Chapter 2

Biotechnological Production of Oligosaccharides — Applications in the Food Industry

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Abstract

Oligosaccharides are carbohydrates, composed of up to twenty monosaccharides linked by glycosydic bonds, widely used in food and pharmaceutical industries. These compounds can be obtained by extraction from natural sources (milk, vegetables, fruits), and by chemical or biotechnological processes. In the last case, chemical structures and composition of the generated oligosaccharides depend on the type and source of enzymes, and on process conditions, including the initial concentration of substrate. Among the various functions of nondigestible oligosaccharides, one that has attracted attention is its prebiotic potential. The intestinal benefits of prebiotics, such as fructooligosaccharides and inulin as well as their symbiotic association with probiotic bacteria, encompass prevention and treatment of infectious diseases, including viral or bacterial diarrhea, and chronic inflammatory diseases such as ulcerative colitis. Other benefits attributed to prebiotics and probiotics include treatment of inflammatory intestinal and irritable bowel syndrome, prevention of cancer, and modulation of the immune system, mineral absorption and lipid metabolism. Fructooligosaccharides (FOS), galactooligosaccharides (GOS) and chitooligosaccharides (COS) have been widely studied for their prebiotic properties. Moreover, novel oligosaccharides with potential prebiotic activity are currently under investigation. This review will focus mainly on the biotechnological production, health benefits and applications of non-natural oligosaccharides in the food industry.

Keywords: oligosaccharides, biotechnological production, applications, bioactivity

1. Introduction

Consumers all around the world are increasingly aware and concerned about safety and the quality of food. Besides the push towards replacement of chemical additives by those obtained from natural sources, this awareness has led to a rising demand for enrichment of foods with bioactive compounds that have beneficial effects on human health [1]. Therefore, nowadays, a variety of gluten free and products enriched with dietary fiber, or containing probiotics and/or prebiotic and functional oligosaccharides are available in the market [2].

Oligosaccharides are carbohydrates, composed of up to twenty monosaccharides linked by glycosydic bonds, widely used in food and pharmaceutical industries. These compounds are obtained from natural sources and through chemical or biotechnological processes [3,4].

Among the various functions of non-digestible oligosaccharides, one that has attracted attention is its prebiotic potential. A prebiotic can be defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" [5]. An oligosaccharide to be regarded as prebiotic must not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract; and must be assimilated selectively by one or by a limited number of beneficial microorganisms in the colon, promoting benefic luminal or systemic effects. To improve colonic function, live microorganisms can be administered in adequate amounts, being known as probiotics; and to be used in food, these organisms must be able to survive passage through the gut; to proliferate and to colonize the digestive tract; and must be safe and effective [6,7].

The intestinal benefits of prebiotics, such as fructooligosaccharides and inulin as well as their symbiotic association with probiotic bacteria, encompass prevention and treatment of infectious diseases, including viral or bacterial diarrhea, and chronic inflammatory diseases such as ulcerative colitis [8]. The mechanisms of action of probiotics against gastrointestinal pathogens consist mainly on competition for nutrients and sites of access, production of antimicrobial metabolites, changes in environmental conditions, and modulation of the immune response of the host. Other benefits attributed to prebiotics and probiotics include treatment of inflammatory intestinal and irritable bowel syndrome, prevention of cancer, and modulation of the immune system, mineral absorption and lipid metabolism [8,9].

Oligosaccharides can be obtained by extraction from natural sources (milk, vegetables, fruits), and by chemical or biotechnological processes [10,11]. Mixtures of oligosaccharides with different degrees of polymerization and glycosidic linkages are usually formed in the enzymatic processes. Chemical structures and composition of these mixtures depend on the type and source of enzymes, and on process conditions, including the initial concentration of substrate [11,12]. Depending on the initial substrate, production of oligosaccharides can involve different steps: hydrolysis of glycosidic bonds giving rise to monomers, followed by generation of disaccharides and other oligomers through the action of transferases [13,14].

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2. Fructooligosaccharides

Fructans are carbohydrates in which one or more fructosylfructose links constitute the majority of glycosidic bonds [15]. These carbohydrates can be of the inulin-type with β-(2,1)-D-fructofuranosyl units, found in plants and synthesized by fungi. Additionally, there are the levan-type fructans with β-(6,2)-D-fructofuranosyl units, found in plants and synthesized by bacteria [16].

Levan is a polymer with very high molecular weight that can reach 10^7 Da [17]. In contrast to levan, inulin from chicory consists of a mixture of oligomers and polymers with a degree of polymerization (DP) that varies from two to approximately sixty units (Figure 1; Table 1) [18]. Around 10% of the fructan chains in native chicory inulin have a DP in the range between two and five [5].

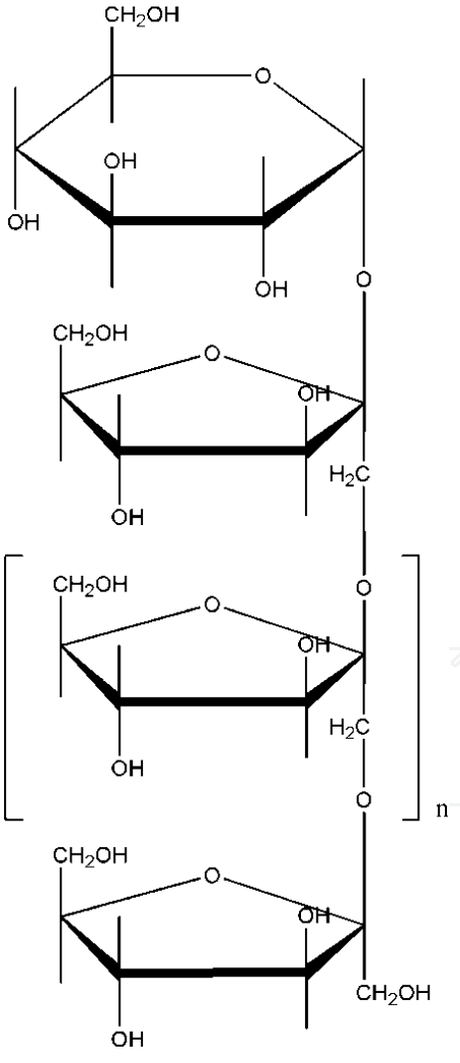

Figure 1. Structure of inulin, a linear fructosyl polymer linked by β-(2,1) bonds (n=3-65), attached to a terminal glucosyl residue by an α-(1,2) bond.

Prebiotics	Chemical structure	Properties	Applicability / Health benefits	Reference
FOS	Fructosyl units linked by β-(2,1) bonds, attached to a terminal glucosyl residue by α-(1,2) bond (Variants: Inulin type β-1,2 and Levan type β-2,6 linkages between fructosyl units in the main chain) DP=3-10.	Soluble fibers; Gel formation; Sugar replacement; Moderate sweetness; Stable (depending on matrix).	Prevention of intestinal infections and extra intestinal infections (e.g. respiratory tract; Inhibition of pathogens, ordering intestinal flora; Regulation of intestinal immune system; Enhancement of immune response; Stimulation of probiotic growth of Lactobacilli and Bifidobacteria species; Optimization of colonic function and metabolism; Production of short chain fatty acids; Increase of mineral absorption; Reduction of food intake and obesity management and control of diabetes type 2; Prevention of cancer.	[8,24,25, 61,67,69, 72,170, 176-186]
Inulin	Mixture of linear fructosyl polymers and oligomers (DP = 3-65) linked by β-(2,1) bonds, attached to a terminal glucosyl residue by α(1—2) bond.	Soluble fiber; Water adsorption; Gel formation; Modifier of viscosity, texture, colour and sensory aspects of food formulations; Replacement for fat and sugar; Low calorimetric value; Moderate sweetness.	Stimulation of probiotic growth; Lowering effect on cholesterol LDL and triglycerides levels; Influence on inflammatory markers and development of gut associated lymphoid tissue (GALT); Regulation of intestinal immune system; Enhancement of immune response; Increase of mineral absorption (Calcium, Iron and Magnesium); Prevention of cancer.	[8,19,67, 176,187-195]
GOS	Mixture of galactopyranosyl oligomers (DP= 3-8) linked mostly by β-(1,4) or β-(1,6) bonds, although low proportions of β (1,2) or β-(1,3) linkages may also be present. Terminal glucosyl residues	Stable in acidic conditions and in higher temperatures; Soluble; Cryoprotector activity; Low ability to crystalyze; Incorporated in various functional foods.	Stimuli of probiotic growth; Reorder intestinal flora; Regulation of intestinal immune system; Reinforcement of intestinal barrier; Inhibition of adhesion of pathogens; Mimic molecular receptors, inhibit microbial adherence;	[8,105, 196-206]

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Prebiotics	Chemical structure	Properties	Applicability / Health benefits	Reference
	are linked by β-(1,4) bonds to galactosyl units.		Prevent infections (e.g. *Clostridium difficile* diarrhea); Prevention of cancer; Enhance mineral absorption; Reduce food intake, helping obesity management. Use in diabetic foods, free from carbohydrates that increase the level of postprandial glucose; Use in specialized foods for individuals intolerant to lactose.	
Lactulose	Galactosyl β-(1,4) fructose.	Sweetener, sugar replacement;	Induces growth of *Bifidobacterium* both *in vitro* and *in vivo*; use as laxative in the treatment of constipation; Optimization of colonic function and metabolism, reducing colon pH and ammonia concentration; Increased mineral absorption; Treatment of portal systemic encephalophathy and chronic constipation; Uses in diabetic and dairy foods.	[205, 207-211]
COS	Chitin: β-(1,4) linked N-acetyl-D-glucosamine residues; Chitosan: β-(1,4) linked D-glucosamine polymer. DP=2-8	Antimicrobial activity of chitosan depends on degree of polymerization, amino groups content and degree of acetylation; Chelation of metal trace elements and essential nutrients; Flocculation and adsorption capacity mainly because of the cationic macromolecular structure.	Antimicrobial and antioxidant activity; Use as food preservative; Use as dietary supplements in functional foods; Prebiotic activity; Hypocholesterolemic;	[149, 212, 213]

Prebiotics	Chemical structure	Properties	Applicability / Health benefits	Reference
XOS	Xylose oligomers connected by β-(1,4) linkages (DP=3-6).	Stable in a large range of pH values (2,5-8,0); Thermal stability (up to 100°C); Antioxidant effects; Antifreezing activity; Low cariogenicity; Low calorimetric value; Low glycemic index.	Inhibition of pathogens growth, reordering intestinal flora; Stimulation of probiotic growth; Reinforcement of intestinal barrier; Optimization of colonic function and metabolism; Obesity management, reduction of food intake and weight.	[159, 162, 179, 214-216]
IMO	Glucosyl residues linked to maltose or isomaltose by α-(1,6) glycosidic bonds.	Low sweetness; Low viscosity; Bulking properties; Humectant; Prevention of sucrose crystallization.	Optimization of colonic function and metabolism, reduces nitrogenated products; Increase caecum weight; Antidiabetic effects; Improve lipid metabolism and obesity management.	[2, 217-219]
SOS	Oligomers composed by galactosyl units linked to sucrose by α-(1,6) bonds. Most abundant are raffinose and stachyose.	Stabilizer properties; Cryoprotectant effect.	Prevention of pathogen proliferation.	[2, 156, 213, 220, 221]

FOS: Fructooligosaccharides; GOS: Galactooligosaccharides; COS: Chitooligosaccharides;

XOS: Xylooligosaccharides; IMO: Isomaltooligosaccharides; SOS: Soybean oligosaccharides

Table 1. Structure and biological activity of prebiotics.

Inulin-type fructooligosaccharides are made up of two or more fructosyl moieties linked by β-(2,1) bonds and united at the non-reducing end to a terminal glucose residue by an α-(1,2) glycosidic bond (Table 1) [19]. The term fructooligosaccharides (FOS) is mainly used for fructose oligomers that contain one glucose unit and from two to four fructose units bound together by β-(2,1) glycosidic linkages [20,21]. Nevertheless, oligofructose and FOS may be regarded as synonyms for the mixture of small inulin oligomers with DP<10 [6,22]; while short chain FOS (sc-FOS) are fructose oligomers mainly composed of 1-kestose (GF_2), nystose (GF_3), and ^{1}F-fructofuranosylnystose (GF_4) (Figure 2) [23-25].

Fructans have storage and protective functions in many commonly consumed plants, being a typical part of the diet. Some food sources are richer in high molecular weight fructans, such as inulin, while others have higher levels of sc-FOS [26].

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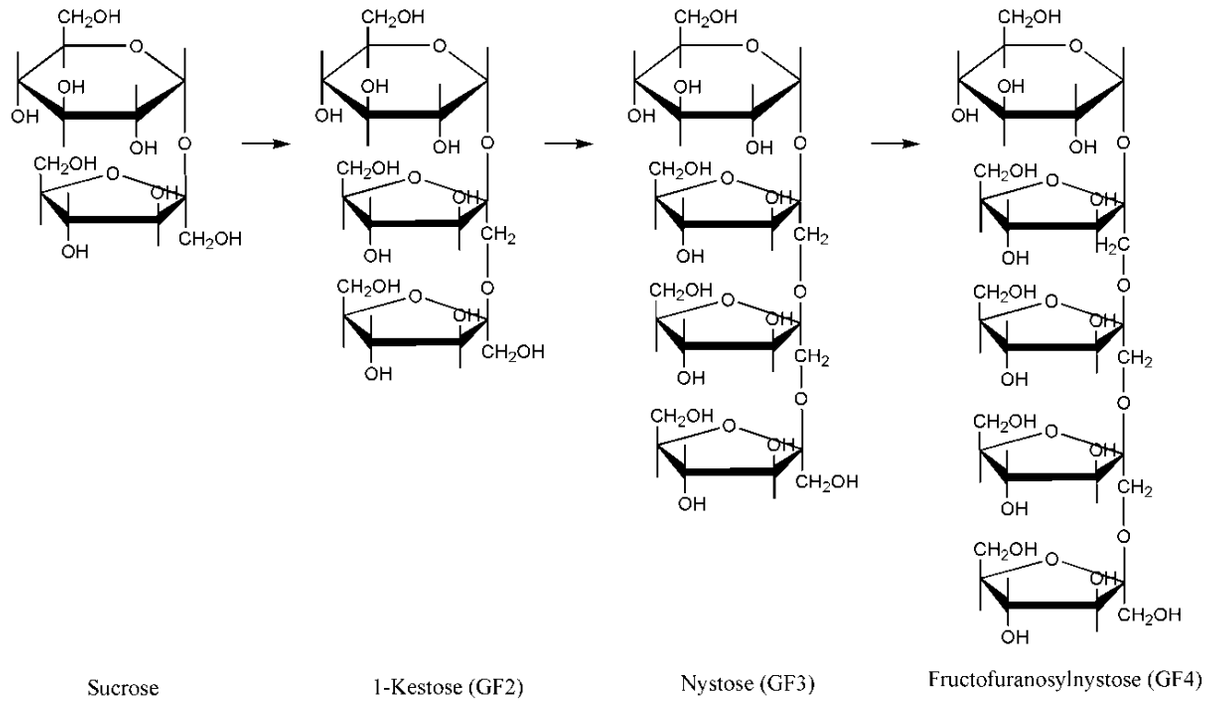

Figure 2. Structures of typical fructooligosaccharides (FOS), derived from sucrose. FOS consist of a glucosyl residue α-(1,2) linked to two or more β-(1,2) fructosyl units. Synthesis of these FOS is catalyzed by fructosyltransferases, requiring a second sucrose molecule as a fructosyl residue donor.

FOS are found in low levels in natural sources such as asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, and rye [27-29]. Apart from usually occurring in low concentrations, seasonal conditions also limit their large-scale production from these sources [30].

For this reason, enzymatic processes are used for the industrial production of FOS. One route involves the controlled hydrolysis of long chain fructans (Table 2) [31,32], which results in a large amount of FOS mostly without glucose in their structures. The other route is the synthesis from sucrose, which leads to sc-FOS that contain a molecule of glucose in their structures [11, 33]. The present review will focus on the synthesis of FOS from sucrose.

FOS are produced from sucrose by the action of microbial enzymes with high transfuctosylating activity: β-D-fructosyltransferase (FTase, EC 2.4.1.9) and β-fructofuranosidase (FFase, EC 3.2.1.26) (Table 2) [34]. Since FTase possesses almost only the transfructosylating activity, it is able to cleave the β-1,2 linkage of sucrose, transferring the fructosyl group to an acceptor molecule, with the resulting formation of fructooligosaccharides and release of glucose [35]. This enzyme shows little affinity towards water as an acceptor, therefore the hydrolase activity of FTase is very low [36].

FFase can catalyze both hydrolytic and transfructosylating reactions, nevertheless, transfructosylation only takes place when sucrose concentrations are higher than 500 g L^{-1} [27,34,36-38]. The production of FOS by the action of FFase on sucrose can occur either by reverse hydrolysis or by transfructosylation [36].

Type of prebiotics	Obtention source	Enzyme processing	Microbial producer	Industrial product and manufacturer	References
FOS	Enzymatic reactions: fructosyltransferases using sucrose as a substrate or from inulin using microbial endoinulinases.	Fructosyltransferases or β-fructofuranosidases; Levansucrases; Endoinulinases.	*B. macerans* *Z. mobilis* *L. reutri* *A. niger* *A. japonicus* *A. foetidus* *A. sydowi* *A. pullans* *C. purpurea* *F. oxysporum* *P. citrinum* *P. frequentans* *P. spinulosum* *P. rigulosum* *P. parasitica* *S. brevicaulis* *S. cerevisiae* *K. marxianus*	Neosugar Actilight NutraFlora P-95 - GTC Nutrition Raftilose P95 - Orafti Group	[21,29, 170, 176, 222]
Inulin	Natural product, extraction from plants	Not applicable	Not apllicable	Inulin-S – SigmaAldrich Fibruline - Trades S.A. Fibrex - Danisco Sugar Frutafit CLR DP8, Fruta- fit HD DP10, Frutafit TEX DP5, Inulin TEX – Sensus Inulin GR, HP, HP-gel, HPX, LS, ST, Raftilin ST, Raftilose P95, Raftiline HP - Orafti Group	[189, 194, 222]
GOS	Enzymatic transgalactosylation reactions, using lactose as substrate; Fermentation process.	β-Galactosidases	*Aspergillus sp.* *Bacillus sp.* *B. circulans* *Kluyveromyces sp.* *B. bifidum*	Vivinal GOS Syrup - Bolculo Domo or Friesland Foods Domo	[105,120, 198, 202-205, 223-227]

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Type of prebiotics	Obtention source	Enzyme processing	Microbial producer	Industrial product and manufacturer	References
			S. singularis *S. thermophilus* *C. laurentii*	Purimune - GTC Nutrition Oligomate 55NP - Yakult Pharmaceutical Inc. Cup Oligo H-70® Kowa Company BiMuno - Clasado Ltd.	
Lactulose	Thermal-alkaline isomerisation of lactose; Enzymatic transgalactosylation of fructose.	β-Galactosidases β-Glycosidases	*A. oryzae* *S. fragilis* *K. lactis* *P. furiosus* *S. solfataricus*	Sigma Aldrich Discovery Fine Chemicals Solvay	[209, 210, 228-230]
XOS	Enzymatic degradation of xylans	Endo-β-1,4-xylanases, exo-β-1,4-xylosidases, α-glucuronosidases, α-L-arabinofuranosidases, acetylxylan esterases, ferulic acid esterases and p-coumaric acid esterases.	*T. reesei* *T. harzianu* *T. viride* *T. koningii* *T. longibrachiatum* *P. chyrosporium* *G. trabeum* *A. oryzae*	Xylooligo™- Suntory Ltd. YOGHURINA - Suntory Ltd. MARUSHIGE GENKISU - Marushige Ueda Co. L-ONE - Enzamin Laboratory Inc. SUKKIRI KAICHO Lotte Co.	[152, 159, 232]
COS	Enzymatic or chemical depolimerization and deacetylation of chitin and chitosan	Chitosanases and other non-specific enzymes (papain, and lysozyme)	*S. coelicolor* *B. pumilus* *Bacillus sp.* *S. kurssanocii*	Qingdao BZ-Oligo Co, Ltd. BioCHOS. AMSBIO	[213 232-235]
SOS	Directly extracted from soybean	Not applicable	Not applicable	Soya-oligo - The Calpis Food Industry Co.	[152]
IMO	Enzymatic hydrolysis of starch	α-Amylases or pullulanases, β-amylases and α-glucosidases in sequence. Pullulanases	*A. niger* *Bacillus spp.* *B.subtilis* *B. stearothermophilus*	Isomalto-900 - Showa Sangyo	[12, 152, 159, 236]

Type of prebiotics	Obtention source	Enzyme processing	Microbial producer	Industrial product and manufacturer	References
			and T. maritima		
			A. carbonarious		
			L. mesenteroides		

Table 2. Obtention and industrial production of prebiotics.

FOS are produced at industrial scale from concentrated sucrose solutions using fungal transfructosylating enzymes mainly from strains of *Aspergillus niger, Aspergillus oryzae* and *Aureobasidium pullulans* [27,29,30]. Moreover, production of FTase from bacteria (*Lactobacillus*) and yeasts (*Rhodotorula*, *Candida*, *Cryptococcus* sp) has been reported [39,40]. The main enzymes used for industrial production of FOS generally give rise to a mixture of molecules with the inulin-type structure, ^{1}F-FOS, whereas those from yeasts usually form levan-type FOS (^{6}F-FOS) or neoFOS (^{6}G-FOS) [41].

The enzymes from *Aureobasidium pullulans* and from *Aspergillus niger* are highly regiospecific in the fructosyl transfer reaction, transferring one fructosyl moiety from sucrose to the 1-OH of the furanoside of another fructose molecule or fructooligosaccharide, with high selectivity [27]. This synthesis is a complex process in which several reactions occur simultaneously, both in parallel and in series, because sc-FOS are also potential substrates of FTase [42].

Catalytic and physicochemical properties of the producing enzymes, as well as production conditions and composition of FOS are different, depending on the microbial strain. For instance, fungal FTases have molecular masses ranging between 180,000 and 600,000, and are homopolymers with two to six monomer units [43].

Fructosyltransferase from *Aureobasidium pullulans* was submitted to preparative scale chromatographic separation on a weak anion-exchanger [42]. The molecular weight of the enzyme determined by size-exclusion chromatography was 570,000. Analysis of the action of FTase on a FOS substrate (Actilight 950P) showed that sucrose was the only donor of fructosyl moiety used in the transfer reaction catalyzed by the enzyme, while the acceptor could be another molecule of fructose or FOS [42].

A transferase isolated and purified from *Aspergillus aculeatus* exhibited pH and temperature optima of 6.0 and 60°C, respectively, remaining stable with no decrease in activity after 5 h under such conditions [44]. The enzyme was monomeric with a molecular mass of 85 kDa. On the other hand, FFase I from *A. pullulans* DSM2404 had a molecular weight of 430,000 [45]. The biocatalyst from *A. aculeatus* showed both transfructosylation and hydrolytic activity, and the transfructosylation ratio increased to 88% at 600 mg mL^{-1} of sucrose [44]. Conditions such as sucrose concentration (400 mg mL^{-1}), temperature (60°C) and pH (5.6) favored synthesis of high levels of GF_3 and GF_4. The major products were GF_2 after 4 h and GF_4 after 8 h of reaction. Prolonged incubation for 16 h resulted in the conversion of GF_4 into GF_2 due to hydrolase activity.

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The theoretical yield of FOS from sucrose is 75% if 1-kestose is the only FOS produced [46]. However, production yields of FOS are typically low (55–60%) due to the hydrolytic activity which gives rise to glucose and fructose as reaction byproducts [27] and/or to the fact that glucose acts as an inhibitor of the enzymes, reducing the reaction efficiency [36,47,48]. To improve FOS production yields, glucose oxidase has been used to remove glucose via transformation to gluconic acid [49] and glucose isomerase has been used to interconvert glucose to fructose [46]. Nevertheless, it is necessary to seek for strains among the microbial diversity with high transfructosylating activity, able to produce high yields of oligosaccharides and low yields of monomeric sugars [35].

In addition, the supply of sc-FOS is limited compared to their increasing demand in the food industry, because enzymes such as fructosyltransferases are not widely commercially available [50]. For this reason, the production of FOS is usually carried out in a two-stage process, in which the first stage consists of the microbial production of the enzyme with transfructosylation activity, while the second involves the reaction of the produced enzyme with sucrose (substrate) to generate FOS [29].

A commercial pectinase preparation from *Aspergillus aculeatus*, Pectinase Ultra SP-L, contains FTase [51,52] besides being composed of different pectinolytic and cellulolytic enzymes. The preparation, used in the food industry to reduce the viscosity of fruit juices [42,53], was the only commercially available source of FTase according to [42].

Enzymes from *Aspergillus japonicus*, *Aspergillus aculeatus* (Pectinex Ultra SP-L) and *Aureobasidium pullulans* were used to determine the reaction conditions required to obtain high yields of sc-FOS [34,51,54]. High concentrations of sucrose (600-850 g L^{-1}), pH (4.5–6.5), temperature (50–60°C), reaction time (3–5 h) and high ratios of transferase and hydrolase activities of the enzyme favored transfructosylation over hydrolysis reaction [44,53].

In a recent study, twenty-five commercial enzyme preparations used in the food industry were screened for transfructosylation activity. Three preparations showed high transfructosylation activity from sucrose, high ratios of transferase over hydrolase activity, selectivity for the synthesis of sc-FOS and did not hydrolyze the produced sc-FOS after a 12 h reaction time [55]. Among these enzymes, a cellulolytic enzyme preparation, Rohapect CM, catalyzed the synthesis of sc-FOS with relatively high production yield (63.8%), under cost-effective conditions of temperature (50°C), sucrose concentration (2.103 M) and enzyme concentration (6.6 TU/mL), which could provide a process with potential application at industrial scale [50].

The synthesis of FOS from sucrose is economically advantageous because sucrose is less expensive than inulin; however, the use of enzymes as catalysts for industrial processes is expensive. Furthermore, the recovery of soluble enzymes for reuse is not economically feasible. In contrast, enzyme immobilization usually confers high storage and long-term operational stability, facilitates the recovery and reuse of the biocatalyst, allowing a cost-efficient use of the enzyme in continuous operation, among other advantages [56,57].

In this context, the commercial enzyme preparation from *Aspergillus aculeatus* (Pectinex Ultra SP-L) has been studied for production of FOS in free and immobilized form. Immobilization of the enzyme onto Eupergit C led to retention of enzyme activity for 20 days of batch

operation, and both free and immobilized enzyme produced FOS from sucrose with a yield around 57% [58]. Similarly, production of FOS using the enzyme preparation immobilized onto epoxy-activated Sepabeads EC (Sepabeads EC-EP5) reached a yield of 61% after 36 h of reaction [59].

Synthesis of FOS by dried alginate entrapped enzymes (DALGEEs) was recently reported [60]. FTase from *Aspergillus aculeatus*, contained in Pectinex Ultra SP-L, was entrapped in alginate gel beads, which were then submitted to dehydration. The dried alginate biocatalysts were evaluated for the synthesis of FOS from sucrose in a continuous fixed-bed reactor. A 40-fold enhancement of the space-time-yield of the fixed-bed bioreactor was observed when using DALGEEs compared with conventional gel beads. The fixed-bed reactor packed with DALGEEs presented excellent operational stability since the composition of the outlet was nearly constant during at least 700 h, with an average FOS concentration of 275 g/L.

A partially purified β-fructofuranosidase from the commercial enzyme preparation Viscozyme L was covalently immobilized on glutaraldehyde-activated chitosan particles [61]. Thermal stability of the immobilized biocatalyst was around 100-fold higher at 60°C when compared to the free enzyme. The biocatalyst also showed a high operational stability, which allowed its reuse for at least 50 cycles without significant loss of activity. The average yield of FOS production from sucrose was 55%.

An alternative to the enzymatic production of FOS is the use of either free or immobilized whole cells in bioreactors [62]. Production of these oligosaccharides via fermentation processes has the advantage of obviating purification of FOS-producing enzymes from the cell extracts [29,63,64].

An integrated one-stage method for production of FOS via sucrose fermentation by *Aureobasidium pullulans* was developed and optimized with experimental design tools. To maximize production of FOS, temperature and agitation speed were optimized. A production yield of FOS from sucrose of 64% was obtained in 48 h of fermentation under the optimum conditions (32°C and 385 rpm) [62].

Two filamentous fungi, *Cladosporium cladosporioides* and *Penicillium sizovae*, with mycelium-bound transfructosylating activity were recently isolated. *C. cladosporioides* and *P. sizovae* provided maximum FOS yields of 56% and 31%, respectively. *C. cladosporioides* synthesized a mixture of FOS (^{1}F-FOS, ^{6}F-FOS and ^{6}G-FOS, including a non-conventional disaccharide (blastose)) with different glycosidic linkages, which could afford certain benefits regarding their bioactivity [41].

Two food companies in Japan and Korea use different commercial processes for the continuous production of FOS with immobilized cells of *Aspergillus niger* and *Aureobasidium pullulans*, respectively, both entrapped in calcium alginate gel [27,63]. Calcium alginate has also been employed to immobilize mycelia of *A. japonicus* aiming to establish FOS-producing processes [65,66].

Immobilization of whole cells of *Aspergillus japonicus* ATCC 20236 onto different lignocellulosic materials was also undertaken to produce fructooligosaccharides. Cells immobilized in the

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different support materials showed FOS production and FFase activity ranging from 128.35 to 138.73 g/L and from 26.83 to 44.81 U/mL, respectively. Corncobs were the best support for immobilization, providing the highest results of microorganism immobilization, FOS and FFase production. In addition, use of immobilized cells led to higher FOS productivity and yield, as well as higher transfructosylation over hydrolysis ratio of FFase than free cells [64].

Several important health benefits are associated with the consumption of FOS as food ingredients. These include modulation of colonic microflora; improvement of the gastrointestinal physiology; activation of the immune system; enhancement of the bioavailability of minerals; reduction of the levels of serum cholesterol, triglycerides and phospholipids; and prevention of colonic carcinogenesis [34,44,67,68].

Among the different FOS, 1-kestose is considered to have better therapeutic properties than those with higher degree of polymerization [69]. The chain length is an important factor influencing the physiological effect of the oligomer in the host [69] and fermentation by bifidobacteria and lactobacilli species [70].

In this context, fermentation of oligosaccharides was evaluated using pure FOS mixtures containing three FOS species (GF_2, GF_3 and GF_4). Only two oligosaccharides (GF_2 and GF_3) were consumed by *Lactobacillus* strains. Moreover, none of the investigated strains metabolized the GF_4 species, suggesting an intracellular metabolism after the FOS transport [70]. This transfer apparently involves an ATP-dependent transport system with specificity for a limited scope of substrates [71].

Moreover, β-fructofuranosidase activity enables bifidobacteria to degrade FOS. Nevertheless, this property is strain-dependent. Some strains consume both fructose and oligofructose, with different preferences and degradation rates [72].

FOS can be used as calorie-free and non-carcinogenic sweeteners. 1-Kestose has enhanced sweetening power compared to other sc-FOS, and 1-kestose-rich sc-FOS syrups can be used as sugar for diabetics [27,73].

Other types of FOS, such as the levan-type and the neo-FOS, have very promising properties; however, they are not yet commercially available [53,74,75].

3. Galactooligosaccharides

Lactose is a disaccharide formed by the condensation of glucose and galactose molecules, and is the most important component of mammalian milk, present in a concentration range from 2.0% to 10%. Lactose can be obtained at industrial scale from whey during cheese production, with dry weight around 80-85%, using crystallization techniques [76-78]. In the past, whey was considered a waste, although, nowadays, it is used to produce whey powders products, improving economic and environmental aspects of the by-products [79].

Lactose presents a great importance for food and pharmaceutical industries, being used in various food products such as chocolate, confectionary and other processed products, as well

as carrier of medicines in dry powder inhalation preparations, excipient of tablets [80]. In humans, lactose can cause abdominal discomfort due to its maldigestion, which reaches approximately 70% of the world's adult population [81]. β-Galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23) plays an important role in human health because it is able to catalyze the hydrolysis of lactose in glucose and galactose, and because of that, it is often referred to as lactase. In addition, the transglycosylation reaction can also occur, in which galactooligosaccharides (GOS) are produced, and their structures can differ in regiochemistry of glycosidic linkage and degree of polymerization (Figures 3-5; Tables 1 and 2) [82,83].

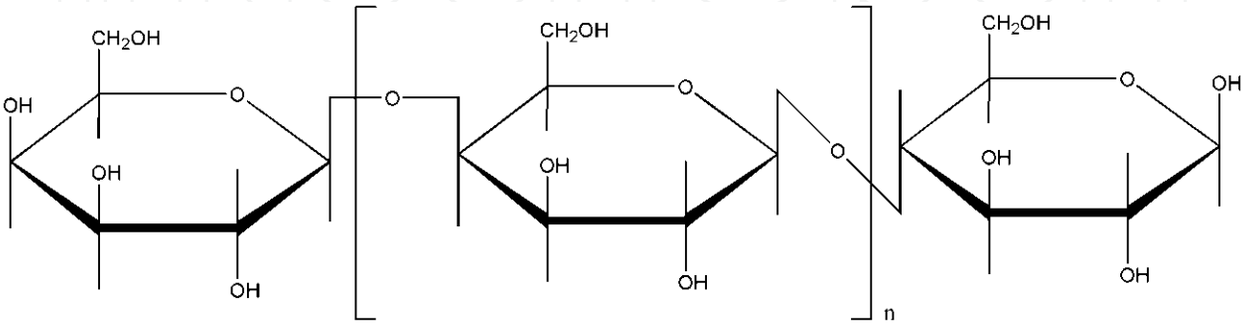

Figure 3. Structure of a galactooligosaccharide (GOS) derived from lactose, a β-(1,4) linked galactosyl oligomer (n=1-4), attached to a terminal glucosyl residue by a β-(1,4) bond. GOS are synthesized by the reverse action of β-galactosidases on lactose in higher concentrations.

Despite the fact that enzymes such as β-glycosidases and β-glucosidases, which also hydrolyze carbohydrates, are able to catalyze transglycosylation reactions, β-galactosidase is the most used enzyme in dairy industry to produce GOS. β-galactosidases from *Kluyveromyces* sp. and *Aspergillus* sp. are the most used in industry because products from those microorganisms are considered as GRAS [84].

Galactooligosaccharides can be defined as a mixture of substances produced from lactose, with two to eight saccharide units, in which one of the units is a terminal glucose and the remaining units are galactose and disaccharides comprising two units of galactose [85]. Several of these GOS are recognized as prebiotics, because they are non-digestible saccharides and can be used selectively by bifidobacteria and lactobacilli in human intestine, and thus improve host health [86, 87].

Conversion of lactose into GOS is catalyzed by β-galactosidases in a kinetically controlled reaction that involves competition between hydrolysis and transgalactosylation. The thermodynamically favored hydrolysis of lactose, which generates D-galactose and D-glucose, competes with the transferase activity that produces a complex mixture of galactose-based di- and oligosaccharides. Transgalactosylation involves direct galactosyl transfer (intramolecular reaction) to D-glucose yielding regio-isomers of lactose, and indirect transgalactosylation (intermolecular) giving rise to disaccharides, trisaccharides, and tetrasaccharides, and eventually longer GOS. The interaction in the active site of the enzyme differs with the acceptor. When the acceptor is water, glucose and galactose are formed; whereas if the acceptor is a sugar, reaction results in GOS [86, 87].

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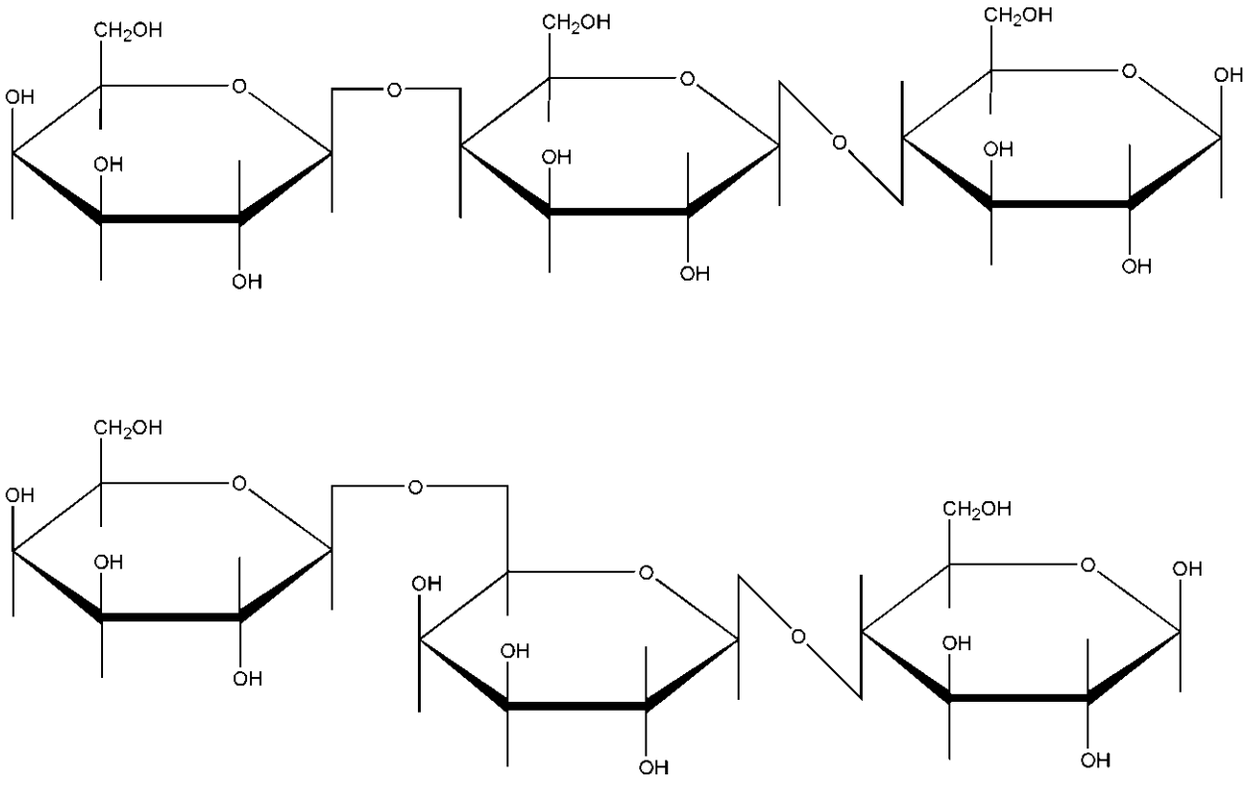
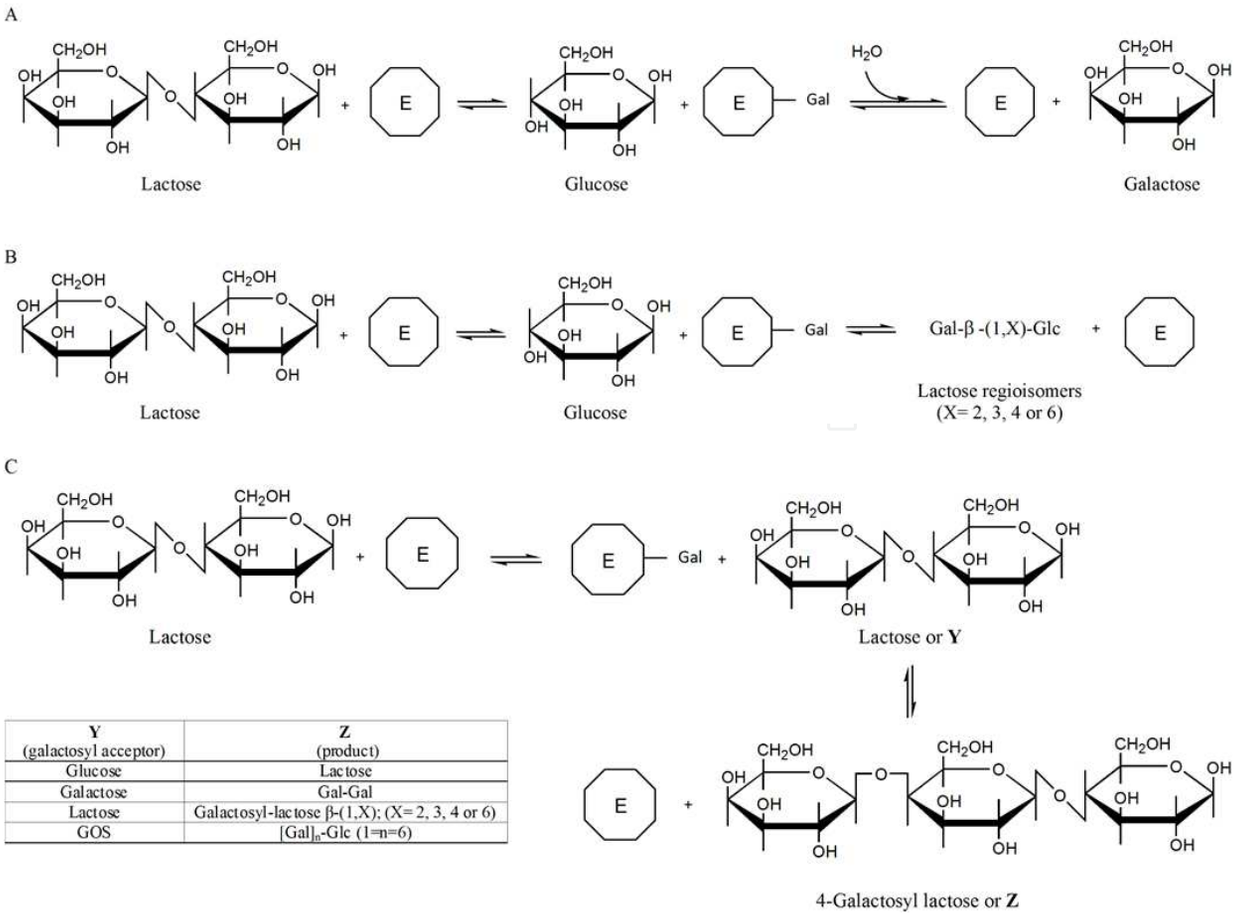


Figure 4. Examples of structures of galactooligosaccharides: 4-galactosyl lactose (top) and 6-galactosyl lactose (bottom) are represented, showing usual regiochemistry differences in galactosyl linkages.

Therefore, high lactose concentrations and low water contents are favorable for GOS synthesis, being the initial lactose concentrations the most important factor, independently of the enzyme source. In general, higher lactose concentrations than 30% are necessary to favor synthesis over hydrolysis [87]. However, at the same lactose concentrations, different yields of GOS can be obtained, because β-galactosidases from different sources, with different structures and/or mechanisms, exhibit different selectivity for water and saccharides. Moreover, GOS yields depend on process conditions, such as temperature, reaction time, pH and enzyme/substrate ratio [88]. However, GOS production can be affected by glucose and/or galactose that are recognized as inhibitors of hydrolysis for many β-galactosidases [89,90].

The reaction time and initial concentration of lactose are considerably important to favor GOS production, since they are simultaneously synthesized and hydrolyzed by β-galactosidase, being regulated by the kinetics of synthesis and hydrolysis. Additionally, lactose concentration can increase formation of GOS due to increased availability of galactosyl and decreased availability of water [82,91]. Additionally, reverse micelle systems, in which the enzyme is entrapped in an aqueous micelle surrounded by organic solvent, provide decrease of the thermodynamic activity of water [92,93]. Chen et al. 2003 [93] reported that the transgalactosylation capability of low concentrations of β-galactosidase and lactose, operating in reverse micelles system, was similar to high concentrations of enzyme and substrate in an aqueous system. Authors also showed that GOS production decreases with the increase in water content.



Y (galactosyl acceptor)	Z (product)
Glucose	Lactose
Galactose	Gal-Gal
Lactose	Galactosyl-lactose β-(1,X); (X= 2, 3, 4 or 6)
GOS	$[Gal]_n$-Glc (1=n=6)

Figure 5. Enzymatic synthesis of GOS by transgalactosylation reactions. Transgalactosylation is the transfer of the galactosyl residue, after the cleavage of lactose, to an acceptor molecule containing a hydroxyl group. When the acceptor is water (A), a galactose is formed by lactose hydrolysis, whereas if the acceptor is a sugar, a disaccharide or a GOS may be formed. In intramolecular transgalactosylation (B), galactosyl donor and acceptor are the same (glucose), only linkage position changes. In intermolecular transgalactosylation (C), there is an enzymatic transfer to another nucleophilic acceptor (Y), which can be all the sugars present in the reaction media, resulting in GOS mixtures.

Production of GOS can be improved increasing the reaction temperature. Lactose has relatively low solubility at room temperature, which increases with increasing temperature. Therefore high temperatures are desirable since they allow the increase of lactose concentration [94,95]. Besides the possibility to increase the solubility of subtracts and products, high temperature is advantageous due to the reduced risk of microbial contamination, lower viscosity and improved transfer rates [96]. However, this is not a general rule, Boon et al. (1998) [97] reported that the increase of initial lactose concentration achieved at high temperature does not influence GOS yield using β-galactosidase from *Pyrococcus furiosus*. Another problem of carrying out GOS synthesis at high temperature is the occurrence of Maillard reaction and enzyme inactivation. Bruins et al. (2003) [95] noted that in addition to enzyme inactivation with the increase of temperature (80°C or above), Maillard reactions almost doubled the rate of enzyme inactivation. Therefore, the development of new thermostable enzymes, through recombinant DNA technology, has been undertaken in order to improve the GOS yield

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[98-102]. Hansson et al (2001) [103] verified an increase of GOS yield due to an increase of transgalactosylation/hydrolysis ratio by changing a phenylalanine residue to tyrosine in β-glucosidase from *Pyrococcus furiosus*, using site directed mutagenesis.

Another strategy to decrease water activity, and carry out catalysis with both high lactose concentration and temperature, demonstrated by Maugard *et al.* (2003) [104], is the use of microwave irradiation. GOS was produced using immobilized β-galactosidase from *Kluyveromyces lactis* along with organic solvents. In these conditions the selectivity for GOS synthesis was increased 217-fold, compared to a reaction carried out under conventional heating.

Similarly to temperature, pH value can affect the GOS yield, possibly through the control of synthesis and degradation [105] According to Huber *et al.* (1976) [106], that studied β-galactosidase from *Escherichia coli* K-12, higher pH values than 7.8 increased transgalactosylation/hydrolysis ratio, which decreased at lower pH values than 6.0. In contrast, Hsu *et al.* (2006) [107] observed that β-galactosidase from *Bifidobacterium longum* CCRC 15708 exhibits its maximum activity at pH 7.0. This enzyme was stable between pH 6.5-7.0, and after three hours in these conditions, 20% of its activity was lost.

In general, oligosaccharides, including galactooligosaccharides, are produced using sucrose or starch, whey, among other substrates with high quality and low cost. The process designed to convert raw material into oligosaccharides must be inexpensive and focused on increasing the productivity and stability of enzymes. In this context, immobilization of biocatalysts can reduce the process costs due to some advantages; such as possibility to reuse the biocatalyst, applying a series of batchwise or continuous reactions; the biocatalyst can exhibit more stability than the native counterpart; besides this, immobilization can reduce costs of downstream, since separation of the biocatalyst from the product can be minimized [108-110]. Recently, several authors have employed immobilized β-galactosidase to produce GOS, applying different strategies with promising results [111-114]. Urrutia et al (2013) [111] immobilized *Bacillus circulans* β-galactosidase in glyoxyl agarose. The enzyme did not lose the synthetic capacity, and retained 92% of its activity along 10 reaction batches, producing 1956 g GOS/g protein at the end of 10 batches. Palai et al (2014) [112] immobilized β-galactosidase in hydrophobic polyvinylidene fluoride and the reaction for GOS production was carried out with partial recirculation loop. Both GOS concentration and selectivity for GOS production increased with increasing initial lactose concentrations, with maximum GOS production of 30% at 50°C, and feed flow rate of 0.5 mL/min. A novel economic and efficient method to produce GOS through cellulose-binding fusion β-galactosidase was developed by Lu et al (2012) [113]. A fusion protein, formed by β-galactosidase from *Lactobacillus bulgaricus* L3 and a cellulose binding domain were employed for immobilization by adsorption onto microcrystalline cellulose. The immobilization was conducted with efficiency of 61% and the maximum GOS yield was 49% (w/w). Moreover, enzymatic activity of 85% and yield over 40% (w/w) were maintained after twenty batches. Warmerdam *et al.* (2014) [114] carried out GOS production in a packed-bed reactor using commercial β-galactosidase (Biolacta N5) immobilized on Eupergit C250L. GOS productivity was six-fold higher in one run in the packed-bed reactor than observed in one run in a batch reactor.

Smart polymers have been studied to develop GOS production processes. Poly-N-isopropyl acrylamide is a thermo-responsive poly-N-isopropyl acrylamide (PNIPAAm), which presents good solubility in water and distinct phase transition at its lower critical solution temperature (LCST). It is applied in different areas, such as medicine, biotechnology, and engineering [115,116]. Based on these advantages, Palai et al (2014) [117] developed a useful bioconjugate between PNIPAAm and β-galactosidase. The constructed PNIPAAm-β-galactosidase (PNbG) can be used in catalysis and, after that; it can be easily separated from the solution by heating at a temperature above its LCST. Further on, Palai et al (2015) [118] continued the GOS production research using this bioconjugate. A maximum GOS yield of 35 % was obtained at pH 6 and 40°C. An increase in GOS yield was observed when the temperature was risen from 30 to 40°C. At 45°C or above, after prolonged time, enzyme deactivation occurred. Moreover, bioconjugates could be reutilized at least ten times; and the separation was done by simple decantation after addition of 0.05 M NaCl and heating at 40°C.

The use of resting or living cells for GOS production appears to be interesting due to its low cost when compared to the use of purified enzyme. Despite the complexity of biocatalysis processes involving whole cells, glucose and galactose can be consumed by them. The consumption of the monosaccharides is interesting because their presence in foods is undesirable, since they do not exhibit prebiotic effect, increase caloric value of food, and can inhibit the activity of certain β-galactosidases [119].

Nevertheless, the use of whole cells can be exploited in order to selectively improve GOS production [120]. Beta-galactosidase form *Aspergillus oryzae* was used to produce GOS from lactose, followed by fermentation with *Kluyveromyces marxianus* cells, that consumed mono and disaccharides. GOS with 95% purity containing mostly tri- and tetrasaccharides were obtained [120]. Association of β-galactosidase and cells can be applied to develop GOS enriched food products. During yogurt manufacturing, GOS was produced by addition of a commercial β-galactosidase, since starter and probiotic culture were not able to provide it. Thus, this yogurt with low lactose content can be useful for lactose intolerant people. Moreover, GOS was stable during storage, probably because it was not metabolized by microbial culture and enzyme was inactivated by yogurt pH [121].

Products containing GOS were launched for the first time in Japan in the 1980s. Due to their various and important health benefits, applications of GOS gradually increased worldwide. These oligosaccharides can be found in diverse products such as yogurt, bakery products, beverages, snack bars among others [122]. GOS are able to stimulate the growth of bifidobacteria and lactobacilli in the lumen despite other members of the microbiota that were considered potentially harmful. These oligosaccharides can prevent bacterial adherence due to their properties of mimicking host cell receptors in which bacterial adhesion occurs [123]. GOS can hinder the development of colon cancer, effect which can be attributed to their capacity of delaying fermentation processes, and reducing the activity of genotoxic bacterial enzymes associated with this disease [124]. Mineral absorption can be stimulated by GOS administration, and their effect on calcium absorption was verified. GOS can be used to alleviate constipation, which is relatively common in elderly people and pregnant women. It occurs due to increased bacterial growth and fecal weight; besides this, short fatty acids stimulate

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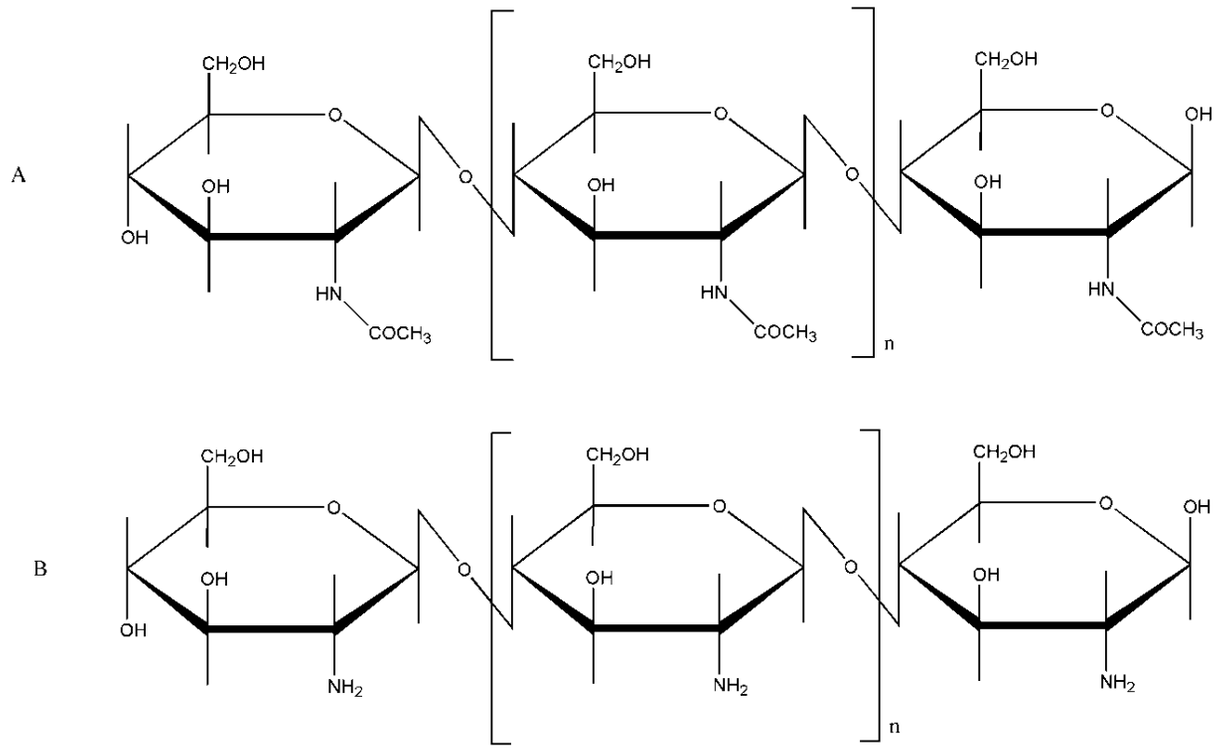

Figure 6. Structures of chitooligosaccharides (n= 3-7), A – Chitin (β-1,4-linked N-acetyl-D-glucosamine residues); B – Chitosan (β-1,4-linked D-glucosamine polymer).

intestinal peristalsis and increase osmotic pressure of fecal weight. Moreover, GOS have been reported as indirectly acting on mucosal and systemic immune activity, and also as having protective effects against allergic manifestations [125].

4. Chitooligosaccharides

In the last years, studies of production and application of chitooligosaccharides (COS) have increased due to their biodegradability, biorenewability, biocompatibility, physiological inertness and hydrophilicity, properties that serve as a basis for the use of COS as functional food or to preserve food from degradation.

Chitin is one of the most abundant natural compounds on earth and its production is mainly based on the extraction from marine species (shrimps, crabs, lobsters, krills, etc.) [126]. Chitin is a copolymer of N-acetyl-D-glucosamine and D-glucosamine units linked by β-(1,4) glycosidic bonds, where N-acetyl-D-glucosamine units are predominant in the polymeric chain as shown in Figure 6A [127]. Chitin obtained from natural sources has a complex composition, containing several minerals, proteins, lipids, pigments and other compounds. Chitosan, an important derivative from chitin, is the deacetylated form of chitin, where N-acetyl groups are removed by chemical methods (Figure 6B).

A considerable amount of residues from processing of fish and crustaceans, rich in chitin and chitosan, are considered hazardous wastes and at the same time have high potential commercial value as raw material [128]. It is possible to obtain chitooligosaccharides from those residues, after prior demineralization and deproteinization by acid and alkali treatments [129].

Chitooligosaccharides are produced by chemical methods or by enzymatic methods from chitosan, produced by alkaline N-deacetylation. At industrial scale, the chemical route is used to produce chitooligosaccharides; however, this methodology presents several disadvantages such as high cost, low yield due to indiscriminate breaks of the polymer chain, production of toxic compounds due to modification on the chitin structure, as well as, corrosion and environmental hazards [130].

The enzymatic process is an attractive solution to overcome the above-mentioned disadvantages, due to their specific action on the substrate, despite the economic costs. Enzymatic hydrolysis of chitin or chitosan involves several enzymes: chitinase, chitosanase, lysozyme and cellulase [131]

According to Mourya *et al.* (2014) [132], various specific enzymes as chitosanases, chitinases and other nonspecific enzymes can hydrolyze chitin and chitosan. Action of chitinases and chitosanases are related to the degree of acetylation of the biopolymers. A novel flow chart for COS production from chitin employing chitinases and chitosanases has been reported (Figure 7) [130].

Chitnases are chitinolytic enzymes hydrolyzing the glycoside bonds between the sugars, which have the unique capacity to hydrolyze the GlcNAc-GlcNAc (2-acetamido-2-deoxy-β-D-glucose) links. Pre-treatment with acid solution is necessary to break down the crystalline structure of chitin and increase the availability of substrate to the action of enzymes. Chitosanases are enzymes that hydrolyze chitosan, classified according to the substrate specificity towards chitosan, which act specifically on the deacetylated (D–D) bonds [133].

In recent years, many scientific papers reported the application of chitinolytic enzymes, from different microorganisms, for the hydrolysis of chitin and chitosan. Enzymes for hydrolysis can be free or immobilized in non-toxic and inert supports.

Fernandes de Assis *et al*. 2010 [134] reported that COS yields of 54% were obtained after 10 minutes of hydrolysis reaction. Initial concentration of chitosan was 1% and the final oligomers concentration was 5.43 mg/mL. Production yields decreased when hydrolysis reaction time exceeded 10 minutes.

Gao *et al.* 2012 [135] determined that the optimal enzyme/chitosan ratio was 7.3 U/mg chitosan at 55°C to produce COS from chitosan employing chitinases from *Bacillus cereus*, achieving a hydrolysis yield of 76%. The yields of COS (GlcN)2, (GlcN)3 and (GlcN)4 were 13.2; 32.6 and 30.2%, respectively.

Ming *et al*. 2006 [136] producing chitooligosaccharides, reported pH range 4.5-6 as the optimal for chitinase activity, reaching 20 g/L of chitooligossacharides from an initial concentration of 50g/L of chitosan, which means a system with 40% of yield in the conversion of chitosan into chitooligosaccharides. Also, employing free and immobilized chitosanase from *Bacillus*

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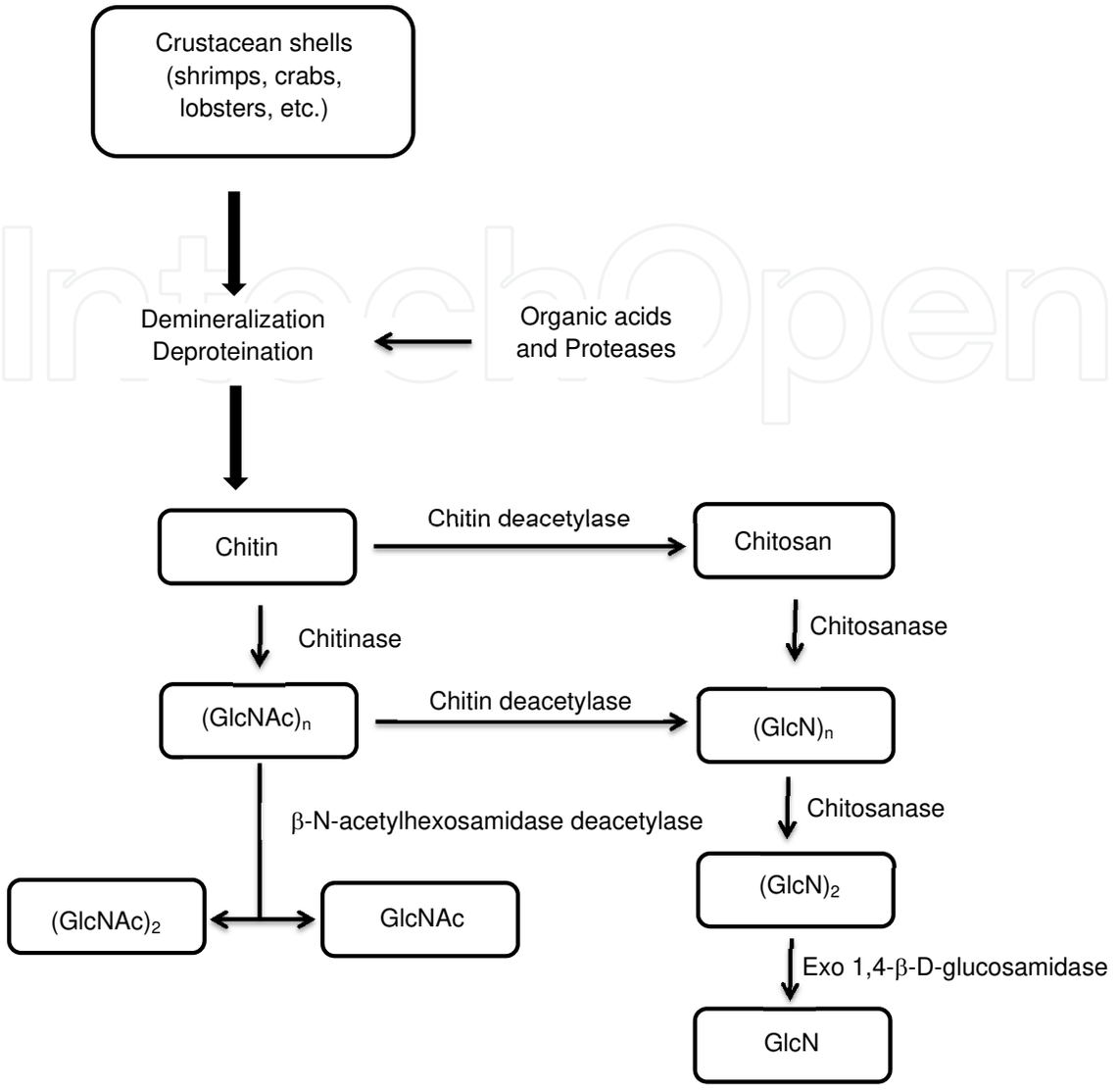

Figure 7. Global flow chart for production of COS by enzymatic hydrolysis of chitin and chitosan (adapted from Jung and Park 2014 [130]).

pumilus, Kuriowa *et al*. 2009 [137], produced chitooligosaccharides in batch and continuous systems. In a system with free enzyme at batch conditions a concentration of 2.8 g/L was achieved, from an initial concentration of 5g/L after 40 minutes of treatment. Another system used was a membrane reactor with cutoff 2000Da. Enzyme concentration of 940 U/L, 40 minutes of residence time and 35°C were reported as the optimal conditions to attain 2.6 g/L of chitooligosaccharides. The membrane bioreactor with the free enzyme was able to maintain a constant rate of chitooligosaccharides production for 96 hours, after that time concentration decreased due to inactivation of enzymes. In order to extend the period of operation, the use of immobilized enzymes was evaluated in the membrane bioreactor. The maximum total concentration of chitooligosaccharides was 2.3 g/L with 620 U/L of immobilized chitinase during 1 month, however it is important to point out that yield was 46%, lower when compared to free enzyme tests.

COS can be applied as food preservatives due to their antimicrobial activity and as functional food, mainly in prebiotics and to help the absorption of important minerals, as calcium. Antimicrobial activity of COS depends on the degree of polymerization (DP) and the degree of deacetylation (DD) as summarized in Table 1.

Inhibitory effects of COS were tested on both Gram (-) and Gram (+) bacteria, including *Escherichia coli, Pseudomonas fluorescens, Salmonella typhimurium, Vibrio parahaemolyticus, Listeria monocytogenes, Bacillus megaterium, Bacillus cereus, Staphylococcus aureus, Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus bulgaricus* [138]. Solutions containing 1% (w/v) COS with different molecular weights inhibited bacterial growth by 1-5 log cycles. For Gram (-) bacteria the antimicrobial activity was inversely proportional to the molecular size of oligomers, which means higher antibacterial activity was found with lower molecular weight of oligomers (1 kDa). This phenomenon was not observed for Gram (+) bacteria.

The proposed mechanism of antibacterial activity for COS with DP>12 was cellular lysis [139]. This would be due to the cationic charges of COS that could link to the negative charges present in the cell walls, leading to the formation of large bacterial clusters, which might block the nutrition transport across the bacterial cell and result in death of the bacteria. Highly deacetylated COS were shown to be more effective at inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus fecalis* and *Samonella typhimurium* than COS with low degree of deacetylation [140].

It has been suggested that COS are able to pass through the bacterial cell wall and be incorporated in the cytoplasm of Gram (+) bacteria [141]. Those low molecular weight compounds can have importance in gene expression related to regulation of stress, autolysis and energy metabolism.

Chitooligosaccharides with DP 4 were demonstrated to have higher antimicrobial effect on four bacteria species (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus lactis*, *Bacillus subtilis*) and six fungi (*Saccharomyces cerevisiae*, *Rhodotorula bacarum*, *Mucor circinelloides*, *Rhizopus apiculatus*, *Penicillium charlesii*, *Aspergillus niger*) [142]. At the same time, degrees of deacetylation over 90% were shown to be more efficient in the inhibition of microbial growth. In addition, chitooligosaccharides with low molecular weight were able to cross the cell wall and interact with DNA in the cytoplasm suppressing the growth of microorganisms. Highly deacetylated COS have many free amines, which can bond to negatively charged residues at the cell wall, leading to formation of aggregates of microorganisms. Those aggregates precipitate, resulting in death of the microorganism.

Chitooligosaccharides can be employed as preservatives due to their antioxidative properties. Antioxidant activity of chitooligosaccharides depends on their degree of deacetylation and molecular weights [143]. It was shown that 90% deacetylated medium molecular weight COS have the highest free radical scavenging activity for DPPH, hydroxyl, superoxide and carbon centered radicals [144]. Antioxidant properties are closely related to the amino and hydroxyl groups, which can react with unstable free radicals to form stable macromolecule radicals [145,146].

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According to Halden *et al*. 2013 [147] COS could be applied as feed additives or hypocholesterolemic agents. Based on their study, hypercolesterol concentration in blood is directly related to the generation of reactive oxygen species. Thus, chitooligosaccharides can be used to scavenge the free radicals on the body, triggering the enhanced synthesis of catalase and superoxide dismutase and decreasing lipid peroxidation.

COS were conjugated with phenolic acid (PAC-COS) to improve the antioxidant properties of the oligosaccharides in the presence of reactive oxygen species (2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (OH) and nitric oxide (NO)) [148]. The increase on the antioxidant activity is associated to the structure of phenolic acids and the substitutions on the aromatic ring of the side chain.

Chitooligosacharides can be considered as prebiotics because they are non-digestible food ingredients with beneficial effects on probiotic bacteria (*Lactobacillus* and *Bifidobacterium*) present in the gastrointestinal tract [5]. In fact, prebiotic activities of COS preparations (0.1 to 0.5%) with varying degree of polymerization (2 to 8) were reported [149]. Assays were conducted with three strains of probiotic bacteria, *Bifidobacterium bifidum KCTC 3440*, *Bifidobacterium infantis KCTC 3249* and *Lactobacillus casei KCTC 3109*.

However, an opposite effect was shown on the population of *Lactobacillus* and *Bifidobacterium* when chitooligosaccharides were tested as prebiotic agents in healthy rats [150]. Chitooligosaccharides have been demonstrated to have a weaker prebiotic effect over *Lactobacillus* and *Bifidobacterium* when compared with other oligosaccharides as fructooligosaccharides, mannanoligosaccharides; and galactooligosaccharides [151].

Chitooligosaccharides from marine species, mainly shrimps and crabs, are produced and commercialized by several companies (Table 2), such as:

- Qingdao BZ-Oligo Co., Ltd: Monomers of chitosan oligosaccharides are obtained by enzymatic hydrolysis, chemical derivatization and column chromatography. The degree of polymerization is from 2 to 10.
- BioCHOS: Preparation of chitooligosaccharides (CHOS) made by controlled enzymatic degradation of chitosan.
- AMSBIO: Preparation of a series of chitosan-oligosaccharides from dimer to hexamer by hydrolysis of chitosan from crab shells. All oligomers are chromatographically pure, not less than 98%, confirmed by high performance liquid chromatography.

5. Novel oligosaccharides

Typical oligosaccharides like FOS and GOS in particular have been widely studied for their prebiotic effects. However, a number of other non-digestible oligosaccharides (NDOs), to which less rigorous study has been so far applied, have at least indications of prebiotic potential. Those with the most accumulated evidence to date are isomalto-oligosaccharides (IMO), soybean oligosaccharides (SOS), xylo-oligosaccharides (XOS) and lactosucrose.

Together with FOS, GOS, and lactulose, all of these oligosaccharides are recognized in the Japanese functional food regulation system as ingredients with beneficial health effects [152].

A great interest resides on the identification, evaluation and commercialization of new products with improved functional properties and benefic health effects such as higher ability to modulate microbiota. Arabinoxylo-oligosaccharides (AXOS), levan-type FOS, gentio-oligosaccharides (GenOS) and pectin-derived oligosaccharides (POS) are examples of these new potential products.

5.1. Isomalto-oligosaccharides

Isomalto-oligosaccharides are usually found as a mixture of oligosaccharides with predominantly α-(1,6)-linked glucose residues with a degree of polymerization (DP) ranging from 2–6, and oligosaccharides with a mixture of α-(1,6) and occasionally α-(1,4) glycosidic bonds such as panose (Figure 8; Table 1) [152].

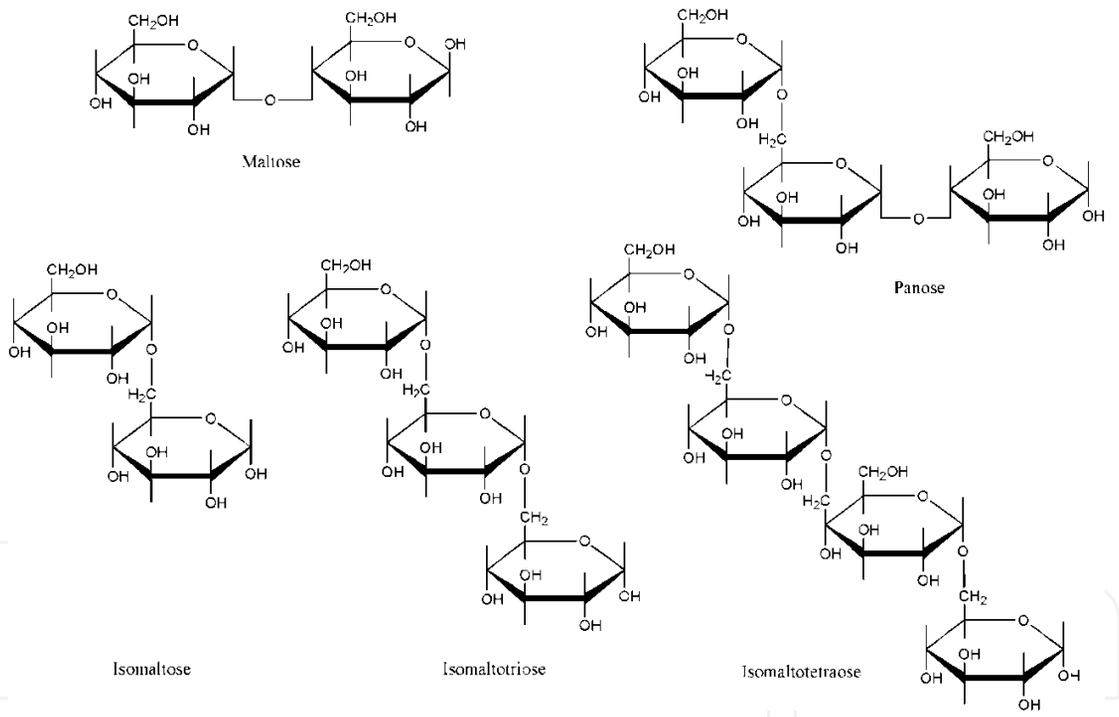

Figure 8. Examples of structures of isomalto-oligosaccharides. Glucosyl residues are linked to maltose or isomaltose by α-(1,6) glycosidic bonds.

Isomalto-oligosaccharides, like malto-oligosaccharides, are produced using starch as the raw material. Isomalto-900, a commercial product, is produced from cornstarch and consists of isomaltose, isomaltotriose and panose. Starch dextrans are easily converted to IMO, which are the market leaders in the dietary carbohydrate sector of functional foods in Japan. However, unlike malto-oligosaccharides, there is evidence to suggest that isomalto-oligosaccharides induce a bifidogenic response [11].

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IMO occur naturally in various fermented foods and sugars such as sake, soybean sauce and honey. They are a product of an enzymatic transfer reaction, using a combination of immobilized enzymes. Initially, starch is liquefied using α-amylase (EC 3.2.1.1) and pullulanase (EC 3.2.1.41), and, in a second stage, the intermediary product is processed by both β-amylase (EC 3.2.1.2) and α-glucosidase (EC 3.2.1.20). Beta-amylase first hydrolyzes the liquefied starch to maltose. The transglucosidase activity of α-glucosidase then produces isomalto-oligosaccharides mixtures which contain oligosaccharides with both α-(1,6)- and α-(1,4)-linked glucose residues (Table 2) [153].

In recent years, much research has been focused on improvement of the efficiency of IMO production by screening for new and better enzymes for high yield IMO synthesis. Efforts also have been made to develop novel processes such as synthesis of IMO from sucrose using free or immobilized dextransucrase and dextranase, and efficient conversion of maltose into IMO using immobilized transglucosidase, or using an enzyme membrane reactor [153,154].

IMO are mild in taste and relatively inexpensive to produce. These oligosaccharides have desirable physicochemical characteristics such as relatively low sweetness, low viscosity and bulking properties. IMOs have been developed to prevent dental caries, as substitute sugars for diabetics [155], or to improve the intestinal flora [152].

Several companies currently manufacture isomaltooligosaccharides, of which Showa Sangyo (Japan) is the major producer. Of the emerging prebiotic oligosaccharides, IMO are used in the largest quantities for food applications. In Japan, the volume of IMOs manufactured is estimated to be three times greater than for either FOS or GOS [152]. Among other oligosaccharides, which are widely used as food ingredients or additives [156] based on their nutritional and health benefits [157], IMO are interesting due to availability, high stability and low cost [154].

Unlike other prebiotic oligosaccharides, considerable digestion of IMO occurs during intestinal transit. A large portion of this ingredient reaches the colon and intestinal enzymes degrade the remainder, leading to a rise in blood glucose levels [154]. Thus, a part of the IMO survives gastric transit to be fermented by the intestinal microbiota [152]. *In vitro* fermentation studies have shown that IMO promote the selective proliferation of bifidobacteria in the fecal microbiota [158]. However, further controlled human feeding studies employing culture and molecular techniques are required to determine the impact of IMO on the intestinal microbiota.

Beneficial effects of IMO consumption have been reported in a few human feeding studies investigating health parameters in specific populations. IMOs stimulate bowel movement and help to decrease total cholesterol levels with an intake of 10 g/d in elderly people [158].The limited data for physiological effects showed only improved defecation pattern (frequency and stool bulk via increases in microbial biomass) and lowering of total cholesterol levels [158,159]. In conclusion, the data for the bifidogenic effects of isomalto-oligosaccharides are less consistent than for other typical oligosaccharides like inulin or oligofructose [155].

5.2. Soybean oligosaccharides

Unlike other oligosaccharides, soybean oligosaccharides are extracted directly from the raw material and do not require enzymatic manufacturing processes. These α-galactooligosaccharides include and consist of galactosyl residues linked to the glucose moiety of sucrose by α-(1,6) bonds (Figure 9,Table 1) [2].

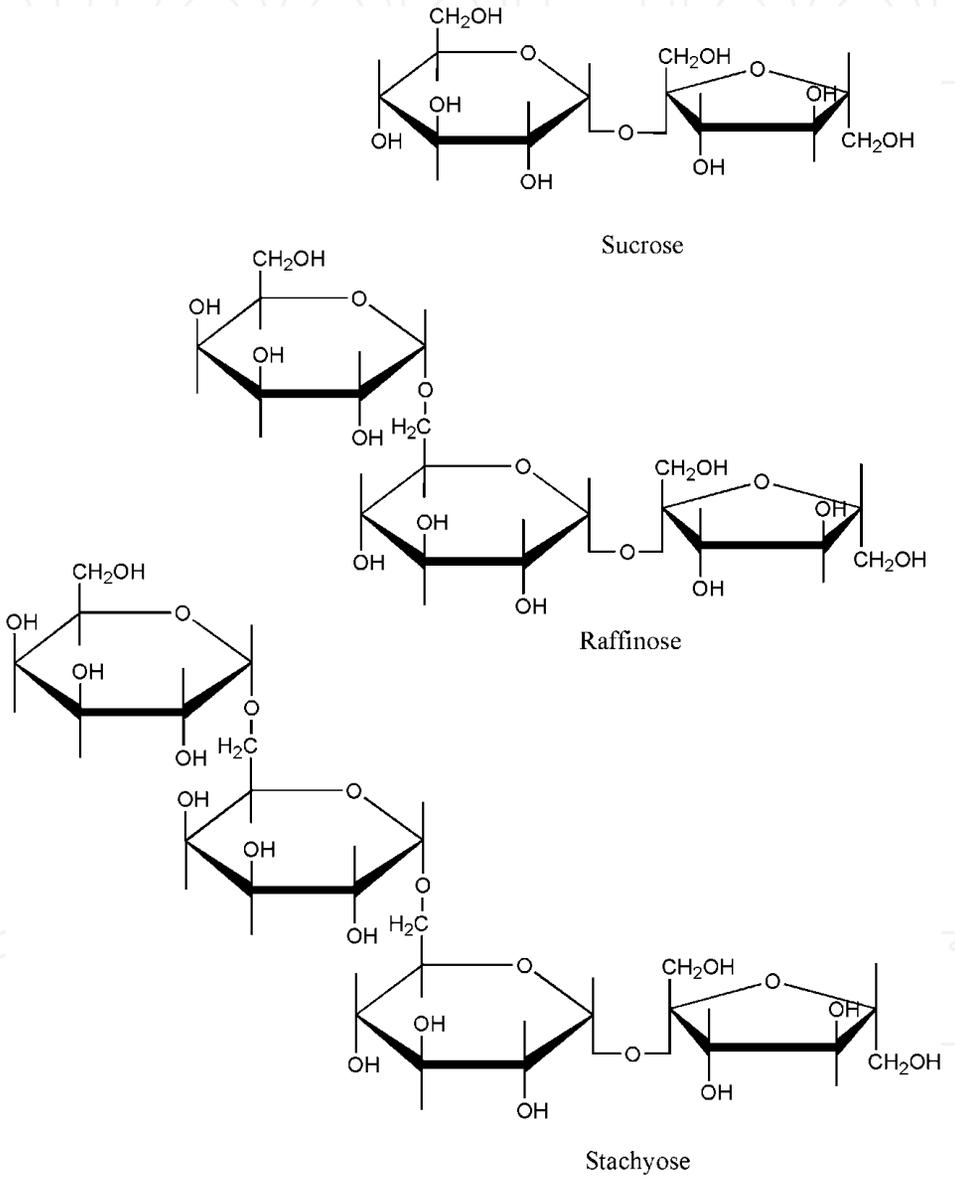

Figure 9. Examples of the main soybean oligosaccharides, raffinose and stachyose, derived from sucrose, showing galactosyl residues linked to sucrose by α-(1,6) bonds.

Soybean whey, a by-product from the production of soy protein isolates and concentrates, is composed mainly of raffinose (DP 3), stachyose (DP 4) and verbascose (DP 5), as well as sucrose, glucose and fructose. The most abundant sugars are extracted from the soybean whey

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and concentrated to produce soybean oligosaccharide syrup (Table 2), rather than being commercially synthesized using enzymatic processes [158].

Raffinose and stachyose are resistant to digestion, since α-galactosidase activity (required to hydrolyze these carbohydrates) is not present among human digestive enzymes and, therefore, reach the colon intact, where they act as prebiotics, stimulating the growth of bifidobacteria. Apart from being acknowledged as non-digestible, human studies on the effects of these oligosaccharides are scarce. Their physiological actions appear to be similar to the other galactooligosaccharides; they are bifidogenic and promote other effects expected from this change in colon microbiota. Calpis Co. (formerly known as Calpis Food Industry Co.) produces soybean oligosaccharides in Japan [11].

5.3. Xylo-oligosaccharides

Xylo-oligosaccharides (XOS) are sugar oligomers of xylose units linked by β-(1,4) linkages (Figure 10,Table 1). The number of xylose residues can vary from 2 to 10, but mainly consist of xylobiose, xylotriose and xylo-tetraose [152], which are found naturally in bamboo shoots, fruits, vegetables, milk and honey [160]. In addition to xylose residues, xylans are usually found in combination with arabinofuranosyl, glucopyranosyl uronic acid or its 4-O-methyl derivative (2- or 3-acetyl or phenolic substituents), resulting in branched XOS with diverse biological properties [153].

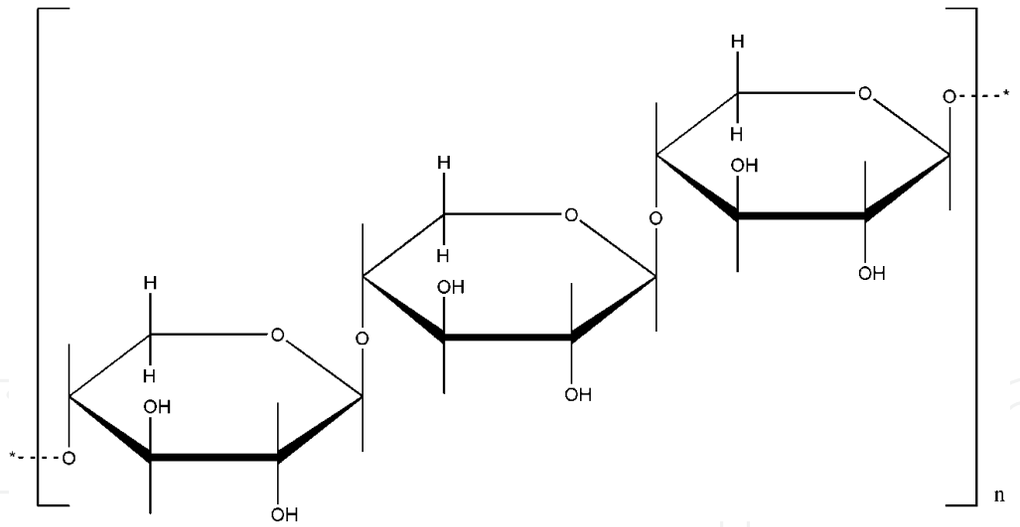

Figure 10. Partial structure of xylo-oligosaccharides (n=3-6) produced by enzymatic hydrolysis of xylan hemicelluloses, catalyzed by β-xylanases.

Their production at an industrial scale is carried out from lignocellulosic materials (LCMs). XOS can be used for several purposes, among them, food-related applications. The LCM for XOS production comes from a variety of feedstocks (from forestry, agriculture, industry or urban solid wastes) that show similarities in composition. The raw material for xylo-oligosaccharide synthesis is the polysaccharide xylan, which is extracted mainly from corncobs besides hardwoods, straws, bagasses, hulls, malt cakes and bran [160].

XOS production from LCM is not simple or economical because it depends on two treatment steps. The first step is the xylan extraction from LCM, which includes a chemical pretreatment. Although there are multiple treatments for xylan extraction (alkaline hydrolysis using NaOH, KOH, $Ca(OH)_2$, ammonia or a mixture of bases, oxidizing agents, salts or alcohols to remove lignin or pectic substances), there is no favorite consensus among them. Once the extracted xylan is in a soluble form, the second step includes the xylanase enzymatic reaction or the hydrolytic degradation of xylan by steam, water or dilute solutions of mineral acids [160]. For the enzymatic production of XOS, xylan is enzymatically hydrolysed to xylo-oligosaccharides by endo-β-1,4-xylanases (EC 3.2.1.8) (Table 2). Enzyme complexes with controlled exo-xylanase and/or β-xylosidase activity are required, to avoid the production of xylose, which may cause inhibition effects in XOS production. For food related applications, a DP of 2–4 is the most desirable [160]. Therefore, development of efficient and economical xylanase based bioprocesses for use in XOS production is necessary. Many microorganisms well known as producers of xylanolytic enzymes may be promising for novel production processes [161].

The process yields predominantly linear β-(1,4)-linked XOS (mainly xylobiose, xylotriose and xylotetraose) as well as some oligosaccharides with branched arabinose residues. For the production of food-grade XOS, a refining step is necessary. Vacuum evaporation increases the XOS concentration and removes volatile compounds such as acetic acid and the flavours of their precursors. In order to obtain higher-purity oligosaccharide products, the monosaccharide xylose and high molecular mass carbohydrates, as well as non-saccharide components can be removed from the oligosaccharides using membrane filtration techniques, organic solvent extraction, adsorption in different materials and chromatographic separation techniques used for XOS purification. Chromatographic methods, however, are not suitable for economic reasons for large-scale production of XOS intended to be used in the food industry [153].

XOS can be metabolized by bifidobacteria and lactobacilli in pure culture. In relation to human health, XOS selectively enhanced the growth of bifidobacteria thus promoting a favorable intestinal environment [152]. XOS is a promising oligosaccharide class that stimulates increased levels of bifidobacteria to a greater extent than do FOS or other oligosaccharides [161]. However, to date well-controlled animal and human feeding studies to confirm the prebiotic activity of XOS are still scarce. While they show promise, more research is required before XOS can conclusively be claimed as prebiotics. Besides the potential prebiotic effect, immunostimulating effects, antioxidant activity, anti-allergy, anti-infection and anti-inflammatory properties were reported for XOS [162-164].

In addition to the beneficial health effects, XOS have interesting physicochemical properties; they are only moderately sweet, have an acceptable odor, are noncariogenic and low caloric, stable over a wide range of pH values (2.5–8.0), even the relatively low pH value of gastric juice, and temperatures up to 100°C. Most oligosaccharides can be hydrolyzed, resulting in the loss of nutritional and physicochemical properties at acidic pH values, when treated at high temperatures for short periods, or when submitted to prolonged storage under room conditions. These properties make them suitable for incorporation into many food products such as in combination with soymilk, soft drinks, dairy products, sweets and confectionaries [158].

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XOS show a remarkable potential for practical utilization in many fields, including pharmaceuticals, feed formulations and agricultural applications. Nevertheless, their most important market developments correspond to food-related applications, however, their comparatively high production costs impair market development of these oligosaccharides, and further improvements in process technology are necessary [11].

5.4. Arabinoxylo-oligosaccharides

Arabinoxylo-oligosaccharides (AXOS) are an example of a novel prebiotic dietary fiber. They can be isolated from wheat bran and consist of xylan chains with a variable substitution of arabinose side chains (Table 3) [158]. On an industrial scale, AXOS are generated through the enzymatic cleavage of AX with endoxylanases, resulting in various molecules differing in DP (between 3 and 67) and degree of substitution of arabinosyl residues [165].

Novel oligosaccharides	Chemical structure	References
Lactosucrose	4-galactosyl sucrose	[2, 156]
Arabinogalactooligosaccharides	Galactan oligomers β-(1,3) or (1,6) attached to arabinofuranose residues.	[19]
Arabinoxylooligosaccharides	Xylan randomly attached to arabinofuranose residues by α-(1,3) or α-(1,2) linkages.	[165, 237]
Arabinooligosaccharides	Arabinosyl units linked by α-(1,5) bonds.	[2]
Pectic oligosaccharides	Linear backbone of α-(1,4) linked D-galacturonic acid units randomly acetylated and/or methylated.	[171, 172, 238, 239]
Galacturonan	Linear chain of α-(1,4) linked D-galacturonic acids	
Rhamnogalacturonan	α-(1,4) linked galacturonic acid and α-(1,2) linked rhamnose units	
Mannan oligosaccharides	Mannose α-(1,6) linked backbone and α-(1,2) and α-(1,3) linked branches.	[176]
Oligodextrans	Glucosyl units linked by α-(1,4) bonds.	[176, 240]
Gentiooligosaccharides	Glucosyl units linked by β-(1,6) bonds.	
Beta-glucan oligosaccharides	Glucosyl units linked by β-(1,3/1,4) or β-(1,3/1,6) bonds.	
Cyclodextrins	α-(1,4) linked cyclic – glucosyl units.	

Table 3. Novel oligosaccharides with prebiotic activities.

The fiber properties include an improvement of bowel habit and positive change of the fermentation in the colon, whereas they were also shown to possess bifidogenic properties [158]. There are indications that AXOS have an effect against type II diabetes. AXOS decrease postprandial glucose levels and insulin response, and increase postprandial ghrelin in healthy humans [156,166].

This bifidogenic effect is strongly influenced by the complexity of the AXOS molecules and decreases with increasing average DP and degree of substitution [72,166]. Genome sequence

analysis reveals that several bifidobacterial strains contain genes possibly coding for enzymes involved in the debranching of side groups and in the cleavage of the xylose backbones of AXOS [72]. This kind of specialization together with the potential to degrade xylose backbones intracellularly could explain the selective growth stimulation of bifidobacteria by AXOS.

5.5. Novel fructooligosaccharides

There is an increasing interest in novel molecules with prebiotic and physiological effects. Some fungi are able to synthesize levan-type FOS containing fructosyl units linked by β-(2,6) linkages (6-kestose being first in the series) (Table 1), or neolevan type FOS containing a fructosyl unit also linked by this type of linkage to a glucose (neokestose, neonystose, or neofructofuranosylnystose). Such FOS have been metabolized by different bifidobacteria strains when supplied as the sole carbon source [167].

Levan-type FOS were synthesized by acid hydrolysis of β-(2,6)-linked polymers containing a glucose at one terminus (levans), these have been produced by several microorganisms growing in sucrose-based medium [168]. The discovery of novel enzymes that synthesize β-(2,6)-linked FOS from sucrose may, however, provide a non-pollutant alternative to acid hydrolysis of levans. Because there is an existing process to produce inulin-type FOS, an enzymatic method involving the hydrolysis of levan to produce levan-FOS may be possible. However, with the lack of an available plant source of levan, as there is for inulin, it is possible to derive an enzymatic process to produce levan-type FOS from microbial levan, using levansucrase (Table 2) and endolevanases [169].

Marx *et al.* 2000 [170] observed that levan-type FOS obtained via the acid hydrolysis of levans were metabolized by different bifidobacteria strains, thus further demonstrating their prebiotic potential. Nevertheless, the levan-type FOS prebiotic properties have not been fully characterized, possibly due to their limited availability.

The production of levan-type FOS has not reached industrial levels [171], despite several reports demonstrating their potential applications as food and feed additives in agriculture as well as their pharmaceutical applications.

5.6. Pectic oligosaccharides

Pectic oligosaccharides (POS) (Table 3) are obtained by pectin depolymerization. Pectins are ramified heteropolymers made up of a linear backbone of α-(1,4)-linked D-galacturonic acid units (which can be randomly acetylated and/or methylated).

POS have been proposed as a new class of prebiotics capable of exerting a number of health-promoting effects. Among these are protection of colonic cells against pathogenic microorganisms [172], stimulation of apoptosis of human colonic adenocarcinoma cells [173] and *in vivo* synergistic empowerment of immunomodulation caused by galactooligosaccharides (GalOS) and fructooligosaccharides (FOS). Other benefits include potential for cardiovascular protection *in vivo*, reduction of damage by heavy metals, antiobesity effects, dermatological applications and antitoxic, antiinfection, antibacterial and antioxidant properties. Additional-

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ly, *in vivo* and *in vitro* studies have confirmed that acidic POS are not cytotoxic or mutagenic, being suitable for use in foods for children and babies [173].

5.7. Gentio-oligosaccharides

Gentio-oligosaccharides (GenOS) consist of 2–5 glucose residues linked by β-(1,6) glycosidic linkages (Table 3). These oligosaccharides are not hydrolysed in the stomach or small intestine and therefore reach the colon intact, thus fulfilling a criterion of a prebiotic [11]. GenOS were further reported to possess bifidogenic activity [153]. GenOS are usually produced from glucose syrup by enzymatic transglucosylation or by biocatalytic glycosylation with cultured cells. Despite the prebiotic potential of GenOS, research on the novel production of GenOS is sparse. Gentio-oligosaccharides are produced in Japan by Nihon Shokuhin Kako [11].

6. Perspectives

Function and application of chitooligosacharides frequently depend on their size, and, therefore, the degrees of polymerization and acetylation. Substrate-enzyme synergisms determine the molecular weight of the generated COS. Gutierrez-Román *et al.* 2014 [174] tested three chitolytic enzymes ChiA, ChiB and ChiC, alone and in combination. In addition, three chitanases were tested in synergism with a chitobiase and a non-catalytic binding protein. When evaluated individually, ChiA was unable to hydrolyze chitin while ChiB and ChiC were able to degrade chitin and generate chitin monomers and dimers. When enzymes were tested pairwise (ChiA-ChiB, ChiA-ChiC, and ChiB-ChiC) the production of dimers and trimers was much higher, and monomers significantly lower than those seen with ChiB or ChiC individually. However, higher concentrations of COS were obtained when the authors tested the four enzymes in combination with non-catalytic binding protein acting on chitin.

Further studies must be focused on the action of the enzymes on substrates with different degrees of polymerization and acetylation and N-acetylation pattern to improve the comprehension of that synergism. In addition, researches involving synergism of non-catalytic binding proteins and hydrolytic enzymes should be developed in order to increase the understanding of oligomers syntheses [127]. Consequently, to produce size-specific chitooligosaccharides by enzymatic hydrolysis, further studies on genetic modification are necessary to overproduce enzymes and non-catalytic binding proteins, which will have a great impact on the quality of oligomers obtained and on the productivity of industrial processes.

Another important challenge in the development of biotechnological processes that employ agro-food industry residues as raw material is the direct fermentation of those raw materials. Obviously, direct fermentation of raw materials is closely related with the aforementioned aspects, since fermentative processes involve microbial growth and enzymatic hydrolysis, and process conditions that in many cases are different from physiological conditions. Moreover, it is important to give attention to screening of new enzymes from extremophile microorganisms, which usually catalyze reactions under non-physiological conditions such as high salinity, high temperature and low water activity [175].

As important part of the biotechnological process, bioreactors and enzyme (free or immobilized) are essential and need special attention to improve yields and productivities. Free enzymes in batch systems are the most conventional technology employed in the production of oligosaccharides by enzymatic hydrolysis. However, it has several important drawbacks, because enzymes are unstable, can be employed once and accumulation of products usually reduces their activity. These drawbacks are related directly to the quality of the product and the yield of the process. Development of novel technologies in order to solve those snags employing immobilized enzymes in column reactor and membrane systems have been studied. Column reactor packing with immobilized enzymes allows continuous production of oligosaccharides and has important advantages, such as increased operational stability of the enzyme and reduced accumulation which otherwise could lead to enzyme inhibition. The poorer affinity of immobilized enzymes is the main disadvantage of the application of column reactors at industrial scale. Studies should be directed towards the improvement of enzyme-support affinity. Membrane reactors are considered a new and attractive technology to produce oligosaccharides, in which enzymes are confined in the reaction side and continuously reused, with obvious implications for the efficiency and economy of the process. Low-cost and low-energy consumption are other important advantages to increase its utilization. The main limitation for industrial application of membrane reactors are fouling and polarization phenomena, which decrease considerably permeate flux, containing the produced oligosaccharides [176]. The main challenge to be studied in order to implement this technology advantageously in the industry is how to reduce the effect of these problems without affecting the stability of enzymes.

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