Enzyme Engineering for Oligosaccharide Biosynthesis

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Abstract

Oligosaccharides are compounds of great interest in the food and pharmaceutical industry, mainly due to their prebiotic action and other health-promoting effects, in addition to other properties, acting as low-caloric sweeteners, antioxidants, or antimicrobial agents. Oligosaccharide functionality depends on their chemical structure, which relies on the nature of the building sugar residues, the linkage type, and the degree of polymerization. Their production is based either in the partial hydrolysis of polysaccharides or in the synthesis from other sugars with lower degree of polymerization. This methodology is described along the first part of this chapter, making special emphasis on the enzymatic-based technology, which shows clear advantages over chemical methods. Glycoside hydrolases and glycosyltransferases are the two enzyme classes involved in oligosaccharide production. They operate as highly specialized molecular tools for the generation of the different types of oligosaccharides. In the second part of the chapter, the strategies employed for improving the properties of these enzymes are explained. Enzyme engineering, through site-directed mutagenesis or directed evolution, has succeeded in delivering enzymatic variants with enhanced oligosaccharide yields, a different product profile or a wider substrate specificity. Moreover, immobilization of enzymes on solid supports through different methods has allowed their reutilization during repeated batches or their implementation in continuous systems, and, in some instances, it was also accompanied by an increased activity or stability.

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Overall, this chapter provides an overview about the facts and potentials of oligosaccharide production methodologies.

Keywords
Prebiotic oligosaccharide • Glycoside hydrolase • Glycosyltransferase • Glycosynthase • Transglycosylation • Site-directed mutagenesis • Directed evolution • Protein immobilization

2.1 Introduction

Oligosaccharides are low molecular weight carbohydrates with a degree of polymerization (DP) between 2 and 10 that may be linear or branched (Weijers et al. 2008). They are crucial for the development, growth, and function of organisms, being present in essential biomolecules as glycolipids and glycoproteins (Raman et al. 2005). Oligosaccharides show a broad structural diversity. In eukaryotes, they are synthesized in the endoplasmic reticulum and Golgi apparatus by several glycosyltransferases. In prokaryotes, N-linked oligosaccharides are synthetized in the periplasmic space, whereas *O*-linked oligosaccharides are produced in the cytoplasm and surface appendages, such as pili and flagella (Szymanski and Wren 2005; Weijers et al. 2008). The structural stability of these compounds depends on the nature of the sugar residues, their degree of polymerization and the linkage type.

Oligosaccharides have been commercialized since the 1980s as low-calorie bulking agents. More recently they have received much interest in food and pharmaceutical industry because of their prebiotic properties and the increased consumer interest in health-promoting products (Patel and Goyal 2011). They are also used in food industry as jelly agents, antioxidants, and humectants and in the pharmaceutical industry for drug delivery. The chemical diversity of different oligosaccharides and their applications is shown in Table 2.1.

2.2 Oligosaccharide Production

The two major methods for the production of oligosaccharides are depolymerization of polysaccharides and the synthesis from precursors of lower DP. Both procedures can be carried out by either chemical or enzymatic technologies (Courtois 2009). Chemical hydrolysis of polysaccharides uses diluted or concentrated solutions of strong acids (up to 2 M), such as hydrochloric, sulfuric, trifluoroacetic, formic, or nitrous acid, at temperatures between 50 °C and 90 °C. Oligosaccharides from fucans, pectins, and galactans are produced by this method (Courtois 2009). The reaction time of the process has been significantly reduced using microwave-induced acid hydrolysis, obtaining gluco-oligosaccharides from branched glucan of *Leuconostoc mesenteroides* (Patel and Goyal 2011). An alternative method that employs higher temperatures (150–200 °C) for hydrolysis in the absence of acid has been used for the production of XOS (Aachary and Prapulla 2011; Carvalheiro et al. 2004; Chen et al. 2014; Samala et al. 2015). Chemical synthesis of oligosaccharides involves the union of at least two sugar molecules by glycosidic bonds. This bond is formed by a nucleophilic displacement of a leaving group attached to the anomeric carbon of a sugar moiety (Fig. 2.1a). The reaction is enhanced in the presence of an activator, generally a Lewis acid, which assists the departure of the leaving group (Weijers et al. 2008). Traditional strategies for chemical oligosaccharide synthesis require extensive protecting/deprotecting steps and/or leaving group manipulation between each glycosylation stage, making the whole process cumbersome and decreasing the efficiency and yield (Fig. 2.1b). An alternative method of chemical synthesis emerged with the development of the armed-disarmed approach by Fraser-Reid and coworkers, based on the chemoselectivity principle (Fraser-Reid et al. 1990). With this method the

Table 2.1 Chemical description of different oligosaccharides, applications, and properties

Substrate	Oligosaccharide type	Chemical description	Applications/properties	References
Lactose	Galactooligosaccharides (GOS)	(D-Galactose)$_n$ connected by ß-(1,3), ß-(1,4), or ß-(1,6) linkages and bonded in most cases to a terminal glucose moiety at the reducing end	Functional food (immune stimulation, prebiotic, anti-adhesive effect against pathogens, calcium absorption improvement)	Crittenden and Playne (1996), Hernot et al. (2009), Macfarlane et al. (2008), Moreno et al. (2014), Whisner et al. (2013)
	Lactulose and lactulose-derived GOS (Lu-GOS)	Lactulose: 4-O-β-D-galactopyranosyl-D-fructose Lu-GOS: (D-galactose)$_n$-fructose	Pharmaceutical (anti-constipation agent, treatment for hepatic encephalopathy) Functional food (prebiotic effect)	Cardelle-Cobas et al. (2011), Crittenden and Playne (1996), Hernandez-Hernandez et al. (2012), Prasad et al. (2007), Schuster-Wolff-Bühring et al. (2010)
	Lactosucrose	O-ß-D-Galactopyranosyl-(1,4)-*O*-*α*-*D*-glucopyranosyl-(1,2)-ß-D-fructofuranosidase (lactosyl fructoside)	Functional food (prebiotic effect and increased mineral absorption)	Crittenden and Playne (1996), Mu et al. (2013)
Sucrose/inulin	Palatinose/isomaltulose	[6-*O*-α-D-Glucopyranosyl-D-fructose]$_n$	Functional food (prebiotic effect) Food additive (substitute sweetener)	Crittenden and Playne (1996)
	Fructooligosaccharides (FOS)	(D-Fructose)$_n$ linked in most cases to a terminal sucrose at the reducing end ^{1}F-FOS: D-fructose units interconnected through ß-(1,2) linkages (inulin type) ^{6}F-FOS: D-fructose units interconnected through ß-(2,6) linkages (levan type) ^{6}G-FOS: D-fructose units connected to the C6 of the terminal glucose (neoseries)	Functional food (prebiotic effect, relieve constipation) Industrial uses (processed food adjuvant, cosmetics)	Closa-Monasterolo et al. (2013), Crittenden and Playne (1996), Monsan and Ouarné (2009), Sabater-Molina et al. (2009)

(continued)

Table 2.1 (continued)

Substrate	Oligosaccharide type	Chemical description	Applications/properties	References
Starch	Maltooligosaccharides (MOS)	(D-glucose)$_n$ connected by α-(1,4) linkages	Industrial sweetener	Moon and Cho (1997)
	Isomaltooligosaccharides (IMOS)	(D-glucose)$_n$ connected by α-(1,6) linkages	Functional food (prebiotic effects, anti-cariogenic effects) Industrial applications (food additives)	Goffin et al. (2011)
	Gentiooligosaccharides (GeOS)	(D-Glucose)$_n$ connected by ß-(1,6) linkages	Bifidogenic activity	Rycroft et al. (2001)
	Cyclodextrins (CDs)	Cyclic maltooligosaccharides formed by 6–12 glucose units α-CDs: 6 units ß-CDs: 7 units γ-CDs: 8 units	Biotechnological applications in food, pharmaceuticals, and chromatographic techniques, as carrier and stabilizer of liposoluble vitamins, polyunsaturated fatty acids, and drugs, and for emulsion of fat oils	Del Valle (2004), Li et al. (2007b), Singh et al. (2002)
	ß-glucan oligosaccharides	(D-Glucose)$_n$ connected by ß-(1,3) linkages	Stimulatation of immunity and decreased infectious complications in humans and animals	Otaka (2006)
Others	Human milk oligosaccharides (HMOs)	Oligosaccharides formed by D-glucose, D-galactose, *N*-acetylglucosamine, L-fucose, and sialic acid as basic building blocks	Prebiotic effect	Bode (2009)
	Chitooligosaccharides (COS)	(D-Glucosamine)$_n$ connected by β-(1,4) linkages	Antimicrobial, antiviral, antioxidant, and immunostimulant	Kim and Rajapakse (2005)
	Xylooligosaccharides (XOS)	(D-Xylose)$_n$ connected by ß-(1,4) linkages	Immunomodulatory properties, antimicrobial activity, and cosmetic effects	Aachary and Prapulla (2011)
	Feruloyl xylooligosaccharides (FeXOS)	Xylooligosaccharides esterified with trans-ferulic acid at position *O*-4 from some α-D-xylopyranosyl residues	Pharmaceutical (protection against oxidative damage of low-density lipoprotein and erythrocytes)	Ishii and Tobita (1993), Katapodis and Christakopoulos (2008)

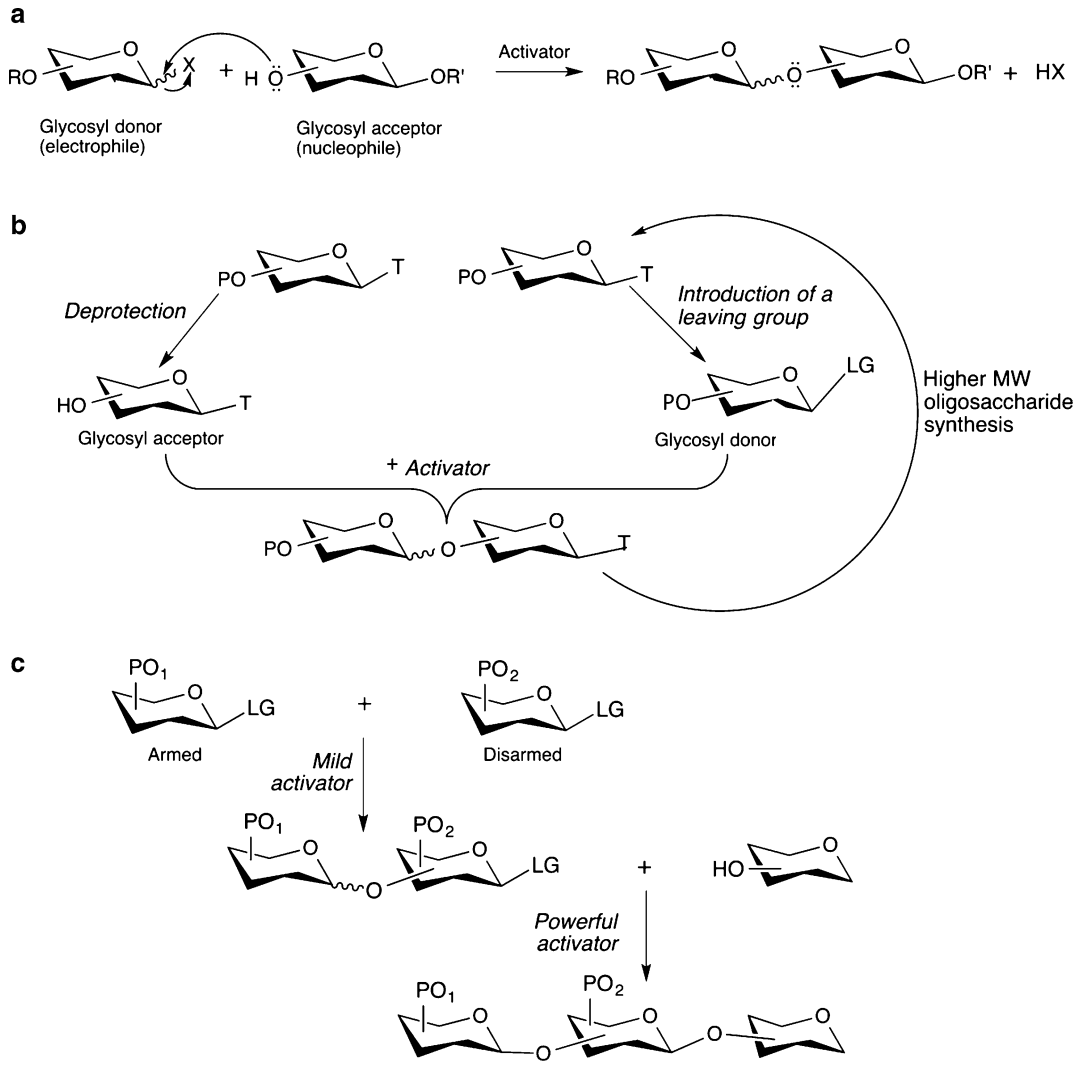

Fig. 2.1 Strategies for chemical synthesis of oligosaccharide. (**a**) Nucleophilic substitution between the glycosyl donor (electrophile) and the glycosyl acceptor (nucleophile). The leaving group is represented as X. (**b**) Traditional strategy for chemical oligosaccharide synthesis, involving protecting/deprotecting steps and the introduction of leaving groups. *PO* protecting group, *T* temporary stable anomeric substituent, *LG* leaving group. (**c**) Armed-disarmed method for oligosaccharide synthesis. The protecting groups (PO_1 and PO_2) modulate the reactivity of the glycosyl molecules. The departure of the leaving group (LG) from the "armed" sugar is promoted by a mild activator, whereas for the "disarmed" sugar, a powerful activator is required

glycosyl donor reactivity is modulated entirely through the choice of protecting group, and the resulting disaccharide can then be used directly in a subsequent glycosylation (Fig. 2.1c). This procedure has been applied in the solid-phase synthesis in which the assembly of the different glycosyl molecules occurs in donor building blocks attached to a solid support, reducing the protection steps and facilitating product isolation (Weishaupt et al. 2010). This solid-phase method is used for making carbohydrate microarrays, with applications in biological and medical research (Park et al. 2013).

The methods of chemical synthesis are expensive and their yields are low. Their use is therefore restricted to obtain high-added-value

oligosaccharides for analytical or medical purposes. For large-scale industrial oligosaccharide production, the use of enzymes offers considerable advantages, as will be discussed in the following sections. The enzymes used are glycosylhydrolases (GHs) and glycosyltransferases (GTs), which are classified in different families, based on sequence homology (Lombard et al. 2014). GHs hydrolyze glycosidic bonds, but in some instances they also catalyze transglycosylating reactions. Both possibilities involve the cleavage of a glycosidic bond. Within the active site of GHs and GTs, different residues form distinct pockets, or subsites, which dock the glycosyl residues framing the glycosidic bond that is going to be cleaved. These subsites determine substrate specificity. According to the nomenclature established by Davies et al., the subsites are designated with integer numbers from $-n$ to $+n$, numbered from the nonreducing to the reducing end of the substrate, with the cleavage occurring between subsites −1 and +1 (Davies et al. 1997).

2.2.1 Enzymatic Hydrolysis of Polysaccharides

Enzymatic hydrolysis is more attractive than chemical hydrolysis because it is environmentally friendly, it does not use hazardous chemical products, it allows an easy control of depolymerization extent, and it does not produce undesired side products (Chapla et al. 2010; da Silva et al. 2014). Oligosaccharides such as xylooligosaccharides (XOS), feruloyl xylooligosaccharides (FeXOS), maltooligosaccharides (MOS), gentiooligosaccharides (GeOS), fructooligosaccharides (FOS), chitooligosaccharides (COS), and cyclodextrins (CDs) can be obtained by enzymatic hydrolysis from polysaccharides.

XOS are released from xylan, a widely available polysaccharide, by endo-ß-(1,4)-xylanase (EC 3.2.1.8) and exo-ß-(1,4)-xylanase (EC 3.2.1.37) (Maalej-Achouri et al. 2009). Xylanases from different microorganisms are used, although fungi are generally considered more efficient producers than bacteria or yeasts (Ahmed et al. 2009). Wheat bran arabinoxylan has been used as a substrate to obtain XOS by combining the action of endo- and exo-xylanases and α-l-arabinofuranosidase (EC 3.2.1.55), yielding 0.3 g of XOS per g of soluble polysaccharides (Manisseri and Gudipati 2010). Commercial xylanase from *Trichoderma viride* released xylobiose, xylotriose, and xylose from the grass *Sehima nervosum* (Samanta et al. 2012). Xylan from alkali-treated corncobs was hydrolyzed by a xylanase from *Aspergillus foetidus* to obtain a mix of XOS with DPs from 2 to 5, with a yield of 6.7 mg XOS/g of raw corncobs (Chapla et al. 2010; Chapla et al. 2012). Thermophilic xylanases (Xyn A/B/C and Xyn W belonging to family GH10) from *Humicola insolens* yielded xylose, xylobiose, and xylotriose using brewing industry waste as a source of xylan (Du et al. 2013). FeXOS result from the hydrolysis of xylan esterified with trans-ferulic acid, which is present in a high proportion of vegetal waste material (Katapodis and Christakopoulos 2008). Endoxylanases from diverse sources show different substrate binding efficiencies to partially feruloyl-substituted xylan. *Thermoascus aurantiacus* GH10 endoxylanase produced FeXOS using as substrate wheat bran dietary fiber previously treated with *Bacillus subtilis* GH11 endoxylanase (Katapodis and Christakopoulos 2008).

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19), belonging to the GH13 family (Lombard et al. 2014), is employed to produce α-, ß-, or γ-CDs from starch or starch derivatives (Li et al. 2007b). In the first step the enzyme cleaves an α-1,4 glycosidic bond at position n from the nonreducing end of an amylose chain (n value being between 9 and about 100) (Terada et al. 1997). The resulting oligosaccharide of n units forms a covalent intermediate with the enzyme through the C-1 of the residue at the reducing end. Subsequently, this C-1 undergoes an intramolecular nucleophilic attack by the 4-hydroxyl group of the nonreducing end, releasing a cyclodextrin of n glucosyl units (Li et al. 2007b). Archaea and bacteria may secrete CGTases in order to take over the starch present in the medium since cyclodextrins cannot be metabolized by competing microorganisms

(Leemhuis et al. 2010). Most CGTases characterized so far yield a mixture of α- and ß-CDs as major products. A specific production of ß-CD was achieved with a CGTase from *Paenibacillus illinoisensis* (Lee et al. 2013). Interesting properties of γ-CDs, such as higher water solubility and bioavailability, have fostered the search of CGTases that synthetize this product (Li et al. 2007b). For instance, CGTases from *Bacillus sp., Bacillus thuringiensis, Paenibacillus macerans*, and *Bacillus clarkia*, yield γ-CDs with a high specificity (Goo et al. 2014; Lee et al. 2013; Li et al. 2007b; Wu et al. 2012).

FOS can be obtained from inulin or levan by enzymatic hydrolysis with either inulinases or levanases, respectively (both types of enzymes belonging to the GH32 family). Like other carbohydrate hydrolases, inulinases may have endo or exo action. Endoinulinases (2,1-ß-D-fructan fructanohydrolases; EC 3.2.1.7) specifically hydrolyze internal ß-(2,1) glycosidic bonds releasing inulotriose, inulotetraose, and inulopentaose (Mutanda et al. 2014). Exoinulinases (ß-D-fructohydrolases; EC 3.2.1.80) cleave the terminal fructosyl residue from inulin and are used to obtain high-fructose syrup (Apolinário et al. 2014). Many yeast and bacterial strains produce inulinase, for instance, species belonging to *Pichia*, *Cryptococcus*, *Candida*, *Yarrowia*, *Debaryomyces*, *Xanthomonas*, and *Pseudomonas*. However, molds like *Aspergillus* and *Penicillium* and the yeast *Kluyveromyces* are more frequently used (Paixão et al. 2013). *Bacillus licheniformis* endolevanase is able to produce FOS containing ß-(2,6) linkages from levan, with a yield of 97 %, based on total sugar content (Porras-Domínguez et al. 2014).

Chitinases are GH18 and GH19 glycoside hydrolases (EC 3.2.1.14.) (Lombard et al. 2014) which hydrolyze ß-1,4 linkages between N-acetylglucosamine units from chitin yielding COS (Kim and Rajapakse 2005). Hexa-N-acetylchitohexaose was produced by chitin hydrolysis using the chitinase ASCHI6 isolated from *Aeromonas schubertii* (Liu et al. 2014). Chitinases from *Bacillus cereus* produced a mix of COS with DPs between 2 and 5 (Wang et al. 2012).

2.2.2 Enzymatic Synthesis of Oligosaccharides

The enzymatic synthesis of oligosaccharides can be carried out by using either glycosidases with transferase capacity or glycosyltransferases. In both cases, the catalytic mechanism implies the transfer of a glycosyl residue from one of the two sugar substrates involved in the reaction (glycosyl donor) to the other substrate (glycosyl acceptor).

Glycosidases (EC 3.2.1) are enzymes with the capability to hydrolyze glycosidic bonds. Depending on the stereochemistry of the product compared to that of the glycosyl donor, glycosidases are classified into retaining or inverting enzymes. The inverting mechanism occurs in a single step involving two critical residues: One acts as general base, activating the water molecule that carries out the nucleophilic attack on the anomeric carbon. The other acts as general acid and protonates the leaving sugar. Instead, retaining glycosidases proceed through a double nucleophilic substitution mechanism. In the first step, a nucleophilic residue in the active site attacks the anomeric carbon of the substrate, and the leaving group is protonated by an acid/base catalyst. This results in a covalent intermediate between the enzyme and the glycosyl donor, which is subsequently released by the nucleophilic attack of a water molecule activated by the acid/base catalyst. The retaining mechanism confers glycosidases the potential to catalyze transglycosylation reactions, which occur when a second sugar molecule enters the catalytic site of the covalent intermediate and acts as glycosyl acceptor (Fig. 2.2) (Davies and Henrissat 1995).

Glycosyltransferases (EC 2.4) catalyze the transfer of sugar moieties from donor to acceptor molecules thereby forming glycosidic bonds. The acceptor substrate may be as simple as a monosaccharide or as complex as a heteropolysaccharide, oligosaccharide, protein, nucleic acid, or lipid (Lairson et al. 2008; Weijers et al. 2008). These enzymes are responsible for the synthesis of most cell-surface glycoconjugates. Like glycosidases, glycosyltransferases can be retaining or inverting, although their detailed molecular mechanism is

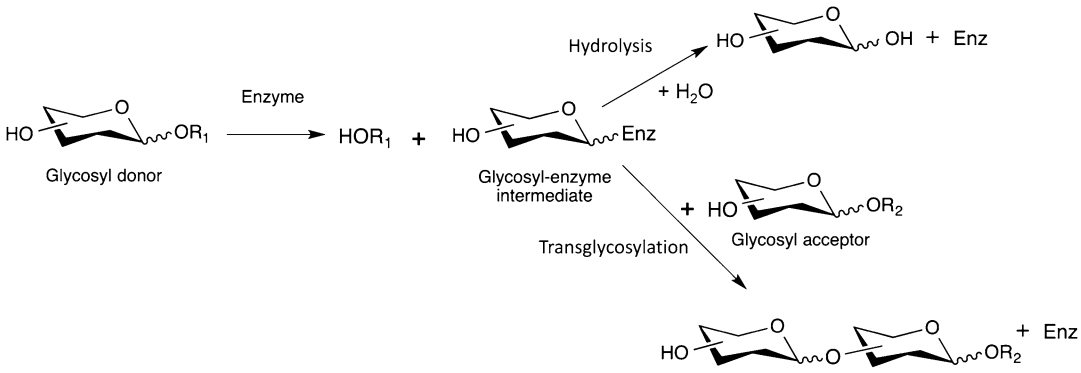

Fig. 2.2 Synthesis of oligosaccharides with nonactivated donors can be carried out by retaining glycosidases. The enzyme (*Enz*) forms a covalent intermediate with a glycosyl group from the glycosyl donor. This group can be transferred to a water molecule (hydrolysis) or to a glycosyl acceptor (transglycosylation), releasing the free enzyme to start a new cycle of catalysis

not completely understood. Inverting glycosyltransferases utilize a direct displacement S_N-2-like mechanism assisted by an enzymatic base catalyst. Retaining glycosyltransferases operate through a double-displacement reaction involving a covalently bound glycosyl-enzyme intermediate, except for members from families GH4 and GH109 which use an elimination/addition mechanism with a transient oxidation, and retaining ß-hexosaminidases that use an intramolecular nucleophile (Lairson et al. 2008; Mark et al. 2001).

Enzymes with transglycosylating activity have been classically classified as *non-Leloir* or *Leloir* transferases depending on the nature of the glycosyl donor. Enzymes that use activated donor carbohydrates bound to nucleosides mono- or diphosphate (CMP, UDP, GDP, or TDP) are classified as *Leloir.* Enzymes that use nonactivated carbohydrate donors or monophosphate-substituted sugars are classified as non-*Leloir.* In this chapter, however, we have employed a more practical classification into two groups of enzymes, based on their capability to synthesize oligosaccharides using either activated or nonactivated donors.

2.2.3 Synthesis of Oligosaccharides with Nonactivated Donors

Lactose is the substrate for the synthesis of galactooligosaccharides (GOS) and related compounds like lactosucrose and lactulose, which represent about 40 % of annual oligosaccharide production (Gänzle 2012). β-Galactosidases transfer the galactosyl moiety from lactose to an acceptor. Lactose can also act as galactosyl, glucosyl, or fructosyl acceptor through the action of ß-galactosidases, glucansucrases, and fructansucrases, respectively.

GOS synthesis is catalyzed by β-galactosidases, with lactose acting as both glycosyl donor and acceptor. The transglycosylation/hydrolysis ratio depends on the structural conformation of the active site of the enzyme, but it is also influenced by other factors such as lactose concentration (Bruins et al. 2003; Ji et al. 2005), water activity, and, to a minor extent, reaction temperature (Millqvist-Fureby et al. 1998). The chemical structure and DP of the resulting oligosaccharide also rely on structural features of the active site that have not been fully explained yet. The most common products are the trisaccharides 6′-galactosyllactose, 3′-galactosyllactose, and 4′-galactosyllactose. GOS yields obtained with different enzymes vary between 2 % and 70 %, based on the total sugar content (Park and Oh 2010; Torres et al. 2010). Fungi, bacteria, and archaea have been used as enzyme sources for GOS production. Currently, commercial enzymes are obtained from from *Kluyveromyces lactis*, *Streptococcus thermophilus*, *Bacillus circulans*, *Aspergillus oryzae*, and *Bifidobacterium bifidum*. The three former belong to the GH2 family, and synthesize GOS with yields up to 44 %, 25 %, and 39 %,

respectively (Rodriguez-Colinas et al. 2011, Torres et al. 2010, Song et al. 2011). Different β-galactosidases from *Bifidobacterium bifidum* produce GOS with yields ranging between 15–47 % (Goulas et al. 2009). They are used for the production of ß-(1,3) GOS (*B. bifidum*), ß-(1,4) GOS (*B. circulans*), and ß-(1,6) GOS (*Aspergillus oryzae*, *Streptococcus thermophilus* and *Kluyveromyces lactis*) (Goulas et al. 2009; Torres et al. 2010; Rodriguez-Colinas et al. 2011). GH35 ß-galactosidases from *Aspergillus* and *Penicillium* species have rendered 30–45 % GOS yields (Torres et al. 2010), whereas GH42 ß-galactosidase from *Bacillus longum* synthesized GOS with lower (10 %) yields (Torres et al. 2010). Since high lactose concentrations have a strong positive influence in GOS yield, the use of high temperatures is desirable in order to increase lactose solubility (Roos 2009). This can be accomplished with ß-galactosidases that are stable at temperatures between 70 °C and 90 °C (Park and Oh 2010). Moreover, these enzymes usually show high structural resistance, convenient for industrial applications. Therefore, different thermostable β-galactosidases have been studied. GH1 enzymes from *Sulfolobus solfataricus*, *Pyrococcus furiosus*, and *Thermus caldophilus* produced GOS with 53 %, 40 %, and 77 % yield, respectively (Torres et al. 2010). *Thermotoga maritima* β-galactosidase from GH2 family yielded 18 % GOS (Ji et al. 2005), whereas that from *Geobacillus stearothermophilus*, belonging to the GH42 family, yielded only 2 % GOS (Placier et al. 2009). The enzymes from *Sulfolobus solfataricus*, and *Pyrococcus furiosus* produce ß-(1,3) and ß-(1,6) GOS (Torres et al. 2010), whereas those from *Thermus sp., Thermotoga maritima*, and *Geobacillus stearothermophilus* show a higher preference for the synthesis of ß-(1,3) GOS (Akiyama et al. 2001; Marín-Navarro et al. 2014; Placier et al. 2009).

Lactulose is synthesized by transgalactosylation when the enzymatic hydrolysis of lactose occurs in the presence of fructose. In this case the galactosyl moiety from lactose is linked to the C-4 atom of fructose. The yield depends on the enzyme and the ratio of concentrations of lactose and fructose. Lactulose is produced in the early stage of the reaction and can be consumed in the final stages. Because of this, it is necessary to stop the reaction or separate the enzyme from the product (Panesar and Kumari 2011). Some ß-galactosidases and ß-glycosidases have been used for lactulose production. GH1 ß-glycosidases from *Aspergillus oryzae, Pyrococcus furiosus*, and *Sulfolobus solfataricus* yielded 30, 44, and 12.5 % lactulose relative to lactose, respectively (Panesar and Kumari 2011; Kim et al. 2006; Wang et al. 2013). Lactulose has also been synthesized from lactose in the absence of fructose by combining a ß-galactosidase from *Kluyveromyces lactis* and a glucose isomerase from *Streptomyces murinus*, with a conversion yield of 19 % (Wang et al. 2013). Another method for lactulose production using only lactose as substrate has made use of a thermostable cellobiose-2-epimerase with isomerase activity, from *Caldicellulosiruptor saccharolyticus*, yielding 58 % lactulose (Kim and Oh 2012).

Lactosucrose has been obtained with levansucrase from *Zymomonas mobilis* using a 1:1 mass ratio of lactose and sucrose as substrate, achieving a conversion efficiency of 28 %. Since this enzyme is inhibited by the glucose released in the reaction, the former yield could be increased up to 43 % by combining the action of levansucrase with glucose oxidase thus transforming the produced glucose into D-gluconolactone, which does not exert inhibitory effect (Han et al. 2009). Trisaccharides derived from lactulose (Lu-GOS) have been synthesized by transglycosylation using the ß-galactosidase from *K. lactis*, with a yield of 14 % from total carbohydrate content (Martinez-Villaluenga et al. 2008). The galactosyl unit from a lactulose molecule was transferred to another lactulose at two different positions releasing two types of Lu-GOS.

Fructosyltransferases (FTase, EC 2.4.1) and ß-fructofuranosidases (FFase, EC 3.2.1.26) with high transfructosylating activity, belonging to GH32 and GH68 families, are the enzymes involved in the synthesis of FOS from sucrose, which acts both as donor and acceptor of the fructosyl group (Dominguez et al. 2014; Ganaie et al. 2013). Fructosyl units can be connected through ß-(2,1) linkages (inulin-type FOS or ^{1F}FOS, as 1-kestose) or ß-(2,6) linkages (levan-type FOS or

^{6F}FOS, as 6-kestose). In neoseries FOS or ^{6G}FOS, a ß-(2,6) link connects a fructosyl unit to the glucosyl moiety of sucrose, as in neokestose (Linde et al. 2012; Porras-Domínguez et al. 2014). Commercial FOS have been produced by enzymes from fungi and bacteria, with different yields and product specificity. Several fungal strains, mostly from the genus *Aspergillus* and *Penicillium*, produce intracellular and extracellular FTases that synthesize mainly inulin-type FOS, with yields ranging between 25 % and 70 % from total sugar content (Dominguez et al. 2014). FOS mixtures include 1-kestose, 1-nystose, and 1-fructofuranosyl nystose as predominant oligosaccharides (Ganaie et al. 2013). There are fewer reports on the production of levan-type FOS as a major product of enzymatic transfructosylation. GH68 levansucrase from *Zymomonas mobilis* synthesizes a mixture of 6-kestose, 1-kestose, and neokestose with different ratios depending on the reaction conditions and a 6-kestose maximum specificity of 49.1 % of total FOS (Vigants et al. 2013). In contrast, *Schwanniomyces occidentalis* GH32 FTase produces FOS with a 16.4 % yield, 6-kestose representing 75 % of total FOS (Álvaro-Benito et al. 2007). Production of neoseries FOS has been reported by FTases from *Xanthophyllomyces dendrorhous*, *Penicillium oxalicum*, and *Penicillium citrinum* with 57 %, 19 %, and 11 % conversion yields to neokestose, respectively (Ning et al. 2010; Xu et al. 2015; Hayashi et al. 2000).

IMOS can be produced by dextransucrases (EC 2.4.1.5) belonging to the family GH70 (Lombard et al. 2014). These enzymes catalyze the transfer of the glucose moiety from a sucrose molecule to a glucose acting as acceptor. *Leuconostoc mesenteroides* dextransucrase is able to produce a mixture of IMOS with DPs between 4 and 9. Maximum conversion yields (between 70–90 %, in terms of glucose equivalents) were achieved with a sucrose/maltose ratio of 2:1 (Lee et al. 2008). α-Glucosidases (EC 3.2.1.20) are exo-glycosidases grouped in GH13 and GH31 families that catalyze the release of glucose from the nonreducing end of short oligosaccharides. Some α-glucosidases with transglycosylating activity, such as the enzymes from *Aspergillus niger, S. cerevisiae*, or *Xanthophyllomyces dendrorhous*, synthetize IMOS (DPs between 2 and 4) from maltose with conversion yields between 30 % and 60 %, from total sugar content (Duan et al. 1995; Fernández-Arrojo et al. 2007).

In the case of GeOS synthesis, a few examples have been reported. A mixture of GeOS with different polymerization degrees (between 3 and 9) has been achieved with a combination of ß-glucosidase and ß-(1,6)-glucanase (GH5) from *Penicillium multicolor.* In this case, ß-glucosidase synthesizes gentiotriose using gentiobiose as substrate, and this trisaccharide is elongated by ß-(1,6)-glucanase yielding the final GeOS mix (Fujimoto et al. 2009). GH70 alternansucrase (EC 2.4.1.140) from *Leuconostoc mesenteroides* is capable of synthesizing GeOS with DPs between 3 and 5 in presence of gentibiose (Côté 2009).

Fucosyl-*N*-acetylglucosamine, one of the components of HMOs, can be synthesized using fucosidases with transglycosylating capability such α-L-fucosidases AlfB and AlfC from *Lactobacillus casei* that yield fucosyl-α-1,3-N-acetylglucosamine and fucosyl-α-1,6-N-acetylglucosamine, respectively, using *p*-nitrophenyl-α-L-fucopyranoside (pNP-fuc) as fucosyl donor and *N*-acetylglucosamine (GlcNAc) as acceptor (Rodriguez-Diaz et al. 2011).

2.2.4 Synthesis of Oligosaccharides with Activated Donors

Synthesis of oligosaccharides with activated donors is carried out by two main groups of enzymes (Fig. 2.3). Phosphorylases can act as glycosyltransferases in their reverse reaction using phosphorylated glycosyl donors, whereas Leloir glycosyltransferases use glycosyl donors activated with nucleotide mono- or diphosphate (CMP, UDP, GDP, or GTP).

In their forward reaction, phosphorylases catalyze the phosphorolysis of glycosyl linkages at the nonreducing end of carbohydrates, releasing a monosaccharide 1-phosphate in the presence of phosphate. In the reverse reaction, these enzymes catalyze the transfer of the activated monosaccharide to a carbohydrate acceptor. In one particular case, the enzyme α-1,4-glucan-maltose-1-P

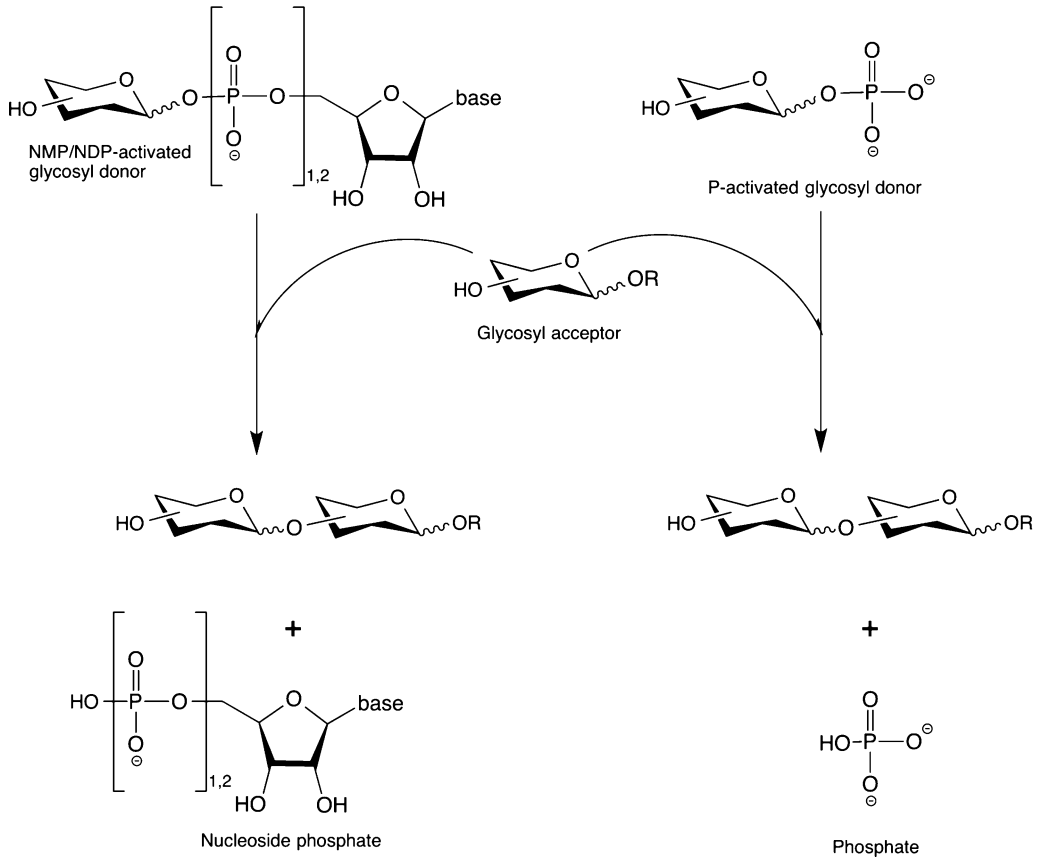

Fig. 2.3 Synthesis of oligosaccharides with activated donors is carried out by phosphorylases (right side) and Leloir glycosyltransferases (left side). The activated glycosyl donor is a phosphorylated sugar or a nucleotide mono- or diphosphate (CMP, UDP, GDP, or GTP), respectively

maltosyltransferase (EC 2.4.99.16) has been described to release maltose 1-phosphate from glycogen (Nakai et al. 2013), which would allow the transglycosylation of a disaccharide in a single step in the reverse reaction. Reversible phosphorolysis of α-glucosides is catalyzed by phosphorylases belonging to glycoside hydrolase families GH13 and GH65 and to glycoside transferase families GT4 and GT35. ß-Glucosides, ß-galactosides, and ß-manosides are substrates of phosphorylases belonging to families GH94, GH112, and GH130, respectively. Family GH94 also includes a chitobiose phosphorylase capable of transferring N-acetyl-glucosamine moieties (Honda et al. 2004). Despite the wide diversity of potential natural sugars able to act as glycosyl acceptors, little use of phosphorylases to produce oligosaccharides has been made due to the reduced number of enzymes characterized so far (Nakai et al. 2013).

GH65 kojibiose phosphorylase (EC 2.4.1.230) from *Thermoanaerobacter brockii* catalyzes the synthesis of kojibiose (α-D-glucopyranosyl-(1,2)-D-glucopyranose) by transglycosylation using ß-D-glucose-1-phosphate as glycosyl donor and D-glucose as acceptor, yielding inorganic phosphate. This enzyme is also able to use other glycosyl acceptors such as L-sorbose, maltose, and glucosyl-maltose to yield glucosyl-l-sorbose, glucosyl-sucrose, and glucosyl-maltose, respectively, as novel oligosaccharides (Chaen et al. 2001). Maltose phosphorylase (EC 2.4.1.8) from *Propionibacterium freudenreichii* catalyzes the transglycosylation of ß-D-glucose from ß-D-glucose-1-phosphate to different acceptors such as D-glucose, D-glucosamine, *N*-acetylglucosamine, mannose, 2-deoxy-D-glucose, and 2-deoxy-D-xylose (Aisaka et al. 1996), making this enzyme suitable for the

synthesis of oligosaccharides with a broad chemical diversity.

Leloir enzymes require the previous synthesis of an activated substrate, which is a nucleotide mono- or diphosphate glycoside. Such compounds are synthesized by the consecutive action of a kinase, which phosphorylates the sugar, and a nucleoside transferase/pyrophosphorylase (Weijers et al. 2008). Therefore, the use of this methodology requires the implementation of in situ coupled reactions either in vitro or in vivo (Weijers et al. 2008). Leloir transferases are classified in two different superfamilies depending on their structure: GT-A and GT-B, and both of them include retaining and inverting enzymes.

The requirement of a nucleotide-activated donor makes *Leloir* glycosyltransferases less attractive than those using nonactivated sugars for industrial production of low-added-value oligosaccharides. However, these enzymes show a high promiscuity, accepting a rather wide range of substrates which leads to the synthesis of a diversity of products. Indeed, *Leloir* glycosyltransferases are able to synthesize most human milk oligosaccharides (HMOs), such as trisaccharides (sialyllactose and fucosyllactose) or other complex oligosaccharides (lacto-*N*-biose derivatives). These compounds are very interesting to be used as functional ingredients in commercial infant formula because of their prebiotic and immunogenic effects (Bode 2009). The synthesis of these oligosaccharides requires the combined action of several enzymes and cofactors. This has been carried out both by in vitro and whole-cell approaches, being the latter more convenient for industrial purposes, due to the high costs associated to enzyme purification and cofactor synthesis (Table 2.2).

2.3 Enzyme Engineering

As it has been described in previous sections, glycosidases and GTs have proven to be useful tools for oligosaccharide synthesis in vitro. However, sometimes native enzymes do not provide good performance, specificity, or stability. In this case, one can either look for new enzymes in nature or change the properties of an existing enzyme by protein engineering. The procedures used to improve enzyme performance are mutagenesis, either random or site directed (Bornscheuer 2013), and immobilization (Homaei et al. 2013).

2.3.1 Directed Evolution

Directed evolution attempts to mimic the natural selection process by recursive rounds of mutation, recombination, and selection (Kittl and Withers 2010) Random mutagenesis carried out by error-prone PCR is generally used to obtain the initial library of mutants. The major limitations of this approach are the inherent bias of existing mutagenesis methods (Wong et al. 2006) and the requirement in many instances of technically complex high-throughput screening methods. However, this approach is a powerful tool for molecular engineering of enzymes, and it does not require any previous information about the structural-function relationships of the enzyme of interest. Directed evolution has successfully been used to increase the transglycosylating activity of a ß-glycosidase (GH1) from *Thermus thermophilus*. Mutant F401S/N282T, using either maltose or cellobiose as acceptors and *o*-nitrophenyl-β-D-galactopyranoside as donor, showed molar yields of trisaccharides up to 60 % and 75 %, respectively, whereas the wild-type yield was only 8 %. Galactosyl transfer to maltose ocurred with a major β-(1,3) regioselectivity, whereas with cellobiose both β-(1,3) and β-(1,6) isomers were synthesized. These mutations may reorient the glycone at the −1 subsite allowing a better transfer to the acceptors (Feng et al. 2005). A similar approach was used to obtain α-L-arabinofuranosidase (GH51) from *Thermobacillus xylanilyticus* with better transglycosylation and lower hydrolytic activity with mutants N344P and N344Y (Arab-Jaziri et al. 2013). α-L-Fucosidase (GH29) from *Thermotoga maritima* was converted into α-L-transfucosidase. The change of activity required mutations at only three positions (T264A, Y267F, L322P). This was carried out by a first step of random

Table 2.2 Oligosaccharide production with *Leloir* glycosyltransferases

Final product	Method	Involved enzymes/microorganisms[f]	Reaction scheme	References
Sialyllactose	Whole cells	(1) *Corynebacterium ammoniagenes* for UTP synthesis (2) CTP synthetase* (3) CMP-NeuAc synthetase* (4) α-2,3-sialyltransferase* *Recombinantly produced by two *E. coli* strains	UTP[1] + ATP + Gln → [2] CTP + ADP + Pi + Glu CTP + NeuAc → [3] CMP-NeuAc + PPi CMP-NeuAc + lactose → [4] sialyllactose + CMP	a
NeuAc (sialyllactose precursor)	Whole cells	(1) GlcNAc epimerase* (2) NeuAc synthetase* *Recombinantly produced by one *E. coli* strain	GlcNac → [1] ManNAc ManNAc + PEP → [2] NeuAc + Pi	b
Sialyllactose	Enzymatic	(1) GlcNAc 2-epimerase (2) NeuAc aldolase (3) CMP-NeuAc synthetase (4) α-2,3-sialyltransferase (5) CMP kinase (6) Acetate kinase (7) Polyphosphate kinase	GlcNac → [1] ManNAc ManNAc + pyruvate → [2] NeuAc NeuAc + CTP → [3] CMP-NeuAc + PPi CMP-NeuAc + lactose → [4] sialyllactose + CMP + *two CTP regeneration systems*: (I) CMP + ATP → [5] CDP + ADP CDP + acetylPi → [6] CTP + Acetate ADP + acetylPi → [6] ATP + acetate (II) CMP + polyPn → [7] CDP + polyPn-1 CDP + polyPn-1 → [7] CTP + polyPn-2	c
Fucosyllactose	Enzymatic/whole cells	(1) GDP-D-mannose-4,6-dehydratase* (2) GDP-L-fucose synthase* (3) Fucosyltransferase *Purified enzymes or recombinantly produced by *S. cerevisiae* or *E. coli*	GDP-mannose → [1] GDP-4-keto-deoxymannose GDP-4-keto-deoxymannose + NADPH → [2] GDP-L-fucose + $NADP^+$ GDP-L-fucose + lactose → [3] fucosyllactose	d
Lacto-*N*-biose I	Enzymatic	(1) Sucrose phosphorylase (2) UDP-glucose-hexose-1-phosphate urydyltransferase (3) Lactobiose *N*-phosphorylase (4) UDP-glucose-epimerase	Sucrose + Pi → [1] Glc1P + Frc Glc1P + UDP-Gal → [2] UDP-Glc + Gal1P Gal1P + GlcNAc → [3] LNB + *UDP-Gal regeneration system:* UDP-Glc → [4] UDP-Gal	e

a Endo et al. 2000; b Ishikawa and Koizumi 2010; Koizumi et al. 2000; c Han et al. 2012; d Albermann et al. 2001; e Nishimoto and Kitaoka 2007; f Numbers in brackets indicate the corresponding step in the "Reaction scheme" column. *Glu* glucose, *Gln* glucosamine, *Gal* galactose, *Frc* fructose, *NeuAc N*-acetylneuraminic acid, *PEP* phosphoenol pyruvate, *GlcNAc N*-acetyl-D-glucosamine, *ManNAc N*-acetyl-D-mannosamine, *PolyP* polyphosphate, *Pi* phosphate, *PPi* pyrophosphate, *Glc1P* glucose 1-phosphate, *Gal1P* galactose 1-phosphate, *LNB* lacto-*N*-biose, *NADPH* reduced nicotinamide dinucleotide phosphate, *$NADP^+$* oxidized nicotinamide dinucleotide phosphate

mutagenesis after which selected mutations were combined to yield a 32-fold increase in the transferase/hydrolytic ratio while keeping 60 % of the overall wild-type enzyme activity (Osanjo et al. 2007). In another instance, aiming to prevent product hydrolysis, cyclodextrin glucanotransferase (GH13) from *Thermoanaerobacterium thermosulfurigenes* was subjected to error-prone PCR, searching for mutant versions of the enzyme with lower hydrolytic rate. Two mutations were identified, located at the outer region of the active site (W232R and S77P). These mutations lowered the hydrolytic activity up to 15-fold while keeping the cyclization activity (Arnold et al. 2009). Significant increase of GOS production was achieved by mutagenesis of ß-galactosidase (GH42) from *Geobacillus stearothermophilus*. A random mutagenesis approach led to the isolation of mutant R109K with a sixfold higher yield than that obtained with the wild-type enzyme. Saturation mutagenesis at this position allowed the characterization of mutations R109V and R109W, which caused an even higher (around 11-fold) yield increment, up to 23 % from total carbohydrate content (Placier et al. 2009). Random mutagenesis of ß-fructofuranosidase from *Schwanniomyces occidentalis* increased 6-kestose production around twofold over the parent type, with the mutations Q78L and F523V. While Gln78 is located close to the active site, Phe523 resides out of the catalytic domain and probably exerts a long-distance effect (de Abreu et al. 2011). Kojibiose phosphorylase from *Thermoanaerobacter brockii* was also improved by random mutagenesis. Two mutants, S676N and N687I, showed higher productivity of kojioligosaccharides than the wild type (Yamamoto et al. 2006).

2.3.2 Site-Directed Mutagenesis

Knowing the structure and reaction mechanism of a given protein allows designing specific mutations to improve its activity or change its substrate specificity. Work done with glycosyl hydrolases has had as a major objective to increase the transglycosylation/hydrolysis ratio. Site-directed mutagenesis of ß-glucosidase (GH1) from *Pyrococcus furiosus* at residues within the catalytic pocket revealed that the F426Y mutant had an increased GOS yield (up to 45 % from total carbohydrate content) compared to the wild type (40 %). Moreover, the double mutant M424K/F426Y had better transglycosylating properties at low substrate (lactose) concentrations compared to the wild type and the corresponding single mutants (Hansson et al. 2001). In another GH1 enzyme, the ß-galactosidase from *Sulfolobus solfataricus*, wild-type GOS production (50.9 %) was increased up to 58 % and 62 % as a consequence of mutations F359Q and F441Y, respectively (Wu et al. 2013). This F441 is homologous to the aforementioned F426 and F401 from the GH1 β-glycosidases from *P. furiosus* and *T. thermophilus*, respectively (Feng et al. 2005; Hansson et al. 2001), all resulting in a higher GOS yield when mutated. The observed effect may be caused by a reorientation of the glycone at the −1 subsite to a position more favorable for transglycosylation. On the other hand, the F359Q mutation may increase the affinity of the +1 subsite for the lactose molecule acting as glycosyl acceptor (Wu et al. 2013).

Within invertases belonging to the GH32 family, different studies with plant and yeast enzymes showed that substitution of one or the two asparagines within the so-called β-fructofuranosidase motif (WMNDPGN), harboring the catalytic nucleophile, resulted in increased FOS synthesis (Alvaro-Benito et al. 2010; Lafraya et al. 2011; Ritsema et al. 2006; Schroeven et al. 2008). One of the most dramatic changes was observed with the invertase from *Saccharomyces cerevisiae*, where N21S and N24S amino acid replacements caused a sixfold increase of 6-kestose synthesis. The combination of the aforementioned changes with the W19Y substitution (also within the β-fructofuranosidase motif) further increased the synthesis of 6-kestose up to tenfold, compared to the wild type. Another conserved motif within hydrolytic GH32 enzymes is the "ECP" motif harboring the acid/base catalyst. The substitution of Pro by Val in this motif also increased 6-kestose

synthesis by the invertase from *S. cerevisiae* and the β-fructofuranosidase from *S. occidentalis* by sixfold and twofold, respectively (Alvaro-Benito et al. 2010; Lafraya et al. 2011). GH32 enzymes from yeasts synthesize mainly 6-kestose, whereas their plant homologues produce mainly 1-kestose. Although the −1 subsite is rather conserved, the +1 subsite is more divergent between the two groups. The second Trp in the "WGW" motif in hydrolytic plant enzymes has been proposed to be part of the sucrose acceptor subsite, and its replacement by Tyr in the invertase from *Arabidopsis thaliana* doubled the transferase capacity of the enzyme (Altenbach et al. 2009). Not only the transglycosylation yield but also substrate and product specificity can be modulated by site-directed mutagenesis. Donor and acceptor substrate selectivity has been altered in a plant GH32 enzyme, transforming a fructan-fructan fructosyltransferase into a sucrose-sucrose fructosyltransferase (Lasseur et al. 2009; Van den Ende et al. 2009). On the other hand, the introduction of the N228A substitution changed the FOS product profile of the N21S mutant of *S. cerevisiae* invertase, from a highly specific 6-kestose synthesis to an equimolar production of 6-kestose and 1-kestose. This Asn 228, together with Trp 291, was proposed to build the acceptor sucrose-binding platform in yeast enzymes (Lafraya et al. 2011). Mutations at an equivalent residue to this Asn 228 in the β-fructofuranosidase from *S. occidentalis* also resulted in a broader product spectrum, further confirming this hypothesis (Alvaro-Benito et al. 2012).

Two GH18 family chitinases were converted into transglycosylases: *Bacillus circulans* chitinase A1 (*Bc* ChiA1) and *Trichoderma harzianum* chitinase 42 (*Th* Chit42) were mutated at the conserved aspartates that act as stabilizers of the general acid/base glutamate. Mutants D200A and D202A of *Bc* ChiA1, as well as D170N and D170A of *Th* Chit42, retained the ability to catalyze the transglycosylation reaction but lost the hydrolytic activity (Martinez et al. 2012).

Glycosyltransferases have an enormous potential for the synthesis of novel, nonnatural, relevant carbohydrate structures (Hancock et al. 2006). However, methodological difficulties in synthesizing the required activated substrates hamper the production of oligosaccharides by this procedure. Few studies have dealt the molecular engineering of glycosyltransferases, aiming to change the donor specificity of the enzymes. Mutation Y289L in bovine ß-(1,4)-galactosyltransferase (GT7) broadened the profile of donor substrates from a strict specificity for transglycosylation of UDP-galactose to include also UDP-N-acetyl-galactosamine and UDP-*N*-acetylglucosamine, without compromising galactosyltransferase activity (Ramakrishnan and Qasba 2002). Mutation of residue R228K in the same enzyme increased glucosyltransferase activity (15-fold) and decreased galactosyltransferase activity (30-fold) (Ramakrishnan et al. 2005). Similarly, the P234S mutation of a human α-(1,3)-galactosyltransferase (GT6) changed the donor specificity from UDP-galactose to UDP-N-acetyl-galactosamine (Ramakrishnan and Qasba 2002).

2.3.3 Glycosynthases

Withers and colleagues (1998) mutated the nucleophile of a retaining exo-acting glycosidase (a β-glucosidase) belonging to the GH7 family. The resulting mutant was inactive as hydrolase. Using as substrate an activated glycoside fluoride donor, such as α-glucosyl or α-galactosyl-fluoride, with anomeric conformation opposite to that of the substrate of the wild-type enzyme, the mutant enzyme was able to carry out transglycosylation. These new types of enzymes were called glycosynthases (GS). The same year, using the same approach, an endo-acting glycosynthase, able to catalyze the glycosylation of different glucoside acceptors with α-laminaribiosyl fluoride, was constructed from a GH16 β-endoglucanase (Malet and Planas 1998). This methodology has been applied to many other enzymes, belonging to different GHs families, with good results in improving the yield of oligosaccharide synthesis (Cobucci-Ponzano et al. 2011). The mutated enzymes include exo-acting glycosidases as glucosidases, mannosidases,

galactosidases, fucosidases, and xylosidases and endo-acting glycosidases as endoglucanases, endoxylanases, laminarinases, and mannanases. In all these cases, substitution of the nucleophile by a residue with a shorter side chain (such as Ala, Gly, or Ser) made the resulting mutant unable to hydrolyze glycosidic bonds but still performed transglycosylation when incubated with activated glycosyl donors. Most GS derived from β-retaining GHs prefer α-glycosyl-fluorides donors and proceed through a single-displacement mechanism (α → β). Hyperthermophylic GS obtained from β-retaining GHs use aryl β-glycosides and require an excess of formate that acts as a nucleophile, forming an α-glycosyl formate intermediate in a double-displacement mechanism (β → α → β). In contrast, for GS derived from α-retaining GHs, β-glycosyl-azides work better as activated donors than β-glycosyl-fluorides (Cobucci-Ponzano et al. 2011). Some more recent examples are the GH13 cyclodextrin glucanotransferase E284G from *Bacillus* sp. (Li et al. 2014) and the GH35 ß-galactosidase E233G from *Bacillus circulans* (Henze et al. 2014) which used α-maltosyl-fluoride and α-D-galactopyranosyl-fluoride as donors, respectively. The latter mutant has been used to produce galacto-*N*-biose or lacto-*N*-biose with good yields (40–90 %) (Henze et al. 2014).

In some instances, consecutive rounds of directed mutagenesis have been applied to further improve the properties of a first generation of GS. One approach has been site-saturation mutagenesis of the nucleophilic residue to select the mutation (Ala, Ser, or Gly) that leads to higher transglycosylating efficiencies (Cobucci-Ponzano et al. 2011). Random mutagenesis has also been used to improve the transglycosylating activity of glycosynthases from families GH1 and GH52 up to 27- and 45-fold, respectively (Ben-David et al. 2007; Kim et al. 2004). Other studies have pursued more complex goals, aiming to change the substrate specificity of the enzyme through site-directed mutagenesis. This was the case of *Humicola insolens* Cel7B E197A mutant, a glycosynthase whose properties were altered by additional mutations at the +1 catalytic subsite. Mutations H209A, H209G, and A211T, combined with the original E197A mutation, were designed in order to widen the substrate specificity to include N-acetyl-glucosaminyl acceptors. However, the resulting mutants were unable to transfer α-lactosyl fluoride onto *N*′,*N*″-diacetylchitobiose, although cellobiose and *N*′-acetylchitobiose still remained as good acceptors (Blanchard et al. 2007a). In contrast, a change in the regioselectivity was observed using α-lactosyl fluoride as substrate donor and *O*-allyl N′-acetyl-2″-azido-ß-chitobioside as acceptor. While E197A and the triple mutant E197A/H209A/A211T preferentially catalyzed the formation of a ß-(1,3)-linked tetrasaccharide between the two disaccharides, the product specificity of the double mutants E197A/H209A and E197A/H209G was switched to a ß-(1,4) linkage (Blanchard et al. 2007b).

Honda and Kitaoka described in 2005 the first glycosynthase derived from an inverting glycosidase, the exo-oligoxilanase (GH8) from *Bacillus halodurans*. This glycosynthase was constructed by saturation random mutagenesis at the catalytic base D263. Nine of these mutants synthesized a xylotrisaccharide from xylose and α-xylobiose fluoride, with the D263C mutant showing the highest yield (Honda and Kitaoka 2006). An alternative method to convert the *Bacillus halodurans* exo-oligoxilanase into a glycosynthase was the mutagenesis of the Y198 residue, which is known to bind the water molecule acting as nucleophile in the hydrolytic reaction. This caused a drastic decrease in the hydrolytic activity and a small increase in the fluoride-releasing activity, which made the transglycosylation catalyzed by the Y198F mutant accumulate a larger amount of product than that achieved with the D263C mutant (Honda et al. 2008; Kitaoka et al. 2008). Another glycosynthase has been produced from the inverting 1,2-α-L-fucosidase (GH95) from *Bifidobacterium bifidum* by mutating the catalytic acid residue. The D766G mutant synthesized 2′-fucosyllactose from β-fucosyl-fluoride and lactose (Wada et al. 2008).

2.3.4 Enzyme Immobilization for Oligosaccharide Synthesis

Enzyme production is expensive for most industrial applications. Enzymes are labile and sensitive to denaturation by chemical agents and

physical conditions, which hinders their use. Moreover, unlike conventional chemical catalysts, most enzymes operate in aqueous solutions making it difficult to separate them from the final product for convenience or to reuse the enzyme in a new reaction (Homaei et al. 2013). Immobilization of enzymes offers a possible solution to these problems and allows their implementation in continuous flow reactors. There is an ample variety of immobilization methods, generally classified in three main types: covalent or noncovalent surface binding, physical entrapment, and self-aggregation by cross-linking (Mateo et al. 2007; Sheldon and van Pelt 2013). There is no universal method, or support to immobilization, because it depends on the nature of the enzyme, the chemical characteristics of the substrate, and the operating conditions. Compared to the free form, immobilized enzymes are generally more stable (to temperature, pH, etc.) and easier to manipulate (Homaei et al. 2013). Sometimes, immobilization changes the kinetic properties of an enzyme altering its activity or substrate specificity (Rodrigues et al. 2013). The immobilization of oligosaccharide-producing enzymes is a promising strategy for large-scale production of these compounds, and there are some examples in bibliography with different supports and enzymes.

Because of the importance of GOS in the food market, immobilization of ß-galactosidases to improve the yield of GOS production has been assayed by different groups (Panesar et al. 2010). *Aspergillus oryzae* ß-galactosidase was immobilized using three different techniques: adsorption on celite, covalent coupling to chitosan with glutaraldehyde, and self-aggregation by cross-linking. The best activity was reported by covalent binding to chitosan, yielding a higher GOS yield (17.3 % of the total sugar) than the free enzyme (10 %) (Gaur et al. 2006). In other instances, total GOS production remained unchanged, but the possibility of reusing the enzyme made the process interesting. That was the case of *Bacillus circulans* ß-galactosidase immobilized in glyoxal-derivatized agarose, which yielded the same GOS production and profile than the free enzyme but allowed carrying out ten repeated batches (Urrutia et al. 2013), or covalent immobilization of *Thermotoga maritima* ß-galactosidase on the surface of epoxy-activated magnetic beads which allowed four cycles of reutilization without a significant degree of inactivation (Marín-Navarro et al. 2014). Immobilized β-galactosidases have also been used in continuous operation systems for GOS production (Klein et al. 2013).

FOS-, COS-, IMOS-, and XOS-producing enzymes have also been subjected to immobilization by different methods. *Aspergillus flavus* fructosyltransferase was entrapped on chitosan and alginate showing better performance with the latter support. The thermal stability of the enzyme increased, and immobilization allowed work with a continuous system, accumulating up to 63 % (w/w) of FOS after 7 days of reaction (Ganaie et al. 2014). Immobilization of chitosanase from *Bacillus pumilus* by multipoint covalent attachment with glutaraldehyde, using as support amylose-coated magnetic nanoparticles, allowed reusability of the enzyme, producing COS with a yield of 40 % of the used chitosan (Kuroiwa et al. 2008). Endo-dextranase from *Penicillium* sp. was covalently attached to an epoxy-activated disk and used to produce IMOS. The immobilized enzyme retained more than 77 % of its activity for 78 days including storage time and repeated uses every 10 days, with a total operational time of 90 h (Bertrand et al. 2014). Alginate fiber entrapment enabled repeated use during ten batches of the *Leuconostoc mesenteroides* dextransucrase for IMOS production (Tanriseven and Doğan 2002). Production of XOS by xylan hydrolysis, using immobilized xylanases, has been successfully assayed. *Talaromyces thermophilus* xylanase, entrapped in gelatin and cross-linked by glutaraldehyde, yielded the same product profile than the free enzyme but allowed up to 13 successive cycles of hydrolysis (Maalej-Achouri et al. 2009). Xylanase B from *Thermotoga maritima* fused to a histidine tag was fixed covalently to a nickel-chelate epoxy support. The double functionality of this resin allows a specific attachment of the enzyme to the support through the histidine-nickel interaction and also has the advantage of a stable, covalent binding mediated by the epoxy groups. Immobilization improved the thermostability of the xylanase and allowed its use in a continuous process (Li et al. 2007a).

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