cells (Newburg et al., 2005). Recently, it has been shown that the different oligosaccharide fractions (acidic oligosaccharides, neutral high-molecular-weight oligosaccharides, and neutral low-molecular-weight oligosaccharides) from human milk have also the ability to block the binding of enteropathogenic *E. coli* (O119), *Vibrio cholerae*, and *S. fyris* to Caco-2 cells (Coppa et al., 2006). Additionally, five HMOs including LNT are able to bind in vitro to toxins A and B expressed by *Clostridium difficile* (El-Hawiet et al., 2011). Two disaccharides present in the structures of HMOs and human milk glycoproteins, fucosyl-α1,3-GlcNAc (3FN) and fucosyl-α1,6-GlcNAc (6FN)

(Rodriguez-Diaz et al., 2011, 2012a, 2013), have shown to possess antiadhesive properties against the enteropahogenic *E. coli* (O86) and 6FN was also able to block the binding of the enteropathogenic *E. coli* (O127a) to HT29 cells (Becerra et al., 2015a).

### 4.2 Viral Pathogens

The two main causes of viral diarrhea worldwide are human rotaviruses (RVs) and human noroviruses (NoVs). Both human NoVs and RVs interact with HBGAs through a surface protruding or spike protein domain (P region of VP1 and VP4, respectively) (Tan and Jiang, 2014). As HMOs mimic the HBGAs structures, it is not surprising that HMOs have shown to be effective against these two intestinal viral infections. The effectiveness of human milk as antiadhesive against human NoVs has been shown at several levels. It was first demonstrated that human milk fraction containing glycoproteins but not soluble HMOs, was able to block the binding of human NoVs in vitro. This inhibition was only seen when the milk of secretor positive donors (individuals that have 2-linked fucosyl-oligosaccharides, e.g., the 2′FL) was used (Jiang et al., 2004). This observation was corroborated with a clinical follow-up of breast-fed newborns, where the incidence of NoVs diarrhea was smaller in the babies fed with milks with higher concentrations of lacto-*N*-difucohexaose (LDFH-I), another 2-linked fucosyl-oligosaccharide (Morrow et al., 2004a,b). Recently, the molecular and structural bases of this antiadhesion process are being deciphered using crystallization and surface plasmon resonance (SPR) approaches (Schroten et al., 2016; Shang et al., 2013; Weichert et al., 2016).

The role of HBGAs as receptors in human RVs was discovered much later than in NoVs (Huang et al., 2012), but it was already known that human milk mucin and lacthaderin were able to inhibit rotavirus infection and prevent gastroenteritis in the mice model (Yolken et al., 1992) and this preventive effect disappeared when the mucin and lacthaderine were deglycosylated (Yolken et al., 1992). These experimental data were confirmed with a clinical follow-up in humans that showed that protection against rotavirus is associated with the glycoprotein lactadherin independently of products of the secretory immune system (Newburg et al., 1998). Recently, HMOs have shown to be effective in preventing rotavirus infections also in the piglet model (Hester et al., 2013; Li et al., 2014).

### 4.3 Protozoan Parasites

In a similar way to the bacteria and viruses seen before, the protozoan parasite *Entamoeba histolytica*, which is the third leading cause of death by parasitic diseases, contains Gal/*N*-acetylgalactosamine (GalNAc) lectins that allow its attachment to intestinal epithelial cells (Saffer and Petri, 1991). Thirteen isolated and pooled HMOs significantly reduce *E. histolytica* attachment to enteric cell lines, and LNT inhibits its cytotoxicity in a dose-dependent manner (Jantscher-Krenn et al., 2012). These data would explain the fact that breast-fed infants are at lower risk to acquire *E. histolytica* infections than formula-fed infants (Islam et al., 1988).

## 5 Immune Modulation Properties

Although the HMOs may indirectly affect the immune system of the newborn through changes in the intestinal microbiota and intestinal epithelium cell response, in vitro studies suggest that HMOs are also able to directly modulate the immune system. HMOs could act both locally in mucosal-associated lymphoid cells and at systemic level, as about 1% of them are absorbed and reach the systemic circulation (Gnoth et al., 2001; Rudloff et al., 2006, 2012). After the treatment of T cells isolated from umbilical cord with certain sialylated HMOs, an increase in the amounts of $CD4/CD3^+$ and $CD3^+/CD8^+$ lymphocytes producers of interferon-γ, and $CD3^{+/}CD8^+$ lymphocytes producers of interleukin-13 was observed (Eiwegger et al., 2004). It has been suggested that these HMOs influence lymphocyte maturation, promoting a change in the T cell response toward a more balanced Th1/Th2 response. Moreover, it has been observed that sialylated HMOs are able to reduce the production of interleukin-4 in a subset of lymphocytes from patients with peanut allergy, leading to the conclusion that some HMOs contribute to allergy prevention (Eiwegger et al., 2010). Regarding this, a recent study using a mouse model of allergy to cow's milk and the treatment of children with atopic dermatitis with a combination of *B. breve* and prebiotic galacto- and fructooligosaccharides, demonstrated the ability of this supplement to increase the levels of galectin 9, which was associated with prevention of allergy symptoms (De Kivit et al., 2012). Following the same line, another study in a mouse model of food allergy showed that daily oral treatment with 2'FL and 6'SL attenuated food allergy symptoms, including diarrhea and hypothermia, through the induction of $IL\text{-}10^+$ T regulatory cells and indirect stabilization of mast cell (Castillo-Courtade et al., 2015).

The immunomodulatory properties of fucosylated HMOs have been additionally studied for lacto-*N*-fucopentaose III (LNFP III), which has shown immunosuppressive properties by activating the secretion of antiinflammatory cytokines and by the inhibition of proliferation of $CD4^+$ naïve T lymphocytes (Terrazas et al., 2001). Furthermore, LNFP III stimulates macrophage activity in vitro in several ways, increasing the secretion of prostaglandin E2, IL-10, and TNF-α, and conferring the ability to activate natural killer cells (Atochina and Harn, 2005). Some of the HMOs which contain Lewis antigens also showed reduction of cell–cell interactions mediated by selectins (Bode et al., 2004b,c).

Sialylated HMOs are also able to reduce the rolling and leukocyte adhesion in a dose-dependent manner. The 3′SL and 3′S-3′FL were identified as the key compounds and contribute to fewer inflammatory diseases in breast-fed children (Bode et al., 2004b). Similarly, sialylated HMOs reduce the formation of platelet-neutrophil complex and neutrophil activation in an ex vivo model with human blood (Bode et al., 2004b). All these data would indicate that sialylated HMOs could be interacting with selectins, a family of lectins involved in these processes.

Despite all the evidence that suggests that HMOs have an immunomodulatory role in the development of the immune system of infants, so far there have been only few analyses of the effects of HMOs on immune cells isolated from neonates. Peripheral blood mononuclear cells (PBMC) isolated from pig neonates were treated with different single HMOs, with defined mixtures of HMOs and with complex mixtures purified from human milk using an ex vivo model (Comstock et al., 2014). The results showed that stimulated PBMC with complex mixtures isolated from human milk produces more IL-10 than unstimulated cells and PBMC stimulated with fucosylated HMOs proliferated less than unstimulated cells. In the same study, it was analyzed whether there were changes in the phenotype of T lymphocytes, in response to costimulation of PBMC with HMOs and phytohemagglutinin (PHA) or lipopolysaccharide (LPS). Costimulated PBMC with complex mixtures of HMOs and PHA proliferated more than stimulated PBMC with only PHA. Similarly, costimulated PBMC with sialylated HMOs and LPS proliferate more and they had fewer $CD4^+$ T lymphocytes. This reduction in the population of T helper cells indicates a change in the balance of the T response to effector functions, and together with the increased proliferation suggests a better immune competence (Comstock et al., 2014).

Actually, it is unknown which are the receptors or signaling pathways involved in the signal transduction mediated from HMOs for the production of cytokines in lymphocytes or macrophages stimulation. Many lectins are involved in immune responses and their binding specificity to glycans suggests that HMOs could interfere in this process. For example, siglecs (sialic acid binding Ig-like lectins) are receptors from the cell surface, which recognize sialic acid. Sialylated HMOs are bound by these lectins, which affect the response of the immune system in several manners (Crocker et al., 2007). Other lectins as galectins bind β-galactosides and modulate the immune response (Rabinovich et al., 2002). HMOs are β-galactosides and generally, they have galactose in its nonreducing end. Therefore, they could be involved in signaling pathways mediated by galectins.

## 6 Biosynthesis of Related HMOs Structures

Considering the great number of benefits attributed to HMOs on infant health, there is a growing interest in the synthesis of these compounds. In addition, the evaluation of the impact of HMOs on infant development and the research about the underlying mechanisms of their activity have been limited mainly by the lack of sufficient quantities of structurally defined HMOs. Due to the inherent difficulties to obtain HMOs from human milk, different strategies have been aimed at the synthesis of HMOs. As the complex structure of oligosaccharides makes classical chemical synthesis difficult (Perugino et al., 2004), most of these strategies rely on biotechnology. First biotechnological approach would be to use lactating mammary glands as a biosynthetic reactor for the production of biologically active oligosaccharides and glycoconjugates. Human α1,2-fucosyltransferase (FUT1) has been

expressed in mice yielding likely amounts of secreted LNT in milk (Prieto et al., 1995), and human α1-3/4-fucosyltransferase (FUT3) has been transiently expressed in goat mammary glands and expressed in transgenic mice (Xu et al., 2004). Other transgenic animals, such as rabbits and pigs have also been developed and the expression of human FUT1 resulted in the production of 2′FL (Prieto, 2012).

Engineered microorganisms have been largely used to synthesize high amounts of complex carbohydrates. This biotechnological strategy uses genes encoding key enzymes that are overexpressed in suitable bacterial hosts. Other strategies include biocatalytic approaches using enzymes that either degraded polysaccharides or GTs and glycosidases that synthesized oligosaccharides from simple sugars (Armstrong and Withers, 2013).

### 6.1 Engineered Microorganisms

Whole cell systems are promising approaches for large-scale and cost-effective production of HMOs. The main bottleneck of these in vivo strategies is the integration in one strain of the three components required for the HMOs synthesis: (1) expression of functional GTs; (2) supply of sufficient amounts of nucleotide-activated sugars, and (3) supply of acceptor substrates. Sugar nucleotide synthesis has been successfully enhanced by overexpression of the UDP-glucose pyrophosphorylase GalU in the probiotic bacterium *L. casei* BL23, which produced higher levels of UDP-glucose and UDP-galactose than the wild-type strain (Rodriguez-Diaz and Yebra, 2011). Similarly, the genes *glmM* and *glmS* coding for the phosphoglucosamine mutase and glucosamine-6P synthase enzymes, respectively, were overexpressed in *L. casei* resulting in high levels of UDP-GlcNAc (Rodriguez-Diaz et al., 2012b). Several strategies have been used for efficient GDP-L-fucose supply during the synthesis of fucosyloligosaccharides in *E. coli.* GDP-fucose in this bacterium can be synthesized from the glycolytic intermediary fructose-6-phosphate, which is transformed first into GDP-mannose by the activities of the mannose-6P isomerase ManA, the phosphomannomutase ManB, and the mannose-1P guanylyltransferase ManC. Subsequently, this sugar nucleotide is converted into GDP-L-fucose by the enzymes GDP-mannose-4,6-dehydratase and NADPH dependent GDP-L-fucose synthase, encoded by the genes *gmd* and *wcaG*, respectively. Overexpression of different combinations of those five enzymes (Lee et al., 2009), regeneration of NADPH by expression of glucose-6P dehydrogenase or NADH kinase (Lee et al., 2011b), and the increment of GTP by modulating the guanosine nucleotides synthetic routes, resulted in the enhancement of GDP-L-fucose in recombinant *E. coli* strains(Lee et al., 2012). This sugar nucleotide can also be produced from cytosolic L-fucose by the bifunctional fucose kinase/fucose-1P guanylyltransferase FKP isolated from *Bacteroides fragilis* (Coyne et al., 2005). The FKP enzyme overexpressed in *E. coli* together with enzymes from the GTP biosynthetic pathway produced GDP-L-fucose in a recombinant *E. coli* strain (Zhai et al., 2015). Inactivation of the genes encoding the fucose isomerase FucI

and the fuculose kinase FucK from the utilization pathway of L-fucose increased the flux toward GDP-L-fucose synthesis (Baumgartner et al., 2013).

Most of the successful methods described until now for HMOs synthesis use recombinant *E. coli* strains as bacterial host. An *E. coli* strain deficient in β-galactosidase activity (*lacZ*$^-$) and overexpressing β-1,3-*N*-acetylglucosaminyltransferase *lgtA* gene and/or β-1,4-galactosyltransferase *lgtB* gene of *Neisseria meningitidis*, converted lactose into different HMOs, such as lacto-*N*-triose, LN*n*T, and lacto-*N*-neohexaose (Priem et al., 2002). Similarly, expression in *E. coli* of genes encoding an α-2,3-sialyltransferase and a CMP-NeuAc synthase, respectively, from *N. meningitidis* resulted in the efficient production of 3′-sialyllactose (Priem et al., 2002). *H. pylori futA* gene (α-1,3-fucosyltransferase) and the *N. meningitidis lgtB* gene were also engineered in *E. coli*, which produced tetrasaccharides carrying the Lewis x motif. This recombinant *E. coli* also expressed in the cytoplasm the rhizobial chitin-synthase NodC and the *Bacillus circulans* chitinase, and synthesized chitinbiose. This oligosaccharide is glycosylated by FutA and LgtB producing the tetrasaccharide Galβ1-4(Fucα1-3)GlcNAcβ1-4GlcNAc (Dumon et al., 2006). 3′-sialyllactose could also be synthesized by an engineered *E. coli* strain deficient in neuraminic acid aldolase and expressing a CMP sialic acid synthase from *Neisseria* and a 3′-sialytransferase (*ctsII*) from *H. pylori* (Antoine et al., 2005). Recently, LNT was produced from lactose and glucose or glycerol by using an engineered *E. coli* strain expressing *lgtA* from *N. meningitidis* and the *wbgO* gene, encoding a β-1,3-galactosyltransferase from *E. coli* O55:H7 (Baumgartner et al., 2014). The production of LNT by this latest recombinant *E. coli* was improved by using galactose as the only carbon and energy source in a fed-batch cultivation system (Baumgartner et al., 2015).

All those successful approaches required engineered *E. coli* strains with GT genes belonging to pathogens, which are unreliable sources for some applications, for example, food industry. The number of GT candidates has increased in the past years with the increasing number of bacterial genomes sequenced. However, association of GT genes with their functionality is not an easy task. The GTs sequences do not predict their substrate or linkage specificities and to determine them, a thorough experimental work is required, including efficient screening protocols for enzyme assays. Regarding this, the use of high-throughput screening methods allowed the recovery of 3′-sialyltransferases with increased efficiency (Aharoni et al., 2006), which opens new perspectives for the selection of new enzyme activities after mutagenesis. Additionally, many GTs are membrane-associated proteins, which complicate their expression in bacterial host. In the case of mammalian GTs, their genes are difficult to express in *E. coli*, however some exceptions have been reported (Seto et al., 1999). Human GTs A and B catalyze the transfer of *N*-acetylgalactosamine or Gal to the structure Fucα1-2Galβ-OR to synthesize the blood group A and B antigens, respectively, and soluble forms of both enzymes were successfully expressed in *E. coli* (Seto et al., 1999).

## 6.2 Enzymatic Approaches

In biological systems, GTs and GHs are responsible for the synthesis and hydrolysis of carbohydrates, respectively (Hancock et al., 2006). These enzymes have been extensively studied for synthesizing biologically relevant carbohydrate structures, such as HMOs.

### 6.2.1 Glycosyltransferases (GTs)

GTs catalyze the formation of glycosidic bonds by the transfer of a saccharide, usually a monosaccharide, from a donor substrate to an acceptor substrate (Lairson et al., 2008). Two main groups of GTs can be distinguished: GTs belonging to the Leloir pathway and non-Leloir enzymes. The first ones use nucleotide sugars as donor substrates; while the non-Leloir GTs use other substrates as donors, such as sugar phosphates, sugar lipids, and saccharides. The acceptor in both types of enzymes can be saccharides, proteins, lipids, or nucleic acids. Thus, GTs exhibit the greatest chemical diversity with respect to substrates and products of any enzyme class. GT reactions are, with some exceptions, stereospecific and regiospecific, and the transfer can occur with either retention ($\alpha \rightarrow \alpha$) or inversion ($\alpha \rightarrow \beta$) of configuration at the anomeric carbon of the transferred sugar (Palcic, 2011; Weijers et al., 2008).

Non-Leloir GTs which use phosphorylated glycosyl donors are named sugar phosphorylases. Most of them are glucosyl phosphorylases and use glucose-1-P as the donor molecule and a wide range of glycosides as acceptors, producing glycosyl glucosides, and inorganic phosphate (Weijers et al., 2008). Another group of non-Leloir GTs uses sucrose as glycosyl donor. They transfer either glucose or fructose, and they are accordingly named GTs (or glucansucrases) or fructosyltransferases. Therefore, they form glucose-based oligosaccharides (glucans) or fructose-based oligosaccharides (fructans), respectively (Plou et al., 2002).

GTs of the Leloir pathway use activated nucleotide sugars as donors and monosaccharides or oligosaccharides as acceptors. The saccharides produced are potential acceptors in subsequent GTs reactions and complex oligosaccharides can be synthesized. Additionally, a variety of lipids and proteins can be also glycoside acceptors resulting in diverse glycocomplexes. The advantage of using GTs to synthesize oligosaccharides is that they present high specificity toward the acceptor and do not hydrolyze the product. However, large amounts of sugar nucleotides have a high cost and therefore limit the application of GTs on an industrial scale (Weijers et al., 2008). Some regeneration methods for these substrates have been developed, but still it required expensive high-energy compounds, such as ATP or phosphoenolpyruvate (Chen, 2015a).

The most widely used GT for oligosaccharides synthesis is a bovine milk β-1,4-galactosyltransferase (β-1,4-GalT). This enzyme, belonging to the family of

galactosyltransferase GalT, catalyzes the transfer of galactose (Gal) from UDP-Gal donor to terminal GlcNAc acceptors, producing Galβ-1,4GlcNAc-R (LacNac-R) structures (Palcic, 2011).

Although several GTs have been characterized (Palcic, 2011; Weijers et al., 2008), few of them have been used to synthesize HMOs. The GTs used in the synthesis of these oligosaccharides are mainly represented by fucosyltransferases and sialyltransferases, which use sugar nucleotides as donor substrates (Albermann et al., 2001; Chen, 2015b; Yu et al., 2005, 2006). Using recombinant α-1,2-fucosyltransferase (FucT2) isolated from *H. pylori* in a reaction with GDP-fucose and in the presence of lactose as acceptor, 2′FL was produced with 65% yield (Albermann et al., 2001). Similarly, the recombinant WgbL FucT2 from *E. coli* O126 is able to fucosylate LNB, LacNAc, and lactose (Engels and Elling, 2014). A one-pot reaction strategy was also tested for 2′FL production with WgbL, which includes the synthesis of the GDP-Fuc donor substrate from L-fucose and ATP by the bifunctional fucokinase/GDP-fuc pyrophosphorylase from *B. fragilis* (Engels and Elling, 2014). This enzyme has been recently used in an efficient fucosylation reaction that also contains the pyrophosphatase PmPpA and the *Thermosynechococcus elongatus* α-1,2-fucosyltransferase. PmPpA hydrolyzes the generated pyrophosphate from the GDP-fuc pyrophosphorylase activity and shifts the reaction toward the synthesis of GDP-fucose. In a next reaction step, this activated sugar is transferred to LNT by the fucosyltransferase to produce lacto-*N*-fucopentaose I (Zhao et al., 2016). Using this strategy, more than 1 g of LNFP I was obtained with a 95% yield.

### 6.2.2 Glycosyl hydrolases (GHs)

GHs, also called glycosidases, are a widespread group of enzymes that are responsible for the cleavage of glycosidic linkages. Exoglycosidases as well as endoacting enzymes are involved in a series of important biological events of different nature (Gloster et al., 2008). Most glycosidases used for synthetic purposes are exoglycosidases which catalyze glycosyl transfer to the nonreducing terminal monosaccharide unit of acceptor substrates. The glycosyl donor can be a monosaccharide, oligosaccharide, or activated glycoside (Zeuner et al., 2014).

According to the anomeric configuration of the donor, the reaction mechanism may be given either by retaining (α → α, β→β) or inverting (α→β, β→α) (Weijers et al., 2008). Inverting glycosidases use a single-step mechanism in which the leaving group is directly displaced by the nucleophilic water molecule yielding the product with an inverted anomeric configuration. The retaining mechanism consists of a double-displacement reaction in two subsequent steps. In the first step, one carboxyl group functions as general acid while the other acts as a nucleophile forming a glycosyl-enzyme intermediate. In the second step, the same residue that previously acted as general acid functions as a general base activating the incoming acceptor (water, alcohol, or other sugar), which hydrolyzes the glycosyl-enzyme intermediate producing a new glycosidic linkage (Mccarter and Withers, 1994). Through this last mechanism, the carbohydrate synthesis is promoted by transglycosylation reactions (Naumoff, 2011).

In transglycosylation reactions, the glycosyl donor should contain a good anomeric leaving group. This donor must be fast reacting to keep reaction times short, thus allowing less time for product hydrolysis. In addition, a donor that binds strongly to the enzyme (low Km) also minimizes product hydrolysis (Van Rantwijk et al., 1999). In several cases, aryl donor substrates resulted in higher yields than di- or trisaccharide donors; which might be due to the aryl aglycone being a better leaving group than the mono- or disaccharides. *p*-Nitrophenyl (pNP)- and *o*-Nitrophenyl (oNP)-monosaccharides are donor substrates widely used by sialidases, α-L-fucosidases and β-galactosidases in transglycosylation reactions (Zeuner et al., 2014).

β-galactosidases, sialidases, and α-L-fucosidases show broad acceptor specificity. β-galactosidases can catalyze transgalactosylation of several substrates including mono-, di-, and oligosaccharides of both anomeric configurations, methyl/aryl glycosides and both primary and secondary alcohols and phenols. The sialidases catalyze the sialyl transfer mainly to α-galactosyl residues, α-glucosyl residues, and monomers of glucose and methyl galactopyranoses of both anomeric configurations (Zeuner et al., 2014). α-L-fucosidases generally use monosaccharides or *p*NP-monosaccharides acceptors (Osanjo et al., 2007; Rodriguez-Diaz et al., 2013).

Glycosidase-catalyzed synthesis can occur either by condensation reaction, which is a thermodynamically controlled reverse hydrolysis activity where the donor substrate is only a monosaccharide, or by kinetically controlled transglycosylation activity, where an activated donor, for example, a disaccharide or a *p*NP monosaccharide is used. Different from the reverse hydrolysis, where the maximum yield is determined by the reaction equilibrium, in transglycosylation the reaction direction can be shift to the glycosylated acceptor product if the substrate is more reactive than the product. Additionally, the reaction times in this case are generally shorter than in reverse hydrolysis (Van Rantwijk et al., 1999).

The transglycosylation product is also substrate for the glycosidase and thus can undergo subsequent hydrolysis or function as a donor substrate, and the rate is dependent on the regioselectivity in the product formation because not all regiosomers are equally good substrates (Berteau et al., 2004). Therefore, the efficacy of transglycosylation depends to a large extent on the kinetic synthesis/hydrolysis ratio, which describes how efficiently the glycosyl-enzyme intermediate is transformed into product. Besides, secondary hydrolysis proceeds via the same intermediate; consequently, secondary hydrolysis will be one of the main drawbacks if that ratio is low (Van Rantwijk et al., 1999).

GHs have the disadvantage that the regioselectivity may not be observed in all cases and the product can be hydrolyzed during the reaction leading to a low yield (Cobucci-Ponzano and Moracci, 2012; Crout and Vic, 1998). Interestingly, they have several advantages, such as an easy access due to the wide availability of many organisms and specially microorganisms (Cabezas et al., 1983), their tolerance to organic solvents (Palcic, 1999), the potential

to transfer block oligosaccharides in one step (Wang and Huang, 2009), and the use of donor substrates readily available, such as compounds of o/pNP-sugars (Rodriguez-Diaz et al., 2013). Therefore, this kind of enzymes is increasingly attractive for development of compounds with biological relevance, such as HMOs. The production of valuable compounds, including HMOs, through transglycosylation reactions has been achieved mainly with β-galactosidases (EC 3.2.1.23), sialidases (EC 3.2.1.18), and α-L-fucosidases (EC 3.2.1.51) (Zeuner et al., 2014).

#### 6.2.2.1 β-galactosidases

Structures, such as LacNAc and LNB as well as prebiotic galacto-oligosaccharides (GOS) have been synthetized by β-galactosidases (Intanon et al., 2014; Zeuner et al., 2014). Although these glycosidases have been isolated and characterized from yeast, fungi, and bacteria, the major industrial enzymes are obtained from *Aspergillus* spp. and *Kluyveromyces* spp. (Intanon et al., 2014). The cloning and characterization of β-galactosidases from different species, such as *Kluyveromyces lactis, Aspergillus oryzae, Bacillus circulans,* and *Lactobacillus* spp. has allowed the identification of different specificities in the production of GOS (Rodriguez-Colinas et al., 2014; Splechtna et al., 2006). The synthesis of these by transgalactosylation reactions uses lactose as β-galactosyl donor as well as acceptor substrate. However, although lactose has also been utilized for LacNAc and LNB production, generally for their synthesis p/oNP-Gal is used (Zeuner et al., 2014). The use of a β-galactosidase from *B. bifidum* in transgalactosylation reactions with pNP-Gal and GlcNAc substrates, mainly produced β-(1,4)-linked disaccharide (LacNAc), with β-(1,6)-linked disaccharide (allo-LacNAc) as minor product (Yoon and Rhee, 2000).

#### 6.2.2.2 Sialidases

Sialidases catalyze the specific release of terminal sialic acid residues α-linked to glycoproteins, glycolipids, and polysaccharides, resulting in hydrolysis or *trans*-sialylation. Although the use of sialidases in the synthesis of oligosaccharides has been much lower as compared to β-galactosidases, some studies have shown that these enzymes isolated from various sources (mainly microorganisms) have the ability to produce some sialylated structures, such as different isomers of sialyl-*N*-acetyllactosamine, which are found in human milk (Ajisaka et al., 1994; Schmidt et al., 2000).

#### 6.2.2.3 α-L-fucosidases

α-L-fucosidases are responsible of cleaving fucosidic bonds in oligosaccharides and glycoconjugates. According to the CAZY database, all α-L-fucosidases hydrolyzing fucosyl derivatives with retention of the anomeric configuration belong to the GH29 family, whereas inverting α-L-fucosidases are classified in family GH95 (Cobucci-Ponzano et al., 2003; Tarling et al., 2003). GH29 α-L-fucosidases has been used generally used to carry out transfucosylation reactions. These enzymes are widely available from different sources

including microorganisms, plants, insects, mollusks, and mammals (Henrissat, 1991), but few studies have described the synthesis of carbohydrates by transfucosylation activity. α-L-fucosidases used in transfucosylation reactions have been isolated from fungi, such as *Aspergillus niger* (Ajisaka and Shirakabe, 1992) and *Penicillium multicolor* (Ajisaka et al., 1998), from thermophilic bacteria, such as *Thermus sp.* (Eneyskaya et al., 2001) and *Thermotoga marítima* (Osanjo et al., 2007), as well as from soil metagenomes (Lezyk et al., 2016). The hydrolysis of the products and the formation of different isomers in those reactions were the main impairments to obtain optimal yields. Contrarily, α-L-fucosidases from *L. casei* have shown high specificity and regioselectivity to synthesize biologically important fucosyl-*N*-acetylglucosamine disaccharides (Fig. 15.2) (Becerra et al., 2015b; Rodriguez-Diaz et al., 2013).

### 6.2.2.4 Glycosynthases and *trans*-glycosidases

Glycosidases have been proven to be extremely valuable in carbohydrate synthesis; however, the use of these enzymes is subject to two key limitations, namely, the challenge of driving the reaction in a thermodynamically disfavored direction and the enzymatic degradation of the reaction product (Hancock et al., 2006). In order to overcome such limitations and

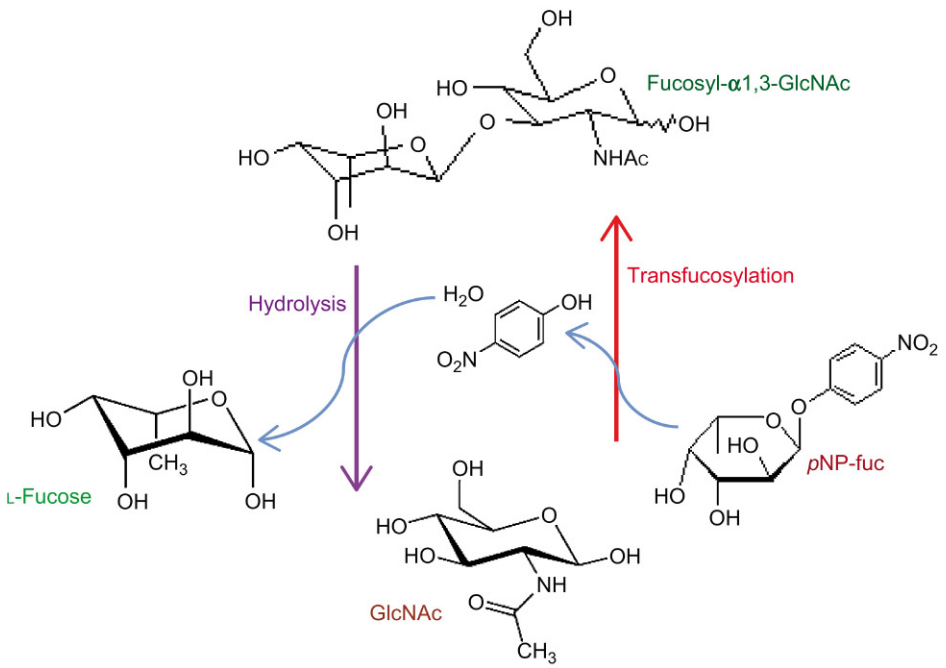


**Figure 15.2: Hydrolysis and Transfucosylation Reactions by α-l-Fucosidase AlfB From *Lactobacillus casei*.**

*GlcNAc*, *N*-acetyl-glucosamine; *pNP-fuc*, *p*-Nitrophenyl-fucopyranoside.

improve performance in the synthesis, many glycosidases have been extensively subjected to enzyme engineering procedures, which generate either glycosynthases or *trans*-glycosidases depending on the strategy used.

The strategy used to generate a glycosynthase is based on the mutation of catalytic residues, so that the active site of the enzyme would be affected (Hancock et al., 2006), preventing the hydrolysis and thus allowing the formation of new glycosidic bonds, and therefore promoting the reaction to the direction of synthesis (Mackenzie et al., 1998). To achieve this type of variants, usually the nucleophile residue (Asp or Glu) located in the active site of the enzyme is replaced by another residue (typically Ala, Ser, or Gly) (Bojarova and Kren, 2009). This technology was introduced since 1998, with a β-glucosidase from *Agrobacterium* sp. (a retaining exo-β-glucosidase). The variant produced efficiently catalyzes the formation of β-glycosidic bonds with α-glucosyl fluoride (donor substrate) in the presence of suitable acceptor substrates (Mackenzie et al., 1998). Based on this technology, two α-L-fucosidases, which belongs to *T. maritima* and *Sulfolobus solfataricus,* were converted into α-L-fucosyntases (Cobucci-Ponzano et al., 2009). These mutant enzymes are efficient to transfer α-L-fucosyl residues to various acceptor molecules, but intrinsically they fail to control the regioselectivity and acceptor specificity, making the purification of the produced oligosaccharides difficult. A generated α-1,2-L-fucosynthase from the α-1,2-L-fucosidase BbAfcA (an inverting enzyme) of *B. bifidum* synthesized 2′FL with β-L-fucopyranosyl fluoride as donor and lactose as acceptor substrate (Wada et al., 2008). The glycosynthase generated from the α-1,3/4-L-fucosidase BbAfcB was able to transfer fucose residue through an α(1,3/4)-linkage to the GlcNAc residue present in LNB and LacNAc structures (Sakurama et al., 2012). As occurs with the galactosynthase from *Bacillus circulans*, both α-L-fucosynthases allowed the synthesis of various HMOs structures. Besides, the synthesis was carried out with relatively good yields, with perfect control of the regioselectivity, and high substrate specificity (Li and Kim, 2014; Sakurama et al., 2012).

*Trans*-glycosylation activity can also be enhanced by using *trans*-glycosidases obtained by directed evolution (Osanjo et al., 2007). This strategy refers to a variety of methods to improve or alter the enzymatic function, which are based on the principles of natural selection (Johannes and Zhao, 2006; Kittl and Withers, 2010). These methods focus primarily on creating libraries of mutant genes from a wild-type gene through random mutagenesis, followed by selection of the "best variants" and recombination of these to achieve mutant enzymes with desired characteristics, which are called *trans*-glycosidases (Johannes and Zhao, 2006; Kittl and Withers, 2010). The first *trans*-glycosidase obtained by directed evolution has been from *T. maritima* α-L-fucosidase, called α-L-transfucosidase, achieving the production of pNP-α-Gal-1,2-Fuc with a relatively high yield (Osanjo et al., 2007). Recently, *trans*-fucosidases developed from *B. longum* α-L-fucosidase have shown ability to synthesize compounds, such as lactodifucotetraose, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, and lacto-*N*-difucohexaose I as well as more complex HMOs like

fucosylated para-lacto-*N*-neohexaose (F-p-LNnH) and mono- or difucosylated lacto-*N*-neohexaose (F-LNnH-I, F-LNnH-II, and DF-LNnH) (Saumonneau et al., 2016).

Glycosynthases as well as transglycosidases have shown to be very efficient for the production of carbohydrates with relevant bioactivity. Thus, it is important to exploit these new enzymatic tools as an alternative for the synthesis of oligosaccharides, such as HMOs on a large scale.

## 7 Biotechnological Applications

### 7.1 Oligosaccharides in the Milk of Other Mammals: Infant Formulas

HMOs are present in human milk at high concentrations, but they are not present or present in low concentrations in infant formulas. These are based in bovine milk (BMOs), which contains significantly lower amounts of oligosaccharides and with lesser complex structures than the HMOs. The concentration of oligosaccharides in bovine colostrum is about 1 g/L and decreases in mature milk to about 0.03–0.06 g/L (Urashima et al., 2013). Unlike human milk, bovine milk free oligosaccharides and *N*-glycans from proteins are highly sialylated and to a lesser extend fucosylated. In addition to *N*-acetylneuraminic acid (Neu5Ac) BMOs also contain *N*-glycolylneuraminic acid (Neu5Gc), which has never been detected in HMOs to date. Similarly, acidic BMOs containing phosphate are present in bovine milk but not in human milk. Another difference with human milk is that bovine milk exclusively contain type-2 oligosaccharides, for example, lacto-*N*-neotetraose (LN*n*T) and their derivatives, but does not have LNT, the type-1 most abundant HMO as already mentioned earlier (Urashima et al., 2013). Oligosaccharides from the milk of other domestic animals, such as goats, sheep, pigs, horses, and camels have been characterized and they revealed many similarities with bovine milk, including their presence in low concentrations and that about 80%–90% of the total oligosaccharide pool is sialylated (Albrecht et al., 2014). Some other differences have also been found. For example, the variety of oligosaccharides in goat milk is higher than in cow milk, and the concentration of fucosylated oligosaccharides in porcine milk is higher than in bovine milk (Urashima et al., 2013).

In an attempt to resolve the low amount of oligosaccharides present in bovine milk, some infant formulas have been supplemented with prebiotic oligosaccharides, including GOS, fructooligosaccharides, and/or polydextrose. The addition of these oligosaccharides to infant formulas stimulates the growth of *Bifidobacterium* species (Ben et al., 2008; Knol et al., 2005; Scalabrin et al., 2012). Similarly, the growth of *Lactobacillus* was also stimulated in infants fed with formulas containing GOS (Ben et al., 2008). However, those oligosaccharides are not present in human milk, they have important structural differences with HMOs and the fructose monomer is not found in human milk. Regarding this and despite their beneficial effects, the stimulating probiotics grow in infants gut; further studies are needed to evaluate the long-term health effects on newborns fed with those nonhuman oligosaccharides.

The synthesis of individual and structurally defined specific oligosaccharides that are indeed present in human milk appears to be currently the trend of the food industry. Biotechnological approaches using engineered microorganisms and enzymatic approaches resulted in the availability of the smallest HMOs, tri- and tetrasaccharides, on a large scale, allowing preclinical and clinical studies. 2′FL industrially produced has been used in a preclinical study in farm piglets (Hanlon and Thorsrud, 2014), and LN*n*T alone or in combination with that trisaccharide have been used in clinical studies with infants and they have been evaluated as safe ingredient for infant foods by the European Food Safety Authority.

### 7.2 Pharmaceutical Compounds

Structural and functional studies about HMOs are likely to be very helpful for the development of pharmaceutical products, which might be effective in protecting newborns from infectious diseases or indicated a variety of diseases in adults. Oligosaccharides present in glycoproteins and glycolipids on the cell surfaces, play important roles in the molecular recognition and communication among human cells and between these and pathogen microorganisms. The development of specific oligosaccharides that may interfere with the binding of pathogens to host cells could be an important glycan-based therapeutic research area. As well, in different types of diseases, such as cancer, neurodegenerative disorders, or arthritis, the cells are abnormally glycosylated (Xu et al., 2005). Synthesis of specific oligosaccharides for avoiding the interaction of cancerous cells with healthy cells could reduce the risk of metastasis. Similarly, the design of carbohydrate-based vaccines targeting specific glycan epitopes on pathogens surfaces or cancerous cells is also a biotechnological challenge. Several polysaccharide vaccines are already commercially available or in phase of development (Astronomo and Burton, 2010; Krasnova and Wong, 2016).

## 8 Conclusions

Human milk glycans, including free oligosaccharides, glycoproteins, glycolipids, and GAGs, have an important role on infant health and development. They are substrate for certain *Bifidobacterium* and *Lactobacillus* species, which have probiotic properties and are present in the gastrointestinal tract of newborns. Those glycans have also antiinfective properties against many enteric pathogens, including bacteria and their toxins, protozoan parasites, and viruses. Additionally, they play important functions in the modulation of the newborn immune system. Considering all these relevant bioactivities associated with milk oligosaccharide, great interest has been aroused to product them by using biotechnological approaches. The most promising strategy to get HMOs in good quantities relies on the use of engineering microorganisms expressing appropriate enzymes and implementing a fed-batch cultivation system. Another strategy with relevant results for synthesizing all kind of HMOs involves transglycosylation reactions carried out with glycosidases, such as β-galactosidases,

sialidases, and α-L-fucosidases. Unlike GTs, these enzymes have the advantage to be found in many microorganisms, to use acceptor and donor substrates readily available and to be easily engineered. This is the case of *trans*-glycosidases, which are obtained by directed evolution and they have been used to efficiently produce oligosaccharides.

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# HANDBOOK OF FOOD BIOENGINEERING
VOLUME 14

# ADVANCES IN BIOTECHNOLOGY FOR FOOD INDUSTRY

Edited by
**Alina Maria Holban** and **Alexandru Mihai Grumezescu**

*Advances in Biotechnology for Food Industry*, a volume in the *Handbook of Food Bioengineering* series, aims to offer an updated perspective regarding the impact of biotechnology in modern food industry, empathizing on new technologies, and innovative approaches in food design and processing. It takes a global approach to describe how food needs are diverse among populations causing the need to develop different, innovative biotechnological processes to ensure efficient food production and processing worldwide. This volume explores how biotechnological processes can increase production and quality of food products including the development of anti-biofilm materials utilized for bioreactors and food processing facilities to decrease the risk of microbial colonization and biofilm formation.

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Food Engineering

ISBN 978-0-12-811443-8
9 780128 114438

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