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Microbial Glycosidases for Nondigestible Oligosaccharides Production

Thais Bezerra, Rubens Monti, Egon B. Hansen and Jonas Contiero

Abstract

There is much interest in the study and production of nondigestible oligosaccharides (NDOs), due to their bioactivities and beneficial effects to the human health. The main approach in the production of NDOs relies on the action of glycosidases performing hydrolysis or transglycosylation of polysaccharides and sugars. In this chapter, a description of the main microbial glycosidases used for NDOs production, their sources, their principal properties, and a description of the production processes with the better results obtained are discussed.

Keywords: glycosidases, transglycosylation, enzymatic hydrolysis, oligosaccharides

1. Introduction

The concept of nondigestible oligosaccharides (NDOs) came from the observation that the human body does not have the necessary enzymes to hydrolyze β-glycosidic linkages present in some sugars of the human diet. Thus, these carbohydrates can arrive intact in the intestine where they are fermented selectively stimulating the growth and/or activity of bacteria in the colon acting as prebiotics [1]. In this context, nondigestible oligosaccharides have received much attention since they have important biological properties promoting health beneficial effects. Stimulation of the intestinal microbiota growth associated with low cariogenic and caloric value are some of these properties. Also noteworthy is a stimulation of the immune system leading to a reduced risk of diarrhea and other infections. The benefits are obtained by a decrease in intestinal pH due to the fermentation of NDOs, decreasing the proliferation of...
pathogenic microorganisms, and an increase of the bifidobacteria population [2]. The bioactive properties of NDOs can be influenced by monosaccharide composition, type of glycosidic linkage, and degree of polymerization [2].

Nondigestible oligosaccharides can be produced using chemical or enzymatic processes. The synthesis using chemical methods are complicated, with numerous protection and deprotection steps required in order to achieve regioselectivity [3]. Other challenges of chemical synthesis are the low yields, expensive chemicals, and impossibility for scale-up. For those reasons with few exceptions, most of the NDOs are produced by enzymatic processes.

The enzymatic production of NDOs can be achieved by two different approaches, the use of glycosyltransferases or glycosidases. Glycosyltransferases catalyze the stereospecific and regiospecific transfer of a monosaccharide from a donor substrate (glycosyl nucleotide) to an acceptor substrate. Some of the difficulties associated with the application of glycosyltransferases are availability of enzymes and sugar nucleotide donors, product inhibition, and reagent costs. These factors decrease the applications of these enzymes in the production of NDOs [4]. The glycosidases offer a good alternative for enzymatic production of NDOs, where they can be synthetized from monosaccharides using transglycosylation reactions, or formed by controlled enzymatic hydrolysis of polysaccharides. Some advantages of the glycosidases in relation to glycosyltransferases are availability, good stability, and the fact that they act on easily found substrates and do not need cofactors [3].

The transglycosylation route can be performed by the use of a good glycosyl donor that can be a disaccharide, in high concentrations. This donor will form an intermediate glycosyl-enzyme that can be intercepted by an acceptor to give a new glycoside or oligosaccharide [3]. When the substrate is a monosaccharide, it will be acting as a donor and acceptor. Some glycosidases used to produce NDOs using this approach are α-galactosidases, β-fructofuranosidase, cyclodextrin glucanotransferase, and α-glucosidase [4].

The production of NDOs by controlled hydrolysis of polysaccharides involves the break of glycosidic bonds, the reaction is acid base catalyzed by an oxocarbenium ion-like transition state and involves two carboxylic groups at the active site [5]. The glycosidases can be divided into inverting or retaining depending on the configuration of the glycosidic linkage after the hydrolysis. Inverting glycosidases operate through direct displacement of the leaving group by water. The two carboxylic groups are responsible for the reaction, one provides base catalytic assistance to the attack of water and the other provides acid catalytic assistance to cleavage of the glycosidic bond. Retaining glycosidases use a double displacement mechanism involving the formation of a covalent glycosyl enzyme intermediate, where one carboxylic group acts as acid catalyst for the glycosylation step and base catalyst for the deglycosylation step [3]. The second carboxylic group acts as a nucleophile and a leaving group. The enzymes inulinase, pullulanase, amylase, xylanase, endogalactanase, rhamnogalacturonase, endogalacturonase, and chitosanase are used for NDOs production using the controlled hydrolysis approach [4].
2. Production of NDOs through glycosyl transfer reaction

2.1. Galactosidases

β-Galactosidases (EC 3.2.1.23) hydrolyze the nonreducing terminal of β-D-galactose residues in β-D-galactosides. The enzyme can be used in the production of galacto-oligosaccharides (GOs) by transgalactosylation reaction in which a galactosyl is transferred into the hydroxyl group of the galactose residue of lactose [6]. Due to the strong prebiotic factor, GOs can modulate the growth of microorganisms of the gut flora, increasing the population of bifidobacteria, this enhancement is associated with beneficial effects, inhibition the growth of potentially pathogens, improvement, elimination, prevention, stimulation mineral adsorption, and decrement cholesterol and lipids [7].

When using concentrated solutions of lactose (40%), high yields of GOs can be achieved. The β-galactosidase of *Pseudozyma tsukubaensis* showed high transgalactosylation capability, yielding of 18.28% of GOs with concentration of 73.12 g/L from a 40% lactose solution [8]. The immobilization of chemically aminated β-galactosidase from *Aspergillus oryzae* onto Purolite® A-109 leads to an increase in the operational stability and transgalactosylation capacity of the enzyme, producing in the optimum conditions (400 g/L lactose, pH 4.5, 50°C) 100 g/L of GOs in a fluidized bed reactor [9]. The utilization of an ultrafiltration membrane bioreactor, allows the synthesis and separation in one system. Using high lactose concentrations (470 g/L) and β-galactosidase from *A. oryzae*, the system yielded 1.88 gGOS/mgE that is 2.44-fold higher than the conventional batch (0.77 gGOS/mgE) [10].

The milk whey, a by-product from the dairy industry, is a valuable substrate for GOs productions due to its lactose content (45–60%). The whey is produced by the processing and manufacturing of raw milk into products such as yogurt, ice cream, butter, and cheese through processes such as pasteurization, coagulation, filtration, centrifugation, chilling, etc. [11]. Depending on the procedure used to precipitate the casein, two types of whey are formed, the acid whey (pH < 5) is obtained after fermentation or addition of organic or mineral acids, whereas the sweet whey (pH 6–7) is obtained by addition of proteolytic enzymes like chymosin [12]. The production of GOs from milk whey using a two-dimensional packed bed bioreactor yielded 97% [13], while a yield of 29.9% of GOs with a concentration of (119.8 mg/mL) was achieved using whey whey as substrate in a 4 h process [14]. When whey permeate was used as substrate in a membrane reactor system, a mixture of GOs with 77–78% of purity was produced [15]. A high lactose conversion was achieved (70–80%), when using whey as a substrate in the production of GOs, yielding 10–20% of total sugars and producing oligomers with DP3, DP4, and DP5 [16]. The GOs production from whey permeate yielded 50% corresponding to 322 g prebiotics/kg whey permeate, presenting tagatose and lactulose in the oligosaccharides mixture [17]. Galacto-oligosaccharides were synthesized by enzymatic transgalactosylation in UF-skimmed milk permeate fortified with lactose (40% w/w). The GOs yields, expressed as a percentage of the initial lactose content, were 41, 21, 13, and 11% with β-galactosidase from *Bacillus circulans*, *A. oryzae*, *Aspergillus aculeatus*, and *Kluyveromyces lactis*, respectively, under optimal conditions [18].
2.2. β-fructofuranosidases

The β-D-fructofuranosidases catalyze the hydrolysis of β-D-fructofuranoside residues at the nonreducing end of β-D-fructofuranosides [19]. Fructooligosaccharides (FOs) can be produced by transfructosylation of sucrose by β-fructofuranosidases, which is carried out through the breaking of the β(2-1) glycosidic bond and the transfer of the fructosyl moiety onto any acceptor other than water, such as sucrose or a FO. The sucrose is used as substrate acting as the glycosyl donor and as the glycosyl acceptor in competition with water (hydrolysis) in a glycosyl transfer reaction [20]. Besides the strong prebiotic factor, many bioactivities have been associated with FOs as anti-inflammatory effect on Crohn’s disease and ulcerative colitis, antimicrobial activity against gut flora pathogens, and prevention of colon cancer [21]. A β-fructofuranosidase from *Penicillium oxalicum* was able to produce neokestose from a 500 g/L sucrose solution, giving 94.2 and 224.7 g/L of neokestose and total FOs, respectively [22]. An invertase produced by *Aspergillus niger* using salt-deoiled cake as substrate was able to form kestose during enzymatic hydrolysis using glucose (50%) [23]. *Penicillium sizowae* and *Cladosporium cladosporioides* were used to produce FOs from a 600 g/L sucrose solution with maximum yield of 184 and 339 g/L, respectively [24]. The filamentous fungus *Gliocladium virens* was able to produce 6-kestose with a yield of 3 in media containing 150 g/L sucrose after 4–5 days of culture [25]. An extracellular β-fructofuranosidase from *Rhodotorula dairenensis* produced a varied type of FOs containing β(2→1)- and β(2→6)-linked fructose oligomers with a maximum concentration of 87.9 g/L (75% sucrose conversion) [26]. A fructosyltransferase from *Aureobasidium pullulans* presented maximum transfructosylation rate at 600 g/L [27].

2.3. Cyclomaltodextrin glucanotransferase

Cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) catalyze the cyclization of oligosaccharides composed of D-glucose monomers joined by α(1-4) glycosidic linkages. This enzyme catalyzes mainly transglycosylation reactions leading to the formation of nonreducing cyclic oligosaccharides, named cyclodextrins. The main types are α-, β-, and γ-cyclodextrins consisting of six, seven, and eight glucose monomers in cycles, respectively. The majority of the CGTases usually produce a mixture of α-, β-, and γ-cyclodextrins, and the product ratio can vary depending on condition and reaction time [28].

The CGTase can produce cyclodextrins from starch, amylase, and other polysaccharides by catalyzing different transglycosylation steps: intermolecular coupling and disproportionation and modification of the length of noncyclic dextrins [29]. Between main microbial sources of CGTases, the *Bacillus, Geobacillus, and Paenibacillus* species are highlighted. The optimum temperature and pH for this enzyme range from 4 to 10.3°C and 10 to 85°C, respectively, whereas the molecular weight ranges from 33 to 200 kDa.

The products of the CGTases α, β, and γ-cyclodextrins are not completely digested in the gastrointestinal tract, rising to the colon where they are fermented by the intestinal microflora and for this reason are considered prebiotics. The microbial degradation results in linear malto-oligosaccharides, which are further hydrolyzed and fermented to absorbable and metabolize short-chain fatty acids. Several studies showed that CDs reduce the digestion of
starch and the glycemic index of food. Other bioactivities include hypocholesterolemic and antithrombotic activity [30].

The most frequently used raw material for CDs production is starch. The product inhibition effect of cyclodextrins on CGTases, make the complete conversion of starch a challenge. Strategies to decrease this effect involve the continual removal of CDs by filtration or the precipitation using agents that forms a specific insoluble complex with CDs. Filtration devices can be coupled to the production systems, hollow fiber and [31]. Table 1 shows the yields or concentration of CDs obtained through the action of microbial CGTase on different substrates.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Substrate</th>
<th>Conditions</th>
<th>Yield (%)</th>
<th>Concentration (g/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-cyclodextrin</td>
<td>B. circulans STB01</td>
<td>5% maltodextrin; 9 h; 50°C</td>
<td>25</td>
<td>4.3</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>B. lehensis</td>
<td>Cassava starch; 55°C; 35 h</td>
<td>–</td>
<td>0.32</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>P. macerans</td>
<td>5% soluble starch; 10 h; 45°C; pH 5.5</td>
<td>–</td>
<td>10.3</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>T. thermosulfurigenes</td>
<td>10% paselli SA2; 0.1 U/mL; pH 5.9; 60°C; 8 h</td>
<td>33</td>
<td>13.0*</td>
<td>[35]</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>B. lehensis</td>
<td>Cassava starch; 55°C; 35 h</td>
<td>–</td>
<td>6.33</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp. C26</td>
<td>Starch</td>
<td>26.5</td>
<td>10.6</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>B. firmus strain 37</td>
<td>5% starch; 24 h</td>
<td>–</td>
<td>15.3</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td>B. firmus strain 37</td>
<td>10% maltodextrin; 24 h</td>
<td>–</td>
<td>21.6</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp. C26</td>
<td>4% starch; 72 h; 50°C</td>
<td>–</td>
<td>8.2</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>B. circulans STB01</td>
<td>5% maltodextrin; 9 h; 50°C</td>
<td>58</td>
<td>10.1</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>B. firmus strain 37</td>
<td>5% corn starch; 3 days; 60°C</td>
<td>–</td>
<td>15.0</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>B. firmus strain 37</td>
<td>5% maltodextrin; 3 days; 60°C</td>
<td>–</td>
<td>10.1</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>10% dextrin; 90 min; 50°C; pH 8</td>
<td>–</td>
<td>6.0</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Thermoanaerobacter sp.</td>
<td>4% soluble starch; 30 s; 60°C; pH 6</td>
<td>7.9</td>
<td>1.3</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>A. gottschalkii</td>
<td>10% starch; 24 h; 40°C; pH 8</td>
<td>45</td>
<td>–</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>B. macerans</td>
<td>Soluble starch</td>
<td>24</td>
<td>4.7</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>P. macerans</td>
<td>5% soluble starch; 10 h; 45°C; pH 5.5</td>
<td>–</td>
<td>4.1</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>T. thermosulfurigenes</td>
<td>10% paselli SA2; 0.1 U/mL; pH 5.9; 60°C; 8 h</td>
<td>54</td>
<td>20.0*</td>
<td>[35]</td>
</tr>
<tr>
<td>γ-cyclodextrin</td>
<td>B. lehensis</td>
<td>Cassava starch; 55°C; 35 h</td>
<td>–</td>
<td>1.02</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>B. cereus</td>
<td>5% starch; 1 h; 20% CGTase</td>
<td>81.9</td>
<td>1.6</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>B. circulans STB01</td>
<td>5% maltodextrin; 9 h; 50°C</td>
<td>17</td>
<td>3.0</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>10% dextrin; 90 min; 50°C; pH 8</td>
<td>–</td>
<td>1.5</td>
<td>[40]</td>
</tr>
</tbody>
</table>
Bacillus sp. species are the main microbial source of CGTase, in some cases thermophiles are used to obtain enzymes with unusual characteristics. Most of studies are focused on the β-cyclodextrin or mixture production and higher concentrations are usually obtained for β- and γ-cyclodextrins. The substrate is usually corn starch, although tapioca, cassava, and potato starches are also commonly used. Table 1 presents some examples of cyclodextrins production by microbial CGTases.

**Table 1. Production of cyclodextrins by microbial CGTases.**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Substrate</th>
<th>Conditions</th>
<th>Yield (%)</th>
<th>Concentration (g/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. clarkii 7364</td>
<td>Potato starch</td>
<td>10 h; 50°C; pH 7</td>
<td>72.5</td>
<td></td>
<td>[45]</td>
</tr>
<tr>
<td>P. macerans</td>
<td>5% soluble starch</td>
<td>10 h; 45°C; pH 5.5</td>
<td>–</td>
<td>1.8</td>
<td>[34]</td>
</tr>
<tr>
<td>B. clarkii 7364</td>
<td>15% soluble starch</td>
<td>55°C; pH 12</td>
<td>47</td>
<td></td>
<td>[46]</td>
</tr>
<tr>
<td>T. thermosulfurigenes</td>
<td>10% paselli SA2</td>
<td>0.1 U/mL; pH 5.9; 60°C; 8 h</td>
<td>13</td>
<td>5.0*</td>
<td>[35]</td>
</tr>
<tr>
<td>B. circulans DF 9R</td>
<td>Glucans</td>
<td>24 h; 40°C</td>
<td>21.1</td>
<td>15.1</td>
<td>[47]</td>
</tr>
<tr>
<td>B. macerans</td>
<td>5% soluble starch</td>
<td>22 h</td>
<td>36.9</td>
<td></td>
<td>[34]</td>
</tr>
<tr>
<td>B. macerans</td>
<td>15% potato starch</td>
<td>pH 7; 45°C; 12 h; 2 U/g CGTase</td>
<td>30</td>
<td>–</td>
<td>[49]</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>50 g/L corn starch</td>
<td>pH 7; 45°C; 12 h; 2 U/g CGTase</td>
<td>30–35</td>
<td>–</td>
<td>[53]</td>
</tr>
<tr>
<td>B. macerans</td>
<td>30% potato starch</td>
<td>pH 5.5–8.5; 40–55°C; 120 h; 1000 U/g CGTase</td>
<td>25</td>
<td>40.0</td>
<td>[49]</td>
</tr>
<tr>
<td>B. macerans</td>
<td>7.5% corn starch</td>
<td>48 U/g CGTase; pH 6; 60°C; 24 h</td>
<td>25</td>
<td>–</td>
<td>[54]</td>
</tr>
<tr>
<td>B. circulans 251</td>
<td>10% potato starch</td>
<td>pH 7; 50°C; 45–50 h</td>
<td>40</td>
<td>–</td>
<td>[55]</td>
</tr>
<tr>
<td>Bacillus sp. 277</td>
<td>10% potato starch</td>
<td>400 U/g CGTase; pH 8; 60°C; 12 h</td>
<td>34</td>
<td>–</td>
<td>[56]</td>
</tr>
<tr>
<td>B. clausii E16</td>
<td>1% soluble starch</td>
<td>pH 7.5; 55°C; 24 h</td>
<td>80</td>
<td>–</td>
<td>[28]</td>
</tr>
<tr>
<td>B. macerans</td>
<td>10% tapioca starch</td>
<td>0.4 mmol cyclodecanone; pH 7; 25°C; 5–10 days</td>
<td>91–93</td>
<td>–</td>
<td>[57]</td>
</tr>
<tr>
<td>Mutant CGTase H43T</td>
<td>1% tapioca starch</td>
<td>1% toluene; pH 6; 60°C; 18 h</td>
<td>15.2</td>
<td>–</td>
<td>[58]</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>12.5% wheat starch</td>
<td>20 U/g CGTase; 2% butanol; pH 7.5; 40°C; 6 h</td>
<td>42.5</td>
<td>–</td>
<td>[59]</td>
</tr>
<tr>
<td>Thermoanaerobacter sp.</td>
<td>5% soluble starch</td>
<td>60°C; pH 6</td>
<td>29</td>
<td>74.0</td>
<td>[60]</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>5% soluble starch</td>
<td>500 U/g; 65°C; pH 6; 24 h</td>
<td>22</td>
<td>–</td>
<td>[61]</td>
</tr>
<tr>
<td>E. coli NV601</td>
<td>5% soluble starch</td>
<td>60°C; pH 6</td>
<td>30</td>
<td>75.0</td>
<td>[60]</td>
</tr>
</tbody>
</table>

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### 2.4. Alpha-glucan acting enzymes

Alpha-glucans are polysaccharides consisting of glucose units connected by α(1-4) or α(1-6) glycosidic linkages. Pullulan, a glucan produced by the fungus *A. pullulans* of α(1-4) linked maltotriose repeats connected by α(1-6) linkages, amylopectin, formed by shorter α(1-4) glucan chains connected by α(1-6) branch points, and dextran are some examples of alpha-glucans [62].

Enzymes that act as hydrolyzing or debranching alpha-glucans are suitable for nondigestible oligosaccharides production. Pullulanase, dextransucrase, and starch acting enzymes can be used in the preparation of maltooligosaccharides and isomalto-oligosaccharides. Maltooligosaccharides contain α-D-glucose residues linked by α(1-4) glycosidic linkages, while isomaltooligosaccharides (IMOs) contain two to five glucose units with one or more α(1-6) linkages. While MO may exhibit immunoregulatory activity [63], the intake of IMO decreases serum cholesterol concentrations and improve bowel movement, stool output, and microbial fermentation in the colon [64]. IMOs also upregulate the Th1 response that play a triggering role in allergic diseases, such as rhinitis, asthma, and eczema [65].

Dextran and wheat starches are also observed. The conditions for cyclodextrin production are usually 40–60°C, pH 6–7, and aqueous media, however, depending on the microbial source of the CGTase some unusual condition may be observed, as 25°C or pH 12. In some cases, the organic media is used to decrease the inhibition of the CD. The highest productivity is reported to the production of a mixture by a recombinant CGTase of *Thermoanaerobacter* using soluble starch that yielded 75 g/L.

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Enzymes that act as hydrolyzing or debranching alpha-glucans are suitable for nondigestible oligosaccharides production. Pullulanase, dextransucrase, and starch acting enzymes can be used in the preparation of maltooligosaccharides and isomalto-oligosaccharides. Maltooligosaccharides contain α-D-glucose residues linked by α(1-4) glycosidic linkages, while isomaltooligosaccharides (IMOs) contain two to five glucose units with one or more α(1-6) linkages. While MO may exhibit immunoregulatory activity [63], the intake of IMO decreases serum cholesterol concentrations and improve bowel movement, stool output, and microbial fermentation in the colon [64]. IMOs also upregulate the Th1 response that play a triggering role in allergic diseases, such as rhinitis, asthma, and eczema [65].

Dextran and wheat starches are also observed. The conditions for cyclodextrin production are usually 40–60°C, pH 6–7, and aqueous media, however, depending on the microbial source of the CGTase some unusual condition may be observed, as 25°C or pH 12. In some cases, the organic media is used to decrease the inhibition of the CD. The highest productivity is reported to the production of a mixture by a recombinant CGTase of *Thermoanaerobacter* using soluble starch that yielded 75 g/L.

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associated with dextranase in the production of IMOs lead to oligosaccharide mixtures containing mainly sugars (up to 36%) with DP varying between 10 and 60 together lower and higher molecular weight sugars [75].

Alpha-amylase (EC 3.2.1.1) also can be used to obtain maltooligosaccharides. This enzyme hydrolyses the internal α(1,4) linkages in starch in a random fashion, leading to the formation of soluble maltooligosaccharides, maltose, and glucose. A protein engineering approach of the amylase from Bacillus lehensis G1-produced mutated proteins with an increase in the transglycosylation to hydrolysis ratio of up to 4.0-fold and reduction in the concentration of maltotriose required for use as a donor/acceptor for transglycosylation. A reduction of steric interference and hydrolysis suppression introduced a synergistic effect to produce MOs with a higher degree of polymerization [76]. Amylases from Streptomyces sp. were able to produce mainly maltotriose (55–75%) from soluble starch at 20–30°C pH 6.5 [77]. The Bacillus subtilis strain SDP1 amylase hydrolyses starch to produce maltotriose and maltotetraose along with maltose after prolonged reactions of 5 h [78]. A recombinant alpha-amylase (145 mg/mL) from Streptomyces avermitilis was able to yield maltose (4.49) and maltotriose (1.77 g/L) from 10.0 g/L of soluble starch [79]. An amylase from Bacillus megaterium produced a maltoligomer mixture with high proportion of maltopentaose (G5) and maltotriose (G3) during hydrolysis of starch, amylopectin, and amylose [80]. Malto-oligosaccharide production by commercial α-amylase (liquefying amylase 6 T) using freeze-thaw infusion resulted in a maximum production of 6.5 g/L after 60 min at 1.0% (w/v) enzyme [81]. A productivity of 8.9 g/L of maltopentaose was achieved using a Bacillus sp. AIR-5 amylase and a 40 g/L solution of soluble starch [82]. A S. solfataricus KM1 amylase was able to give an 80% yield of trealose from a 10% amylose solution [83].

Pullulanase (EC 3.2.1.41), a debranching enzyme, hydrolyses the α(1-6) linkage in pullulan and branched polysaccharides, producing maltotriose. An amylopullulanase from the hyperthermophilic archaean Caldivirga maquilingensis was able to act on a wide range of substrates. Assays with the enzyme produced linear MOs (≤G8–G1) from cyclodextrins, amylopectins (DP6-96) from amylose, and amylopectins (DP1-76) from amylopectin and potato starch [84]. A one-step method using neopullulanase and α-amylase for the bioconversion of purified rice starch slurry (30% w/w) resulted in a syrup containing 59.2% of IMO (dry basis) after 72 h of bioconversion (Lin et al. 2011).

Alpha-glucosidase (EC 3.2.1.20), an exo-acting hydrolase, attacks the substrates from the non-reducing end producing α-D-glucose and presents some transglycosylation activity that can be used in the production of oligosaccharides [85]. Liquefied banana slurries were used for IMO synthesis by Transglucosidase L, producing after 12 h of transglucosylation, a yield of 76.6% with a concentration of 70.74 g/L. The IMOs mixture was composed of 53 isomaltotriose, 21 isomaltotetraose, and 26% maltooligoheptaose and larger oligomers [86]. A yield of 58.1% with a concentration of 93 g/L was obtained for IMOs production from an immobilized glucosidase using as substrate a maltose solution (160 mg/mL) in a membrane reactor system [87]. Partially purified a-glucosidase from Aspergillus carbonarius, immobilized on glutaraldehyde-activated chitosan beads in a packed bed reactor, produced isomaltooligosaccharides at a yield of 60% (w/w) using 30% (w/v) maltose solution. Using intact mycelia
attached with polyethyleneimine-glutaraldehyde, a yield of 46% (w/w) was obtained using 30% (w/v) maltose solution [88]. A high yield of IMOs (67%) with concentration of 2 g/L was obtained when 30% (w/v) of soluble tapioca starch was incubated with amylomaltase (120 U) for 0.5 h (pH 7.0; 40°C). While a yield of 53% and concentration of 1.63 g/L was obtained using transglucosidase (6 U) in the same condition for 1 h [89]. When amylomaltase (1.5 U) and transglucosidase (8 U) of were incubated with 20% (w/v) maltotriose for 30 min at 40°C, 9.9 mg/mL of IMOs were produced to with DP 2–7 [90].

3. Productions of NDOs through polysaccharide hydrolysis

3.1. Inulinase

Fructooligosaccharides can be produced by the controlled hydrolysis of fructans. Fructans are fructose-based polysaccharides, representing the major reserve carbohydrates in about 15% of flowering plant species [91]. According to differences in glycosidic linkages they can be classified in many types, being linear inulin the most studied and best-characterized fructan. Inulin consists of β(2-1)-linked fructose units terminating at the reducing end with a glucose residue attached through a sucrose-type linkage [92]. Inulinases can hydrolyze the β(2-1) linkages in inulin and can present endo- or exo-activity. Exo-acting inulinases (EC 3.2.1.80) produce fructose as the main end product, whereas endoinulinases (EC 3.2.1.7) act randomly and hydrolyze internal linkages of inulin to yield FOs and minor amounts of monosaccharides [93].

The highest yield (92%) for the conversion of chicory inulin (50 g/L) in to FOs was reported by the application of a dual system of Xanthomonas sp. and Pseudomonas sp. endoinulinases [94]. On another approach, an endoinulinase from Xanthomonas sp. yielded 86% of FOs from dahlia tubers inulin (10 g/L) after 10 h [95]. A production of 78% and 79% of FOs was achieved from a solution (100 g/L) of chicory inulin and chicory juice, respectively [96]. An endoinulinase produced by Streptomyces rochei E87 yielded 70% of FOs after 3 days of incubation with inulin producing mainly inulotriose [97]. A maximum yield of 75.6% in total of FOs was obtained by hydrolysis of a solution containing 50 g/L of inulin by Pseudomonas sp. inulinase, producing a mixture of oligosaccharides with DP2-7 [98]. A commercial inulinase preparation yielded 96% of FOs from dahlia tubers inulin (pH 6.0; 100 g/L). The product presented FOs with DP ranging from 1 to 6 but the major products were DP3 (23%) and DP4 (24%) [99].

The production of FOs by a inulinase from A. niger immobilized in montmorillonite lead to a yield of 18.32% on aqueous media and 16.03% in organic media [100], while high yields of DP3 (70.3 mM), DP4 (38.8 mM), and DP5 (3.5 mM) FOs were obtained through the enzymatic hydrolysis of inulin (150 mg/ml; 60°C; pH 6.0; 48 h) by other A. niger inulinase (60 U/mL) [101]. When a commercial endoinulinase preparation (Novozym®960) from A. niger was used in the production of FOs from inulin (60°C; pH 6.0), a productivity of F3 (70.3mM), F4 (38.8mM), and F5 (12.43 mM) was achieved [102]. Inulinases from K. marxianus NRRL Y 7571 produced DP2 (11.89%) and DP3 (20.83%) oligomers using inulin (20%) as substrate at 24 h at 50°C [103]. A maximum FOs production of 11.9 g/L.h and specific productivity of 72 g/g.h
were observed when a mutant X. campestris pv. phaseoli grown in a 5 L fermenter containing 3% inulin and 2.5% tryptone [104]. A continuous production of FOs from inulin was carried in a bioreactor packed with immobilized cells of Escherichia. coli expressing a Pseudomonas sp. endoinulinase. Under the optimal operation conditions, continuous production of FOs was achieved by 150 g/L.h (17 days; 50°C) [105]. Continuous production of FOs from chicory juice (100 g/L) was carried out using the polystyrene-bound endoinulinase in an enzymatic reactor achieving an oligosaccharide yield of 82% [106]. Aspergillus ficuum endoinulinase (10 U/g) yielded 50% of FOs from Jerusalem artichoke inulin (50 g/L; 45°C; pH 6.0) after 72 h. With Jerusalem artichoke the yield reached 89% and the maximum IOS production was up to 80% after 72 h [107].

3.2. Xylanases

Xylan is also a heteropolysaccharide with a backbone formed by xylose homopolymer subunits linked through β(1-4) linkages. This polymer can be found in the hemicellulose fraction of lignocellulosic materials associated with lignin and cellulose. Through the hydrolysis of xylan with xylanases, xylooligosaccharides (XOs) can be produced. The intake of XOs is associated with many health benefits as improvement of bowel function, immunomodulatory, and anti-inflammatory activities, preventive effects on cancer and inhibitory effects on carcinogenesis, antimicrobial, antiallergic, and antioxidant activities [108].

The xylanase (β-1,4-d xylan xylanohydrolase, EC 3.2.1.8) is the main enzyme applied for xylan hydrolysis and XOs production, due its action on the main chain of xylan and release of oligosaccharides. Before the enzymatic hydrolysis of xylan, the hemicellulosic materials can be submitted to a pretreatment to enhance the xylan availability. Many types of pretreatments that can be performed, one approach uses NaOH or H₂SO₄ solutions associate with high temperatures to disrupt the hemicellulose structure. Between the substrates used for XOs production agroresidues and food by-products are highlighted due to their high contents of hemicellulose [109].

Hydrolysis of alkali pretreated corncob powder using a commercial endoxylanase produced 81 ± 1.5% of XOs in the hydrolysate equivalent to 5.8 ± 0.14 mg/mL of XOs. Reaction parameters for the production of XOs from corncob using endoxylanase from A. oryzae MTCC 5154 were optimized and an XOs yield of 10.2 ± 0.14 mg/mL corresponding to 81 ± 3.9% with 73.5% xylobiose [110]. The optimization of the XOs production from corncob using the thermostable endoxylanase from Streptomyces thermovulgaris TISTR1948, showed that for an enzyme concentration of 129.43 U/g of substrate, 53.80°C, and pH 6.17, the yield of XOs reached 162.97 mg/g of substrate or 752.15 mg/g of hemicellulose in KOH-pretreated corn cob [111]. When corncob was hydrolyzed with a xylanase from Aspergillus foetidus MTCC 4898 a yield of 67.7% was obtained after 8 h of reaction time using 20 U of xylanase at 45°C [112]. A commercial xylanase produced 1.208 mg/mL of xylobiose and 0.715 mg/mL of xylotriose, using 5.83 U for 16.59 h of incubation (pH 5.91; 40.8°C) [113]. Steam-exploded liquor of corncobs was treated using a thermostable xylanase from Paecilomyces thermophila J18 resulting in a XOs yield of 28.6 g/100 g xylan [114]. After a pretreatment with H₂SO₄ (60°C; 12 h), the corncob was hydrolyzed by xylanase, yielding 67.7% of XOs with
Three commercial xylanase preparations (Rapidase Pomaliq, Clarex ML, and Validase) were evaluated for the enzymatic production of pentoses from the hemicellulose fraction of corn husks and corn cobs. Rapidase Pomaliq produced 104.1 g of XOs for each kg of corn husks or 133 g of XOs for each kg of corn cobs (480 min of reaction at pH 5.0 and 50°C) [116]. Tobacco stalks were hydrolyzed by xylanase producing a XOs yield of 8.2% after 8 h and 11.4% after 24 h reaction period [118]. Another process yielded 7.28 and 4.52 g/L of XOs from wheat straw and rice straw xylan, respectively, after hydrolysis with a from A. foetidus MTCC 4898 [119]. Using xylanases from two glycoside hydrolase families, yields of 60% and 40% were obtained for rye bran arabinoxylan hydrolysis by GH10 and GH11, respectively [120]. Wheat straw xylan was hydrolyzed using a variant of the alkali-tolerant Bacillus halodurans S7 endoxylanase A, resulting in 36% conversion of the xylan to predominantly xylobiose [121]. The XOs produced from garlic straw hemicelluloses hydrolyzed with xylanase secreted by B. mojavensis were composed of xylobiose, xylotriose, and xylotetraose, together with a small amount of xylolentaose and xylohexose yielding 29 ± 1.74% after 8 h [122]. Xylan extracted of Mikania micrantha was hydrolyzed by a recombinant Paenibacillus xylanase, yielding 68% of XOs [123]. Oil palm empty fruit bunch fiber was hydrolyzed by Aspergillus terreus xylanase with a maximum 262 mg of xylobiose was produced from 1.0 g of pretreated fiber [124]. Several crop by-products were subjected to an enzymatic treatment to obtain a XOs through the action of a Buzyme 2511 (R). The hydrolysis lead to a concentration of 5.3 (apple pomace), 1.3 (white poplar), 2.9 (giant cane), and 6.5 g/L (grape stalk) [125]. The enzymatic hydrolysis of hard shell almond yielded 34.0% of XOs with 70% of purity [126]. A process for producing XOs from Sehima nervosum grass through enzymatic hydrolysis yielded 11 g/100 g xylan of xylobiose [127]. The treatment of wheat bran with the commercial xylanase preparation enzymes, produced a yield of approximately 31.2% of XOs, with a purity of 95% (w/w) and degree of polymerization of 2–7 [128]. Viscose fiber mills were used as substrate in the production of XOs yielding 68.9% after enzymatic hydrolysis [129]. When sugarcane bagasse was hydrolyzed with a crude xylanase secreted by Pichia stipites, XOS accumulated with a maximum yield of 31.8% of the total xylan was achieved at 12 h, which contained 29.8% xylobiose, 47.1% xylotriose, and 18.4% xylotetraose [130]. The hydrolysis of sugarcane bagasse with a B. subtilis xylanase produced xylotriose (X3), xylotetraose (X4), and xylolentaose (X5) and also is less amounts xyooligomers (X11). The process yielded was 113 and 119 mg/g sugarcane bagasse for 7 and 8 h, respectively [131]. In another approach using sugarcane bagasse treated with hydrogen peroxide, the enzymatic hydrolysis by crude extracts from Thermoascus aurantiacus produced a maximum yield of 37.1 with 2.6% of substrate and xylanase load of 60 U/g [132]. A productivity of 2.36, 2.76, 2.03, and 2.17 mg/mL of X2, X3, X4, and X5, respectively, was obtained after hydrolysis of sugarcane bagasse by Streptomyces rameus L2001 xylanase [133]. A maximum yield of 5.96% was obtained for the
conversion of sugarcane bagasse being xylobiose and xylotriose the main products [134]).

The enzymatic hydrolysis of *Camellia oleifera* shell pretreated with NaOH produced 1.76 g/L of xyooligosaccharides (DP 2–6) [135].

### 3.3. Pectinases

Pectins are components of the cell walls of most higher plants, this heteropolysaccharide is characterized by a high content of galacturonic acid (GalA) monomers bonded together by α(1-4) linkages, showing acetylation or esterification with methyl groups. They are composed of homogalacturonans, xylogalacturonanes, rhamnogalacturonans, arabinans galactans, and arabinogalactans. Depending on how these polysaccharides are associated, pectin can be classified as homogalacturonan and rhamnogalacturonans I and II [136].

Studies using piglets showed that POs can modulate the grown of microbial communities in the ileum increasing, for example, the *Lactobacillus* counts [137, 138]. POs were also able to interfere with the toxicity of Shiga-like toxins from *E. coli* O157:H7, which play a key hole in diarrhea and hemorrhagic colitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura [139].

Enzymes that act on pectins with a hydrolyzing or debranching activity have the potential to produce nondigestible oligosaccharides. The pectinolytic enzymes can be divided into: pectinesterases, pectin-methylesterases, and depolymerases being this last one more suitable for POs production. Endopolygalacturonases are depolymerases produced by various microorganisms such as bacteria, yeasts, and molds. They are also found in some plants and especially in fruits. In general, they release mono-, di-, and tri-galacturonic acid by a multiple attack mechanism single chain. Rhamnogalacturonases produce linear oligomeric compounds of alternating rhamnose and galacturonic acid (4–6 residues) with galactose residues connected to some or all the rhamnose residues. Galactanases can be divided in to endo-β-1,4-galactanases and exo-β-1,3-galactanases. The difference between these enzymes lies in their ability to hydrolyze the β(1-3), β(1-4), or β(1-6) linkages between the galactose residues [136].

Because of its high pectin content, potato, sugar beet, and apple by-products are often used as substrate for POs production. The hydrolysis of sugar beet pectin by combining endopolygalacturonase and pectinmethylesterase produced POs with a DP 1–9, with a maximum yield of trigalacturonic acid of 3.7% [140]. POs were obtained by the action of commercial enzymes on the potato rhamnogalacturan, with a yield of 93.9 and 66.2% using Depol 670L and endo-β-1,4-galactanase, respectively. The hydrolysates yielded up to 50.6% of oligomers with DP of 13–70. Major oligosaccharides obtained with Depol 670L were DP 5 (26.3%) and DP6 (24.9%), whereas the endo-β-1,4-galactanase were DP3 (19.0%), DP5 (10.6%), and DP8 (12.6%) [141]. A high yield (93.9%) of POs was achieved using multienzymatic preparation (Depol 670 L) to hydrolyze a potato pulp by-product rich in galactan-rich rhamnogalacturan I. Main products were oligosaccharides with DP of 2–12 (79.8–100%), whereas the oligomers with DP of 13–70 comprised smaller proportion (0.0–20.2%) [142]. A pool of pectinases was used to produce POs with degree of polymerization from 2 to 8 and six different rhamnogalacturonide structures. Total recoveries were 200 (homogalacturonides) and 67 mg/g (rhamnogalacturonides) [143]. The use of commercial pectinase preparations (Endopolygalacturonase M2,
Pectinase, Viscozyme L, Pectinex Ultra SP-L, Pectinase 62 L, and Macer8 FJ) to produce POs from polygalacturonic acid. Best results were obtained with endopolygalacturonase M2 after 2 h of reaction, yielding 58, 18, and 13% of DP3 > DP2 > DP1, respectively [144].

In some cases, other food by-products were applied in the production of POs. A initial amount of 100 kg of orange peel can yield 7.5 kg of gluco-oligosaccharides, 4.5 kg of galacto-oligosaccharides, 6.3 kg of arabinono-oligosaccharides, and 13 kg of oligogalacturonides [145]. Through the action commercial enzymes (EPG-M2, Viscozyme, and Pectinase) on onion skins a yield 5.6% of pectic oligosaccharides (POS) was obtained [146].

### 3.4. Chitosanase

Chitin is a polysaccharide formed by N-acetyl-glucosamine monomers, joined by β(1-4) linkages and chitosan is the N-deacetylated form of chitin. Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the hydrolysis of β(1-4) glycosidic bond in chitosan to produce glucosamine oligosaccharides [147]. Studies using pigs indicated a modulating effect of chito-oligosaccharide (COs) inhibiting growth of harmful bacteria in the gut [67]. Strong antibacterial activity was also reported with complete inhibition of *E. coli* growth with a 0.5% solution [148]. They can also inhibit the growth of tumor cells by exerting immunoenhancing effect [149] and stimulate the growth of *Lactobacillus* sp. and *B. bifidium* KCTC 3440 indicating considerable bifidogenic potential [150].

A chitosanase (EC 3.2.1.132) from *Aspergillus* sp. Y2K showed preference for higher deacetylated chitosan as substrate, producing chitotriose, chitotetraose, and chitopentaose as the major products after hydrolysis with a total yield of 115% [151]. The chitosanolytic enzymes of *Metarhizium anisopliae* produced dimers (0.2 g/L), trimers (0.19 g/L), tetramers (0.06 g/L), and pentamers (0.04 g/L) from chitosan hydrolysis [147]. The enzymatic hydrolysis of chitosan by a chitosanase from *Bacillus* sp. yielded 60% of COs with 95% of purity [152], whereas *Bacillus pumilus* BN-262 chitosanase yielded above 80% in a UF membrane reactor [148]. Through the action of a *B. pumilus* BN-262 chitosanase, a COs productivity of 20 and 15 g/L was obtained in a batch and membrane reactor, respectively [153]. The hydrolysis with *B. pumilus* chitosanase yielded 52% of COs, producing mainly pentameric and hexameric chitosan oligosaccharides was steadily produced at 2.3 g/L (46% yield) for a month [154].

### 4. Concluding remarks

Glycosidases are widely applied in the production of nondigestible oligosaccharides presenting easy-handed processes with high efficiency. The application of molecular biology tools to produce enzymes with new characteristics has increased the yield and productivity of NDOs. The immobilization of the enzymes and application of membrane and batch reactors are also highlighted for improvements in the production processes. Nowadays alternative substrates have been used frequently in co-products and by-products from food and agroindustry. This approach can lead to a decrease in the cost of the process and help in the correct management of these residues.
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