

Microbial Production of Bioactive Pigments, Oligosaccharides, and Peptides


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1 Introduction

Nowadays, an epidemiologic transition has come about that consists of a pattern of changes in disease and mortality in the population, wherein the provenance of particular infection pathologies, neonatal diseases, nutritional deficiencies, among others, are reduced but at the same time the identification of disease groups, such as chronic nontransmissible disease (Valenzuela et al., 2014), including those that affect the circulatory system, neoplastic disease, diabetes, and obesity, is increasing. Therefore, currently there is a search for new strategies against these pathologies by way of the use of new bioactive compounds. Some bioactive compounds that may prevent diseases, such as cancer, atherosclerosis, cataracts, multiple sclerosis, and cardiovascular diseases, also have attributed to them an important role in lipid regulation and antioxidant and antiinflammatory activity, and, on the other hand, are commonly used in cosmetics as stabilizers and bulking agents (Cardenas-Toro et al., 2015; Patel and Goyal, 2011). Usually, these compounds that are found in nature, in plants, fruits, animals, and microorganisms, have been chemically synthesized, but the use of chemically synthesized bioactive compounds as food additives or in functional cosmetics has been severely regulated in recent years, which has increased interest in obtaining them from natural sources (Sun et al., 2016). However, due to the beneficial effects in human health, alternative ways of producing larger and more specific amounts have been developed, in which production using microbiologic techniques has an important role. Microorganisms, such as fungi, bacteria, yeast, and microalgae have been employed to produce molecules with added value, and bioprocesses in solid and SmF with application of agro-industrial waste has been evaluated. Employing microorganisms for the generation of added-value products seems to be an extremely relevant tool for the pharmaceutical, chemical, and food industries, including complex material production, such
as proteins, nucleic acids, polysaccharides, and even cells, and down to low-molecular weight molecules possessing biological activities, some of these compounds are synthesized in a natural way from a wide variety, including molecules such as pigments, oligosaccharides, and bioactive peptides (Demain, 2000a). Nevertheless, the development of methodologies to recover, purify, and increase yields is necessary. The aim of this chapter is to discuss the microbial production of pigments, their types and characterization, coloring properties, and applications, as well as the microbial production of oligosaccharides and bioactive peptides, recent studies, bioengineering aspects, chemical and physical characteristics, and industrial applications.

2 Bioactive Compounds

Nutritional problems, current climate change, and preservation of the environment have induced the development of new alternatives for the renewable exploited resources and the production of bioactive compounds (Cardenas-Toro et al., 2015). Bioactive compounds are secondary metabolites produced by microorganisms in an active-culture cultivation (Singh Nee Nigam and Pandey, 2009). Employing microorganisms in metabolite-production technologies poses benefits, as they have the ability to achieve a broad variety of reactions; the flexibility to adapt to sundry environments; the capacity to be transplanted from the nature to a laboratory flask or even an industrial fermenter; the ability to be grown using cheap carbon and nitrogen sources and the faculty to produce from these added-value molecules; the facility to be genetically engineered to improve characteristics or increase production and modify activities and structures, resulting in the metabolite production of completely unique compounds; and the skill to produce active enantiomers specifically, in contrast with chemical synthesis, which regularly produces active and inactive enantiomer mixtures (Demain, 2000b).

Microbial production of metabolites, such as antibiotics, proteins, oligosaccharides, antioxidants, pigments, peptides, and phenolic compounds has been studied by a large number of researchers. To improve yields and the quality of these important molecules, the production, extraction, and possibly application conditions have been evaluated.

2.1 Carotenoids

Carotenoids are phytochemicals found in fruits and vegetables, and their increased consumption is associated with several health benefits (Shi et al., 2000). The useful properties of these compounds have lead the cosmetic, pharmaceutical, and food industries to include them in nutraceuticals and drugs, since some carotenoids may prevent diseases, such as cancer or atherosclerosis (Guyomarc’h et al., 2000); because of this, the production of carotenoids is a topic of great relevance today. A key issue in the production of carotenoids is the cost, since the profitability of this process depends on this. Production by microorganisms offers this benefit. Furthermore, commercial production of carotenoids using microorganisms is highly efficient, because they are easily manipulated in the processing schemes (Taskin et al., 2011).
2.1.1 Natural source

Carotenoids are one of the most diverse and widely distributed classes of natural pigments. They are synthesized by all photosynthetic, and many nonphotosynthetic organisms, including bacteria, fungi, algae, and higher plants (Araya-Garay et al., 2012; Cardoso et al., 2016; Garrido-Fernández et al., 2010). β-Carotene, lycopene, lutein, and astaxanthin are the most important carotenoids, and their extraction from natural sources, such as tomatoes, papayas, watermelons, and carrots has been studied (Chen and Zhong, 2015; Devitt et al., 2010).

The red color of tomatoes is due to an accumulation of lycopene. Lycopene is a liposoluble carotenoid with antioxidant, antiproliferative, and prodifferentiation activities (Heber and Lu, 2002). Anese et al. (2015) have studied the effect of ultrasound treatment, oil addition, and storage of lycopene in tomato pulp. The authors reported that a decrease of approximately 35% lycopene content occurs at storage times of longer than 15 days, due to isomerization and oxidation reactions. Furthermore, the content and stability of β-carotene present in carrots, carrot juice, and orange juice has been evaluated. Picouet et al. (2015) have studied the effects of thermal and high-pressure treatment on carotene content, and high-pressure processing and mild heating processes were shown to induce modifications in carotene content, color, acidity, and °Brix. Carotenoid content, antioxidant and cell-growth activities of tomato waste also have been evaluated (Stajčić et al., 2015).

Animals are unable to synthesize carotenoids, but they can accumulate carotenoids so that they contribute to health and behavior (Cazzonelli and Pogson, 2010). The effect of dietary pigments on the coloration and behavior of fishes and birds has also been reported on (Baron et al., 2008; Sun et al., 2012).

Carotenoids are very important chemical compounds because they protect cells against photooxidative damage. They are the most widespread group of naturally occurring pigments. For these reasons, they have been associated with multiple applications in the environment, food and nutrition, and disease control, and have been identified as potent antimicrobial agents. At the present time, more than 750 structurally different yellow-, orange-, and red-colored carotenoids are found in both eukaryotes and prokaryotes, with a worldwide market value of $919 million (Kirti et al., 2014). Therefore, meeting consumer demand for microbial carotenoids with low-cost and high-quality alternatives is necessary.

2.1.2 Microbial production

Carotenoids are industrially and biotechnologically important pigments produced from bacteria, fungi, plants, and microalgae (Table 4.1) (Chen et al., 2016; Colet et al., 2014; Cutzu et al., 2013; Mannazzu et al., 2015; Xie et al., 2016; Yoo et al., 2015). Nowadays, production of bioactive metabolites from microorganisms is easier to handle and less costly than chemical synthesis or extraction from plants, (Cardoso et al., 2016) and can generate biomass
rich in natural pigments (Lee et al., 2016; Rodrigues et al., 2014). Also, agro-industrial waste, such as molasses, sugarcane, bagasse, corn steep liquor, corn bran, rice bran, whey, grape must, and so on (Buzzini and Martini, 2000; Čertík et al., 2013; Roadjanakamolson and Suntornsuk, 2010; Schneider et al., 2013; Valduga et al., 2014) is a viable alternative as a substrate of low cost for microorganism growth (Freitas et al., 2014). However,
carotenoid production by microorganisms depends not only on the substrate, but also on temperature, pH, aeration, and light (Lee et al., 2004; Mantzouridou et al., 2008; Valduga et al., 2011, 2009; Zhang et al., 2014).

There are two methods for microbial production: solid-state fermentation (SSF) and submerged-state fermentation. Michelon et al. (2012) reported on the extraction of carotenoids from Phaffia rhodozyma and showed that the highest specific concentration of carotenoids (190.35 µg/m) resulted from the combined techniques of diatomaceous earth and enzymatic lysis cell disruption. Roadjanakamolson and Suntornsuk (2010) evaluated β-carotene production with Rhodotorula glutinis, using rice bran, and reported 1.65 mg β-carotene/kg rice bran. Polyurethane foam has been employed in lycopene production by R. glutinis (Hernández-Almanza et al., 2014). Some red yeast, such as R. glutinis and Rhodosporidium toruloides, are efficient producers of β-carotene, torulene, and torularhodin due a relatively rapid growth rate (Braunwald et al., 2013; Dias et al., 2015; Lee et al., 2014). Blakeslea trispora has been employed in lycopene-specific production, mutation breeding, plus and minus strain ratios, carbon-to-nitrogen ratios, inhibition of lycopene cyclase, and other production factors (Choudhari and Singhal, 2008; Choudhari et al., 2008; López-Nieto et al., 2004; Wang et al., 2014). Wang et al. (2014) developed a novel method for producing lycopene from B. trispora (ATCC 14060 and 14059) through SmF, with the addition of 2-isopropylimidazole completely inhibiting lycopene cyclase, which limited the accumulation of β-carotene and other carotenoids. Also, Qiang et al. (2014) reported on the mutation breeding of the lycopene-producing strain B. trispora by a novel atmospheric and room temperature plasma, which showed a maximum lycopene concentration of 26.4 ± 0.2 mg/L.

Furthermore, some bacteria and microalgae have the capacity to produce natural pigments. Astaxanthin is the carotenoid that helps in preventing diabetes by reducing blood glucose; this pigment has been produced by some microalgae and yeast. Liu et al. (2016) investigated the production of lipids and astaxanthin by Chlorella zofingiensis under different culture conditions. The highest content of astaxanthin achieved was 4.89 mg/g dry weight.

Carotenoid production by bacteria is a bioprocess rarely studied, however, Sowmya and Sachindra (2015) evaluated carotenoid production by Formosa sp. KMW, a marine bacteria of the Flavobacteriaceae family. The authors reported that carotenoid extract yield and carotenoid content (0.97 ± 0.03 mg/L) were higher when the culture was grown under conditions that included shaking and light. Moreover, Giuffrida et al. (2016) characterized C50 carotenoids produced by strains of the cheese ripening bacterium Arthrobacter arilaitensis. Based on the UV-visible spectra, the mass spectra, and the elution order, eight carotenoids were identified, including all-E-decaprenoxanthin, all-E-sarcinaxanthin, 9-Z-decaprenoxanthin and 15-Z-decaprenoxanthin, and four others.

Microbial production is an area widely studied; however, cell-disruption techniques, extraction methodologies, and purification and quantification of this compounds should be studied to take advantage of biological and functional properties.
2.1.3 Biological properties

Carotenoids occur in some foods under two types of conditions: in “equilibrium” with other antioxidants in the thermodynamically controlled networks that serve as color indicators of good antioxidant status, and as active antioxidants by uptake radical network with regeneration of kinetic control. It has been shown that carotenoids are beneficial to human health; some, such as the \( \beta \)-carotene and \( \alpha \)-carotene that are precursors of vitamin A, also help prevent some chronic diseases and cancer because they retard oxidation of cells (Martini et al., 2010). The beneficial properties of carotenoids come from their rich structure conjugated double bonds, which block singlet oxygen and interrupt chain reactions by removing radicals (Yamaguchi et al., 2010). Carotenoids are also essential for maintaining many physiologic activities including growing bones (Chen et al., 2015).

2.1.3.1 Anticancer activity

Lim et al. (2014) have indicated that \( \beta \)-carotene has chemotherapeutic potential for the treatment of neuroblastoma. Another carotenoid that may protect against cancer is lycopene (Borel et al., 2015); furthermore, it helps strengthen the immune system by eliminating reactive oxygen species to inhibit lipid peroxidation (Gammone et al., 2015). Zhou et al. (2015b) reported that astaxanthin can improve cognition, because it protects neurons by inhibiting the nuclear factor \( \kappa B \) (NF-\( \kappa B \)) and regulates tumor necrosis factor \( \alpha \).

2.1.3.2 Antiinflammatory properties

Recent studies have shown that lycopene also has antiinflammatory properties (Hazewindus et al., 2014). The antioxidant effect of lycopene reduces the expression of proinflammatory cytokines and chemokines expression in macrophages (Simone et al., 2011).

2.1.3.3 Antioxidant activity

It has also been shown that the antioxidant properties of carotenoids are determined by various factors (chemical structure, including the number of conjugated double bonds, and the type of structural terminal groups and substituents containing oxygen) and the position it occupies in the lipid membrane (Fig. 4.1).

Astaxanthin is the carotenoid with the highest antioxidant capacity (Zhou et al., 2015a). It also helps in preventing diabetes by reducing blood glucose (Preuss et al., 2011). Also, Campoio et al. (2011) have demonstrated that this carotenoid could prevent oxidative stress in humans. Other carotenoids, such as canthaxanthin and fucoxanthin, also have beneficial health effects. Fucoxanthin, like other carotenoids, has antioxidant properties, along with antiobesity and antiinflammatory effects (Fung et al., 2013), and it has the ability to protect against oxidative stress caused by UV-B radiation (Heo and Jeon, 2009), such as fucoxanthin, canthaxanthin also provides protection against UV radiation (Brizio et al., 2013).
2.1.3.4 Other properties

It has been shown that a diet rich in β-carotene and α-carotene reduces the risk of developing type 2 diabetes (Sluijs et al., 2014). Carotenoids also prevent eye diseases. Lutein and zeaxanthin are concentrated in the macula, which is the central part of the retina, and they benefit eye health because they have antioxidant potential and absorb harmful blue light radiation (Berrow et al., 2011; Vishwanathan and Johnson, 2013), thus retarding the development of age-related macular degeneration. For this reason these carotenoids are recognized in macular pigment (Bone et al., 2001; Junghans et al., 2001). Piermarocchi et al. (2012) reported that treatment with lutein and zeaxanthin significantly improves visual acuity, contrast sensitivity, and visual function.

However, little or no water solubility causes food carotenoids to not have the desired bioavailability, while carotenoids from dietary supplements are better absorbed due to the oil content present in the supplement (Verrijssen et al., 2015).

2.1.4 Trends and applications in the food industry

Carotenoids are molecules with the ability to absorb light, and due to this property we can see colors ranging from yellow to red; this feature allows for different applications (Rodrigues et al., 2012). Some of the most common carotenoids, such as β-carotene, astaxanthin, canthaxanthin, lycopene, fucoxanthin, lutein, and zeaxanthin, are used for animal feed. In the same vein, astaxanthin is used in aquaculture in the diets of salmon and shellfish, mainly to
obtain desired coloration to meet consumer needs in the market. Also, it has been discovered that it helps the biological functions of fish, such as reproduction (Kalinowski et al., 2013; Kistler et al., 2002). In poultry farming, carotenoids are used in chicken feed to fatten poultry and to add pigmentation to egg yolks and chicken skin (Sánchez et al., 1999).

Carotenoids are natural pigments, and this feature has been used in the cosmetics industry in the manufacture of new products, such as facial and body emulsions (Solari et al., 2011). Besides their coloring properties, carotenoids are natural antioxidants, and as such play an important role in medicine and in the pharmaceutical industry, in which they have been used as markers in monitoring cells, and as antioxidants and antitumor agents. In the food industry they have been used as additives and colorants in some foods and beverages, such as orange juice, lemons, nectars, butter, and sausage.

2.1.5 Recovery and purification strategies

Carotenoid extraction is one of the crucial steps in using these compounds in the food, pharmaceutical, and cosmetic industries. The productivity of these processes depends on growth conditions, as well as the recovery of intracellular pigments (Hiranvarachat et al., 2013; Monks et al., 2013). Due to high conjugation of double bonds in their molecules, carotenoids are decomposed by the effects of light, temperature, and air. The light and heat favor the photochemical reactions that change the original structure of the carotenoid, while air, due to oxygen, promotes oxygenation of the double bonds for epoxide functionality, hydroxyl, and peroxides (Aburai et al., 2015; Jørgensen and Skibsted, 1990; Meléndez-Martínez et al., 2004). For these reasons, the extraction, storage, handling and analysis of carotenoids should be performed in conditions with an absence of light, at room temperature or less, and in the absence of oxygen. In addition it should be done as quickly as possible, and from fresh tissue, to avoid degradation by a combination of these adverse factors (Eh and Teoh, 2012; Pérez Gálvez and Garrido Fernández, 1997).

There are different methods for extracting carotenoids, and theses are divided into two main methods: mechanical and nonmechanical. These methods in turn are divided into physical, chemical, and enzymatic (Lee et al., 2012; Middelberg, 1995). Conventional extraction of carotenoids is carried out with solvents. Acetone is the most widely used solvent for the extraction of carotenoids. This solvent penetrates the food matrix well and dissolves both carotenes and xanthophylls efficiently, and subsequent partitioning to an apolar solvent occurs more easily. Other solvents, such as hexane, petroleum ether, methanol, and ethanol, have also been utilized (Durán and Alvarez, 2000; Monks et al., 2013; Yin et al., 2013). Thus, mixtures of solvents have been preferred. Another methodology that uses solvents is extraction with fluids under pressure. The pressurized liquids have the advantage of increased solubility with increasing temperature due to the greater diffusion of analyte from the solid matrix to the bulk solvent, and of reducing the viscosity of the solvent, which facilitates the penetration of the solvent in the matrix (Amosova et al., 2014; Cardenas-Toro et al., 2015).
Supercritical fluids are used in another methodology for the extraction of carotenoids. Supercritical fluids are widely accepted for extraction, purification, crystallization, and fractionation operations in many industries (Goto et al., 2015). Using this method, extracts can be obtained faster and in a solvent-free manner. These advantages are due to the volatility of supercritical fluids and their improved transport properties (Peredo-Luna et al., 2009). Supercritical fluids are alternatives for the extraction of carotenoids because they can minimize the purification step and reduce extraction time (Macías-Sánchez et al., 2009). Microwave-assisted extraction (MAE) is a methodology that has been proposed as an alternative method for the extraction of carotenoids. MAE involves the use of microwave energy to heat a solvent in contact with a sample to allow release of a bioactive compound (Hiranvarachat et al., 2013). Using MAE, Ho et al. (2015) obtained a yield of 13,592 mg carotenoids/100 g of extracted all-trans-lycopene, showing that MAE is promising because it allows for removal in a very short time and also lowers the amount of solvent needed compared to more conventional methods of solvent extraction (Hiranvarachat and Devahastin, 2014).

The use of hydrolytic enzymes to break down cell walls, thus helping to release intracellular contents, is another method for the recovery of carotenoids (Choudhari and Ananthanarayan, 2007). Treatment with lytic enzymes has gained attention because they disrupt the structural integrity of the cell wall, thus generating cell lysis and the release of intracellular compounds. Enzymes must be very specific; because of this different enzymes are required for the release of different compounds of interest (Middelberg, 1995; Strati et al., 2014).

The increase in performance obtained using ultrasound-assisted extraction is attributed to the effect of acoustic cavitation produced in the solvent as a result of the ultrasonic wave passing through it (Luengo et al., 2014). This method exerts a mechanical effect, enabling greater penetration of the solvent into the tissue, so that it increases the contact surface area between the solid and liquid phase (Dey and Rathod, 2013). Ultrasonically assisted extraction has been used to extract carotenoids from different plant materials in recent years, showing a high extraction efficiency (Ordóñez-Santos et al., 2015). Compared to some other extraction techniques, such as MAE and supercritical fluid extraction, the ultrasonic method is less expensive and much easier in practice (Eh and Teoh, 2012; Yolmeh et al., 2014).

On the other hand, mechanical methods are used for cell disruption in samples that are more difficult to break. This method consists of an agitator with blades, which can be of different materials. The agitator rotates in a container full of beads and fixed cells; it also has a cooling system so that the carotenoids remain stable (Bury et al., 2001; Lee et al., 2012; Middelberg, 1995). This type of cell-disruption method does not involve toxicity risks and does not use solvents, which also makes it suitable for industrial quantities (de Medeiros et al., 2008).

Some researchers have extracted carotenoids by combining and comparing different methods. Michelon et al. (2012) conducted experiments for removing the pigments of
P. rhodozyma. The results produced by these authors indicate that cell disruption shows no significant difference in the concentration of carotenoids among extraction techniques involving ultrasonic waves (the study used 88.38 mg/g and 56.75%), immersion in liquid nitrogen (84.68 mg/g and 54.38%), and maceration with diatomite and enzymatic lysis (88.80 mg/g and 54.03%). On the other hand, Bury et al. (2001) showed that a bead mill and high-pressure homogenization are equally suitable for the extraction. Nevertheless, Gu et al. (2008) compared three methods of extracting carotenoids (ultrasonic, grinding, and assisted extraction with HCl) showing that there is significant difference for the HCl method.

After the extraction, separation, and quantification of carotenoids is performed, quantitation can be accomplished by spectrophotometric methods (Dere and Güneş, 1998). For the separation and identification of these compounds, the most commonly used method is high-resolution liquid chromatography (Luterotti et al., 2013; Valdivielso et al., 2015).

2.2 Fructooligosaccharides (FOS)

A range of dietary oligosaccharides, such as lactosucrose, galactooligosaccharides (GOS), isomaltooligosaccharides (IOS), and fructooligosaccharides (FOS), have shown evidence of prebiotic properties (Lamsal, 2012; Peshev and Van den Ende, 2014).

Fructans have been classified into three types, based on the type of linkage present in the polysaccharide: inulin, levan, and graminan (Roberfroid, 2005). Fructans of DP ≤ 10 are generally described as FOS (IUB-IUPAC, 1982). FOS, which are also known as oligofructose (OF), belong to the category of nondigestible carbohydrates, have an average DP of 4.8, and are mainly composed of 1-kestose (GF₂), 1-nystose (GF₃), and 1-β-fructofuranosyl-nystose (GF₄), in which fructosyl units are linked in the β-(2-1) position of a sucrose molecule (Chen et al., 2014; Mussatto et al., 2009a). A glucose molecule (G) is present at the end of the fructose chain joined by an α (1,2) bond. The formula GFₙ indicates the degree of polymerization by the number of fructose molecules that are bound to the glucose (Fig. 4.2) (Niness, 1999).

The metabolism of individual FOS by strains of lactobacilli has been studied and FOS fulfill all the classification criteria of prebiotics (Gibson and Roberfroid, 1995). For example, although FOS are indigestible and are not absorbed in the upper part of the gastrointestinal tract, they do promote the proliferation of beneficial bacteria because of their fermentation from desirable products, such as lactic acid and short-chain fatty acids (SCFA) (Macfarlane and Macfarlane, 2003; Wong et al., 2006), and also because they induce microbial competition for nutrients and attachment sites between beneficial microbiota and putrefactive pathogens (Jia et al., 2008), and in consequence induce positive effects in the health of the host (they improve gut absorption of Ca²⁺ and Mg²⁺, lowering blood lipid levels, preventing urogenital infections, reducing the risk of colon cancer, and providing a reduction in total cholesterol and triglycerides) when consumed in recommended dosages. FOS
have been tested in vivo to meet all the requirements for the current criteria for prebiotics (Roberfroid, 2007; Sims et al., 2014).

Because of FOS potential to impact health due to their novel properties and the high demand from consumers to have functional-food products, the development of the industrial methods and strategies for the production of FOS aiming toward the biotechnological growth and promoting technological development have been covered in this chapter.

2.2.1 Natural source

FOS can be found in different natural sources; they occur in various families of monocotyledonous and dicotyledonous plants as storage oligosaccharides (Li et al., 2014). In the monocotyledonous family Liliaceae, garlic (*Allium sativum*) and onion (*Allium cepa*) both contain a good quantity of FOS (Kumar et al., 2015). In onions, FOS help to promote osmotic adjustment during the formation of bulbs. Burdock root (*Arctium lappa* L.) usually contains abundant amounts of FOS, which are usually considered its main bioactive component (Corradini et al., 2013; Li et al., 2013). Yacon (*Smallanthus sonchifolius* Poepp. Endl) is a root crop that is considered a rich source of FOS, which are present in high amounts in the tissue (~60%–70%, dry basis) (Corradini et al., 2013; Fernández et al., 2013; Hirinos and Evallos, 2003). FOS also occur in the roots of chicory (*Cichorium intybus* L.) and in the bulbs of Jerusalem artichoke (*Helianthus tuberosus*) (Bornet et al., 2002; Courtin et al., 2009; Durieux et al., 2001; Kovács et al., 2013). They also exist naturally in the medicinal plant *Morinda officinalis* (Yang et al., 2010). Apart from these sources, FOS are also present in
other plants, such as wheat, rye, and barley; in fruits, such as bananas and tomatoes; and in other substances, such as honey and agave syrup (Bornet et al., 2002; Lopez et al., 2003; Muñiz-Márquez et al., 2015).

### 2.2.2 Microbial production

FOS are produced by enzymes with transfructosylation activity, namely fructosyltransferase (FTase) (EC 2.4.1.9) and β-fructofuranosidase (FFase) (EC 3.2.1.26), which are present in different species of plants, as well as bacteria (Bacillus. Lactobacillus. Zymomonas), yeasts (Saccharomyces. Rhodotorula. Candida), and fungi, mainly of the genera Aureobasidium spp., Penicillium spp., Aspergillus spp., and Fusarium spp. (Dominguez et al., 2012; Ganaie et al., 2013; Maiorano et al., 2008; Mussatto et al., 2013; Sangeetha et al., 2004; Yoshikawa et al., 2006; Yun et al., 1995) (Table 4.2).

The transfructosylation of sucrose takes place via the cleavage of the β-2,1-glycosidic bond and the transfer of the fructosyl moiety onto an acceptor, such as sucrose or FOS. This reaction produces FOS which units are linked by a bond at the β(2,1) position of sucrose, while the glucose that is liberated in the reaction mixture acts as an Ftase inhibitor (Dominguez et al., 2013). As for the enzymes that catalyze this reaction, there is a difference of opinion in the nomenclature by different authors who refer to both the FOS-producing enzyme FFase (EC 3.2.1.26) (Nguyen et al., 1999; Sheu et al., 2001; Yoshikawa et al., 2006) and FTase (EC 2.4.1.9) (Antošová and Polakovič, 2001; Maiorano et al., 2008; Patel et al., 1994; Sangeetha et al., 2004; Tanriseven and Gokmen, 1999; Yun et al., 1995). Both have been reported as being FOS-producing enzymes in the literature, and it has also been shown that these both have hydrolyzing activity ($U_h$) and transfructosylating activity ($U_T$), which varies greatly in its ratio ($U_T/U_h$) depending on the source of these enzymes.

### Table 4.2: Microorganisms use and yields in recent works about FOS production.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Yield (%)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>A. japonicus ATCC 20236</td>
<td>66.00</td>
<td>Mussatto and Teixeira (2010)</td>
</tr>
<tr>
<td>A. japonicus ATCC 20236</td>
<td>70.00</td>
<td>Mussatto and Teixeira (2010)</td>
</tr>
<tr>
<td>A. niger SI 19</td>
<td>54.94</td>
<td>Ganaie et al. (2013)</td>
</tr>
<tr>
<td>A. niger 6640</td>
<td>51.11</td>
<td>Zeng et al. (2016)</td>
</tr>
<tr>
<td>A. flavus NFCC 2364</td>
<td>63.40</td>
<td>Ganaie et al. (2013)</td>
</tr>
<tr>
<td>A. terreus NFCC 2347</td>
<td>24.17</td>
<td>Ganaie et al. (2013)</td>
</tr>
<tr>
<td>A. pullulans</td>
<td>64.10</td>
<td>Dominguez et al. (2012)</td>
</tr>
<tr>
<td>A. pullulans DSM 2404 [89]</td>
<td>69.00</td>
<td>Yoshikawa et al. (2006)</td>
</tr>
<tr>
<td>P. expansum MUM 02.14 [97]</td>
<td>58.00</td>
<td>Prata et al. (2010)</td>
</tr>
<tr>
<td>P. islandicum MTCC 4926 [2]</td>
<td>43.56</td>
<td>Ganaie et al. (2013)</td>
</tr>
</tbody>
</table>
Enzyme activity is defined in different ways according to different authors:

- The amount of enzyme that transfers 1 µmol of fructose per minute (Chen and Liu, 1996; Dorta et al., 2006; Maiorano et al., 2008).
- The amount of enzyme that liberates 1 µmol of glucose per minute (Nemukula et al., 2009; Sangeetha et al., 2005a,b).
- When 1 µmol of nitrophenol is released per minute from p-nitrophenol-α-D-glucopyranoside (Wang and Rakshit, 1999).
- The amount of enzyme that produces 1 µmol of kestose per minute (da Silva et al., 2011; Maiorano et al., 2008).
- The amount of enzyme required for the hydrolysis of 1 µmol of sucrose per minute (Ganaie et al., 2013).

Another enzyme that has been utilized recently for the production of FOS is levansucrase (EC 2.4.1.10), because of its ability to directly use the free energy of cleavage of nonactivated sucrose to transfer the fructosyl group to a variety of acceptors, including monosaccharides (exchange), oligosaccharides (FOS synthesis), or a growing fructan chain (polymer synthesis) (Tian and Karboune, 2012; Tian et al., 2014; Tian et al., 2014).

The industrial production of short-chain FOS is expanding quickly due to the importance of these compounds in the food and pharmaceutical industries.

Because of this, several studies have been conducted on the production of FOS, aiming to optimize the development of more efficient production processes and their potential as food ingredients.

The improvement of FOS yield and productivity can be achieved by the use of different fermentative methods, different microbial sources of FOS-producing enzymes, and the optimization of nutritional and culture parameters. Among those fermentative methods, SmF has been the most worked on and developed for several years, while SSF is still being developed but has been receiving special attention in recent years; both are growing rapidly and gaining strength due to their potential. Both fermentative methods are reviewed later.

2.2.2.1 FOS production by SmF

Conventionally, FOS production is a two-stage process; the first stage consists of the microbial production of the enzyme with transfructosylation activity, and the second stage consists of the reaction of the extracted enzyme with sucrose (the substrate) to produce FOS under controlled conditions (Mussatto et al., 2009b; Sangeetha et al., 2005a).

The variables studied to define generally the best operating conditions for the production of the enzyme are the source of carbon and nitrogen, their concentration, the time of cultivation, and agitation and aeration. Other important factors are the addition of various minerals, small amounts of amino acids, and polymers and surfactants (Dominguez et al., 2012; Maiorano et al., 2008).
Mineral salts in the fermentation medium have been found to improve FOS production by microbes. The effect of salt concentrations on the synthesis of FOS has been studied. For example, da Silva et al. (2011) evaluated important factors for the production of FOS at three different levels; that is, carbon and nitrogen sources; percentage of sucrose and yeast extract, respectively; inoculum percentage; pH; temperature; agitation; concentration of urea; and the average concentration of the mineral salts $K_2HPO_4$, $(NH_4)_2SO_4$, $MgSO_4$, $ZnSO_4$, and $MnSO_4$. In cases where the sucrose concentration proved to be a positive parameter for the formation of FOS, it was because the enzymes catalyze the reaction of transfructosylation at high substrate concentrations. The improved productivity conversion was 54.7% and 223 g/L total FOS with an initial concentration of 400 g/L sucrose. In terms of mineral salts, $MnSO_4$ proved to be the only mineral that presented a significant stimulus effect for the production of FOS. On the other hand, the component $K_2HPO_4$ is described as a source of micronutrients for cell growth, as well as being a buffer solution.

Also, Nemukula et al. (2009) isolated, purified, and characterized a transferase from *Aspergillus aculeatus*. They examined the influence of pH, temperature, reaction time, and enzyme and sucrose concentration on the formation of short-chain FOS. The enzyme showed both transfructosylation and hydrolytic activity with the transfructosylation ratio increasing to 88% at a sucrose concentration of 600 mg/mL. In this study incubation time had a critical effect on the yield of FOS, as the major products were $GF_2$ after 4 h and $GF_4$ after 8 h, and a prolonged incubation of 16 h showed the conversion of $GF_4$ into $GF_2$ as a result of selfhydrodase activity.

Afterward, Belghith et al. (2012) evaluated the carbon source, nitrogen source, temperature, and initial pH of the growth medium in submerged liquid cultures to optimize the production of FOS from an isolated thermophilic levansucrase from *Bacillus* sp. The optimal temperature and pH of the levansucrase were 50°C and 6.5, respectively, and its activity increased fourfold in the presence of 50 mM $Fe^{2+}$. A crude enzyme of *Bacillus* sp. rich in levansucrase was established for the synthesis of FOS and levan.

One consideration is the effect of pH on the production of FTase and microorganism growth. It has been reported that a pH of 5.5 has been found to be the best initial value for the production of *A. oryzae* FTase CFR202 (Sangeetha et al., 2005a,b), *A. japonicus* JN19 (Wang and Zhou, 2006), and *Penicillium purpurogenum* (Wang and Zhou, 2006).

The use of agro-industrial residues is not limited to SSF; a reduction of costs in SmF has been attempted using agro-industrial wastes, such as carbon and nitrogen sources, (Gnaneshwar Goud et al., 2013) and a cost-effective medium formulation for $\beta$-d-FFase production by *Saccharomyces cerevisiae* GVT263 to replace sucrose, peptone, yeast extract, and malt extract has been developed. Among the agro-industrial wastes screened were banana-leaf powder (BL) and groundnut oil cake (GOC); both were promising carbon and nitrogen sources for FFase production, along with $MnSO_4$, inoculum size (IS), and incubation period (IP), which factors...
were selected and optimized. Maximum FFase production showed a ninefold increase in 48 h (from 400 U/mL in basal medium to 3587 U/mL), which was obtained using BL to 4%, GOC 4%, MnSO₄ 0.06%, and IS 0.5%. The use of agro-industrial residues show promise as an application to lower the costs of SmF, as has been done with SSF.

2.2.2.2 Production by SSF

SSF is defined as the growth of microorganisms on moistened solid substrate, in which there is enough moisture present to maintain microbial growth and metabolism but no free-moving water (Rahardjo et al., 2005). SSF has gained significant attention for the development of industrial bioprocesses. Some advantages of this process are the simplicity of operation, high volumetric productivity, product concentration, an initial inexpensive investment, and lower energy requirements. In addition to these factors there is less risk of contamination due to its high concentration of inoculum and low humidity in the reactor, it involves a simpler separation process, and it is ecofriendly, as it mostly utilizes solid agro-industrial wastes as the substrate (source of carbon) (Dominguez et al., 2012; Mussatto and Teixeira, 2010; Mussatto et al., 2009a; Sangeetha et al., 2005a; Thomas et al., 2013).

Agro-industrial by-products have emerged as ripe for exploitation in producing FOS using SSF due to the composition rich in sugars, their organic nature easily assimilated by microorganisms, and the need to find an alternative use for them.

Sangeetha et al. (2004) executed FTase production from Aspergillus oryzae CFR 202 using SSF and various agricultural by-products, such as cereal bran, corn products, sugarcane bagasse, cassava bagasse, and the by-products of coffee and tea processing. Among the various agro-industrial by-products used, rice bran, wheat bran, corn germ, and spent coffee and spent tea were good substrates for FTase production from A. oryzae CFR 202. The FTase had maximum activity at 60°C and at pH 6.0. The FTase was stable up to 40°C and in the pH range 5.0–7.0. Maximum FOS production was obtained with FTase after 8 h of reaction with 60% sucrose.

On the other hand, Mussatto et al. (2013) performed different fermentation assays using coffee silverskin as the support material to maximize FOS and FFase production. The factors evaluated were moisture contents of 60, 70, and 80% with a 240-g/L sucrose solution, spore suspensions of Aspergillus japonicus at $2 \times 10^5$, $2 \times 10^6$, and $2 \times 10^7$ spores/g dry material, and temperatures at 26, 30, and 34°C during 20 h. The moisture content did not influence the FOS and FFase production; however, temperatures of 26–30°C and an inoculum rate of approximately $2 \times 10^7$ spores/g dry material maximized the results (FOS = 208.8 g/L) with productivity of 10.44 g/L h; FFase = 64.12 units U/mL with productivity of 4.0 U/mL h). In another study Mussatto et al. (2009b) evaluated the ability of A. japonicus ATCC 20236 to colonize different synthetic materials (polyurethane foam, stainless steel sponges, vegetal fiber, pumice stones, zeolites, and foam glass) and to produce FOS from sucrose. Cells were
immobilized in situ by absorption, through in direct contact with the carrier particles at the beginning of fermentation. Vegetal fiber was the best immobilization carrier, as *A. japonicus* grew well on it (1.25 g/g carrier), producing 116.3 g/L FOS (56.3 g/L 1-kestose, 46.9 g/L 1-nystose, and 13.1 g/L 1-β-fructofuranosyl nystose) with 69% yield (78% based only on the consumed sucrose amount) and giving also elevated activity of the FFase enzyme (42.9 U/mL). The authors concluded that *A. japonicus* immobilized on vegetal fiber is a potential alternative for high production of FOS at an industrial scale due to similar results obtained compared with *A. japonicus* free cells.

Other efforts have been made to improve the production of FTase by SSF. Muñiz-Márquez et al. (2016) used aguamiel (agave sap) as a culture medium for FTase and FOS production. SSF was carried out while evaluating the parameters of inoculum rate, incubation temperature, initial pH, and packing density to determine the most significant factors through Box-Hunter and Hunter design. The maximum FTase activity (1347 U/L) was obtained at 32°C, using a packing density of 0.7 g/cm³.

An economic analysis and environmental impact assessment of three different fermentation processes for FOS production was made by Mussatto et al. (2015), in which three different fermentation processes were evaluated and compared in terms of economic aspects and environmental impact. The processes included SmF of sucrose solution by *A. japonicus* using free cells, the same process using cells immobilized in corncobs, and SSF using coffee silverskin as a support material and nutrient source. SSF was the most attractive process in terms of both economic and environmental aspects since it is able to generate FOS with higher annual productivity (232.6 t) and purity (98.6%) than the other processes and presented the lowest payback time (2.27 years); and since it is more favorable environmentally, causing a lower carbon footprint (0.728 kg/kg, expressed in mass of CO₂ equivalent per mass of FOS) and the lowest wastewater generation.

2.2.3 Biological properties

FOS possess many bioactive characteristics, such as prebiotic effects, suppressing putrefactive pathogens, reducing the risk of colon cancer, improving mineral absorption, immunomodulatory effects, and decreasing the levels of blood glucose, serum cholesterol, phospholipids, and triglycerides (Bornet et al., 2002; Kumar et al., 2015; Roberfroid, 2002; Watzl et al., 2005).

2.2.3.1 FOS as prebiotics

Lactobacilli constitute only 0.07%–1% of the bowel bacteria population (Balows et al., 1990), while bifidobacteria constitute 25%–30% of the total bacteria population of the bowel (Pedreschi et al., 2003). Several studies demonstrate that some strains of bifidobacteria and lactobacilli are considered very important probiotics for human health (Baldwin et al., 2010;
Liu et al., 2016; Orlando et al., 2012; Zhang et al., 2012). Probiotics are defined as live microorganisms added to the diet that benefits the development of the colon microbiota.

Studies in the last decade have demonstrated that fructans exhibit prebiotic activity (Watzl et al., 2005). FOS in particular cannot be absorbed in the human small intestine but promotes the proliferation of beneficial bacteria, particularly bifidobacteria, in the large intestine (Al-Sheraji et al., 2013; Gibson et al., 1995; Quigley et al., 1999).

Gibson and Roberfroid (1995) demonstrated that FOS are selectively fermented by most strains of bifidobacteria. Additionally, when these strains grow in FOS, bacteroids, clostridia, and coliforms are inhibited (Muñoz et al., 2012). Other studies have shown that some strains of lactobacilli are able to ferment FOS as well (Muñoz et al., 2012; Padalino et al., 2012; Pedreschi et al., 2003).

The beneficial properties of FOS to the host are provided by a chain whose first step starts with prebiotic action; the selective fermentation by beneficial bacteria and the proliferation and production of metabolites are what generate several reactions in the host that lead to the health-promoting properties that are listed here.

2.2.3.2 FOS role in prevention of colon cancer

As noted before, prebiotics, such as fructans allow quantitative and/or qualitative alterations of beneficial microflora, which are able to inactivate mutagenic/carcinogenic/genotoxic compounds (Fotiadis et al., 2008). Bifidobacterium longum and lactulose increased the activity of colonic glutathione S-transferases, which are involved in the detoxification of toxic metabolites and carcinogens (Challa et al., 1997).

FOS fermentation leads to the production of SCFA, such as butyrate, which has immunomodulation properties whose results include upregulation of the apoptosis. Butyrate is a preferred energy source for the mucosal cells and might inhibit neoplastic changes in cancer cells (Pryde, 2002).

Another mechanism against colon cancer unchained by the proliferation of probiotic bacteria is the stimulation of the host’s antitumor immunity (Ghoneum et al., 2004; Lee et al., 2004; Pagnini et al., 2010; Takagi et al., 2008) by stabilizing epithelial tight junctions, inducing epithelial defense production, inducing the antiinflammatory and immunomodulatory capacity of T regulatory cells and of dendritic cells, and stimulating B cells and natural killer cells. Probiotic bacteria also have a role in controlling tumor promotion and progression (Uccello et al., 2012).

2.2.3.3 FOS role in obesity

It is not clear at the present time that manipulating the bifidobacterial populations in the gut will have an impact on obesity, but there is promising evidence suggesting that prebiotics might have an impact on appetite, thus indirectly impacting weight gain.
Verhoef et al. (2011) fed the prebiotic OF to 28 healthy adults for 13 days and studied appetite profiles, energy intake, and the expression of the gut hormones PYY and GLP-1. They found that although OF consumption did not suppress appetite, energy consumption was reduced by 11% on day 13 when consuming 16g OF per day. Here, expression of both gut hormones was increased. The authors suggest that this might be due to production of elevated levels of SCFA by fermentation of OF (Rastall and Gibson, 2014).

A recent systematic review has been conducted on the effect of OF and inulin on appetite regulation, energy intake, and weight loss in children and adults (Liber and Szajewska, 2013). Studies amplifying this have been published by Cani et al. (2006, 2009), who showed that fructan prebiotics could influence satiety in humans. Similarly, Parnell and Reimer (2009) demonstrated that the same type of prebiotics could influence hormonal regulation and therefore appetite in overweight humans. The conclusion is that OF and inulin may have a contribution to make in reducing energy intake and weight loss.

2.2.3.4 FOS role in lipid regulation

Other studies have examined FOS, which was also found to reduce blood lipids (Bornet et al., 2002; Roberfroid, 2002). This was thought to be due to the inhibition of a lipogenic enzyme in the liver, which may be a result of the action of propionate produced from the fermentation of prebiotics by gut bacteria.

For example, acetic acid can stimulate mucin secretion (Wong et al., 2006), and propionic acid can affect lipid metabolism by decreasing de novo fatty acid synthesis (Macfarlane and Macfarlane, 2003).

2.2.3.5 FOS role in mineral absorption

FOS can affect mineral absorption, and in human studies, 15 g per day of OF or 40 g per day of inulin increased apparent calcium absorption (Roberfroid, 2002). Magnesium absorption has been found to increase following ingestion of FOS (Bornet et al., 2002). Also, a study (Mathey et al., 2004) was undertaken to investigate whether isoflavones (IF) and FOS, which are known to modify large-bowel flora and metabolism, may exhibit a cooperative bone-sparing effect. This work was carried out on three-month-old Wistar rats assigned to 12 groups: 2 SH (sham-operated) and 10 OVX (ovariectomized). Animals received a diet for 90 days containing total IF (Prevastein HC, Central Soya) at 0 (OVX and SH), 10 (IF10), 20 (IF20), 40 (IF40), and 80 (IF80) 1 g/g body weight per day. FOS (Actilight, Beghin-Meiji) were given orally to half of the groups, (OVX FOS), (IF10 FOS), (IF20 FOS), (IF40 FOS), (IF80 FOS), and (SH FOS). Bone strength was improved as well. As far as the FOS diet is concerned, addition of prebiotics significantly raised the efficiency of the IF protective effect on both femoral BMD and mechanical properties. Simultaneous FOS consumption improved IF protective effect on the skeleton, with the lowest IF dose becoming efficient.
2.2.3.6 FOS immunomodulatory effect

Some natural dietary components that have the capability to stimulate the suppressed immune system could be ideal candidates to boost the immune system, since they are derived from natural sources (Wichers, 2009). Recent animal studies clearly suggested that nondigestible carbohydrates, including fructans, have a strong influence on the immune system (Nauta and Garssen, 2013). Prebiotic inulin-type fructans elicit additional direct effects, such as immunomodulation, along the gastrointestinal tract (Jeurink et al., 2013). The immune system response could be modulated by inulin-type fructans by an indirect mechanism through fermentation products of gut commensal bacteria or by a direct mechanism via interaction with toll-like receptors, membrane lipids, etc. of mostly innate immune cells (Vogt et al., 2015).

The prebiotic function of fructans reduce the pathogenicity of pathogenic bacteria and leads to decreased inflammatory markers, such as phagocytosis and IL-6 production, by increasing CD3+, CD4+, and CD8+ cell populations (Guigoz et al., 2002).

One study has demonstrated that inulin-type fructans directly modulate the immune response through TLR activation (TLR2) in human PBMCs. The chain length of inulin-type fructans appeared to be an important factor in the ability to switch between the induction of anti-inflammatory and pro-inflammatory cytokines (Vogt et al., 2013).

Macrophages are immune-responder cells that act as bridges between two major arms of the immune system; that is, innate and adaptive immune response. Generally, activation of macrophages is assessed by the production of NO and increased phagocytic activity (Clement et al., 2010; Kardošová et al., 2003; Kumar et al., 2015). Chandrashekar et al. (2011) studied fructans from garlic extract and demonstrated that low-molecular weight inulin-type fructans (DP ≤ 15) were found to be more potent inducers of NO production and phagocytosis by murine peritoneal macrophages than high-molecular weight inulin-type fructans (DP ≥ 25).

In a recent study, Kumar et al. (2015) investigated the immunomodulatory properties of onion FOS. In this study onion FOS (50 µg/mL) significantly increased (~3-fold) the proliferation of mouse splenocytes/thymocytes versus the control group, also enhancing (~2.5-fold) the production of nitric oxide by peritoneal exudates cells (PECs) in Wistar rats, and the intracellular free radicals production and phagocytic activity of isolated murine PECs were also augmented. FOS immunostimulatory activity toward murine lymphocytes and macrophages was demonstrated.

2.2.4 Trends and applications in the food industry

FOS are officially recognized as natural food ingredients and classified as dietary fiber in almost all European countries. FOS are generally used as components of functional foods and have “generally recognized as safe” (GRAS) status from the US Food and Drug Administration (Kovács et al., 2013). Fructans have texture-improvement and
organoleptic properties, and provide health benefits, such as fat and sugar replacement (Apolinário et al., 2014).

FOS were the first bioactive components that were approved for food health and sold as functional ingredients, principally in European and Asiatic countries, such as France, Germany, the Netherlands, and Japan. FOS from sucrose were first introduced into the market by the Meiji Seika Company in 1984 in Japan under the trade name Meioligo. Afterward Meioligo established a joint venture with the Beghin-Maiji companies in France and produced FOS called Actilight. GTC nutrition is an American company that collaborated with Meiji Seika and formulated and marketed FOS as Nutraflora. Orafti Active Food Ingredients USA makes products called Raftiline and Raftilose that contain inulin and inulin-derived products obtained from chicory roots by partial enzymatic hydrolysis of inulin for the generation of FOS with DP 3–7 (Singh et al., 2016).

2.3 Peptides

Bioactive peptides are amino-acidic fragments that have an extension from 2–20 units, although they can extend even more, that are inactive in protein sequences, and that can be generated using oven proteolysis with commercial enzymes or from proteolytic microorganisms, and even through fermentative methods (Vercruysse et al., 2005). They also possess biofunctional properties, and these properties are conferred from the different quantity and variety of amino acids that form the peptide sequence, and besides present multifunctionalities (Harnedy and FitzGerald, 2012).

2.3.1 Natural sources

Peptides can be produced from vegetal or animal-protein sources, can act as a substrate in microbial enzymatic hydrolysis or can be liberated in the fermentative process, and can influence the functional properties for amino acid composition.

From vegetal sources, we find that soybean seeds contain high quantities of protein with great nutritional quality. The principal proteins are glycinin and β-conglycinin, which possess suitable properties for food industry use (Scilingo and Añón, 2004). Their primary use is in oil extraction, and the residues are rich in protein and fiber. They are usually used in animal feed or processed into different products for human consumption, such as soybean protein concentrates (Coscueta et al., 2016), but they even used in functional compound production by microbial transformation, which exhibits generally antioxidant or antihypertensive peptides, employing strains, such as Bacillus subtilis (Sanjukta et al., 2015).

Moreover, wheat cereal represents another vegetal source for this kind of functional molecule. This and another cereals, such as rye, barley, and oats (Martínez-Esteso et al., 2015), present gluten as the structural protein. They can be divided into two main groups, gliadin and glutenin, and also a unique amino-acid composition rich in glutamine and proline has been
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reported, presenting a certain hydrophobicity (Suetsuna and Chen, 2002). The production of antioxidative peptides from defatted wheat germ by solid fermentative process using a *B. subtilis* strain has been reported as well (Niu et al., 2013), along with the antihypertensive effect of wheat germ hydrolyzed with an alkaline protease from *Bacillus licheniformis* (Matsui et al., 2000).

Although rice has a relatively low protein content, it possesses proteins with a higher digestibility potential and higher lysine amounts (Amagliani et al., 2016). Rice protein consists of alkali-soluble glutelins and water-soluble albumins (Lim et al., 1999) that can also act as a substrate in an enzymatic or microbial conversion for peptide production. For example, Zhou et al. (2013) proved the antioxidant effect from rice protein hydrolysates prepared with microbial proteases; antihypertensive peptide generation from rice extracts through fermentation with *Monascus purpureus* mold fermentation also has been demonstrated (Kuba et al., 2009).

Rapeseed represents one of the main oilseed crops and is an attractive edible protein source (Vioque et al., 2000). This product is processed for oil extraction; the residues generally are a press cake or meal, and the protein portion can be availed for protein preparation as a coproduct (Thiyam et al., 2009). The use of rapeseed in SSF with *B. subtilis* for antioxidant peptides has been reported (He et al., 2012), and using the same fermentation type but with coaction between bacteria and enzymatic hydrolysis has promoted antimicrobial peptides production (Xie et al., 2015).

The animal protein represents another peptide source, with milk as the main substrate for peptide production (Dziuba et al., 1999). Milk is fluid secreted by female mammals to supply the nutritional requirements of neonates; its composition varies from species to species (O’Mahony and Fox, 2013). Milk contains about 3.5% protein, in which 80% is an assortment of α-, β-, and κ-caseins (Kitts and Weiler, 2003), and the other 20% is whey proteins that contain β-lactoglobulin, α-lactalbumin, immunoglobulin, and the bovine serum albumin (Yadav et al., 2015). Cows’ milk protein has a different amino-acid composition, the majority being glutamic acid/glutamine, proline, leucine, and lysine in the casein portion, with methionine and cysteine in the whey proteins (Pellegrino et al., 2013). For this reason the peptides from this protein source can induce activities, such as in vitro antithrombotic activity from casein peptides released with *Lactobacillus casei* Shirota (Rojas-Ronquillo et al., 2012), antioxidant peptides from camel milk (Moslehishad et al., 2013), and in vitro antihypertensive peptides from milk fermentation with *Kluyveromyces marxianus* (Li et al., 2015).

The eggs represent another important protein source. They are made up of 9%–11% eggshell, 60%–30% egg white, and 28%–29% egg yolk, and the protein content is 6, 50, and 44%, respectively. The eggshell is almost entirely composed of minerals but is covered with a cuticle that is composed of 90% protein, which is rich in glycine, glutamic acid, lysine,
cysteine, and tyrosine. The egg white is composed of water and protein, which includes ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme. Finally, yolk is made up of α- and β-lipovitellins and phosvitin (Mine, 2007). The antioxidant capacity of egg white–protein hydrolysates generated with a variety of gastric and microbial enzymes has been reported (Chen et al., 2012), along with antihypertensive peptides from the hydrolysis of yolk with a commercial crude extract of Rhizopus (Yoshii et al., 2001).

Proteins derived from meat and fish also have the potential to enhance peptide production. The term meat refers to edible parts removed from bovine, porcine, ovine, and caprine animals, and it makes up the principal source of animal protein for many consumers; it is rich in protein with a positive physiological activities potential (Lafarga and Hayes, 2014). When this kind of product is generated a series of particular wastes can be produced, such as blood, skin, and bones (Jayathilakan et al., 2012). Proteins, such as hemoglobin, collagen, gelatin, and even myofibril from meat and its by-products can be exploited for peptide production (Lafarga and Hayes, 2014). For example, Ryder et al. (2016) produced antihypertensive peptides with a hydrolysis of meat bovine by-products using microbial proteases.

On the other hand, fish and poultry meat and associated by-products can be used for the production of this compound class (Jayathilakan et al., 2012) through microbial fermentation with B. subtilis. Also, fish meat and chicken liver fermentation with Pediococcus acidilactici generated peptides with antioxidant and antimicrobial properties (Chakka et al., 2015; Jemil et al., 2014).

2.3.2 Microbial production

There exist diverse strategies for bioactive peptide production, including enzymatic hydrolysis; biotechnological methods, such as fermentation using proteolytic microorganisms; and a combination of both methodologies. The peptides generated through enzymatic hydrolysis are derived generally from food protein sources of vegetal origin (such as wheat, rice, corn, soybeans, pumpkin, and sorghum) or from animal protein (such as milk, gelatin, bovine blood, meat, eggs, and some fish, such as tuna, sardines, and salmon), among other sources that have been employed as substratum, in a partial degradation with digestive, vegetal, and even microbial proteases (Möller et al., 2008).

Proteinases represent an enzyme group that have the capacity to hydrolyze the peptide bond in proteins. These enzymes possess a certain specificity linked to the amino-acid sequences surrounding the rupture site and also act in a wide pH and temperature range (Rani et al., 2012). Given these parameters, along with required hydrolysis time, which affects peptide size and composition directly and therefore the biological activity that can be exhibited, this methodology is considered a fast, easy, and safe way of bioactive protein hydrolysate production. Another advantage is the possibility of exploiting agro-industrial proteic residues intended for disposal such as substrate to generate high-value products (de Castro and Sato, 2015).
Lactic acid bacteria (LAB) is relevant for bioactive peptide production by fermentative processes, including the submerged fermentation process, that ground their hydrolytic capacity in a complex proteolytic system whose principal component is a proteinase set attached to the bacterial wall, whose main task is to transform proteins into oligopeptides; specific transporters conduct the oligopeptides inside the cell and an endopeptidases group transforms oligopeptides into free amino acids or low-molecular weight peptides (Chaves-López et al., 2014). The capacities of this microorganism class are exploited by the food industry to obtain fermented products, and over time it has been reported that a variety of microorganisms have the capacity for peptide generation: *Lactobacillus helveticus*, *Lactobacillus lactis* ssp. *diacetylactis*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus lactis* ssp. *cremoris*, and *Streptococcus salivarius* ssp. *thermophilus* (Hernández-Ledesma et al., 2011). But we can also highlight the use of other microorganisms, such as *Kluyveromyces marxianus* (Li et al., 2015), *S. cerevisiae*, and even *Aspergillus egypticus* and *A. oryzae* (Inoue et al., 2009; Zhang et al., 2006). Peptide production from these processes is highly influenced by criteria, such as media composition and the conditions under which the fermentation is done. On the other hand, the use of SSF has improved peptide production in rapeseed with *B. subtilis* strains or soybeans (He et al., 2012; Sanjukta and Rai, 2016).

It also has been demonstrated that the peptidic molecules present in biological activity in traditional fermented foods made from milk, soybeans, and even meat products, show that the fermentative step is essential not only for flavor generation, but also for active metabolites, such as peptides with antioxidant, antihypertensive, antitumor, or antimicrobial activities. (Fadda et al., 2010; Moslehishad et al., 2013; Rho et al., 2009; Xu et al., 2015). Table 4.3 shows a list of peptide production using the fermentative process.

### 2.3.3 Biological properties

#### 2.3.3.1 Antimicrobial peptides

Due to pathogenic microorganism resistance to antibiotics that has developed from indiscriminate antibiotic use and the mutagenic capacity of these microorganisms, the need to find new molecules with antibiotic activity has arisen (Harrison et al., 2014). An example is

<table>
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<th>Functionalities</th>
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<th>Cultivation Condition</th>
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<tr>
<td>Antioxidant/antibacterial</td>
<td><em>B. subtilis</em> A14h</td>
<td>SmF</td>
<td>Flask</td>
<td>Moayedi et al. (2016)</td>
</tr>
<tr>
<td>ACE-I inhibition</td>
<td><em>L. casei</em> ssp. <em>pseudoplantarum</em></td>
<td>SmF</td>
<td>Flask</td>
<td>Vallabha and Tiku (2014)</td>
</tr>
<tr>
<td>ACE-I inhibition</td>
<td><em>Enterococcus faecium</em></td>
<td>SmF</td>
<td>Flask</td>
<td>Martinez-Villaluenga et al. (2012)</td>
</tr>
<tr>
<td>ACE-I inhibition</td>
<td><em>Bacillus pumilus</em> AG1</td>
<td>SmF</td>
<td>Flask</td>
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</tr>
<tr>
<td>ACE-I inhibition</td>
<td><em>Pichia kudriavzevii, K. marxianus</em></td>
<td>SmF</td>
<td>Flask</td>
<td>Chaves-López et al. (2014)</td>
</tr>
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</table>
food-derived peptides, which are mainly composed of hydrophobic and cationic peptides, and which exhibit bactericide properties against pathogenic microorganisms (Saadi et al., 2014). Cell membranes in bacteria are negatively charged, and when they interact with positive peptides, they suffer oligomerization, form pores on the membrane, and finally liberate cell contents (Aoki et al., 2012). Other mechanisms include key process interrupt, protein synthesis, DNA, and cell wall (Nguyen et al., 2011).

2.3.3.2 Antihypertensive peptides

Hypertension constitutes one of the main risk factors for cardiovascular disease, such as stroke, artherioesclerosis, and heart failure, and affects more than one billion people worldwide (Padwal et al., 2015). Peripheral blood pressure is controlled by a series of biochemical routes, which include the renin-angiotensin-aldosterone system (RAAS), the neuro endopeptidase system, and the endothelin converting enzyme (Harnedy and FitzGerald, 2012). The angiotensin-converting enzyme (ACE) plays a critical role in physiological blood pressure regulation. This enzyme transforms angiotensin I into angiotensin II, a potent vasoconstrictor; for this reason the enzyme inhibition represents a principal mechanism for blood-pressure regulation (Ngo et al., 2012). There exist peptides capable of inhibiting ACE and regulating hypertension, generally constituted of short chains from two or three amino acids with proline, lysine, and arginine on extreme C-terminal sites (Mohanty et al., 2015), although tryptophan, tyrosine and phenylalanine, and aliphatic amino acids are in the extreme N-terminal region (Ngo et al., 2012).

2.3.3.3 Antithrombotic peptides

Blood coagulation is a complex chain process involving a series of stimulus responses in conjunction with coagulation factors and enzymes, whose intent is to stop blood fluxes when a vascular tissue injury occurs (Ngo et al., 2012). Venous thromboembolism is a condition linked to trauma, prolonged immobilization, or blood coagulation (Lafarga and Hayes, 2014), and also to manifestations of atherothrombosis, such as hearth failure and stroke, where the platelets plays an important role, and for this reason molecules that avoid platelets aggregation exhibit a potential for clinical use (Shimizu et al., 2009). Peptides that exhibit this potential are present in milk, and this activity has been attributed to the presence of fibrinogen homologues sequences, which bind to receptors, avoiding the thrombus formation. (Mulero Cánovas et al., 2011).

2.3.3.4 Antioxidative peptides

The oxidative stress on cells due to the natural process of respiration (Sarmadi and Ismail, 2010), or other stimuli, such as contamination, UV radiation, chemicals, and tobacco, can promote the formation of substances, such as free radicals (Lafarga and Hayes, 2014) or reactive oxygen species. These substances are unstable and highly reactive chemical species with unpaired electrons (Di Bernardini et al., 2011) that can enhance reactions with main
cellular components, such as lipids, proteins, and ADN, bringing effects at the molecular level, such as lipid membrane oxidation and lost mobility, and enzyme inactivation and sequence mutation in nucleic acids. This promotes the development of cancer and other degenerative diseases ([Hayashi and Cortopassi, 2015]
).

2.3.4 Trends and applications in the food industry

These bioactive molecules possess a variety of potential uses; due to the link between health and food they can be used as nutraceuticals in functional foods, as in the milk drink Calpis or Finish “Evolus”, which contain antihypertensive tripeptides so that their ingestion regulates blood pressure ([Iwaniak et al., 2014]
). Or they can even be incorporated into foods to preserve their integrity, as with those with the most relevant properties, such as antioxidant peptides, which can provide protection against lipid peroxidation and off flavors in meat, and increase the shelf life of meat products ([Rossini et al., 2009]
).

2.3.5 Recovery and purification strategies

The recovery and purification stage in bioprocessing is one of the most relevant. The complexity of the technology employed depends on metabolite localization, type, concentration, purity required, potential use, market demand, and in peptides the charge and molecular mass. Due to these factors this stage affects processing cost directly, representing a 20%–60% of the total cost of processing.

Employing membranes represents one efficient alternative for peptide recovery in cases where a hydrolysis or fermentative production route is employed. A membrane system is a technique employed to purify peptides based on molecular weight ([Kim et al., 2007]
), using a membrane as a filter with the capacity to distribute components and using pressure as a driving force. The membranes can have different pore sizes, which determine their classification; in microfiltration (MF) membranes have pores in the range of 0.2–100 µm and in ultrafiltration (UF) pores are in the range of 0.0–2.0 µm ([Prapulla and Karanth, 2014]
). This technique has the advantage of possible scale-up at a lower price than other separation methodologies ([Chay Pak Ting et al., 2010]
), although during UF the partially or completely retained solutes tend to cause membrane fouling and a lower flux ([Arunkumar et al., 2016]
). The possibility of employing charged membranes can help to separate peptides according to charge and mass when membranes are positively or negatively charged and present a definite pore shape and size, avoiding the aggregation of molecules due to the repulsion between solutes and the membrane ([Saxena et al., 2009]
).

Chromatographic methodologies have also been implemented, in which the separation of peptides is based on their composition or structure. Basically the components of interest are passed through stationary supports, and their interaction promotes the separation from molecules with similar characteristics, either through ion exchange, where molecules can bind to the stationary matrix that has a complementary charge to the interacting molecules,
or through pH affinity, where by changing this parameter compounds with similar affinities can be recovered. Another class of recovery methodology is size-exclusion chromatography, in which the stationary matrix has different sizes of molecular pores; when a sample with molecules of different sizes passes through, the larger-sized ones will not be trapped in the matrix and will be expelled quickly (Prapulla and Karanth, 2014). This technique has an exceptional resolution and selection rate, but the instrumentation is expensive and limits the scale-up (Agyei et al., 2016). A combination of all these methods promotes high efficiency in the purification of complex samples, such as fermentation broths and hydrolysis of food proteins.

3 Genetic Engineering of Bioactive Compound-Producing Microorganisms

Genetic engineering of yeast, bacteria, and fungi by introducing and controlling genes for bioactive metabolites production has been studied. The development of recombinant DNA technology has provided new tools for approaching yield improvement by means of genetic manipulation of biosynthetic pathways (Olano et al., 2014).

Different microorganism types have been engineered to produce carotenoids. Bautista et al. (2005) replaced the zeta-carotene desaturase (ZDS) gene, and both the ZDS and phytoene desaturase (PDS) genes of Synechocystis sp. PCC 6803, with the phytoene desaturase (crtI) gene of Rhodobacter capsulatus, to test the redox function of carotenoids in a secondary electron transfer pathway that oxidizes Chl(Z) and cytochrome b(559) in photosystem II (PS II) when normal tyrosine oxidation is blocked. Carotenoids with shorter conjugated pi-electron systems and higher reduction potentials than β-carotene were produced from Synechocystis sp. PCC 6803 after the transformation. On the other hand, Olson (2014) undertook the engineering of S. cerevisiae for the production of heterologous isoprenoid compounds, which protect cells from oxidative stress and reactive oxidative species in the environment. This author reintroduced the cytosolic catalase T (CTT1) gene and overexpressed the HMG1 gene, improving carotenoid production from 15 ± 3.3 mg/g (dry cell weight) to 22 ± 2.1 mg/g (dry cell weight).

Halophilic organisms are a very interesting microbial group for carotenoid production. Rodrigo-Bañños et al. (2015) mentioned some advantages of halophilic archaea for carotenoid production, among them: (1) different halophilic organisms have high carotenoids production availability; (2) downstream processes for carotenoid isolation from halophilic organisms is relatively quick, easy, and cheap; and (3) carotenoid production by haloarchaea can be improved by genetic modification or by optimization of fermentation aspects, such as nutrition, growth pH, and temperature. Elucidation of the C_{50} carotenoid pathway was recently reported on by Yang et al. (2015). These authors found that the c0507, c0506, and c0505 genes codify for a carotenoid 3,4-desaturase (CrtD), a bifunctional lycopene elongase and 1,2-hydratase (LyeJ), and a C_{50} carotenoid 2″,3″-hydratase (CruF), respectively. These
enzymes catalyze the reactions that convert lycopene to C\textsubscript{50} carotenoid bacterioruberin in *Haloarcula japonica*, which is an extremely halophilic archaeon. Another halophilic microorganism reported as a carotenoid producer is *Haloferax mediterranei* (Rodrigo-Baños et al., 2015).

Some pathogenic microorganisms have genes codifying for enzymes that are very important in the carotenoids pathway; these genes can be cloned in nonpathogenic microorganisms to produce these enzymes and enhance carotenoid production. Maeda (2012) reported that *Staphylococcus aureus*, a pathogenic bacterium that causes opportunistic infection, produces C30 carotenoids. The acyclic C30 carotenoids showed higher radical scavenging activity than did dl-\(\alpha\)-tocopherol (Kim et al., 2016). Different enzymes intervene in the C30 carotenoid synthetic pathway, among them dehydrosqualene synthase (CrtM), which converts farnesyl pyrophosphate to dehydrosqualene; then this compound is converted to the yellow C30 carotenoid 4,4'-diaponeurosporene by the dehydrosqualene desaturase. Introduction of *S. aureus* CrtM and CrtN genes into *B. subtilis*, which is a GRAS organism resulting in yellow pigmentation, accumulated two C30 carotenoids, 4,4'-diapolycopene and 4,4'-diaponeurosporene (Maeda, 2012). Recently, novel structures of C30 and C35 carotenoids, including acyclic, monocyclic, and bicyclic structures, have been produced in *Escherichia coli* (Kim et al., 2016).

Microbial genes have been used to transform plants to increase carotenoid production. Ravanello et al. (2003) mentioned that it was possible to increase total levels of carotenoids, including phytoene and \(\beta\)-carotene, which were augmented 50-fold, with the ratio of \(\beta\)- to \(\alpha\)-carotene being 2:1 in canola (*Brassica napus*) seeds after transformation with the bacterial phytoene synthase gene (CrtB).

Other techniques for FOS production have been implemented using enzyme genetic engineering. Marín-Navarro et al. (2015) discloses a process for the production of 6-kestose, in which a modified invertase expressed by *Saccharomyces* is used, which exhibits improved transfructosylation activity wherein 6-kestose was produced with high specificity representing 95% total FOS. This could make genetic engineering an interesting tool to enhance the activity of the enzymes involved in the synthesis of FOS and to improve their production.

Several reports of engineering metabolics to produce carotenoids have been reported, but just a few studies that described FOSs and bioactive peptides production were in development. Therefore, another important area of study to explore is available.

### 4 Conclusions and Perspectives

To satisfy needs of consumers, bioprocesses must be feasible and cover certain conditions; the most important characteristics are that these biological activities be environmentally friendly, low cost, and high quality. Microbial production presents many challenges, but the most complicated
is scaling recovery and purification techniques, always with the tendency to incorporate methodologies. For example, in peptide production, one challenge is bitter taste and functional-property maintenance for consumption. On the other hand, the liposolubility of carotenoids requires that organic solvents be used in the extraction, but the toxicity of these compounds is inconvenient. Also, cell disruption to release intracellular carotenoids, mix of pigments, yields, stability, and substrate sources are factors that should be optimized. Technologies are emerging for extraction and cellular-wall disruption: genetic engineering to increase yields, agro-industrial waste employed as a source of substrates, bioreactors designed to facilitate production and recovery, and the introduction of inhibitors or regulators of enzymes into biosynthetic pathways for carotenoids. These are all possible solutions; nevertheless, extensive study is necessary.

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**Further Reading**