Glycosidases and Glycosynthases in enzymatic synthesis of oligosaccharides. An overview

Antoni Planas* and Magda Faijes.

Laboratory of Biochemistry, Institut Químic de Sarrià, Universitat Ramon Llull. Via Augusta, 390 - 08017 Barcelona, Spain.

Glicosidasas y Glicosintasas en síntesis enzimática de oligosacáridos. Una revisión.

Glicosidases i Glicosintases en síntesi enzimàtica d'oligosacàrids. Una revisió.

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RESUMEN

Las glicosidasas que actúan con retención de configuración han sido ampliamente estudiadas para la síntesis enzimática regio- y estereoselectiva de oligosacáridos y glicoconjugados como estrategia alternativa a los métodos químicos que requieren elaboradas etapas de protección, activación y desprotección. Este artículo revisa las diferentes metodologías enzimáticas desarrolladas para la síntesis de glicoconjugados: reacciones de transglicosidación catalizadas por glicosidasas "salvajes", analizando los recientes desarrollos en tecnología de medios de reacción, y glicosintasas, nuevas glicosidasas producidas por ingeniería de proteínas que son hidrolíticamente inactivas pero catalizan eficientemente reacciones de condensación y que se están actualmente desarrollando como una nueva herramienta en síntesis enzimática.

Palabras clave: **Síntesis enzimática. Glicosidasas. Glicosintasas. Oligosacáridos. Glicoconjugados.**

SUMMARY

Retaining glycosidases have been extensively studied for the synthesis of oligosaccharides and glycoconjugates as a convenient enzymatic approach to achieve the required regio- and stereoselectivity in glycoside bond formation as compared to chemical methods which require tedious protection, activation and deprotection steps. This paper reviews the different methodologies developed for enzymatic synthesis of glycoconjugates: classical transglycosylation reactions catalyzed by wild-type glycosidases outlining the recent developments in reaction conditions technology, and novel glycosynthases, engineered glycosidases lacking hydrolytic activity but efficiently catalyzing condensation reactions that are emerging as promising tools in the field.

Key words: **Enzymatic synthesis. Glycosidases. Glycosynthases. Oligosaccharides. Glycoconjugates.**

RESUM

Les glicosidases que actúen amb retenció de configuració han estat abastament estudiades per a la síntesi enzimàtica regio- i estereoselectiva d'oligosacàrids i glicoconjugats com estratègia alternativa als mètodes químics, els quals requereixen elaborades etapes de protecció, activació i desprotecció. Aquest article revisa les diferents metodologies enzimàtiques desenvolupades per a la síntesi de glicoconjugats: reaccions de transglicosidació catalitzades per glicosidases "salvatges", analitzant els recents desenvolupaments en tecnologies de medis de reacció, i glicosintases, noves glicosidases produïdes per enginyeria de proteïnes que són hidrolíticament inactives però catalitzen reaccions de glicosidació de forma eficient, i que s'estan desenvolupant actualment com una nova eina en síntesi enzimàtica.

Mots clau: **Síntesi enzimàtica. Glicosidases. Glicosintases. Oligosacàrids. Glicoconjugats.**

1. INTRODUCTION

Oligosaccharides, polysaccharides and glycoconjugates

Carbohydrates are key biomolecules in essentially all living organisms in which they play a variety of biological functions that include (a) structural components of cell walls and reserves of stored energy (oligo- and polysaccharides), (b) mediators of cell-cell interactions acting as partners and effectors in cellular communication between cells, host/symbion, and host/pathogen interactions (glycoconjugate molecules such as glycolipids and glycoproteins), and (c) control components of protein folding (glycoproteins) and structural elements involved in protein turnover and degradation within the cell[1, 2].

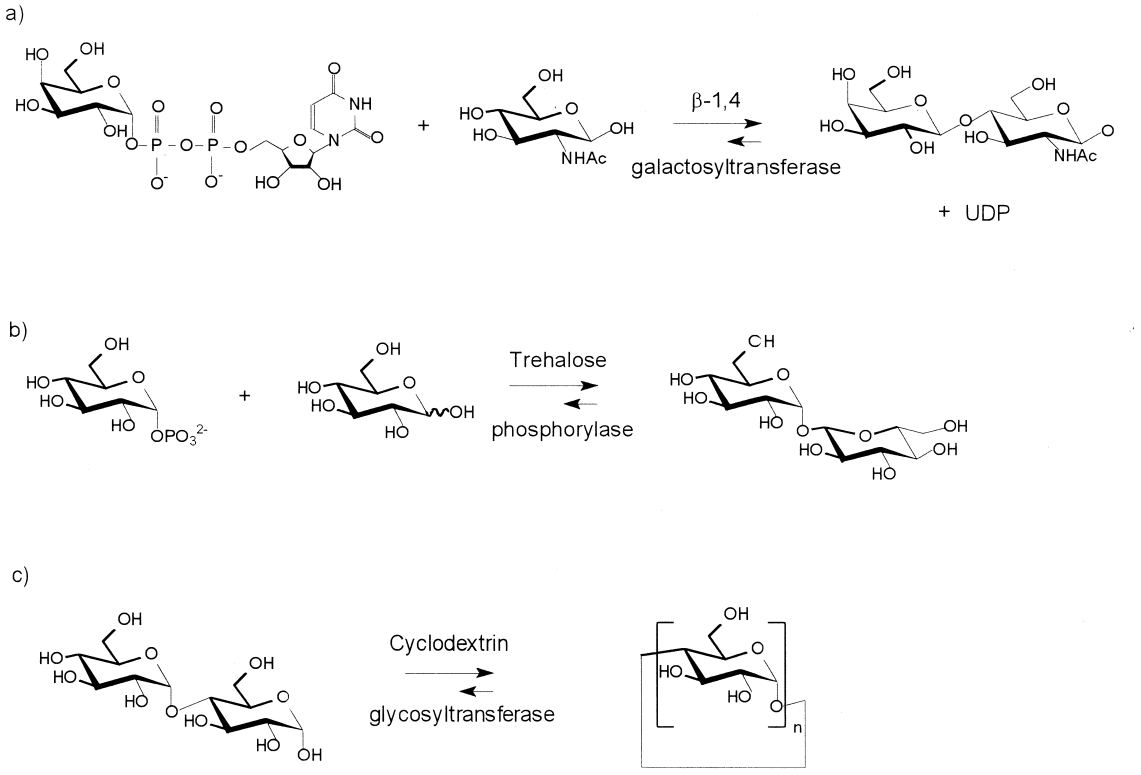
The fast growing field of *Glycobiology* seeks the understanding of the biological roles of carbohydrates and glycoconjugates, and the function of the repertoire of carbohydrate-acting enzymes involved in their biosynthesis, degradation and modification.
In this context, natural oligosaccharides as well as *neo*-glycoconjugates need to be synthesized for biochemical studies in glycobiology and as potential drugs directed to enzymes or receptors involved in their function and metabolism[3]. In addition, other interests in carbohydrate-based compounds relay on their applications as advanced materials due to their biocompatibility, structure-forming capacity, and environmentally benign properties (biopolymers, membranes, sensors, etc)[4].
Formation of glycosidic bonds between monosaccharide units in oligo- and polysaccharides is the key step in the preparation of such compounds, and it demands regio- and stereo-specificity to form defined linkages due to the high functionalization and similar reactivity of the different hydroxyl groups of any monosaccharide unit. Nature utilizes a battery of enzymes, glycosyltransferases and transglycosylases for building up these carbohydrate structures by forming glycosidic bonds in a regio- and stereo-specific fashion. On the other hand, glycosidic bonds are hydrolyzed by the action of glycosidases, also with high selectivity for the type and stereochemistry of the bond to be cleaved. Such structural complexity of carbohydrates has made chemical synthesis of oligosaccharides a challenging and difficult field, where new glycosylation methods are constantly appearing in the search of more general and reliable methods. Because of that, *in vitro* enzymatic synthesis is currently an active research field trying to mimic nature in order to include carbohydrate-acting enzymes with the desired specificity into chemoenzymatic schemes for the preparation of *glyco* compounds.

Glycosidic bond formation:
***in vivo* biosynthesis and *in vitro* synthesis**

In Nature, glycosidic bond formation for the assembly of oligo- and polysaccharides and the glycan structures on glycoconjugates is performed by glycosyltransferases. These enzymes catalyze the coupling (by a transfer reaction) of a glycosyl donor to an acceptor molecule forming a new glycosidic bond (Scheme 1) with a strict regio- and stereo-specificity[1, 5-8]. Depending on the donor molecule, glycosyltransferases are grouped in three main mechanistic classes:

a) *Leloir*-type glycosyltransferases. They require sugar nucleotides as donors for the transfer to an acceptor, and have a high degree of specificity, many of them catalyzing the formation of a unique linkage. Those involved in polysaccharide synthesis are procesive enzymes (*i.e.* glycogen synthase or cellulase synthase) which add sequentially a monosaccharide unit on the non-reducing end of the growing chain, whereas those involved in glycoprotein and glycolipid biosynthesis catalyze a single monosaccharide transfer (*i.e.* galactosyl, fucosyl, mannosyl, N-acetylglucosaminyl, and sialyl transferases).

b) *Non-Leloir* glycosyltransferases. Phosphorylases catalyze the phosphorolysis of polysaccharides. The reaction is reversible and these enzymes are also able to form glycosidic linkages using glycosyl phosphates as donors. The synthetic utility of glycosyl phosphorylases has been focused on few enzymes such as sucrose phosphorylase, trehalose phosphorylase, and plant phosphorylases for the synthesis of sucrose, trehalose and starch derivatives, respectively.



Scheme 1. Oligosaccharide biosynthesis by glycosyl transferases. (a) Leloir-type glycosyl transferase (Gal-UDP shown as sugar nucleotide donor); (b) phosphorylase; (c) transglycosidase.

c) *Transglycosylases.* These enzymes catalyze the glycosyl transfer from an oligosaccharide donor to an acceptor, and often have some residual hydrolytic activity. In terms of reaction mechanisms, glycosidases and transglycosidases fall into the same mechanistic class (see below). Relevant examples for their use in oligosaccharide synthesis are cyclodextrin glycosyltransferase, dextranase and levansucrase.

In vitro syntheses of oligosaccharides and glycan structures are currently performed by chemical methodologies and chemoenzymatic approaches. Chemical synthesis has evolved greatly in recent years because improved glycosyl donors and advanced synthetic methodologies have been developed. The complexity of oligosaccharide synthesis by chemical methods arise from the high functionality of the monosaccharide units where several hydroxyl groups have similar reactivities enabling a number of combinations (regio- and stereo-) to form glycosidic linkages between monosaccharides. Classical methods are based on stereoselective coupling reactions and laborious protecting-group manipulations (for general reviews see[9-12]).

To overcome some of the problems faced in chemical synthesis, enzymatic glycosylation has become an attractive tool in recent years; stereoselectivity is controlled by the enzyme and no protecting groups are required. Both glycoyltransferases and glycosidases are in use as biocatalyst for glycosyl bond formation. Glycosyltransferases, as being the biosynthetic enzymes *in vivo*, can be seen as the first candidates to oligosaccharide synthesis. A number of these enzymes have been cloned and purified for *in vitro* applications. However, some problems with their use as synthetic reagents are: (a) sugar nucleotide mono- or diphosphates as glycosyl donors are expensive to be used in multigram-scale synthesis (partially solved by coupling a biosynthetic pathway to produce *in situ* the activated donor), (b) the inhibitory effect of the nucleotide phosphate released in the condensation reaction (requiring either decomposition by adding a phosphatase, or better coupling an enzymatic cycle for cofactor regeneration), and (c) the limited availability of the enzymes, and the fact that most of the glycosyltransferases are membrane-bound enzymes, difficult to isolate or with reduced stability in solution (enzyme immobilization or protein engineering to express a truncated but active enzyme form have improved their use in some cases). Nevertheless, continuous progress in the study of these enzymes, cloning of new variants, protein engineering to improve stability, and solid-phase applications is changing the potential of glycosyltransferases in enzymatic oligosaccharide synthesis. Elegant examples have been reviewed recently[12-15].

On the other side, glycosidases are degrading enzymes that catalyze the hydrolysis of glycosidic bonds, but their normal hydrolytic reaction can be reversed under appropriate conditions[16]. Therefore, glycosidases have been extensively studied as biocatalysts for oligo- and polysaccharide synthesis. They are stable enzymes, easy to produce, a large number of enzymes from different organisms and with different specificities are available, and the glycosyl donors required are cheap compounds and easy to obtain in a multigram scale.

This paper reviews some of the methodologies developed for the use of glycosidases in the enzymatic formation of *O*-glycosidic bonds in oligo- and polysaccharides: classical transglycosylation reactions catalyzed by wild-type glycosidases outlining the recent developments in reaction conditions technology, and novel glycosynthases, engineered glycosidases lacking hydrolytic activity but catalyzing efficiently condensation reactions that are emerging as promising tools in the field.

2. TRANSGLYCOSYLATION BY RETAINING GLYCOSIDASES. CLASSICAL APPROACHES, NEW STRATEGIES

Glycosidases catalyze the hydrolysis of glycosidic bonds in oligo- and polysaccharides releasing a mono- or disaccharide from the end of the oligosaccharide chain (*exo*-acting enzymes) or cleaving an internal glycosidic bond (*endo*-acting enzymes). They fall into two mechanistic categories according to the stereochemical outcome of the bond cleavage (Scheme 2): inverting enzymes, which hydrolyze the glycosidic bond with inversion of configuration, and retaining enzymes, which do so with net retention of the anomeric configuration. Both types operate by general acid-base catalysis involving Asp, Glu or Tyr as catalytic residues, but differ in their mechanisms as a consequence of their active site topology[17-20]. Inverting glycosidases operate by a single-step mechanism in which a water molecule (with general base catalysis) effects a direct displacement at the anomeric center with protonic assistance by the general acid residue on the departing glycosidic oxygen (Scheme 2a). The catalytic residues are located at approximately 10 Å apart to each other allowing binding of the substrate and a water molecule in a ternary productive complex. By contrast, retaining glycosidases follow a double-displacement reaction via formation and hydrolysis of a glycosyl-enzyme intermediate, and have the catalytic residues closer to each other at *c.a. 5.5* Å (Scheme 2b). In the first step (*glycosylation*) the amino acid residue acting as a general acid protonates the glycosidic oxygen while the deprotonated carboxylate functioning as a nucleophile attacks the anomeric center with concomitant C-O breaking of the scissile glycosidic bond leading to a covalent glycosyl-enzyme intermediate. The second *deglycosylation* step involves the attack by a molecule of water assisted by the conjugate base of the general acid residue which renders the free sugar with overall retention of configuration, and the enzyme returns to its initial protonation state.

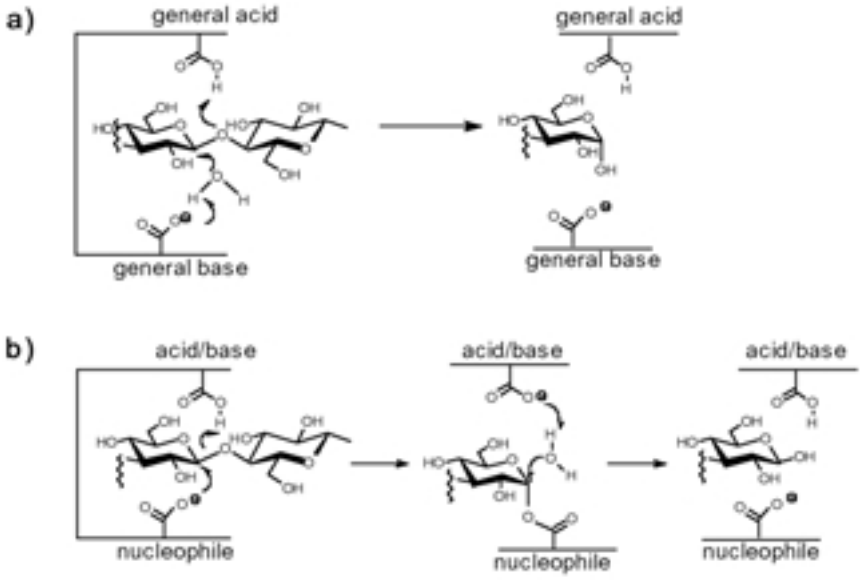
Under conditions that favor reversal of their normal hydrolytic reaction, retaining glycosidases have been extensively used as catalysts in oligosaccharide synthesis. This may be achieved either by displacing the equilibrium towards glycosidic bond formation (*thermodynamically controlled synthesis*) or by using activated glycosyl donors *(kinetically controlled transglycosylation)*.

a) Thermodynamic approach (reversed hydrolysis):

The hydrolysis-synthesis equilibrium is balanced by approximately 4 kcal·mol^{-1} towards bond cleavage under ambient aqueous conditions[16]. The thermodynamically controlled synthesis approach involves the direct shift of the equilibrium towards products (Scheme 3) by altering the reaction conditions such as:

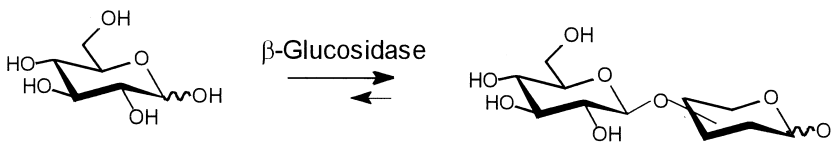
(a) using high substrate concentrations[21], including the use of the acceptor as the reaction solvent as reported for the synthesis of alkyl glycosides by different glycosidases in which the sugar is dissolved in the alcohol acceptor[22-26].

(b) addition of a water-miscible organic cosolvent to reduce the activity of water, often limited to low organic solvent contents due to enzyme unstability or reduced sugar solubility, but with some exceptions. For example, almond β-glucosidase was stable and active for days in 90% aqueous acetonitrile at 40 ºC or *in tert*-butanol at 50 ºC[24, 25, 27]. *Aspergillus oryzae* β-galactosidase retained 90% of activity in di- or monoglyme, 75% in N,N-dimethylformamide, dioxane or acetone, but only 10% of activity in acetonitrile[28].



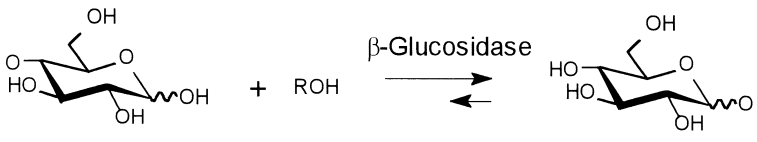
Scheme 2. Enzymatic hydrolysis of glycosidic linkages catalyzed by glycosidases. (a) Inverting glycosidase; (b) retaining glycosidase. The catalytic residues in the enzyme active site are indicated to show their function in the enzyme mechanism.

a)



yield:	β-1,6	β-1,2 + β-1,3	β-1,4	overall
37 ºC	19.7%	8.0%	3.6%	31.3%
55 ºC	25.7%	11.5%	4.2%	41.4%

b)



alcohol	alcohol/water ratio	yield(%)
$-(CH_2)_6-OH$	90:10	61
$-(CH_2)_3-CH=CH_2$	92:8	50
$-(CH_2)_2-Si(CH_3)_3$	55:5.6	11

Scheme 3. Thermodynamically-controlled synthesis (reversed hydrolysis) by wild-type glycosidases.

(c) higher temperatures have shown to increase the synthesis yields as demonstrated with almond β-glucosidase (Scheme 3), where a 10% yield increase was obtained for disaccharides formation from glucose when temperature was raised from 37 to 55 ºC[21], or yields as high as 62% were achieved in alkyl glycoside formation at 50 ºC[24].

Most of the reported examples by the thermodynamic approach are for the preparation of simple glycosides (mainly disaccharides)[22, 29] or glycosides of simple hydrophilic alcohols[23] using *exo*-glycosidases (β-galactosidases, α- and β-glucosidases, α-mannosidases). Yields are often low (< 20%) and purification procedures are difficult, and this methodology is less common than the kinetically-controlled transglycosylation.

b) Kinetic approach *(transglycosylation)*:

As illustrated in Scheme 4, the glycosyl-enzyme intermediate that builds-up in the mechanism of a retaining glycosidase is attacked by a water molecule leading to the hydrolysis product. Under appropriate conditions, a nucleophilic acceptor other than water may intercept the intermediate and form a new glycosidic bond with an anomeric configuration in the product identical to that of the original donor. The kinetically-controlled transglycosylation depends on the more rapid trapping of the reactive intermediate by the glycosyl acceptor than by water. This approach relies on the fast formation of the reactive intermediate from an activated glycosyl donor, and a suitable glycosyl acceptor that reacts as a nucleophile faster than water. Although glycoside formation may be favored kinetically, hydrolysis always remains favored thermodynamically because the newly formed product is itself a substrate for the enzyme, and the equilibrium is slowly displaced towards hydrolysis as represented in Scheme 4. Therefore, reaction time and experimental conditions must be carefully controlled. A large number of examples and conditions have been reported in the literature, and excellent reviews have summarized the different strategies (see, for example Wong and Whitesides[16], Wong and co.[13, 30], Murata and Usui[31], Withers and co.[29], Thiem and co.[32], Fernández-Mayoralas[22], Sheldon and co.[23], and Flitsch[33]). Some of the most common strategies and considerations for efficient kinetically-controlled transglycosylation by wild-type retaining glycosidases can be rationalized as follows:

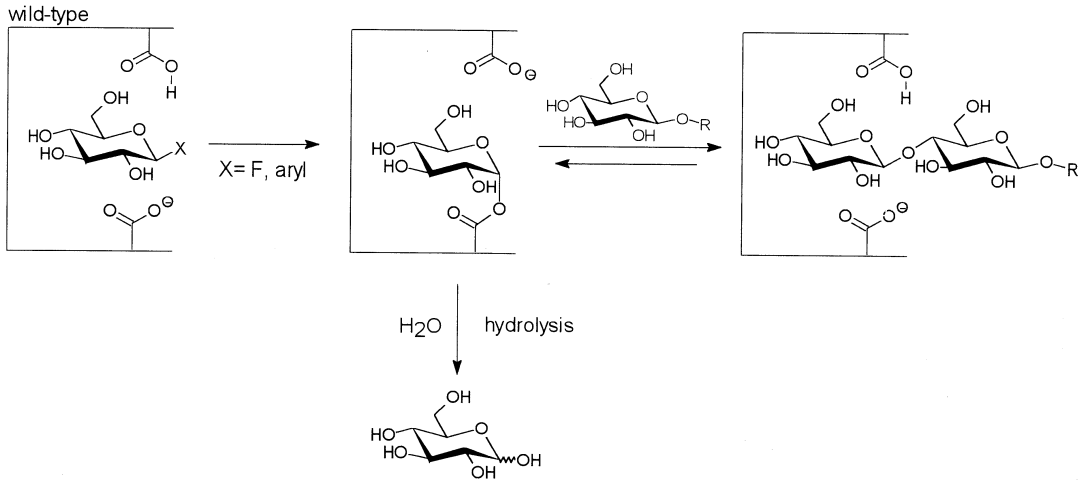
(a) Use of high acceptor concentration, above its Kd for the acceptor site in the enzyme, to occupy the acceptor subsites and favor transglycosylation over hydrolysis of the glycosyl-enzyme intermediate[34].

(b) Use of highly reactive glycosyl donors, most commonly activated donors such as aryl glycosides and glycosyl fluorides[35]. In some cases, disaccharides have been used as donors with *exo*-glycosidases (the overall reaction thus being a glycosyl transfer between sugars), as for example lactose with β-galactosidase and N-acetylglucosamine as acceptor to yield N-acetyllactosamine[34]. However, the glucose released as a leaving group can itself compete as an acceptor. Activated glycosides (Scheme 5, a-b) are a better choice for two reasons. First, the departed aglycone (F^- or a phenol) is a poor acceptor and will not compete with the actual acceptor molecule. Secondly, the activated glycosyl donor is often a better substrate of the enzyme (in terms of k_{cat}/K_M) than the saccharide product formed, thus reducing the competing hydrolysis reaction of the product. In other words, a fast-reacting donor keeps the reaction times short, and hence decreases the time available for product hydrolysis, and tight binding of the donor (low K_M) also inhibits product hydrolysis. In addition to glycosyl fluorides and aryl glycosides, other less common glycosyl donors have also been used:

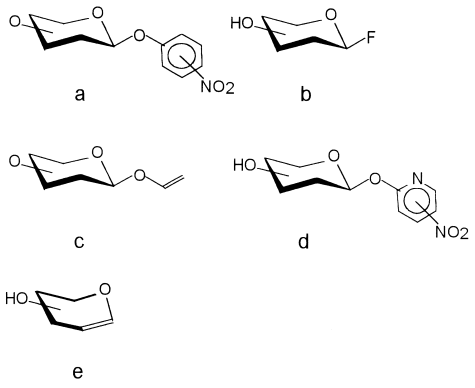
– vinyl-β-galactoside (Scheme 5,c) reacting with methyl α-galactoside catalyzed by *Aspergillus oryzae* β-glycosidase increases the transglycosylation yield at low temperatures (as compared to a *p*-nitrophenyl β-galactoside donor) due to the higher solubility of the vinyl donor at low temperatures[36].

– nitropyridyl glycosides (Scheme 5,d) with β-galactosidase, β-glucosidase and N-acetyl-β-hexosaminidase-catalyzed transglycosylations enable reactions under high donor concentrations due to their high solubility and reactivity, thus resulting in higher yields than those with conventional *p*-nitrophenyl glycosides[37].

– glycals (Scheme 5,e) have also been reported as glycosyl donors in some cases to produce 2-deoxy-glycosides[38, 39].



Scheme 4. Transglycosylation catalyzed by a retaining glycosidase.



Scheme 5. Activated glycosyl donors used in kinetically-controlled transglycosylations by retaining glycosidases: (a) nitrophenyl glycosides, (b) glycosyl fluorides, (c) vinyl glycosides, (d) nitropyridyl glycosides, and (e) glycals.

TABLE I

Enzyme	Donor	Acceptor	Product	Yield (%)
α-galactosidase	α-Gal-OPNP	β-Gal-OMe α-Gal-OMe	α-Gal-1,6-β-Gal-OMe α-Gal-1,3-α-Gal-OMe	18 27
β-galactosidase	β-Gal-OPNP	β-Xyl-OMe β-Xyl-OBn	β-Gal-1,4-β-Xyl-OMe β-Gal-1,3-β-Xyl-Obn	6 21
	β-Gal-OPNP	β-Gal-OMe β-Gal-OMe(6-OAc)	β-Gal-1,6-β-Gal-OMe β-Gal-1,3-β-Gal-OMe β-Gal-1,3-β-Gal-OMe(6-OAc)	6 8 15
β-glucosidase	β-Gal-OPNP	β-Gal-SPh β-Man-SPh	β-Gal-1,3-β-Gal-SPh β-Gal-1,4-β-Man-SPh	9 24

(c) Transglycosylation is a stereoselective process where the α or β linkage formed is dictated by the enzyme, but regioselectivity is not necessarily absolute or predictable. *Endo*-glycosidases (such as cellulases, α-amylases, 1,3-1,4-β-glucanases, xylanases...) are highly regioselective in transglycosylation reactions maintaining the same specificity as that of their corresponding hydrolytic reaction on oligo- and polysaccharides (see below). For *exo*-acting glycosidases, however, mixtures of products with different glycosidic bonds are often observed. In general, the primary hydroxyl group of the acceptor reacts preferentially over secondary hydroxyl groups, resulting in 1,6-glycosidic linkages[22]. Selectivity can be controlled to some extent:

1) selecting an appropriate donor/acceptor combination (Table I). The configuration of the anomeric center of the acceptor, or the aglycone group of the acceptor influences the regioselectivity of glycosylation. In the case of coffee bean α-galactosidase with *p*-nitrophenyl α-galatoside as donor, mainly α-1,6-linked disaccharide is formed with the methyl β-galactoside acceptor, whereas an α-1,3 disaccharide was obtained as major product with the methyl α-galactoside[40]. The effect of the aglycon is illustrated by the *E. coli* β-galactosidase transglycosylation of *p*-nitrophenyl β-galactoside with two acceptors, methyl β-xyloside which gives exclusively the β-1,4 disaccharide, and benzyl β-xyloside which provided the β-1,3 regioisomer as the major product, the yield increasing five-fold in the latter reaction[41]. The sugar acceptor may also induce regioselectivity; mannosyl or galactosyl glycosides as acceptors for the reaction of *Agrobacterium faecalis* β-glucosidase with the *p*-nitrophenyl β-galactoside donor give exclusively the β-1,4 or β-1,3 disaccharides, respectively[38]. A singular example has been reported with the *E. coli* β-galactosidase in which a 6-*O*-acetyl protected monosaccharide acceptor prevents 1,6-glycosidic bond formation, thus redirecting specificity towards β-1,3-glycosyl linkage[42, 43].

2) glycosidases from different species often show altered regioselectivities with preference for different hydroxyl groups of the same acceptor. Galactosylation of D- or L-xylose produces mixture of three regioisomers, β-1,2, β-1,3 and β-1,4, in different ratios depending on the source of the β-galactosidase used. For the *A. oryzae* enzyme, 64% of the total disaccharide products was the β-1,4 isomer, and only 7% of the β-1,2 disaccharide, whereas 58% of β-1,2 and 10% of β-1,4 disaccharides were obtained with the *S. fragilis* β-galactosidase[44].

3) engineering experimental conditions. The type and amount of organic co-solvent added to the buffered aqueous solution may modify the proportion of different regioisomers as observed for the *A. oryzae* and *S. fragilis* β-galactosidases catalyzing the transglycosylation of *p*-nitrophenyl β-galactoside with 3-*O*-methyl glucose[34]. An inclusion complex of the acceptor with a cyclodextrin has been shown to change regiospecificity in a N-acetyl hexosaminidase-catalyzed reaction where β-1,6' and β-1,3 linkages are preferentially formed as compared to β-1,6 and β-1,3 and β-1,4 bonds in the absence of cyclodextrin[31].

In general, synthetic yields are rather low, with better results using the kinetic approach (up to 40-50%) than the thermodynamic approach (commonly lower than 20%). The use of organic cosolvents to decrease the activity (or effective concentration) of water is the most common way to improve yields, but no general rules can be outlined because the type and amount of organic cosolvents varies from one enzyme to another depending on their stability as well as the solubility of the substrates.

To reduce the extend of hydrolysis, some other strategies have been devised which give rise to different, and often complementary, methodologies:

(a) Removal of the transglycosylation product from the reaction mixture. Ideally, if the product formed were continuously and selectively removed from the reaction without affecting the enzyme, high transglycosylation yields would be possible. Some examples illustrate this concept:

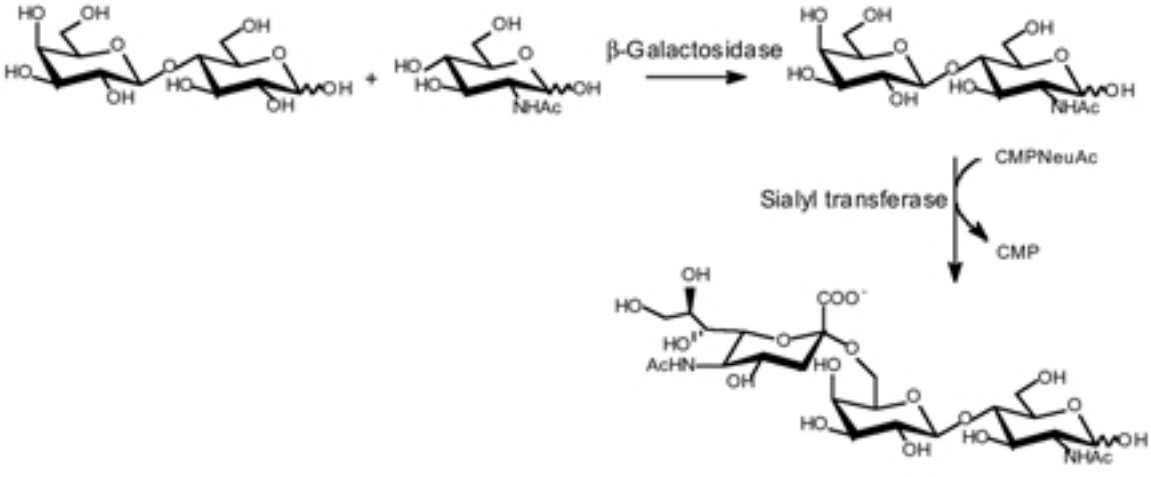
– crystallization or insolubilization of the product *in situ*, as in the case of self-condensation of di-N-acetylchitobiose catalyzed by hen egg white lysozyme under thermodynamically-controlled synthesis in the presence of a high concentration of ammonium sulfate, where an hexamer and heptamer oligosaccharides precipitated[45].

– absorption of the product on an activated charcoal column, but restricted to preferential absorption of disaccharides when reacting monosaccharides donor and acceptor by an *exo*-glycosidase, illustrated by the condensation of glucose by *Penicillium funiculosum* β-glucosidase[46].

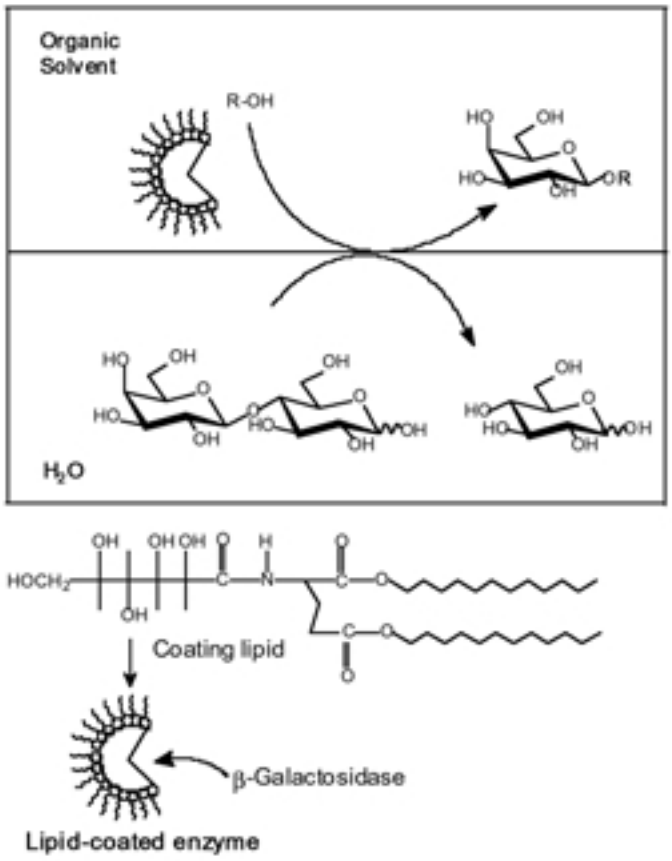
– coupling the reaction to another enzymatic process which will transform selectively the transglycosylation product into another compound that is no longer an hydrolyzable substrate of the glycosidase. *Bacillus circulans* β-galactosidase (Scheme 6) produces preferentially N-acetyllactosamine that, coupled with a α-2,6-sialyltransferase, was converted to a trisaccharide which is not hydrolyzed by the β-galactosidase, thus improving the overall yield[47].

(b) Enzyme immobilization has become a widespread methodology that provides many advantages compared with free enzymes in solution. Immobilization on supports allows re-use of the enzyme, continuous operation and simplified and efficient processes, including higher stability in organic solvents, modified pH range of operation, and higher temperatures. A number of solid supports for enzyme immobilization are available, from natural or synthetic polyhydroxylic matrixes, porous inorganic carriers to a variety of functional polymers[48, 49]. Using *Bacillus circulans* β-galactosidase immobilized on CNBr-Sepharose, the enzyme thermostability and stability in the presence of DMF was increased, and optimization of the *p*-nitrophenyl β-galactopyranoside and benzyl-α-N-acetylgalactosamine transglycosylation afforded a β-1,3-linked disacharide with 62% molar yield in a gram-scale synthesis[50]. With immobilized β-glucosidase, direct alkylation of glucose with hexanol gave a higher conversion yield and product concentration than that obtained with the soluble enzyme[51]. Immobilized β-galactosidases have been extensively exploited for the production of galactooligosaccharides from lactose in the food industry. For example, *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth achieved a maximum galactooligosaccharides production of 27% at 50% lactose conversion with 500 g/L of initial lactose concentration. The thermal stability of the enzyme has increased 25-fold upon immobilization and the half-life was more than 1 year at 40 ºC and 48 days at 50 ºC[52].

(c) Lipid-coated enzymes are stable and active in organic media, and have recently shown to be appropriate for synthetic reactions catalyzed by hydrolases (lipases, proteases, glycosidases). They are prepared by mixing an aqueous solution of the enzyme and an aqueous dispersion of the lipid followed by lyophilization of the precipitate. The resulting complex is insoluble in buffer solutions but is freely soluble in most organic solvents such as benzene, ethyl acetate, isooctane, isopropyl ether, DMSO, and ethanol. The lipid-coated system has been successfully used with glycosidases. Lipid-coated α-mannosidase from *Jack beans* catalyzes the transglycosylation of *p*-nitrophenyl α-mannoside as donor to 5-phenyl-1-pentanol in isopropyl ether at 30 ºC with yields close to 80% in condensation product[53]. With β-galactosidase from *E. coli*, a two-phase aqueous-organic system has been used when the glycosyl donor is a water-soluble saccharide[54] (Scheme 7): the lipid-coated enzyme and hydrophobic alcohol acceptors are in the organic phase whereas lactose as donor is mainly in the aqueous phase. The transglycosylation product was obtained in 66% yield and no galactose (from donor or product hydrolysis) was detected. Supercritical fluids have also been reported as efficient solvents for lipid-coated enzymes. In supercritical carbon dioxide or fluoroform, lipid-coated β-galactosidase gave transglycosylation products in 80-90% yields, with rates faster than reactions in isopropyl ether as organic solvent[55, 56].



Scheme 6. Synthesis of a sialylated trisaccharide by coupling a α-2,6-sialyltransferase to displace the β-galactosidase-catalyzed transglycosylation of lactose with N-acetylglucosamine.



Scheme 7. Two-phase system for transglycosylation with a lipid-coated retaining glycosidase.

Of particular relevance is the *in vitro* synthesis of polysaccharides by *endo*-glycosidases under kinetically-controlled conditions. The regioselectivity is almost absolute as opposed to *exo*-glycosidases, providing a valuable methodology for the preparation of biopolymers. Relevant examples are (Scheme 8):

(a) Artificial cellulose was produced by cellulase-catalyzed polycondensation of β-cellobiosyl fluoride in acetonitrile/acetate buffer[57, 58]. When a crude cellulase was employed for the enzymatic polymerization, the cellulose II allomorph, an stable crystalline form with anti-parallel chain packing, was obtained. Interestingly, metastable crystalline cellulose I with parallel chain packing was obtained with a purified cellulase preparation. Since other enzymes and protein components were present in the crude extract, these results indicate that the relative intermolecular direction of growing glucan chains is controlled in the propagating process of enzymatic polymerization[59]. An unnatural cellulose derivative has also been prepared by enzymatic polymerization of 6-*O*-methyl-β-cellobiosyl fluoride as substrate of cellulase, giving rise to an alternating 6-*O*-methylated cellulose derivative[60]. Similarly, thiocello-oligosaccharides were synthesized using 4-thio-β-cellobiosyl fluoride as donor in acetonitrile/buffer system[61]. The *Humicola insolens* cellulase Cel7B also catalyzes kinetically-controlled transglycosylations and it has been used for the preparation of a bifunctionalyzed fluorogenic cellotetraoside as substrate for the screening of cellulases[62].

(b) Maltooligosaccharides (up to 12 glucose units) were prepared by polymerization of α-maltosyl fluoride using an α-amylase in various organic-water mixture of solvents[63, 64]. Without organic cosolvent, oligosaccharides up to maltopentaose were observed at the early stage of polymerization, but they were finally converted to glucose and maltose. The formation of odd-numbered maltooligosaccharides from a disaccharide donor should be due to enzymatic hydrolysis of products during the reaction.

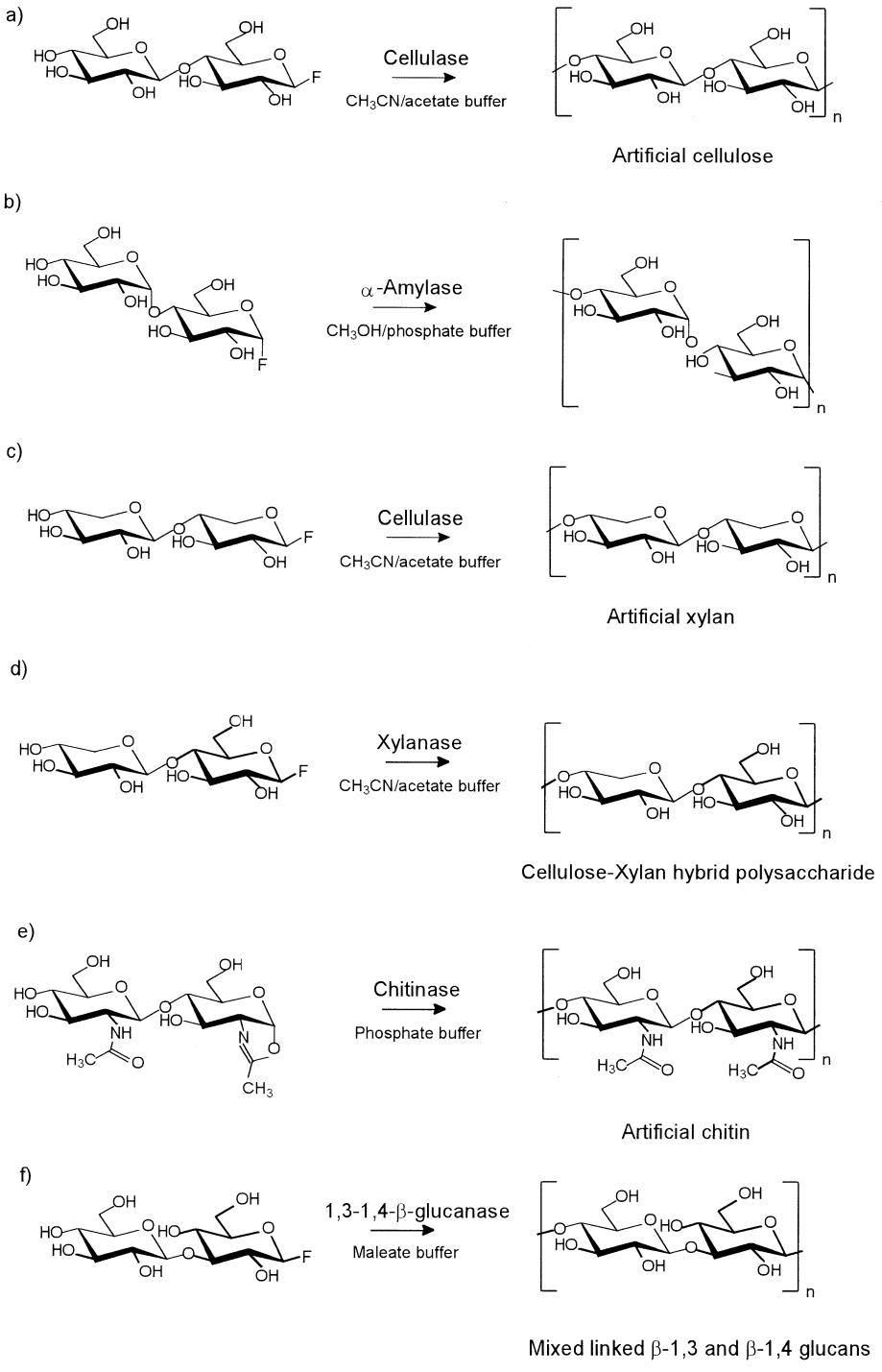
(c) Xylan was also produced by enzymatic polymerization of β-xylobiosyl fluoride in acetonitrile/acetate buffer with a crude cellulase containing xylanase activity[65]. The artificial xylan with all β-1,4 glycosidic bonds differs from the natural xylan (principal component of hemocellulose in plant cell walls) in that the natural polymer also contains other monosaccharides (methylglucuronic acid, L-arabinose,...) as minor units in the side chain.

(d) A hybrid cellulose-xylan polysaccharide was obtained with a xylanase using a disaccharide donor composed of two different monosaccharyl units. Condensation of 4-β-xylopyranosyl-α-glucopyranosyl fluoride gave rise to hybrid polymer with a degree of polymerization up to 12 saccharide units with all β-1,4 glycosidic linkages[66].

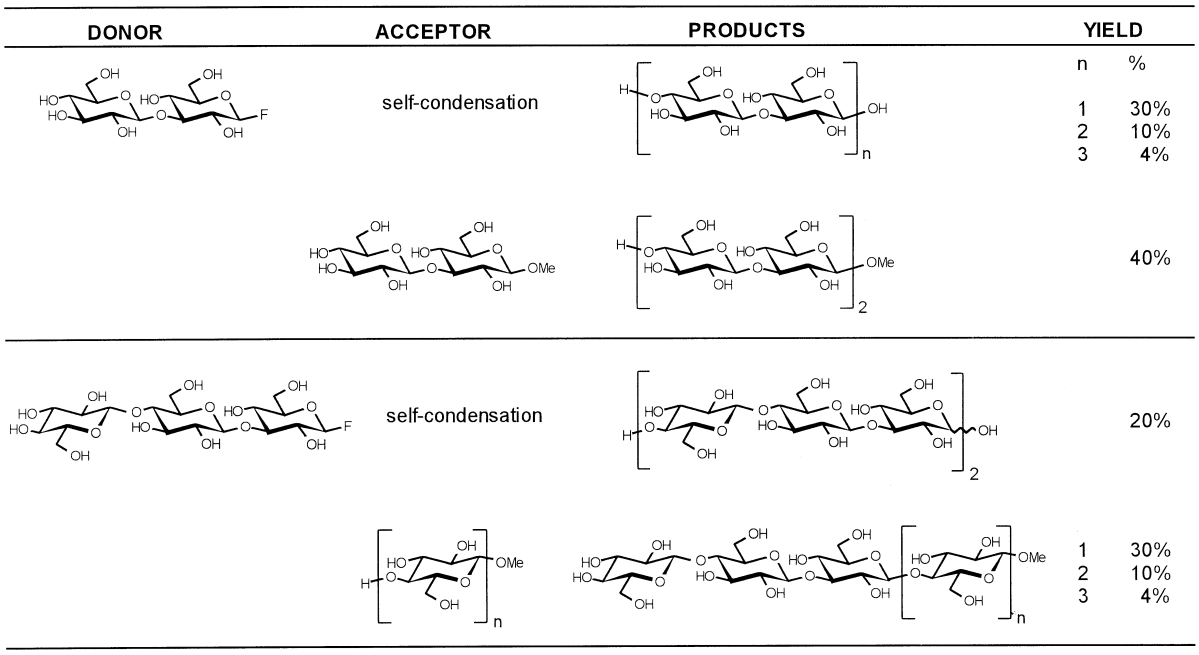
(e) The first enzymatic synthesis of chitin used chitobiose oxazoline as glycosyl donor for polycondensation by chitinase. In this case, the oxazoline derivative has a distor-

ted conformation that mimics the transition state structure in the hydrolytic mechanism of a retaining chitinase. By choosing the appropriate pH value (10.6), the hydrolytic activity of the enzyme is significantly suppressed and self-condensation of the disaccharide derivative leads to an artificial chitin oligomer[59, 67].

(f) Mixed linked β-1,3 and β-1,4 glucans have also been produced by enzymatic transglycosylation catalyzed by *Bacillus licheniformis* 1,3-1,4-β-glucanase. β-glycosyl fluorides are good glycosyl donors but not *p*-nitrophenyl β-glycosides[68]. Self-condensation of β-laminaribiosyl fluoride or β-glucosyl-β-1,4-laminaribiosyl fluoride give a mixture of oligomers, the reactions proceeding up to the formation of an insoluble polymeric material. In the presence of an acceptor saccharide, different transglycosylation products are produced as summarized in Scheme 9, where the newly formed glycosidic bonds are only β-1,4. Glucooligomers containing different combinations of β-1,3 and β-1,4 glycosidic linkages have been produced in this way in our group[68-70].



Scheme 8. Oligosaccharide/polysaccharide synthesis by enzymatic polymerization catalyzed by retaining *endo*-glycosidases.



Scheme 9. Transglycosylation reactions catalyzed by *B. licheniformis* 1,3-1,4-β-glucanase.

3. GLYCOSYNTHASES: NOVEL ENGINEERED GLYCOSIDASES

In spite of the improvements in reaction conditions and technologies reviewed above, the major drawback of retaining glycosidases in glycoside synthesis is hydrolysis of the newly-formed glycosidic linkage, and transglycosylation yields rarely exceed 50%.

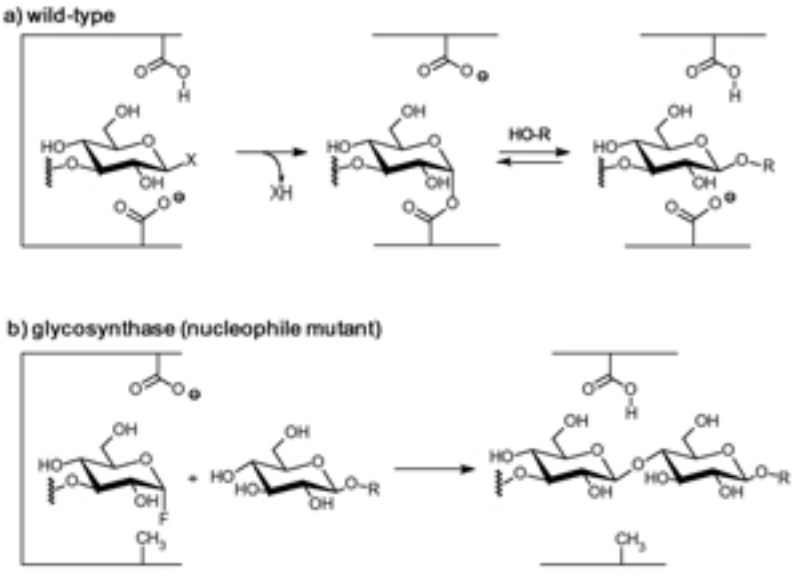
A novel strategy based on the redesign of the enzyme's catalytic machinery is currently being developed. The glycosynthase concept was introduced in 1998 by the Withers' group on an *exo*-glycosidase[71] and extended to *endo*-glycosidases by our group[72]. A glycoynthase is a specifically mutated retaining glycosidase in which site-directed mutation of the catalytic nucleophile by a non-catalytic residue (Ala, Gly or Ser) renders a hydrolytically inactive enzyme, yet able to catalyze the transglycosylation of glycosyl fluoride donors having the opposite anomeric configuration of that of the normal substrates of the parental wild-type enzyme. The transglycosylation products formed are not hydrolyzed, and production yields are as high as 95-98% in some cases.

The rationale of this methodology is depicted in Scheme 10. The upper part of the scheme shows the mechanism of the kinetically-controlled transglycosylation by the wild-type enzyme, in which the covalent glycosyl-enzyme intermediate is the actual donor for glycosyl transfer to an acceptor or hydrolysis by transfer to water. Even though the hydroxyl group of the sugar acceptor is a better nucleophile than water and transglycosylation is kinetically favored, this step is reversible and the process is shift towards hydrolysis. In the glycosynthase (Scheme 10b), mutation of the catalytic nucleophile disables the enzyme as a hydrolase because no glycosyl-enzyme intermediate can be formed; the same glycosyl donors that would be used with the wild-type enzyme (*i.e.* aryl β-glycosides or β-glycosyl fluorides for a β-glycosidase) will not react with the glycosynthase. But an activated glycosyl donor with an anomeric configuration opposed to that of the donor substrate in the wild-type reaction (*i.e.* an α-glycosyl fluoride for a β-glycosidase) would mimic the glycosyl-enzyme intermediate and then be able to react with an acceptor. The cavity created in the active site by mutation of the carboxylate residue acting as nucleophile in the wild-type enzyme by a smaller residue allows binding of the glycosyl fluoride with opposed anomeric configuration. As with the wild-type enzyme, transglycosylation is kinetically favored but the transglycosylation reaction is now irreversible because of the lack of the catalytic nucleophile; the product is no longer hydrolyzed and accumulates to give high transglycosylation yields.

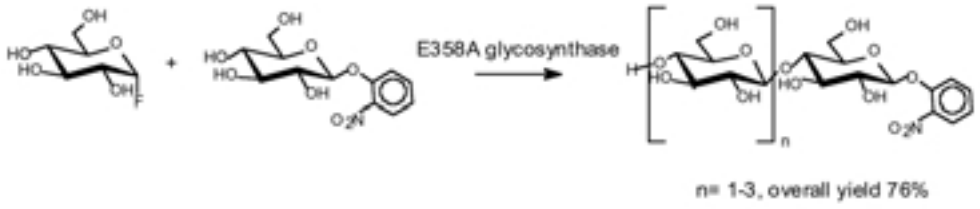
There are currently 8 glycosynthases developed for oligosaccharide synthesis (described below), and many other might be under study as this methodology is showing to be a powerful tool for biocatalysis. Glycosynthases derived from both *exo*- and *endo*-acting glycosidases have been produced with complementary applications in their use. *Exo*-glycosynthases will add a monosaccharide on the non-reducing end of an oligosaccharide acceptor, whereas *endo*-glycosynthases will be able to condense oligosaccharides for the synthesis of complex glycoconjugates as they accept oligosaccharide donors.

Exo-glycosynthases

a) β-Glucosidase from *Agrobacterium* sp. The first reported glycosynthase was that derived from the β-glucosidase/galactosidase from *Agrobacterium* sp.[71]. It is a family 1 glycosidase with a broad specificity. The E358A mutant catalyzes the condensation of β-glucosyl fluoride with a range of carbohydrate acceptors in good to excellent yields. With 2-nitrophenyl β-glucoside as acceptor, a mixture of di-, tri-, and tetra-saccharides were readily isolated in 76% overall yield (Scheme 11). The disaccharide product arising from donor-acceptor condensation was obtained in



Scheme 10. Glycosidase-catalyzed glycosidic bond formation. (a) Wild-type enzyme (kinetically-controlled transglycosylation). (b) Glycosynthase (nucleophile-less mutant enzyme) using an α-glycosyl fluoride donor.



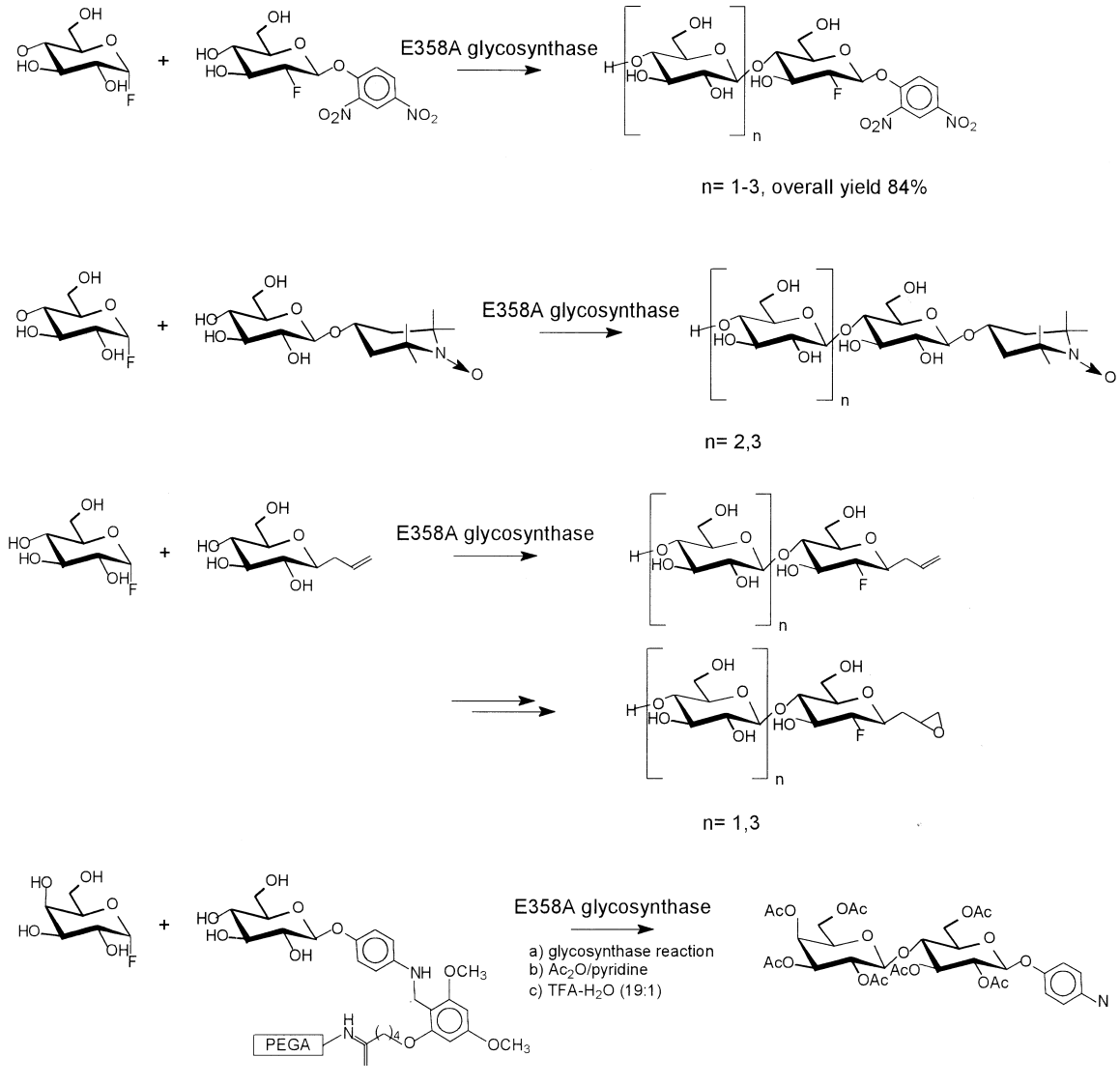
Scheme 11. Glycosynthase reactions catalyzed by E358A β-glucosidase from *Agrobacterium faecalis.*

41% yield, but higher oligomers were also obtained as the result of elongation reaction in which the products themselves act as acceptors to condense with the glucosyl fluoride donor. With α-galactosyl fluoride as donor, single condensation products were obtained because the *galacto* configuration of the donor and the products on their non-reducing end precludes self-condensation of the donor and further elongation reactions of the products. The regiochemistry of the newly formed glycosidic bonds is mainly β-1,4, following the specificity of the wild-type enzyme in the hydrolase activity. However, a xyloside acceptor produces β-1,3-linked oligosaccharides.

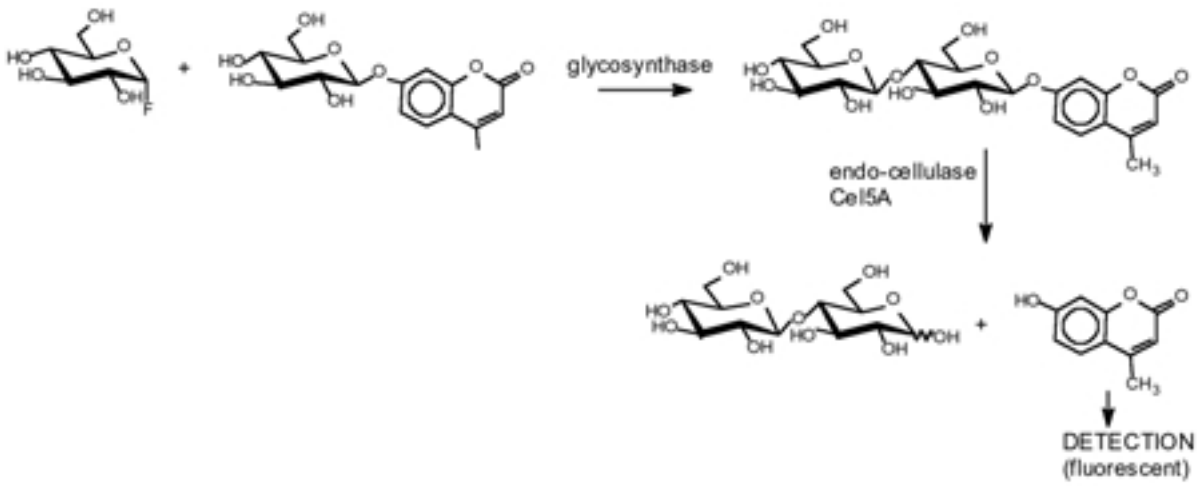
A second variant of this glycosynthase with a serine instead of alanine mutation (E358S β-glucosidase) showed improved glycosynthase activity[73]. It has 24-fold higher synthetic rates and it has been claimed to be due to some favorable hydrogen-bond interaction of the serine hydroxyl group with the anomeric fluoride of the donor in the transition state resulting in enhanced catalysis. Interestingly, an N-acetylglucoside acceptor was glycosylated by the E358S glycosynthase when it was not an acceptor of the E358A enzyme, thus showing that the enhanced reactivity broadens the acceptor specificity.

Some interesting compounds have been prepared by using the *Agrobacterium* glycosynthase as summarized in Scheme 12: 2-deoxy-2-fluoro glycosides as inhibitors of retaining glycosidases[71], spin-labeled cellooligosaccharides as probes for binding studies of cellulose-binding domains of cellulases[74], cellobiosyl *C*-glycosides as precursors for the synthesis of mechanism-based inactivators of cellulases[75, 76], and more recently, a glycopeptide as the first and promising example of solid-phase oligosaccharide and glycopeptide synthesis using a glycosynthase[77].

Based on the *Agrobacterium* β-glucosidase model, a screening strategy for the identification of potential glycosynthase mutants from random mutagenesis has been proposed. It involves the use of a coupling glycosidase which is able to specifically hydrolyze the condensation product produced by the «glycosynthase»[78]. As shown in Scheme 13, the library of mutants, from which a new or improved glycosynthase wants to be identified, is treated with the glycosyl fluoride donor and an acceptor saccharide bearing a fluorogenic aglycon. If a glycosynthase-catalyzed condensation product is formed, it is now the substrate for a coupling glycosidase that is coexpressed in the same cells. The coupling glycosidase has to be carefully selec-



Scheme 12. Some products obtained by the E358A *Agrobacterium* glycosynthase.



Scheme 13. Screening method for glycosynthase mutants by coupling the condensation product to a specific glycosidase with release of a chromophoric aglycon.

ted since it has to be able to hydrolyze the product of the glycosynthase reaction with release of the fluorogenic aglycon, but unable to hydrolyze the acceptor molecule. This approach has been successfully applied to screen a library of single point mutations of the *Agrobacterium* β-glucosidase with the cellulase Cel5A from *Cellulomonas fimi* as the coupling enzyme[78]. This is currently a unique example, but other examples may be developed provided suitable coupling enzymes are selected.

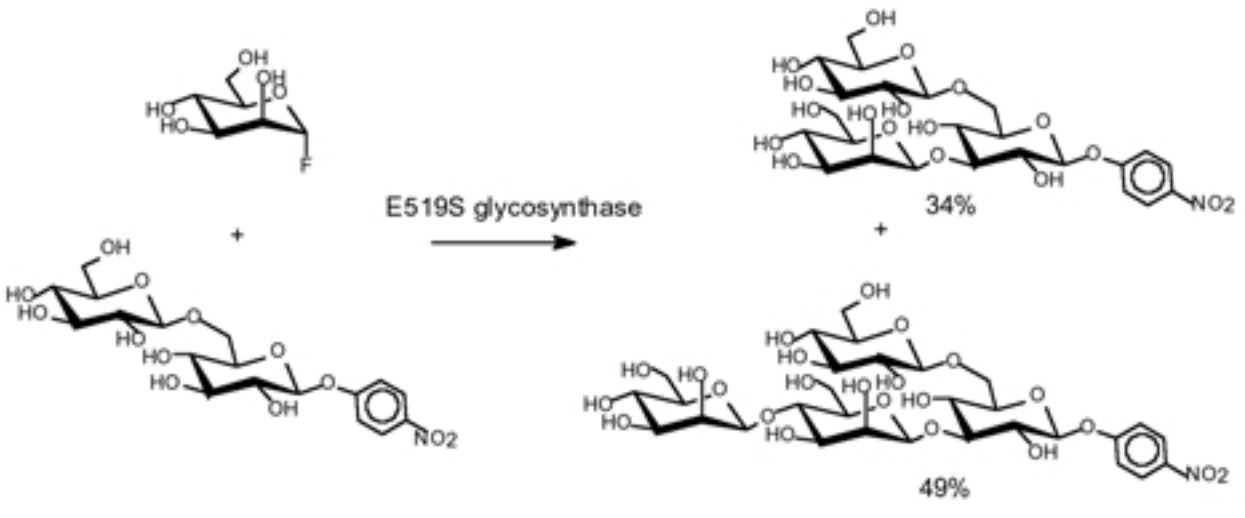
b) β-Glycosidase from *Sulfolobus solfataricus*. The β-glucosidase from the thermophile *Sulfolobus solfataricus* has a broad specificity, including glucosidase, galactosidase, xylosidase and fucosidase activities[79]. The E387G mutant has shown to function as a glycosynthase, catalyzing the condensation of α-glucosyl fluoride with various acceptors[80]. With 2-nitrophenyl β-glucoside as acceptor, one major compound was obtained in 80% yield. It proved to be a β-1,3-linked disaccharide, thus showing a different regioselectivity as compared to the *Agrobacterium* glycosynthase. Depending on the conditions, a mixture of products is obtained, with β-1,3, β-1,4 and β-1,6 glycosidic bonds. Interestingly, branched trisaccharides have also been produced in low yields. However, the complex regioselectivity shown by this enzyme depending on conditions (as a consequence of the broad specificity of the parental wild-type enzyme) may limits its synthetic utility.

c) β-Mannosidase from *Cellulomonas fimi*. Mutants E519A and E519S derived from the retaining β-mannosidase Man2A are glycosynthases capable of constructing β-mannosidic linkages[81]. As previously shown with the *Agrobacterium* β-glucosidase, the serine mutant is a much more efficient glycosynthase than the alanine mutant; whereas only an 8% yield of di- and trisaccharides was obtained upon reaction of α-mannosyl fluoride with *p*-nitrophenyl β-mannoside acceptor using the E519A mutant, an overall 74% yield (21% di-, 32% tri-, 19% tetra- and 1% penta-saccharides) was achieved with the E519S mutant. The selectivity in glycoside bond formation was mainly β-1,4. Remarkably, *p*-nitrophenyl β-gentibioside (Glcβ1,6Glc-*p*NP) as acceptor gave branched tri- and tetra-saccharide products that were β-1,3-linked to the Glc-pNP residue (Scheme 14).

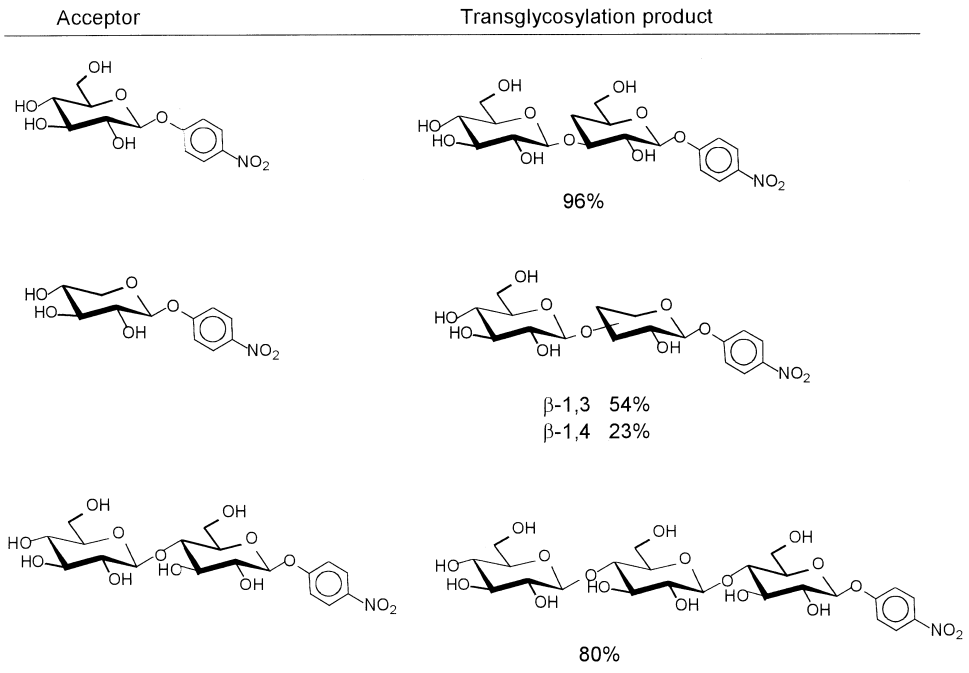
d) β-glucosidase from *Streptomyces* sp. Recently, another family 1 β-glucosidase has been engineered as a glycosynthase by producing the E383A mutant (Faijes, Saura and Planas, unpublished). α-glucosyl and α-galactosyl fluorides were good donors for the glycosynthase-catalyzed condensation with mono- and di-saccharide acceptors (Scheme 15). Interestingly, mainly β-1,3-linked products were obtained with monosaccharide acceptors, but regiospecificity changes to β-1,4-linked products with disaccharide (both cellobioside and laminaribioside) acceptors.

***Endo*-glycosynthases**

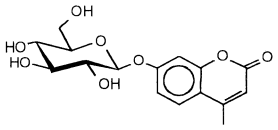
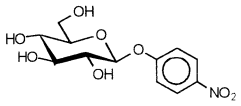
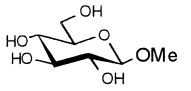
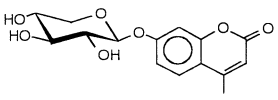
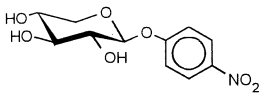
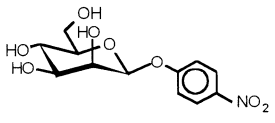
a) 1,3-1,4-β-glucanase from *Bacillus licheniformis*. Short after the first *exo*-glycosynthase was reported (the *Agrobacterium* β-glucosidase), the glycosynthase methodology was successfully extended to the first *endo*-glycosidase in our group[72, 82]. The wild-type 1,3-1,4-β-glucanase from *Bacillus licheniformis* has a strict specificity for hydrolysis of β-1,4 glycosidic bonds in 3-*O*-substituted glucosyl units in mixed-linked β-1,3 and β-1,4 oligo- and polysaccharides[83]. Glu134 was identified as the catalytic nucleophile[84, 85]. The activity of the hydrolytically inactive E134A mutant was rescued by addition of formate as an exogenous nucleophile, and remarkably, a transient α-glycosyl formate intermediate was identified[86], adduct that actually mimics the covalent glycosyl-enzyme intermediate in the mechanism of retaining glycosidases. The E134A mutant 1,3-1,4-β-glucanase was shown to be an efficient glycosynthase catalyzing the condensation of α-laminaribiosyl fluoride with glucosides, cello- and laminaribiosides in excellent yields[72]. The new glycosidic bond formed is exclusively β-1,4 in all cases, illustrating the higher regioselectivity of the *endo*-acting enzymes as compared to the *exo*-acting. The donor and acceptor specificity and enzyme kinetics have been analyzed for this glycosynthase. For a series of donors with increasing degree of polymerization ([Glcβ1,4]$_n$Glcβ1,3GlcαF, n=0-2), reaction rates are higher for the tetrasaccharide than for the disaccharide donor, as expected for an *endo*-glycosynthase, and in agreement with subsite mapping analysis of the wild-type enzyme[87]. The acceptor specificity is summarized in Scheme 16. Methyl β-glucoside is a poor acceptor whereas 4-methylumbelliferyl β-glucoside reacts readily. Therefore subsite

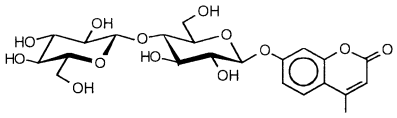
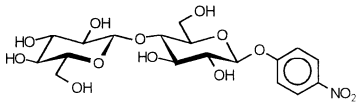
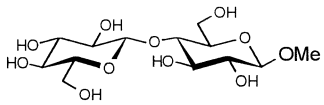
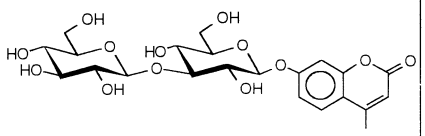
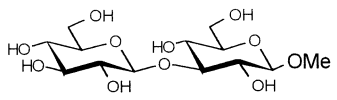
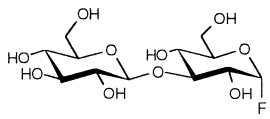


Scheme 14. Formation of branched oligosaccharides by condensation of α-mannosyl fluoride with *p*-nitrophenyl gentibioside catalyzed by the E519S mutant β-mannosidase from *C. fimi.*



Scheme 15. Specificity of the glycosynthase derived from the *Streptomyces* β-glucosidase (E383A) with mono- and disaccharide acceptors.

Acceptor (monosaccharides)	R
	**1.00**
	**0.95**
	**< 0.01**
	**1.10**
	**1.21**
	**0.03**

Acceptor (disaccharides)	R
	**1.29**
	**0.91**
	**1.03**
	**0.41**
	**0.20**
	**0.11**

Scheme 16. Glycosynthase activity of the E134A 1,3-1,4-β-glucanase mutant. Relative yields (R) of transglycosylation at 24 h reactions with α-laminaribiosyl fluoride donor and different acceptors.

+I has low affinity and the acceptor must occupy subsite +II, as also seen from the higher rates with disaccharides. α-Glucosides, sucrose and maltose do not react at all, indicating that an α-linkage is not accepted. As expected, galactosides and 2-acetamido-2-deoxy glucosides are not glycosylated. On disaccharides, cellobiosides are better acceptors than laminaribiosides, probably as a consequence of a competition of the laminaribioside acceptor with the α-donor for the same subsites -II/-I, rather than higher affinity of the cellobiosides for subsites +I/+II[88]. Subsite mapping analysis on the wild-type enzyme has shown that subsites +I/+II can accommodate both laminaribiosyl and cellobiosyl units with similar affinities.
Synthetic applications for the production of mixed-linked oligosaccharides have been developed with this glycosynthase as described below[89, 90].

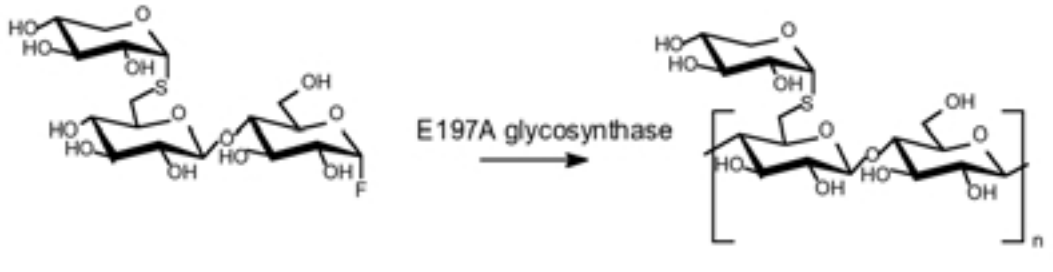
b) Cellulase (1,4-β-glucanase) from *Humicola insolens*. The E197A mutant of Cel7B cellulase from *H. insolens* is another *endo*-acting glycosynthase that catalyzes the transfer of α-cellobiosyl and α-lactosyl fluorides to a variety of substituted mono- and di-saccharide acceptors[91]. With α-lactosyl fluoride, yields are higher than 80% with glucoside and cellobioside acceptors, but also good yields were obtained with other acceptors such as benzyl β-xyloside (51%) or *p*-nitrophenyl β-thiomannoside (61%). Laminaribiosides were also proved to be good acceptors. Based on the X-ray structure of the highly homologous Cel7B from *F. oxysporum* complexed with a non-hydrolyzable thiooligosaccharide substrate analogue, there are no interactions with the protein involving the 6-OH groups of the ligand in the -II subsite. Taking advantage of it, a 6''-bromo and 6''-amino substituted α-cellobiosyl fluorides were polymerized by the glycosynthase. Remarkably, 6''-S-(α-xylopyranosyl)-6''-thio-α-cellobiosyl fluoride was also able to undergo polycondensation resulting in branched oligosaccharide products (Scheme 17). In all cases, the newly-formed glycosidic bonds by the Cel7B glycosynthase are β-1,4. Recently, this glycosynthase has been used for the chemoenzymatic synthesis of a bifunctionalized cellohexasaccharide as specific substrate of cellulases[92].

c) 1,3-β-glucanase from barley. The alanine and glycine mutants of the barley 1,3-β-glucanase (E231A and E231G, respectively) have shown to behave as glycosynthases catalyzing the condensation of α-laminaribiosyl fluoride with *p*-nitrophenyl β-glucoside, or polymerization of the same donor in quantitative yield into oligomers with degree of polymerization higher than 20[93]. Again, the enzyme is regiospecific forming β-1,3 glycosidic linkages.

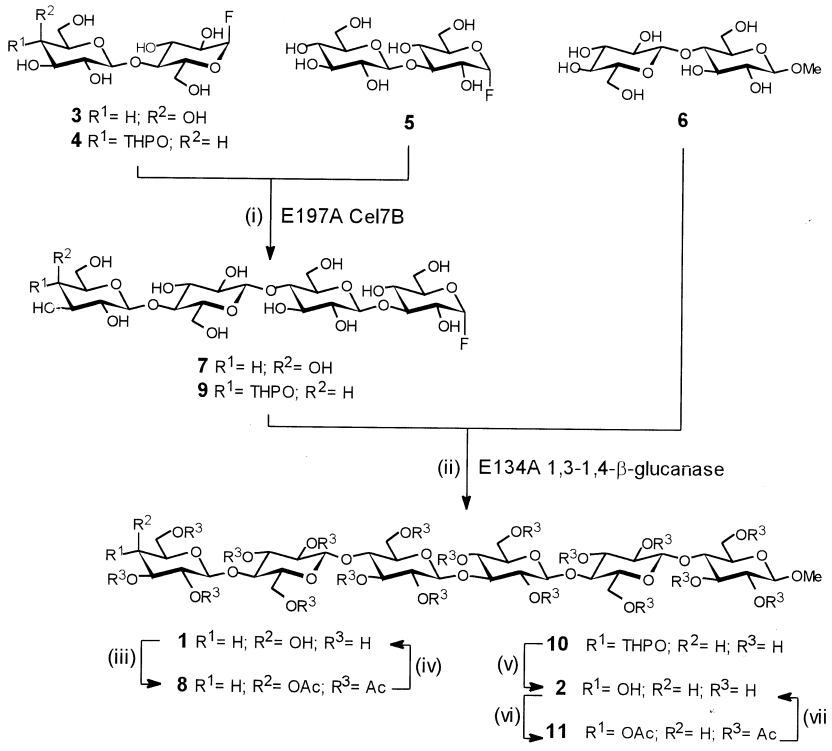
As observed with most of the current glycosynthases, polycondensation reactions often give mixtures of products: the glycosyl fluoride donor and the transglycosylation product may also act as acceptors provided that the configuration of the condensable hydroxyl group on the non-reducing end of the donor has the same stereochemistry as the normal acceptor, then leading to self-condensation of the donor or elongation of the transglycosylation product, respectively. It is therefore important to modulate the different reactions for an efficient use of the glycosynthase methodology in preparative synthesis of target oligosaccharides. As best illustrated with the *endo*-glycosynthases derived from the *B. licheniformis* 1,3-1,4-β-glucanase and the *H. insolens* cellulase, different strategies to control the undesired reactions have been designed:

(a) Selection of a donor with a different configuration of the hydroxyl group that normally acts as acceptor. Whereas α-cellobiosyl fluoride leads to polymerization products (cellooligosaccharides) with the *H. insolens* E197A cellulase, α-lactosyl fluoride gives a single condensation product with the acceptor[91]. With E134A 1,3-1,4-β-glucanase from *B. licheniformis*, α-galactosyl-β1,4-laminaribiosyl fluoride (Galβ1,4Glcβ1,3GlcαF) gives single condensation products with mono- and disaccharide acceptors, whereas the gluco-trisaccharide analogue (Glcβ1,4Glcβ1,3GlcαF) produces an insoluble polysaccharide in the absence of acceptor, or a mixture of condensation and elongation products when an acceptor is present in a 1 to 1 molar donor/acceptor ratio[89]. In both cases, the galactosyl unit in the non-reducing end of the donor prevents self-condensation and elongation reactions ("capped-donor").

(b) Use of a temporary protecting group on the polymerizable hydroxyl group of the donor. As shown for the E197A cellulase, a 4''-tetrahydropyranyl(THP)-protected α-cellobiosyl fluoride is a good donor that is unable to self-condense. After the glycosynthase-catalyzed condensation with methyl β-glucoside or methyl β-cellobioside as acceptors, the THP protecting group in the condensation products is removed by acid treatment. The stepwise application of this strategy has allowed the preparation of cellodextrins having degree of polymerization from 3 to 6 in high yields[94]. The same protecting group has been applied to the E134A 1,3-1,4-β-glucanase in which the corresponding tetrasaccharide donor protected on the 4-OH group in the non-reducing end gave a single condensation product with methyl β-cellobioside acceptor in 85% yields[90]. Another "protecting group" useful for further chemical derivatization of the condensation product is an azi-



Scheme 17. Glycosynthase-catalyzed polymerization by the E197A Cel7B cellulase from *Humicola insolens* to produce a branched oligosaccharide.



Scheme 18. Enzymatic synthesis of the hexasaccharides **1** and **2** by tandem *endo*-glycosynthases (E197A Cel7B cellulase from *H. insolens* + E134A 1,3-1,4-β-glucanase from *B. licheniformis*). (i), (ii) phosphate buffer pH 7.0, CaCl2 0.1 mM, 35 ºC; (iii) Ac_2O/pyridine, 24 h, r.t. (80% from **3**); (iv) MeONa/MeOH, 4h, r.t. (90%); (v) HCl 1M, 30 min, r.t.; (vi) Ac_2O/pyridine, 12h, r.t. (75% from **4**); (vii) MeONa/MeOH, 3h, r.t. (90%).

do group as shown with the E197A Cel7B cellulase. The glycosynthase-catalyzed reaction of 4'-azido-4'-deoxy-α-cellobiosyl fluoride with cellobiose gives a tetrasaccharide product in high yield, which is then an intermediate for the synthesis of a bifunctionalyzed fluorogenic substrates of cellulases[92].

(c) Addition of an excess of acceptor to decrease the probability of the donor to act as an acceptor. In most of the cases, simple addition of a large excess of the acceptor minimizes self-condensation, polymerization and elongation reactions. However, it may be inappropriate when the acceptor is an expensive or elaborated molecule, and a chromatographic step is required to remove the excess of the acceptor from the reaction product.

The potential of glycosynthases in the enzymatic synthesis of complex oligosaccharide is nicely illustrated by the synthesis of mixed-linked hexasaccharide substrates of 1,3-1,4-β-glucanases by coupling two *endo*-glycosynthases of different specificity in a "one-pot" process. Because the E134A 1,3-1,4-β-glucanase and the E197A cellulase show different specificities, towards laminaribiosyl and cellobiosyl donors, respectively, we have prepared the target hexasaccharides shown in Scheme 18 by condensation of the corresponding disaccharide building blocks through sequential addition of the glycosynthases[90]. Both the "capped-donor" and "temporary protecting group" strategies have been used to prevents self-condensation and elongation reactions, and the hexasaccharide products were obtained in 80% overall yields. This stereoselective, rapid, and high-yielding multi-step coupling attests to the power of glycosynthases as synthetic tools for oligosaccharide assembly.

All the above described glycosynthases derive from α-glycosidases, thus accepting α-glycosyl fluoride donors to produce β-glycosidic bonds. Application of the methodology to α-glycosidases seemed to be elusive. Just recently, the first evidence of an α-glycosynthase has been reported: the nucleophile mutant D481G of the α-glucosidase from *Schizosaccharomyces pombe* was found to catalyze the formation of an α-glucosidic linkage from β-glucosyl fluoride and 4-nitrophenyl glucoside to produce 4-nitrophenyl maltoside and isomaltoside in 29 and 41% yields, respectively[95].

CONCLUSION

Retaining glycosidases are extensively exploited as biocatalysts for glycoside bond formation. Classical thermodynamically or kinetically controlled reactions with wild-type enzymes often result in rather low yields, but recent developments in reaction conditions such as the use of immobilized enzymes, lipid-coated enzymes or solvent engineering have clearly improved the practical application of these methodologies. A step forward has just been recently achieved by introducing the 'glycosynthase' concept by engineering retaining glycosidases as efficient transglycosylases with no hydrolase activity. This novel methodology, still applied to a reduced number of enzymes, deserves much attention as new enzymes and synthetic applications are to be developed.

BIBLIOGRAPHY

[1]. Fukuda, M. and Hindsgaul, O. (2000): «Molecular and Cellular Glycobiology», Oxford University Press, Oxford.

[2]. Montreuil, J.; Vliegenthart, J. F. G. and Schachter, H. (1995): «Glycoproteins», Elsevier, Amsterdam.

[3]. Roseman, S. (2001): «Reflections on Glycobiology». *J. Biol. Chem.* **276**, 41527-41542.

[4]. Shoda, S. and Kobayashi, S. (1997): «Recent developments in the use of enzymes in oligo- and polysaccharide synthesis». *TRIP* **5**, 109-115.

[5]. Joziasse, D. H. (1992): «Mammalian glycosyltransferases: genomic organization and protein structure». *Glycobiology* **2**, 271-277.

[6]. Unligil, U. M. and Rini, J. M. (2000): «Glycosyltransferase structure and mechanism». *Curr. Opin. Struct. Biol.* **10**, 510-517.

[7]. Gastinel, L. N. (2001): «Galactosyltransferases: A structural overview of their function and reaction mechanisms». *Trends Glycosci. Glycotechnol.* **13**, 131-145.

[8]. Breton, C. and Imberty, A. (1999): «Structure/function studies of glycosyltransferases». *Curr. Opin. Struct. Biol*. **9**, 563-571.

[9]. Toshima, K. and Tatsuta, K. (1993): «Recent progress in O-glycosylation methods and its application to natural products synthesis». *Chem. Rev*. **93**, 1503-1531.

[10]. Davis, B. G. (2000): «Recent developments in oligosaccharide synthesis». *J. Chem. Soc. Perkin Trans. I*, 2137-2160

[11]. Nicolaou, K. C. and Mitchell, H. J. (2001): «Adventures in carbohydrate chemistry: new synthetic technologies, chemical synthesis, molecular design and chemical biology». *Angew. Chem. Int. Ed. Engl.* **40**.

[12]. Sears, P. and Wong, C. H. (2001): «Towards automated synthesis of oligosaccharides and glycoproteins». *Science* **291**, 2344-2350.

[13]. Gijsen, H. J. M.; Fitz, W. and Wong, C. H. (1996): «Recent advances in the chemoenzymic synthesis of carbohydrates and carbohydrate mimetics». *Chem. Rev*. **96**, 443-473.

[14]. Chen, X.; Kowal, P. and Wang, P. G. (2000): «Large-scale enzymatic synthesis of oligosaccharides». *Curr. Opin. Drug Discovery Dev*. **3**, 756-763.

[15]. Augé, C., Le Narvor, C. and Lubineau, A. (2000): «Solid-phase synthesis with Glycosyltransferases». In *Carbohydrates in Chemistry and Biology* (Ernst, B., Hart, G. W., and Sinaÿ, P., eds.), Wiley-VCH Verlag GmbH, Weinheim, Vol. 2, pp. 705-722.

[16]. Wong, C. H. and Whitesides, G. M. (1994): «Synthesis of glycoside bonds». In *Enzymes in Synthetic Organic Chemistry* (Wong, C. H. and Whitesides, G. M., eds.), pp. 252-311, Pergamon, Oxford.

[17]. Sinnott, M. L. (1990): «Catalytic mechanisms of Enzymic Glycosyl». *Transfer. Chem. Rev.* **90**, 1171-1202.

[18]. Davies, G.; Sinnott, M. L. and Withers, S. G. (1998): «Glycosyl Transfer. In Comprehensive Biological Catalysis» (Sinnott, M. L., ed.), pp. 119-209, Academic Press Ltd., New York.

[19]. Withers, S. G. (2001): «Mechanism of glycosyl transferases and hydrolases». *Carbohyd. Polym.* **44**, 325-337.

[20]. McCarter, J. D. and Withers, S. G. (1994): «Mechanisms of enzymatic glycoside hydrolysis». *Curr. Opin. Struct. Biol.* **4**, 885-892.

[21]. Ajisaka, K.; Nishida, H. and Fujimoto, H. (1987): «The synthesis of oligosaccharides by the reversed hydrolysis reaction of β-glucosidase at high substrate concentration and at high temperature». *Biotechnol. Lett.* **9**, 243-248

[22]. Fernández-Mayoralas, A. (1997): «Synthesis and modification of carbohydrates using glycosidases and lipases». *Top. Curr. Chem.* **186**, 1-20.

[23]. vanRantwijk, F.; Oosterom, M. W. and Sheldon, R. A. (1999): «Glycosidase-catalysed synthesis of alkyl glycosides». *J. Mol. Catal. B. Enzym.* **6**, 511-532.

[24]. Vic, G. and Crout, D. H. G. (1994): «Synthesis of glucosidic derivatives with a spacer arm by reverse hydrolysis using almond β-D-glucosidase. *Tetrahedron Asymm.* **5**, 2513-2516.

(25). Vic, G.; Hastings, J. J. and Crout, H. G. (1996): «Glycosidase-catalyzed synthesis of glycosides by an improved procedure for reverse hydrolysis: application to the chemoenzymic synthesis of galactopyranosyl-(1,4)-*O*-α-galactopyranoside derivatives». *Tetrahedron Asymm*. **7**, 1973-1984.

[26]. Panintrarux, C.; Adachi, S.; Araki, Y.; Kimura, Y. and Matsuno, R. (1995): «Equilibrium yield of n-alkyl-β-D-glucoside through condensation of glucose and n-alcohol by β-glucosidase in a biphasic system. *Enzyme Microb. Technol.* **17**, 32-40.

[27]. Vic, G.; Biton, J.; Le Beller, D.; Michel, J.-M. and Thomas, D. (1995): «Enzymic glucosylation of hydrophobic alcohols in organic medium by the reverse hydrolysis reaction using almond β-D-glucosidase». *Biotecnol. Bioeng.* **46**, 109-116.

[28]. Scheckermann, C.; Wagner, F. and Fischer, L. (1997): «Galactosylation of antibiotics using the β-galactosidase from *Aspergillus oryzae*». *Enzyme Microb. Technol.* **20**, 629-634.

[29]. Vocadlo, D. J. and Withers, S. G. (2000): «Glycosidase-catalysed oligosaccharide synthesis». In *Carbohydrates in Chemistry and Biology* (Ernst, B., Hart, G. W., and Sinaÿ, P., eds.), Vol. 1, pp. 723-844, Wiley-VCH Verlag GmbH, Weinheim

[30]. Koeller, K. M. and Wong, C. H. (2000): «Emerging themes in medicinal glycoscience». *Nature Biotech*. **18**, 835-841.

[31]. Takeomi, M. and Taiich, U. (2000): «Enzymatic synthesis of important oligosaccharide units involved in N- and O-linked glycans». *Trends Glycosci. Glycotechnol.* **12**, 161-174.

[32]. Thiem, J. (1995): «Applications of enzymes in synthetic carbohydrate chemistry». *FEMS Microbiol. Rev.* **16**, 193-211.

[33]. Flitsch, S. L. (2000): «Chemical and enzymatic synthesis of glycopolymers». *Curr. Opin. Chem. Biol.* **4**, 619-625.

[34]. Finch, P. and Yoon, J. H. (1997): «The effects of organic solvents on the synthesis of galactose disaccharides using β-galactosidases». *Carbohydr. Res.* **303**, 339-345.

[35]. Williams, S. J. and Withers, S. G. (2000): «Glycosyl fluorides in enzymatic reactions». *Carbohydr. Res.* **327**, 27-46.

[36]. Chiffoleau Giraud, V.; Spangenberg, P. and Rabiller, C. (2001): «β-Galactosidase transferase activity in ice and use of vinyl-β-D-galactoside as donor». *Tetrahedron Asymm*. **8**, 2017-2023.

[37]. Yasukochi, Inaba, Fukase and Kusumoto (1999): «Nitropyridyl gycosides: new glycosyl donors for enzymatic transglycosyaltion». *Tetrahedron Lett.* **40**, 6585-6589.

[38]. Prade, H.; Mackenzie, L. F. and Withers, S. G. (1998): «Enzymatic synthesis of disaccharides using *Agrobacterium sp.* β-*glucosidase*». *Carbohydr. Res.* **305**, 371-381.

[39]. Trincone, A.; Pagnotta, E.; Rossi, M.; Mazzone, M. and Moracci, M. (2001): «Enzymatic synthesis of 2-deoxy-β-glucosides and stereochemistry of β-glycosidase from *Sulfolobus solfataricus* on glucal. *Tetrahedron Asymm.* **12**, 2783-2787.

[40]. Nilsson, K. G. I. (1987): «A simple strategy for changing

the regioselectivity of glycosidase-catalysed formation of disaccharides». *Carbohydr. Res*. **167**, 95-103.

(41). Fernández-Mayoralas, A. and López, R. (1994): «Enzymatic β-galactosidation of modified monosaccharides: study of the enzyme selectivity for the acceptor and its application to the synthesis of disaccharides». *J. Org. Chem.* **59**, 737-745.

(42). Millqvistfureby, A.; Macmanus, D. A.; Davies, S. and Vulfson, E. N. (1998): «Enzymatic transformations in supersaturated substrate solutions - II - Synthesis of disaccharides *via* transglycosylation. *Biotechnol. Bioeng*. **60**, 197-203.

(43). Macmanus, D. A. and Vulfson, E. N. (2000): «Regioselectivity of enzymatic glycosylation of 6-*O*-acyl glycosides in supersaturated solutions». *Biotechnol. Bioeng.* **69**, 585-590.

(44). Montero, E.; Alonso, J.; Canada, F. J.; Fernández-Mayoralas, A. and Martin-Lomas, M. (1997): «Regioselectivity of the enzymatic transgalactosidation of D- and L- xylose catalysed by β-galactosidases». *Carbohydr. Res*. **305**, 383-391.

(45). Usui, T.; Matsui, H. and Isobe, K. (1990): «Enzymatic-synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate». *Carbohydr. Res*. **203**, 65-77.

(46). Fujimoto, H.,; Hiroko, N. and Ajisaka, K. (1988): «Enzymatic synthesis of glucobioses by a condensation reaction with α-glucosidase, β-glucosidase and glucoamylase». *Agric. Biol. Chem*. **52**, 1345-1351.

(47). Herrmann, G. F.; Ichikawa, Y.; Wandrey, C.; Gaeta, F. C. A.; Paulson, J. C. and Wong, C.-H. (1993): *Tetrahedron Lett.* **34**, 3091.

(48). Fernández-Lorente, G.; Fernández-Lafuente, R.; Armisen, P.; Sabuquillo, P.; Mateo, C. and Guisan, J. M. (2000): «Engineering of enzymes via immobilization and post-immobilization techniques: preparation of enzyme derivatives with improved stability in organic media». *Methods in Non-Aqueous Enzymology*, **36-51**.

(49). Tischer, M. and Wedekind, F. (1999): «Immobilized enzymes: Methods and applications. Biocatalysis from discovery to application». *Top. Curr. Chem*. **200**, 95-126.

(50). Naundorf, A.; Caussette, M. and Ajisaka, K. (1998): «Characterization of the immobilized β-galactosidase C from *Bacillus circulans* and the production of β-1,3-linked disaccharides. *Biosci. Biotechnol. Biochem.* **62**, 1313-1317.

(51). Papanikolaou, S. (2001): «Enzyme-catalyzed synthesis of alkyl-β-glucosides in a water-alcohol two-phase system». *Bioresour. Technol.* **77**, 157-161.

(52). Albayrak, N. and Yang, S.-T. (2002): «Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth. *Biotechnol. Bioeng.* **77**, 8-19.

(53). Mori, T. and Okahata, Y. (1997): «A variety of lipid-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents». *Tetrahedron Lett.* **38**, 1971-1974.

(54). Okahata, Y. and Mori, T. (1998): «Transglycosylation catalyzed by a lipid-coated β-D-galactosidase in two-phase aqueous-organic system. *J. Nutr. Sci. Vitaminol.* **5**, 119-123.

(55). Mori, T. and Okahata, Y. (1998): «Effective biocatalytic transgalactosylation in a supercritical fluid using a lipid-coated enzyme». *J. Chem. Soc. Chem. Commun*. 2215-2216

(56). Mori, T.; Li, M.; Kobayashi, A. and Okahata, Y. (2002): «Reversible control of enzymatic transglycosylations in supercritical fluoroform using a lipid-coated β-D-galactosidase». *J. Am. Chem Soc.* **124**, 1188-1189.

(57). Kobayashi, S.; Kashiwa, K.; Kawasaki, T. and Shoda, S. (1991): «Novel method for polysaccharide sysnthesis using an enzyme: the first in vitro synthesis of cellulose via a non-biosynthetic path utilizing cellulase as catalyst». *J. Am. Chem. Soc.* **113**, 3079-3084.

(58). Karthaus, O.; Shoda, S. I.; Takano, H.; Obata, K. and Kobayashi, S. (1994): «Cellulase-catalyzed glycosylation reactions: simple route towards a highly selective synthesis of oligosaccharides». *J. Chem. Soc. Perkin Trans.* **1**, 1851-1857.

(59). Kobayashi, S.; Sakamoto, J. and Kimura, Y. (2001): «In vitro synthesis of cellulose and related polysacharides». *Prog. Polym. Sci.* **26**, 1525-1560.

(60). Okamoto, E.; Kiyosada, T.; Shoda, S. and Kobayashi, S. (1997): «Synthesis of alternating 6-*O*-methylated cellulose via enzymatic polymerization of a substituted cellobiosyl fluoride monomer catalyzed by a cellulase». *Cellulose* **4**, 161-172.

(61). Moreau, V. and Driguez, H. (1996): «Enzymic synthesis of hemithiocellodextrins». *J. Chem. Soc. Perkin Trans.* **1**, 525-527.

(62). Armand, S.; Drouillard, S.; Schülein, M.; Henrissat, M. and Driguez, H. (1997): «A bifunctionalized fluorogenic tetrasaccharide as a substrate to study cellulases«. *J. Biol. Chem.* **272**, 2709-2713.

(63). Okada, G.; Genghof, D. S. and Hehre, E. J. (1979): «The predominantly nonhydrolytic action of α-amylases on α-maltosyl fluoride». *Carbohydr. Res*. **71**, 287-298.

(64). Kobayashi, S.; Shimada, J.; Kashiwa, K. and Shoda, S. (1992): «Enzymatic polymerization of α-D-maltosyl fluoride utilizing α-amylase as the catalyst: a new approach for the synthesis of maltooligosaccharides». *Macromolecules* **25**, 3237-3241.

(65). Kobayashi, S.; Wen, X. and Shoda, S. (1996): «Specific preparation of artificial xylan: a new approach to polysaccharide synthesis by using cellulase as catalyst». *Macromolecules* **29**, 2698-2700.

(66). Fujita, M.; Shoda, S. and Kobayashi, S. (2002): «Xylanase-catalyzed synthesis of a novel polysaccharide having a glucose-xylose repeating unit, a cellolose-xylan hybrid polymer». *J. Am. Chem. Soc.* **120**, 6411-6412.

(67). Kobayashi, S.; Kiyosada, T. and Shoda, S. (1996): «Synthesis of artificial chitin: irreversible catalytic behavior of a glycosyl hydrolase through a transition state analogue substrate». *J. Am. Chem. Soc*. **118**, 13113-13114.

(68). Viladot, J. L.; Moreau, V.; Planas, A. and Driguez, H. (1997): «Transglycosylation activity of Bacillus 1,3-1,4-β-D-glucan 4-glucanohydrolases». Enzymatic synthesis of alternate 1,3-1,4-β-D-glucooligosaccharides». **J. Chem. Soc. Perkin Trans. 1**. 2383-2387.

(69). Viladot, J. L.; Stone, B. A.; Driguez, H. and Planas, A. (1998): «Expeditious synthesis of a new hexasaccharide using transglycosylation reaction catalyzed by *Bacillus* 1,3-1,4-β-D-glucan 4-glucanohydrolase». *Carbohydr. Res*. **311**, 95-99.

(70). Hrmova, M.; Fincher, G. B.; Viladot, J. L.; Planas, A. and Driguez, H. (1998): «Chemoenzymatic synthesis of 1,3-1,4-β-D-glucooligosaccharides for subsite mapping of 1,3-1,4-β-D-glucan endohydrolases. *J. Chem. Soc. Perkin Trans.* **1**. 3571-3576.

(71). Mackenzie, L. F.; Wang, Q.; Warren, R. A. J. and Withers, S. G. (1998): «Glycosynthases: Mutant Glycosidases for Oligosaccharide synthesis». *J. Am. Chem. Soc.* **120**, 5583-5584.

(72). Malet, C. and Planas, A. (1998): «From β-glucanase to β-glucansynthase: glycosyl transfer to α-glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile. *FEBS Lett.* **440**, 208-212.

[73]. Mayer, C.; Zechel, D. L.; Reid, S. P.; Warren, R. A. J. and Withers, S. (2000): «The E358S mutant of *Agrobacterium* sp. α-glucosidase is a greatly improved glycosynthase». *FEBS Lett.* **466**, 40-44.

[74]. Johnson, P. E.; Brun, E.; MacKenzie, D. A.; Withers, S. G. and McIntosh, L. P. (1999): «The cellulase-binding domains from *Cellulomonas fimi* β-1,4-glucanase CenC bind nitroxide spin-labeled cellooligosaccharides in multiple orientations». *J. Mol. Biol*. **287**, 609-625.

[75]. Howard, S. and Withers, S. G. (1998): «Bromoketone C-glycosides, a new class of β-glucanase inhibitors». *J. Am. Chem. Soc*. **120**, 10326-10331.

[76]. Fairweather, J. K.; Stick, R. V. and Withers, S. G. (2000): «A glycosynthase-assisted synthesis of some epoxyalkyl β-C-oligosaccharides». *Aust. J. Chem.* **53**, 913-916.

[77]. Tolborg, J. F.; Petersen, L.; Jensen, K. J.; Mayer, C.; Jakeman, D. L.; Warren, R. A. J. and Withers, S. G. (2002): «Solid-phase oligosaccharide and glycopeptide synthesis uning glycosynthases». *J. Org. Chem.* **67**, 4143-4149.

[78]. Mayer, C.; Jakeman, D. L.; Mah, M.; Karjala, G.; Gal, L.; Warren, R. A. J. and Withers, S. G. (2001): «Direct evolution of new glycosynthases from Agrobacterium β-glucosidase: a general screen to detect enzymes for oligosaccharide synyhesis». *Chem. Biol*. **8**, 437-443.

[79]. Nucci, R.; Moracci, M.; Vaccaro, C.; Vespa, N. and Rossi, M. (1993): «Exo-glucosidase activity and substrate specificity of the β-glycosidase isolated from the extreme thermophile Sulfolobus solfataricus». *Biotechnol. Appl. Biochem.* **17**, 239-250.

[80]. Trincone, A.; Perugino, G.; Rossi, M. and Moracci, M. (2000): «A novel thermophilic glycosynthase that effects branching glycosylation». *Bioorg. Med. Chem. Lett.* **10**, 365-368.

[81]. Nashiru, O.; Zechel, D. L.; Stoll, D.; Mohammadzadeh, T.; Warren, A. J. and Withers, S. G. (2001): β-Mannosynthase: synthesis of β-mannosides with a mutant β-mannosidase. *Angew. Chem. Int. Ed. Engl*. **40**, 417-420.

[82]. Planas, A. (2000): «Bacterial 1,3-1,4-β-glucanases: structure, function and protein engineering». *Biochim. Biophys. Acta* **1543**, 361-382.

[83]. Malet, C.; Jiménez-Barbero, J.; Bernabé, M.; Brosa, C. and Planas, A. (1993): «Stereochemical course and structure of the products of the enzymic action of endo-1,3-1,4-β-D-glucan 4-glucanohydrolase from *Bacillus licheniformis. Biochem. J.* **296**, 753-758.

[84]. Juncosa, M.; Pons, J.; Dot, T.; Querol, E. and Planas, A. (1994): «Identification of Active Site Carboxylic Residues in *Bacillus licheniformis* 1,3-1,4-β-D-Glucan 4-Glucanohydrolase by Site-directed Mutagenesis». *J. Biol. Chem*. **269**, 14530-14535.

[85]. Viladot, J. L.; de Ramon, E.; Durany, O. and Planas, A. (1998): «Probing the mechanism of Bacillus 1,3-1,4-β-D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues». *Biochemistry* **37**, 11332-11342.

[86]. Viladot, J. L.; Canals, F.; Batllori, X. and Planas, A. (2001): «Long-lived glycosyl-enzyme intermediate mimic produced by formate re-activation of a mutant endoglucanase lacking its catalytic nucleophile». *Biochem. J.* **355**, 79-86.

[87]. Malet, C. and Planas, A. (1997): «Mechanism of Bacillus 1,3-1,4-β -D-glucan 4-glucanohydrolases: kinetics and pH studies with 4-methylumbelliferyl β-glucan oligosaccharides. *Biochemistry* **36**, 13838-13848.

[88]. Planas, A.; Viladot, J. L. and Faijes, M. (1999): «Consequences of removing the catalytic nucleophile in *Bacillus* 1,3-1,4-β-glucanases: synthase activity and novel properties by chemical rescue. In *Recent Advances in Carbohydrate Bioengineering* (Gilbert, H. J.; Davies, G. J.; Henrissat, B. and Svensson, B., eds.), pp. 292-301, The Royal Society of Chemistry, Cambridge.

[89]. Fairweather, J. K.; Faijes, M.; Driguez, H. and Planas, A. (2002): «Specificity studies of *Bacillus* 1,3-1,4-β-glucanases and application to glycosynthase-catalyzed transglycosylation». *Chem. BioChem*. in press **3**, 866-873.

[90]. Faijes, M.; Fairweather, J. K.; Driguez, H. and Planas, A. (2001): «Oligosaccharide synthesis by coupled endoglycosynthases of different specificity: a straightforward preparation of two mixed-linkage hexasaccharide substrates of 1,3-1,4-β-glucanases. *Chem. Eur. J*. **7**, 4651-4655.

[91]. Fort, S.; Boyer, V.; Greffe, L.; Davies, G. J.; Moroz, O.; Chirstiansen, L.; Schülein, M.; Cottaz, S. and Driguez, H. (2000): «Highly efficient synthesis of β(1-4)-oligo- and -polysaccharides using a mutant cellulase. *J. Am. Chem. Soc.* **122**, 5429-5437.

[92]. Boyer, V.; Fort, S.; Frandsen, T.P.; Schülein, M.; Cottaz, S. And Driguez, H. (2002): «Chemoenzymatic synthesis of a bifunctionalized cellohexaoside as a specific substrate for the sensitive assay of cellulase by fluorescence quenching». *Chem. Eur. J.* **8**, 1389-1394.

[93]. Hrmova, M.; Imai, T.; Rutten, S. J.; Fairweather, J.K.; Pelosi, L.; Bulone, V.; Driguez, H. and Fincher, G. B. (2002): «Barley 1,3-β-D-glucan endohydrolase mutants synthesise crystalline 1,3-β-D-glucans. *J. Biol. Chem.* **277**, 30102-30111.

[94]. Fort, S.; Christiansen, L.; Schülein, M.; Cottaz, S. and Driguez, H. (2000): «Stepwise synthesis of cellodextrins assisted by a mutant cellulase». *Israel J. Chem.* **40**, 217-221.

[95]. Okuyama, M.; Mori, H.; Watanabe, K.; Kimura, A. and Chiba, S. (2002): α-Glucosidase mutant catalyzes «α-glycosynthase»-type reaction». *Biosci. Biotechnol. Biochem*. **66**, 928-933.