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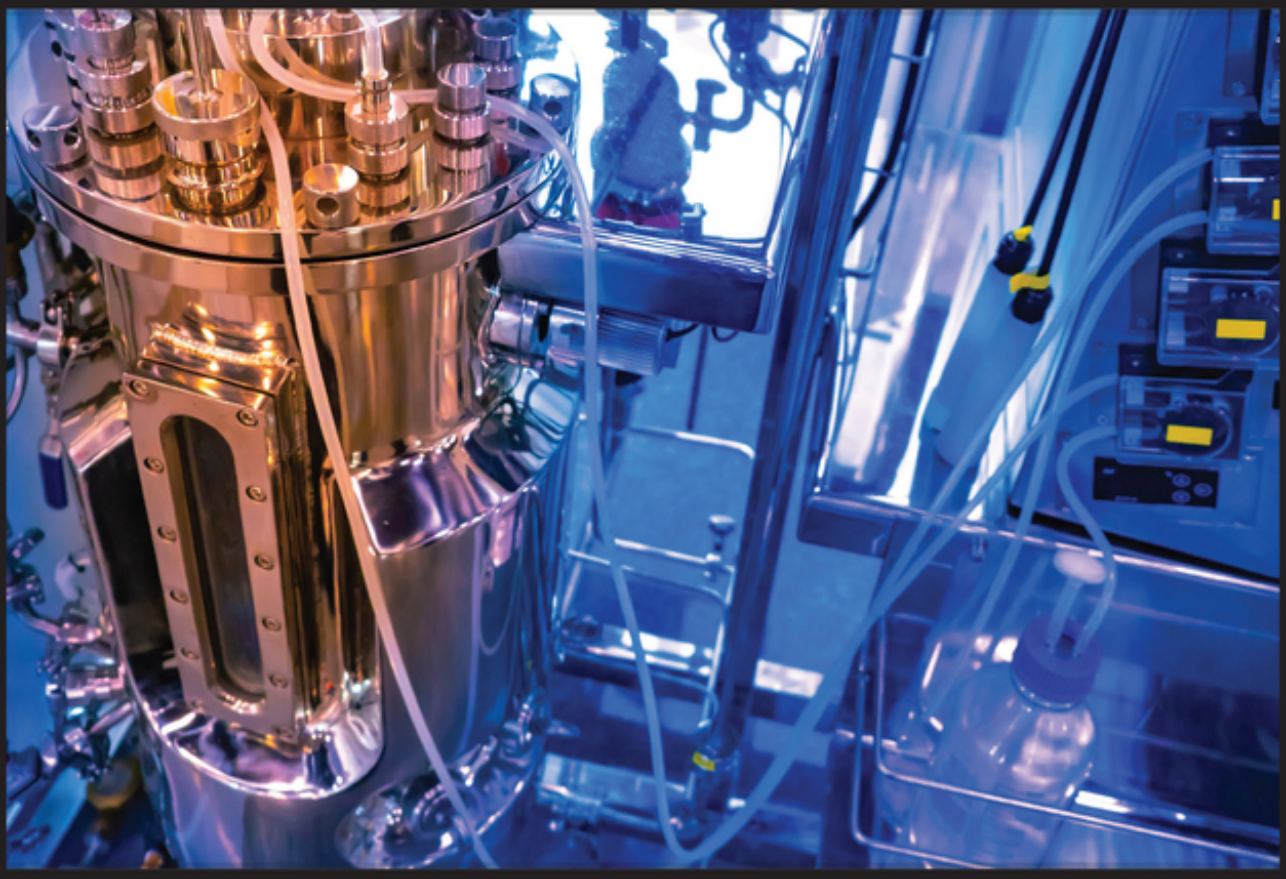
# BIOPROCESSING FOR BIOMOLECULES PRODUCTION



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**Bioprocessing for Biomolecules Production**

# Bioprocessing for Biomolecules Production

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# Part I

# General Overview of Biotechnology for Industrial Segments: An Industrial Approach

# 1

# An Overview of Biotechnological Processes in the Food Industry

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## 1.1 Introduction

The problems related to climate change and the rising costs of supplies and fuel associated with population growth have led to the search of renewable and sustainable technologies in order to provide products that meet market and consumers demand (Zilberman et al. 2013). This technological development is crucial for the implementation of *bioeconomics,* in which biotechnological processes are fundamental for providing economic opportunities for the chemical, food and feed, pulp and paper, textiles, automotives, electronics, and energy sectors (De Buck et al. 2016; Diehl 2017).

Plants and animals have interesting commercial compounds (e.g., flavors, polysaccharide, fatty acids), and research in recent years has focused on extracting those compounds. As a bonus, they have the advantage of being labeled as *natural,* which consumers increasingly desire (Akacha and Gargouri 2015; Nigam and Luke 2016). However, the availability of these resources is insufficient to meet industrial needs, due to seasonality, ecological, social, and political factors, in addition to the low achievable yields that limit the use of several compounds obtained from this source (Felipe et al. 2017).

The limitations encountered in the extraction processes have encouraged the search for alternative methods to obtain commercial products such as chemical synthesis (Longo and Sanromán 2006). Chemical synthesis is characterized as a low-cost process with flexible production and efficiency, and is commonly used in industrial processes (Carroll et al. 2016; Serra et al. 2005). However, the environmental damages resulting from its use, as well as the distaste for products seen as modified or *unnatural,* has resulted in disadvantages for the use of this process (Abu Yazid et al. 2017).

Bioprocesses have thus become a promising alternative for obtaining natural products due to the climatic, seasonal limitations, and the availability of natural sources used in the extraction processes. They are aimed at developing sustainable and efficient industrial processes with the potential to result in commercially applicable products, such as polysaccharides (Arisawa and Watanabe 2017; Barnard et al. 2012).

The fermentation process is one of the earliest methods of using biotechnology, considered a key component of several industrial applications that involve the use of

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biological material. As an example, it contributes to several food additives, besides the range of other high-value-added products, which mainly serve the food industry (Chotani et al. 2017; Lokko et al. 2018). In 2015, the market for fermentation-derived products was valued at US$ 24.3 billion, with an expected annual growth of 7.7%, reaching US$ 35.1 billion in 2020 (Felipe et al. 2017; PRNewswire 2015).

Through technological development, the options for using these biotechnological processes have expanded, making it possible to obtain natural flavors and dyes; emulsifiers; new vitamins, and improved enzymes. Also, the use of genetic modification (GM) techniques allows for the creation of new products, optimization of processes, and new waste-treatment procedures, from "greener" manufacturing processes (Maryam 2017; Nyambok and Robinson 2016).

Thus, considering the advantages of using biotechnological processes for the food industry, the purpose of this chapter is to present an overview of the industrial landscape of final products and food additives obtained from biotechnological processes, as well as general perspectives and economic data of the sectors.

## 1.2 Biotechnological Process Applied to Food Products

Consumer demand for natural food products has encouraged the use of biotechnological processes and their development, in order to obtain economically viable and optimized commercial products (Gupta et al. 2017). In this way, several companies, such as Novozymes, Danisco, and Nestlé, have pursued expansions in the microorganisms-related market to capitalize on that demand. Mergers between companies have also become an alternative to achieve greater development and space in the sector, as in the case of Bayer-Monsanto, Syngenta-ChemChina, and Dow-DuPont (Messeni Petruzzelli et al. 2015; Novozymes 2016).

The presence of biotechnology in food-processing chain occurs with the use of microorganisms for the preservation and production of a variety of food products or aiming at the production of food additives, such as enzymes, vitamins, organic acids, aminoacids, flavor compounds, microbial lipids, proteins, carbohydrates, and several others (Balciunas et al. 2013; Karihaloo and Perera 2010; Kawasaki and Ueda 2017; Ramachandra Rao and Ravishankar 2002). Hence, the main industrial products obtained from biotechnological processes of the sector will be presented in this section.

### 1.2.1 Organic Acids

Organic acids are considered organic compounds with weak acidic properties that do not completely dissociate in the presence of water. Being commonly produced from the primary metabolism of the microorganisms, they present several applications in the industrial sector of food processing, nutrition and feed, pharmaceuticals, and oil and gas stimulation units (Chen and Nielsen 2016; Panda et al. 2016).

Biotechnological methods are the most used to produce organic acids, with the first processes documented in 1823 and 1913, to obtain acetic acid and citric acid, respectively. The application of DNA recombination techniques and metabolic engineering to obtain organic acids has been used with the intention of increasing the efficiency of these biotechnological processes, as well as to meet the global market demand for these bioproducts (Becker et al. 2015).

These products can support shelf-life extension, improve moisture retention, prevent oxidation of lipid components, accelerate solidification and coagulation, and enhance flavor and ion chelation (Kawasaki and Ueda 2017). Organic acids are thus considered one of the most important additives in the food industry, and are widely used in beverages, food, and in the processing of these products (Quitmann et al. 2013).

The most popular organic acids used in food processing are citric acid, acetic acid, formic acid, and lactic acid (Kawasaki and Ueda 2017). In 2011, citric acid was most used in the market, with a bioproduction around 1.75 million tons (Chen and Nielsen 2016). However, by 2016 and 2017, acetic acid dominated the market, with values around US$ 1.8 billion. Both of these organic acids are of great interest in the food and pharmaceutical sectors (Report Buyer 2019).

Global market of organic acids reached US$ 16.8 billion in 2016, dominated by the citric acid and formic acid of microbial origin (Sahu 2017). With the growing demand for natural products, it is expected the value will grow 7% by 2022, with expected income of US$ 20 billion (Shrivastava 2017).

The main companies representing the organic acid market are BASF SE (Germany), Dow Chemical (United States), BP Plc, BioAmber Inc. (Canada), Tate and Lyle Plc (United Kingdom), Archer Daniels Midland (United States), Corbion NV (The Netherlands), Elekeiroz SA (Brazil), Cargill Inc. (United States), Henan Jindan Lactic Acid Technology Co. Ltd. (China), Myriant Corporation (United States), Jungbunzlauer Suisse AG (Switzerland), Nature Works LLC (United States), Celanese Corporation (United States), and Eastman Chemicals Company (United States) (Transparency Market Research 2017a).

### 1.2.2 Flavors

Flavors can be defined as volatile organic compounds (VOCs), which can be subdivided mainly as alcohols, aldehydes, carboxylic acids, furans, fatty acids, esters, ethers, hydrocarbons, ketones, lactones, pyrazines, and terpenes (Longo and Sanromán 2006; Wylock et al. 2015).

Among the processes used for flavor production, chemical synthesis is most used (Chreptowicz et al. 2016). However, due to the negative image associated with the chemical synthesis processes, mainly related to their classifications as "artificial," several companies have sought alternative methods, such as biotechnological processes (Akacha and Gargouri 2015).

The biotechnological processes offer advantages for the generation of volatile compounds, with the potential to obtain high optical purity and not having seasonal interferences (Felipe et al. 2017). In addition, these processes have the appeal of sustainable production, an attractive factor for industries (Berger 2015).

Flavors are considered the largest segment of the food additives market, besides being one of the main components of the global market, registering in 2016 a value of US$ 12.1 billion, characterized as a highly specialized, technical, and innovative category (Mordor Intelligence 2017).

These compounds are mainly used in dairy products, beverages, confectionery products, nondairy ice cream, bakery products, and nutraceuticals. Such compounds may be marketed as powder, liquid, and paste, of which vanilla, vanillin, and fruity flavors are the products with the greatest prominence in the sector. In relation to vanillin, commercial

projections indicate that in 2023 the consumption of this product would be approximately 500 tons (Verma 2016; Market Research Future 2018a).

Companies in the biotech-flavors sector have invested in research and innovation, in order to excel in the market. As an example, Givaudan S.A. (Switzerland) stood out with a market share of 18.7% in 2016 (Goeke et al. 2017; Schroeder and Ruethi 2017). In the same year, Firmenich S.A. (Switzerland) (13.5%), International Flavors and Fragrances Inc. (United States) (12.3%), and Symrise A.G. (Germany) (9.2%) also represented the sector, with market values estimated at US$ 24.7 billion at the end of that period (Leffingwell & Associates Flavor 2017).

### 1.2.3 Polysaccharides

Polysaccharides obtained from biotechnological processes are high-molecular-weight biopolymers, being water-soluble gums with novel and unique physical properties (Bergfeld et al. 2011). With similar application to those polymers extracted from natural sources, biopolymers have stable characteristics, with unlimited availability in the market, and well-controlled production processes on a large scale within a comparatively limited space and production time, thus favoring their production and commercial application (Giavasis 2013).

The industrial application of polysaccharides can take place in several sectors, such as textiles, detergents, adhesives, microbial enhanced oil recovery, wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives (Rütering et al. 2016). In the food industry, polysaccharides are mainly used as thickeners, stabilizers, and gelling agents in a wide range of food products (Ramalingam et al. 2014).

The current global hydrocolloids market was estimated at US$ 5.8 billion in 2017, with projected growth of 3.6% over the period 2017–2025, having an expected value of US$ 7.6 billion at the end of that period (Transparency Market Research 2017b). Among the main commercial microbial polysaccharides, hydrocolloids are xanthan gum, dextran, scleroglucan, pullulan, and levan (Ates 2015).

Among those, xanthan gum is the most significant exopolysaccharide on the market, accounting for 6% of the total market value of hydrocolloids, with annual production of about 30 000 tons (Ates and Oner 2017). Produced from *Xanthomonas campestris*, xanthan gum stands out for its rheological properties, pseudoplasticity, thickening property, and heat stability, acid and alkali, and was the first biopolymer produced on an industrial scale (Li et al. 2016). These factors directly reflect its increased demand of 5–10% per year, demonstrating its importance (Habibi and Khosravi-Darani 2017).

The companies that stand out in recent years are DuPont (United States), Cargill Inc. (United States), Darling Ingredients (United States), Kerry Group (Ireland), CP Kelco (United States), Fuerst Day Lawson (United Kingdom), Ingredion Inc. (United States), Ashland Inc. (United States), DSM N.V. (The Netherlands), and Rico Carrageenan (Philippines) (Market Research Reports Search Engine 2017).

### 1.2.4 Amino Acids

Amino acids play a fundamental role as building blocks of proteins (Mitsuhashi 2014). Nine amino acids are considered essential for human and animal metabolic processes:

*L*-valine, *L*-leucine, *L*-isoleucine, L-lysine, *L*-threonine, *L*-methionine, *L*-histidine, *L*-phenylalanine, and *L*-tryptophan (Leuchtenberger et al. 2005).

Amino acids have several industrial applications in the food, cosmetic, pharmaceutical, and animal feed industries (Tonouchi and Ito 2016). Focusing on the food industry, these compounds are used for dietary supplementation, mainly in food products linked to infants' and children's diets (Mitsuhashi 2014).

The amino acids with the highest commercial interest are *L*-glutamate (monosodium), L-lysine (chloride), *L*-tryptophan, methionine, and *L*-phenylalanine, with glutamate being the most required in the industry (IMARC Group 2017; Tonouchi and Ito 2016).

In 2015, the global market of amino acids was estimated at US$ 4.88 billion, with an expected growth of 6.5% between 2017 and 2023 (Research and Markets 2017). The food amino acids market is expected to grow 7.8% by 2022 due to the market's encouragement of healthy foods and constant process innovations, with projected values for the end of this period of US$ 6.82 billion (Markets and Markets 2017a).

Among the main companies in the food amino acid market are Ajinomoto Co. Inc. (Japan), Kyowa Hakko Kirin Group (Japan), Evonik Industries A.G. (Germany), Sigma-Aldrich Co. LLC (United States), Prinova Group LLC. (United States), and Daesang Corporation (South Korea), among others (Markets and Markets 2017a).

### 1.2.5 Enzymes

Enzymes are considered natural reactions catalysts, presenting the ability to accelerate specific chemical reactions, thus reducing the activation energy required for the process. These proteins have great application in the food and beverage industries, being used to control the brewing process (Singh et al. 2016).

The enzyme market is driven by the need to improve flavoring, texture, and quality of food products. It encourages the search for new technologies in large-scale fermentation processing and the expansion of the food and beverage industries (Verma 2017). The essential enzymes have broad application in the food and beverage industry, such as amylase, cellulase, xylanase, pectinase, protease, and lipase, among others, mainly used in baking, juice production, dairy, and brewing (Kumar et al. 2014; Singh et al. 2016).

Among the main commercial enzymes, amylases stands out in the industrial sector, accounting for about 25% of the global enzyme market, followed by proteases (Khemakhem et al. 2018). Projections made for the sector by the Freedonia Group (2016) suggest growth of global demand for enzymes around 4.6% by 2020. This data represents a total global revenue of US$ 7.2 billion, just for the food and beverage sector, projections of US$ 2.3 billion in 2020.

Among the main companies in this sector, Novozymes (Denmark), DSM N.V. (The Netherlands), AB Enzymes (United States), Amano Enzyme Inc. (Japan), DuPont (Danisco) (United States), Soufflet Group (French), Megazyme (United States), Boli Bioproducts (China) can be highlighted (Hegde 2017). Novozymes is considered the market leader for industrial enzymes, with a 48% share in the 2016, followed by DuPont, which had an estimated share of 19% in the same year (Novozymes 2016).

### 1.2.6 Surfactants

Microbial surfactants, also known as biosurfactants, are amphiphilic surface active molecules featuring the ability to alter the surface and interfacial properties of a liquid,

resulting in the formation of microemulsions (Campos et al. 2013). As an advantage over conventional surfactants, biosurfactants can be produced with less environmental impact when renewable substrates are used, as well as being biodegradable and presenting lower toxicity (Henkel et al. 2017).

The potential for application of these compounds is mainly as emulsification agents in pharmaceutical, food, dye, cosmetic, and agrochemical industries (Sivapathasekaran and Sen 2017). Among the main commercial products are rhamnolipids, sophorolipids, methyl ester sulfonates, alkyl polyglucosides, sorbitan esters, and sucrose esters (Panjiar et al. 2017; Variant Market Research 2017).

The food-processing sector of the biosurfactants market was 5.8% in 2013, mainly due to the demand of the dairy, confectionery, and bakery sectors, being used as stabilizers, foams, gels, and emulsifiers (Ahuja and Singh 2018a).

The sector of biosurfactants is expected to grow by 4.3% in value between 2014 and 2020, with projection of US$ 2.6 billion by 2023 (Grand View Research 2015). Growth in this period is mainly associated with biosurfactants rhamnolipids (forecast of 8% increase), sophorolipids (forecast of $ 3.3 million), alkyl polyglucosides (forecast of 4.5%), and methyl ester sulfonates (forecast of $ 900 million) (Ahuja and Singh 2018a). There is expected to be a consumption of consumption over 6.5 tons by 2023 by the food-processing industry in the United States alone (Ahuja and Singh 2018b).

Companies such as Mitsubishi Chemical Corporation (Japan), Urumqi Unite Bio-Technology Company Ltd. (China), Jeneil Biotech Inc. (United States), Croda International Plc (United Kingdom), Saraya Co. Ltd. (Japan), Cargill Inc. (United States), and Evonik Industries A.G. (Germany), stand out in the biosurfactants sector (Variant Market Research 2017).

### 1.2.7 Pigments

Considered the first sensory parameter observed by the consumers, pigments influence the perception of the food by the consumer, stimulating the appetite and directly impacting in the consumption of these products (Damant 2011; Solymosi et al. 2015). Industrial pigments are divided into synthetic, also called artificial, and natural pigments. However, due to the consumer risk of carcinogenicity, hyper-allergenicity, and other toxicological problems, many of these artificial pigments have been banned by organizations such as the European Food Standards Authority (EFSA), the World Health Organization (WHO), and the US Food and Drug Administration (FDA) (Tuli et al. 2015; Venil et al. 2013). Due to the risks associated with artificial pigments and the market demand for natural products, the production and commercialization of natural pigments have been prioritized (Rodriguez-Amaya 2016).

The microorganisms present the ability to synthesize natural pigments through an efficient and controlled process, resulting in products with potential benefits to consumer's health, presenting potential medicinal properties such as antioxidant, antimicrobial, anticancer, and immunoregulation (Nigam and Luke 2016).

Technological development and genetic engineering methods allowed the improvement of the biosynthesis process, allowing the use of microbiological pigments in the food industry (Kumar et al. 2015). Due to these factors, it is possible to find pigments on the market obtained from biotechnological processes, with the most common being

riboflavin, β-Carotene, lycopene, astaxanthin, and canthaxanthin (Nigam and Luke 2016).

During 2015, for example, the production of pigments reached 2 tons, with a 10% increase expected until 2050. One of the factors involved in this growth is related to their use in food and beverage industries, applicable in bakery and confectionery, beverages, dairy, frozen, meat products, oil and fat, and fruits and vegetables (Grand View Research 2017a). Another factor is related to the risks associated with the consumption of the synthetic pigments, which drive the natural food colors market, having an expected growth in 2025 of US$ 2.5 billion (Grand View Research 2017b).

Main companies in the field of natural dyes include DSM N.V. (The Netherlands), CHR Hansen (Denmark), FMC Corp. (United States), Allied Biotech Corporation (Taiwan), Chenguang BioTech Group Co. Ltd. (China), and Roha Dye Chem Pvt. Ltd. (India), among others (Goldstein Research 2017).

## 1.3 Genetically Modified Organisms (GMO)

A widely adopted GM technique can be applied to yeasts, virus, bacteria, animals, or plants in typically unrelated species (Nsanzabera 2016). As a method of genome manipulation, the GM process is divided according to the material to be modified. Thus, when the process occurs in food, it is known as genetically engineered, genetically modified foods or transgenic, as when the modification occurs in organisms, such as bacteria and yeasts is known as genetically modified organisms (GMOs) when the genetic changes (Dizon et al. 2016).

GMO applications are found in human health, agriculture, environment, food, chemicals, paper, and textile industries (Saxena 2015). Through the genetic changes implemented, it is possible to optimize fermentation processes, thus obtaining higher yields, higher efficiency, and lower process cost (Maryam 2017).

These advantages occur due to improved metabolic potential, greater stability, and specificity of GMOs, which results in better fermentation conditions when compared to the preexisting processes (Han 2004; Mishra et al. 2017). Examples are observed in industrial fermentation processes, such as enzyme production processes, performing genetic alterations in *Bacillus subtilis* to obtain α-amylase and in *Escherichia coli* and *Aspergillus niger* to get rennin, or lipase from *E. coli* with GDSL lipase-encoding gene (Memarpoor-Yazdi et al. 2017). In addition, breweries can use GM yeast with foreign gene encoding glucoamylase (Sewalt et al. 2016).

The GM strains can also be used to obtain bioproducts of industrial interest, such as organic acids from *Yarrowia lipolytica* (Liu et al. 2017), *A. niger* (Hu et al. 2017), *Ustilaginaceae* species (Paulino et al. 2017). Food flavors from *Pseudomonas putida* S12 (Groeneveld et al. 2016) and *Thymus albicans* (Filipe et al. 2017) or polysaccharides from *X. campestris* and *Agrobacterium* strains (Schmid et al. 2015) can also be obtained.

Regarding the global market for GMOs, the projections for 2022 are US$ 6.28 billion, indicating a growth of 14.5% during that period (Markets and Markets 2017b). Among the companies associated with this market are Thermo Fisher Scientific Inc. (United States), Horizon Discovery Group Plc. (United Kingdom), New England Biolabs (United States), Transposagen Biopharmaceuticals Inc. (United States), Genscript Biotech Corporation (United States), Merck KGaA (Germany), Lonza Group Ltd. (Switzerland), Inc.

(United States), Origene Technologies, Inc. (United States), and Integrated DNA Technologies, Inc. (United States) (Market Research Future 2018b).

The global food market trend of GMOs is growing, having an expected annual growth of 3.2% by the year 2021 (Research Nester 2019). It is possible to infer that this growth is closely linked to the use of GMOs, a technique well established in the industrial sector. As a characteristic, this technique is involved in several fermentative processes in the food industry, such as microbiological enzyme production processes, or recombination of yeast strains (Saxena 2015).

However, consumer hesitation is still a hindrance, since the consumer believes that these products can cause health hazards. The lack of information on processes and food safety has led to consumer mistrust of GM products (Gwira Baumblatt et al. 2017). Herewith, it is necessary that the target audiences of food companies overcome their fear, leading to a greater acceptance of the products. Presenting the processing would be a factor of extreme importance for the development of the sector and increase the acceptance of these products.

Thus, it is possible to observe the impact on the development and production of the food sector by the use of GMOs. Associated with the fermentation processes, they gained traction and enabled superior yields and efficiency in the industrial processes. This contributed to a greater growth of the biotechnology market, as well as a greater incentive of investment in the sector.

## 1.4 Future Perspectives of Biotechnological Processes in the Food Industry

Biotechnology is a multidisciplinary field present in the industrial sector, which enables the development of new processes and products, with less environmental impact than preexisting products, better quality, and with acceptable economic feasibility (Gupta et al. 2017). With expected growth of 7.4% by the year 2025, the worldwide biotechnology market is expected to reach US$ 727.1 billion by the end of this period (Grand View Research 2017c).

The development and economic aspects of any technology, as in the case of biotechnological processes, is driven by many factors, from market demand and profit generation to the creation of new process and innovation techniques of preexisting techniques (Felipe et al. 2017; Messeni Petruzzelli et al. 2015). In this context, patents have become tools that drive the increase in the demand of this market, allowing investment firms and researchers to benefit from their discoveries and creations. In addition, the contribution of investors for the generation of new products on the market are also important for the development of this market (Lokko et al. 2018).

Investments in research for the production and use of enzymes in the last four years have been significant, with many of these studies associated with genetic alterations and solution of problems in industrial processes (Adrio and Demain 2014; Singh et al. 2016).

Companies like Novozymes, DSM, and DuPont have shown great interest in the sector (Nielsen and Loft 2015). These companies have been granted 160 patents within the last five years covering a wide range of solutions of problems of industrial processes (Cramer et al. 2018; Jenner et al. 2014; Persson and Banke 2013), including proteins

production (Stocks et al. 2017), ethanol production (Tsang et al. 2014a,b,c,d,e,f), creation of methodologies for the production of dairy product with enzymes that present lactase activity (Henriksen et al. 2017), enzymes applications at fermented milk products (Eisele et al. 2017; Yu et al. 2016), and production of food and beverage products (mainly in brewing) (Sorensen and Miller 2017).

With the aim of meeting the expectations of consumers seeking healthier foods, there is a greater tendency for research in this field, such as regarding the production of sweeteners from natural methods. GMs were made into *Solanum lycopersicum* and *Yarrowia* strains, for example, to obtain optimum processes conditions for the production of steviol gycoside through fermentation (Boer et al. 2018; Bosch et al. 2018). Another interesting sweetener investigation was related to erythritol in fruit juices. The aim of the study was to use microorganisms capable of metabolizing sugars into sugar erythritol. The process is carried out by microorganisms of the genera *Pichia*, *Yarrowia*, *Penicillium*, *Aspergillus*, *Candida*, *Torulopsis*, *Trigonopsis*, *Moniliella*, *Aureobasidium*, and *Trichosponon* spp., with the purpose of producing a fermented drink with low carbohydrate content (Hugenholtz et al. 2015).

Since 2014, there has also been a strong market trend in microorganism GMs. Companies including Ajinomoto® Co. have shown great interest in this sector, producing *L*-amino acids from modified microorganisms belonging to the Enterobacteriaceae family (Cerceau et al. 2016; Doi 2016; Livshits et al. 2016; Mitsuhashi 2014; Rybak et al. 2016; Vasilievich et al. 2016).

Other companies have also shown interest in GMOs, such as Cargill Inc., in order to produce of lactic acid from modified bacteria (Brazeau 2015) and fatty acids by means of modified microorganisms (Hans et al. 2015). In addition, BASF SE studied ways to obtain succinic acid from strains from the family of Enterobacteriaceae, Pasteurellaceae, Bacilli, or Actinobacteria (Hartwig et al. 2017).

The future of biotechnology in the food sector is mainly related to the consumer's perception of the benefits associated with these biotechnologies. Researchers, governments, and the food industry itself all play an important role in educating consumers about the technology, its advantages, and its limitations, and thus demonstrate overall that the food products and additives obtained are safe (Gupta et al. 2017).

## 1.5 Concluding Remarks and Perspectives

Biotechnological products application is diverse, including pharmaceutical, textile, and food industries, among several others. Focusing on the food sector, it is important to highlight the availability of food additives, such as organic acids, flavors, polysaccharides, amino acids, enzymes, surfactants, and pigments, which stand out in the sector and encourage the use of bioprocess routes.

Associated with these processes, several methods of genetic alterations are used in order to optimize preexisting processes as well as to develop new ones. These methods contribute to the development of the food industry, allowing better yields and higher efficiency. Thus, the potential associated with these biotechnological products is interesting for application on an industrial scale, in order to supply the market demand for several commercial food products.

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# 2

# Status of Biotechnological Processes in the Pharmaceutical Industry

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## 2.1 Introduction

Since their major inception in the 1980s, biotech drugs based on natural proteins or produced via biotechnological processes have become the difference between life and death for millions of patients, treating diabetes, cancer, infectious diseases, and many genetic disorders. Especially with the scientific progress concerning recombinant DNA techniques, biotechnology is now present in the pharmaceutical sector. Pharmaceutical biotechnology has been consolidated as a field of study contemplating the use of basic science, such as biology and chemistry, as well the knowledge of applied sciences, which includes engineering and pharmaceutical sciences.

The first step in developing a biotechnological medicament is to genetically modify an organism to introduce a genetic code sequence that produces the chosen protein or induce the production of the desired metabolite by biotechnological process. Therefore, it is a multidisciplinary area that uses industrial and scientific knowledge to produce complex biomolecules (such as antibodies, enzymes, and antibiotics) that are tools for health promotion (Vitolo et al. 2015).

In the past decades, biotechnology provided several contributions to pharmacology and medicine, through the discovery and development of specific and safe medicines for complex and rare diseases, of new diagnosis tools, of new pathways for drug production (semisynthetic drugs) and of potential gene therapies (Lybecker 2016). The traditional synthetic routes for drug synthesis, which established medicines as

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Paracetamol (analgesic), Ketamine (anesthetic), Acyclovir (antiviral), Azidothymidine (AZT, antiviral), have started to show signs of lower productivity and is losing ground to molecular biology and biotechnology, which are now the major source of innovation in the pharmaceutical industry (Lybecker 2016).

The history of polypeptides, proteins, or glycoproteins being used as therapeutic agents started with Pasteur in 1885, when he injected inactivated rabies virus in a child bitten by a rabid dog (Cardoso 2013). In 1904, L-asparaginase, probably the main enzyme used as a drug, was first observed by Lang and Uber (1904) and is the first therapeutic enzyme with antineoplastic properties (Szymanska et al. 2012).

Alexander Fleming reported the first and probably most famous example of antibiotic discovery in 1929. Fleming accidentally found an antibacterial compound produced by a *Penicillium* species that inhibited the growth of some *Staphylococcus* colonies. That compound was named penicillin, and initially it was thought to be produced by a *Penicillium chrysogenum* strain. After full sequencing of the genome of that strain the fungus was reidentified as *Penicillium rubens* (Houbraken et al. 2011). The discovery of antibiotics was a groundbreaking innovation in the twentieth century and helped save a large number of lives in wars.

In the twentieth century, Herbert Boyer and others succeeded at genetically manipulating plasmids of *Escherichia coli* bacteria to produce insulin with the same amino acid sequence as seen in humans. In 1982, insulin was the first genetically engineered drug to be approved for marketing in the United States (Cardoso 2013). Moreover, the first recombinant Hepatitis B vaccine and the first monoclonal antibody (mAb) therapy against liver-transplant rejection were launched in 1986 (Ecker et al. 2015). Fomivirsen was the first oligonucleotide developed for therapeutically purposes, and was approved in 1998 (Orr 2001).

The increasing knowledge of the diseases' mechanisms at a molecular level has enabled the pharmaceutical industry to model and synthesize more and more specific molecules (*National Research Council* 2004). These advances were only possible due to the biotechnological revolution, accomplished with -omic studies (e.g. genomics, functional genomics, proteomics, metabolomics, and cytometry) that provided information about the functional and/or structural identification of tissues and cells; about gene expression patterns, screening of DNA mutations and polymorphisms, and metabolic profiles (Kayser and Müller 2004; Vitolo et al. 2015).

With this information, it is now possible to identify physiological and/or metabolic alterations induced by a disease, and even evaluate the pharmaceutical effect of a medicine on a given organism. Thus, greater understanding of the genetic causes of diseases allows for early detection and treatment, and the new field of gene therapy may enable treatment and even total recovery of a disease (Kayser and Müller 2004).

In terms of the current scenario, biotechnology has already made significant strides for human health. New drugs have been created, especially for rare or still-untreated diseases (Gould Rothberg et al. 2008). The advances of biotechnology enabled the development of biopharmaceuticals, semisynthetic drugs, enzymes, more powerful antibiotics, gene therapies and diagnosis tools (Kayser and Müller 2004; Vitolo et al. 2015).

Of these aforementioned biotechnology advances, biopharmaceuticals (also called biologicals), contribute to a great share in the income of pharmaceutical industries. Biopharmaceuticals can be proteins (including antibodies, enzymes, blood factors) or nucleic acids (DNA, RNA, or antisense oligonucleotides) used for therapeutic or

*in vivo* diagnostic purposes and are produced by means other than direct extraction from a native (nonengineered) biological source (Vitolo et al. 2015). While traditional synthetic drugs are composed of small molecules typically defined as between 100 and 1000 atoms; biopharmaceuticals are large, complex proteins, consisting of thousands of atoms, and being mostly chemically and biochemically unstable. This molecular complexity of biopharmaceuticals and their biosynthesis in living cells make the final product very sensitive to variations in production conditions. Nevertheless, these biotechnology production methods provide safer versions of existing treatments and potentially in unlimited quantities (Geigert 2013).

The *first-generation biopharmaceuticals* are usually made of recombinant proteins with amino acids sequences identical to the natural proteins (Vitolo et al. 2015). The *second-generation* ones, however, have a planned alteration in the amino acid sequence, with the purpose of increasing or decreasing the peak of the product biological activity, increase the half-life or decrease the immunogenicity of the product (Geigert 2013). However, compared to synthetic-route drugs, there are no identical biopharmaceuticals among two different batches or suppliers, because this type of medicine is produced by independent cell lines (Geigert 2013). To overcome this limitation, biopharmaceuticals are submitted to numerous and rigorous tests to attest to their safety, stability, and function.

Not only peptides and nucleotides can be produced through biotechnological process for health promotion. Nowadays, brand-new antibiotics are industrially produced from organisms, such as fungi. In addition, several molecules can be chemically modified using specific enzymes to create more potent analogues of their natural counterparts. For instance, microbial lipases have been applied to kinetic resolution of racemic mixtures in order to produce enantiopure drugs (Carvalho et al. 2006; Łukowska-Chojnacka et al. 2017).

Accordingly, this chapter describes biotech products that are on the market as well as a summary of their production process. An overview of the economic aspects, intellectual property scenario, and perspectives about therapeutically antibodies, biologicals, and enzymes are also discussed.

## 2.2 Main Biotechnological Products in the Pharmaceutical Industry

### 2.2.1 Antibiotics in the Pharmaceutical Industry

Antibiotics are secondary metabolism compounds with low molecular weight (<1500 Da) usually produced during the late-growth phase by microorganisms, including bacteria and fungi. These molecules are one of the most important classes of drugs used in several infectious diseases. They inhibit growth of microorganisms even at low concentrations.

Antibiotics account for a significant share of the pharmaceutical industry economic income. As an example, in 2009 the market of antibiotics corresponded to 46% of sales of anti-infective drugs that also include antiviral drugs and vaccines, as well as 5% of the global pharmaceutical market generating sales of US$42 billion (Hamad 2010; IMS Health. IMS MIDAS 2009).

Biotechnological production of antibiotics is focused on fermentation processes of some microorganisms, including *Penicillium* spp. and bacteria like *Streptomyces* spp. In addition, there are semisynthetic antibiotics that are first produced by fermentation, purified and, then, followed by chemical alterations through organic synthesis tools. Finally, some antibiotics are completely chemically synthesized.

There are different criteria for classification of antibiotics and different applications. With regards to human health, β-lactam antibiotics are probably the most relevant group of antibiotics. Marketed β-lactam antibiotics include penicillin-derived, like ampicillin and floxacillin; cephalosporins, such as cefalothin and cefadroxyl, among other relevant antibiotics. These broad-spectrum antibiotics contain a β-lactam ring in their molecular structures. This ring is composed of one nitrogen and three carbon atoms. Their action mechanism is based on the inhibition of the last stage of bacterial cell wall synthesis (Suárez and Gudiol 2009). In addition, they have been produced by some fungi, such as *P. chrysogenum* and *Acremonium chrysogenum* (Elander 2003).

Apart from production of antibiotics by fungi, several relevant antibiotics are produced by actinomycetes, mainly *Streptomyces* spp. These Gram-positive bacteria produce several medically relevant antibiotics including tetracyclin (*Streptomyces aureofaciens*) and streptomycin (*Streptomyces griseus*).

For antibiotic production, the main biosynthetic pathways are those involved with formation of peptides, polyketides, isoprenes, oligosaccharides, aromatic compounds, and β-lactam rings (Sánchez et al. 2010). These pathways involve the action of individual enzymes or multifunctional polypeptides, including different synthases and synthetases (Demain 1998). Most genes involved with such pathways are usually chromosomal. In only a few cases genes have plasmidial origin. Additionally, genes involved with antibiotic synthesis are usually clustered, but not necessarily as single operons (Sánchez et al. 2010).

The classical method for screening of antibiotics is based on the cultivation of strains in different growth conditions, followed by the extraction of metabolites and evaluation of the bioactivity. If positive results are acquired, more detailed analysis are performed, including the identification of the compounds using chromatography and mass spectrometry. Although these approaches are still important, the availability of genome sequence of different microorganisms allowed the identification of bioactive molecules that were previously not possible. In addition, metabolic engineering techniques based on genome editing, more specifically on gene deletion and overexpression, result in improvements never seen before.

Examples of metabolic engineering of microbial strains to overproduce antibiotics include the overexpression of the malonyl-CoA synthesizing complex (acetyl-CoA carboxylase complex), leading to an increase in actinorhodin production in *Streptomyces coelicolor* (Ryu et al. 2006). Another example is the improvement of oxytetracycline production in *Streptomyces rimosus* M4018 by metabolic engineering of the G6PDH gene in the pentose phosphate pathway (Tang et al. 2011).

### 2.2.2 Enzymes in the Pharmaceutical Industry

Enzymes are biocatalysts with remarkable biotechnological applications. Most enzymes are proteins, except some cases in which they are RNA molecules. They have varied functions and hence several biotechnological uses. Naturally, they act intra,

extracellularly, or on the surface of a cell membrane (Maurer 2001). These proteins are capable of catalyzing reactions under mild reactions conditions regarding pH and temperature. They present high selectivity for the substrate, and therefore, produce high levels of added-value compounds with a low number of toxic byproducts.

The pharmaceutical industry is one of the most important areas of enzyme applications. In this field, some enzymes can be used as drugs, because of their capability to treat diseases, including some types of cancer. Others, due to their major selectivity for substrates, can be used in organic synthesis to obtain enantiopure drugs. Enzymes can also be used as biomarkers for diagnosis purposes. Regarding their production, recombinant enzymes can be produced through homologous or heterologous production using efficient hosts (Yang et al. 2011; Ahmad et al. 2014; Contesini et al. 2017b).

#### 2.2.2.1 Enzymes as Pharmaceuticals

Enzymes have been studied for use as drugs for decades (Vellard 2003). They are of great interest as pharmaceuticals because they frequently bind to their targets with great affinity and specificity, converting target molecules into the desired products. There are many therapeutic enzymes and, in this section, we will describe some representative examples.

L-asparaginase is probably the most important enzyme used as a drug. This enzyme catalyzes the degradation of asparagine, an essential amino acid for leukemic cells, into ammonia and aspartate. This enzyme was first observed by Lang and Uber (1904) and is the first therapeutic enzyme with antineoplastic properties. This enzyme is applied to the treatment of acute lymphoblastic leukemia in combination with other drugs (vincristine and a glucocorticoid) (Szymanska et al. 2012). In addition, L-asparaginase is used in the treatment of other diseases, such as Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticulosarcoma, and melanosarcoma (Broome 1961; Kidd 1953). Since L-asparagine is an essential amino acid for many tumor cells, its conversion into aspartic acid and ammonia is the basis for its medical use. Therefore, malignant cells are deprived of important growth factors, often resulting in cell death. L-asparaginase is produced by several microorganisms, including bacteria and fungi. However, only L-asparaginases from *E. coli* and *Erwinia chrysanthemi* have been approved to be used as part of a multiagent chemotherapy to treat acute lymphoblastic leukemia (Verma et al. 2012). This enzyme is commercially available as *Crasnitin*, *Crisantas*, *Pasum*, *Kidrolase*, *Elspar*, *Erwinaze PEG-asparaginase*, and *Pegasparagasum*.

α-Galactosidase A is an important example of enzyme as pharmaceutical due to its use in cases of Fabry disease. Patients with this disease present an X-linked inborn error of glycolipid metabolism. The main cause for this is the absence of the lysosomal α-galactosidaseA that hydrolyzes terminal α-galactoside linkages in different glycolipids. In males, this leads to early death due to occlusive disease of the heart, kidney, and brain. As an alternative, efforts have been made to replace the defective enzyme with normal enzyme obtained from different human and recombinant DNA sources (Mapes et al. 1970; Brady et al. 1973; Desnick et al. 1979). Therefore, some α-galactosidase A is commercialized under different names, including Replagal™ and Fabrazyme®.

Other therapeutic enzymes are Laronidase, Rasburicase, Sacrosidase, Pegaspargase, Pegademase bovine, Dornase *α*, among others (Vellard 2003).

#### 2.2.2.2 Enzymes Used to Obtain Pharmaceuticals

Many enzymes present high selectivity for the substrate allowing the obtainment of specific compounds that frequently are difficult to produce using chemical catalysts. This is the case for enantiopure drugs, as single enantiomers. Since several drugs are optically active due to the presence of one or more chiral centers, if these drugs are chemically synthesized in the absence of a source of stereoinduction, the resulting drug will be a racemate. The problem for the pharmaceutical industries often relies on the fact that, in many cases, enantiomers often show important differences in their activity and pharmacokinetics (Calvey 1995). Furthermore, the separation or resolution of the racemic mixture by chemical methods is highly difficult due to the similar physicochemical properties of both enantiomers.

Enantiopure drugs can be produced by different approaches, including (i) asymmetric catalysis that requires the synthesis of chiral chemical catalysts, or (ii) the use of enzymes capable of recognizing one of the enantiomers from a racemic mixture. These enzymes catalyze enantioselective reactions, facilitating the separation of the enantiomers, in a process called kinetic resolution. This step can be applied to pharmaceuticals or to precursors used in the chemical synthesis of the drug. The process can be carried out by extracellular enzymes or whole cells as the biocatalysts. Among the extracellular enzymes, hydrolases, mainly lipases, are the most used enzymes for obtaining enantiomerically pure compounds. On the other hand, oxidases and reductases are frequently used within the cell that are applied to the synthesis of enantiomerically pure drugs since these enzymes often requires intracellular cofactors such as adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NAD(P)H), and flavin adenine dinucleotide (FADH) (Jeon et al. 2017; Sikora et al. 2017; Tramontina et al. 2017).

Lipases naturally hydrolyze oils and fat. However, in the presence of hydrophobic organic solvents, such as hexane, these enzymes are capable of catalyzing esterification reactions. Therefore, lipases from different organisms can catalyze hydrolytic and synthetic enantioselective reactions. For example, a lipolytic extract from *Aspergillus niger* has been studied for the enantioselective esterification of the racemic nonsteroidal anti-inflammatory drugs ibuprofen and ketoprofen (Carvalho et al. 2006). Studies have also been focused on the enantioselective hydrolysis of naproxen methyl ester by *Candida rugosa* immobilized-lipase showing remarkable values of enantioselectivity (Sayin et al. 2014).

Whole cells are also utilized in the production of enantiopure drugs, mainly using oxidases/reductases. In this case, although it is based on enzymatic reactions, the biocatalyst is considered the whole cell. One interesting example is the production of enantiopure (+)- and (−)-pinoresinol by kinetic resolution. (+)-Pinoresinol showed to be effective against different diseases, including cancer (Sepporta et al. 2013). In the work of Ricklefs et al. (2016), an *E. coli*-based whole-cell system was developed based on the heterologous expression of three enzymes, a reductase from *Forsythia intermedia*, a vanillyl-alcohol oxidase from *Penicillium simplicissimum* and a bacterial laccase from *Corynebacterium glutamicum*, showing interesting results.

#### 2.2.2.3 Enzymes Used for Diagnostics Purposes

Several diseases associated with many components of the enzyme metabolism systems are now widely applied in clinical exams as biomarkers for diseases. For example, the presence of some of the proteins in the serum indicates that tissue or cellular damage