Journal of Molecular Catalysis B: Enzymatic 60 (2009) 97–105

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Review

# Monolith enzymatic microreactor at the frontier of glycomic toward a new route for the production of bioactive oligosaccharides

C. Delattre [a,b,*], M.A. Vijayalakshmi [a]

[a] CBST, Vellore Institute of Technology (VIT), Deemed University, Vellore 632014, Tamil Nadu,India
[b] GREENTECH, Biopôle Clermont Limagne, 63360 Saint Beauzire, France

ARTICLE INFO

*Article history:*
Received 11 November 2008
Received in revised form 24 April 2009
Accepted 28 April 2009
Available online 6 May 2009

*Keywords:*
CIM disk
Enzymatic reactor
Glycomic
Monolith microreactor
Oligosaccharides

ABSTRACT

Recent research in the area of bioactive carbohydrates has shown the efficiency of oligosaccharides as signal molecules in a lot of biological activities. Newly observed functions of oligosaccharides and their abilities to act as specific regulatory molecules on various organisms have been more and more described. A successful development of these bioactive molecules in future needs efficient processes for specific oligosaccharides production. To exploit them for putative industrial scale up processes, two main strategies are currently investigated: the synthesis (chemical or bioconversion processes) and the polysaccharide cleavage (chemical, physical or biological processes). Nevertheless, if new manufacturing biotechnologies have considerably increased the development of these functional molecules, the main drawback limiting their biological applications is the complexity to engender specific glycosidic structures for specific activities. In the recent years, new enzymatic reactors have been developed, allowing the automatic synthesis of oligosaccharide structures. This review focuses on the knowledge in the area of bioactive oligosaccharides and gives the main processes employed to generate them for industrial applications with challenges of monolith microreactors.



**Contents**



## 1. Introduction

During the last two decades, a lot of studies have investigated the production of oligosaccharides as putative biological molecules. In fact, in carbohydrates family, oligosaccharidic structures take part in an extensive range of biological functions as cell surface marker biomolecules [1,2]. In this context, these compounds are more and more developed and used in industrial fields such as pharmaceutics, cosmetic, food industries or agronomy [1–12].

Traditionally, oligosaccharides are defined as polymers of monosaccharides with degrees of polymerization (DP) between 2 and 10, but in common way, higher DP up to 30 are often considered as oligosaccharides. These carbohydrate compounds have

* Corresponding author. Current address: GREENTECH, Biopôle Clermont Limagne, 63360 Saint Beauzire, France.
*E-mail address:* cedric_delattre@hotmail.com (C. Delattre).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.04.016

predominantly two origins [2]. Firstly, they can be synthesized by organisms using glycosyltransferases or by chemical processes through specific glycosylation reactions. Secondly, they can derivate from chemical, physical or biological degradation of polysaccharides. A plentiful literature has treated more particularly on the biological activities of this class of oligosaccharide from large depolymerization of polysaccharides [2]. In fact, oligosaccharides and low molecular weight polysaccharides possess a large variety of biological activities on numerous organisms as fungi, bacteria, mammalian, plant and algae [1,2]. Therefore, the literature relating to therapeutic activities, prebiotic activities, elicitor activities of oligosaccharides is still the most abundant [2,13]. More generally, biological activities are dependent on not only structural and biochemical factors such as glycosidic linkages, monosaccharides composition, polymerization degrees (dp) but also substitution degrees with alkyles, acetates, pyruvates, phosphates, or sulfates [1,2,9,14,15].

Consequently, the most active oligosaccharides should be anionic or neutral with DP between 4 and 30 partially pyruvated, phosphated, sulfated or acetylated. Nevertheless, very low amount of specific bioactive structures have been reported. Research on the biological impact of oligosaccharides and derivates such as glyconjugates leads to an important demand for large amount of oligosaccharides from synthetic or natural origins. As a result, ways to pure bioactive oligosaccharides production are more and more looked for in the development of oligosaccharide-based therapies and are required to efficiently improve enzymatic and chemical synthesis processes [2,16–18].

Commonly, biological activities were identified using oligosaccharides mixtures not clearly characterized. The structural variability and complexity of oligosaccharides is a major drawback limiting their applications in the area of neutraceuticals, cosmetics, pharmaceuticals, biofertilizers [2,13]. In fact, even if the chemical or biological synthesis of oligosaccharides is highly efficient and regio- and/or stereospecific, it requires more and more specific substrates and simple protocols with no (or low) industrial compatibilities. For this reason, the polysaccharides depolymerization by enzymatic or chemical processes seems to be the best procedure for the large production of oligosaccharides [2,13]. This review presents a survey of methods for the production of bioactive oligosaccharides with the recent advances in the development of new monolith microreactor.

## 2. Oligosaccharides engineering processes

The potentialities of oligosaccharides are increasingly employed as therapeutic and functional drugs [1,2,12,13]. Nevertheless, in most cases the lack of industrial oligosaccharides production is the main drawback limiting their biological applications [2]. For this reason, development of oligosaccharides engineering strategies is of great importance. Bioactive oligosaccharides are from oligomers engineering with either synthesis (using enzymatic or chemical engineering) or, polysaccharides depolymerization (using physical, chemical or enzymatic methods) [2,13].

### 2.1. Chemical and biochemical oligosaccharides synthesis

In modern biotechnological processes, chemical and biochemical synthesis of oligosaccharidic structures is more complicated than it is described in the synthesis processes of peptides/oligopeptides and nucleic acids due to the higher number of possibilities in the combinations of monomeric units [2]. Consequently, the stereo-specific introduction of glycosidic linkages appears as the main challenge of oligosaccharides block synthesis. Nevertheless, recent advances in enzymatic and chemical synthesis allow to envisage the preparation of a wide range of simples and highly complex oligomers structures.

#### 2.1.1. Chemical glycosylation in oligosaccharides production

For decades, many strategies have been published for the chemical synthesis of specific oligosaccharidic structures [2,19,20]. Usually, the production of oligosaccharides by chemical processes takes place as given in Fig. 1A. As illustrated, the glycosylation reaction is obtained by inter-glycosidic condensation process between a protected glycosyl donor having an excellent leaving group such as halogenides at its anomeric position and a glycosyl acceptor possessing at least one free hydroxyl group. Anomeric fluorides, trichloroacetimidates and thioglycosides are currently employed as glycosyl donor for the high yield production of glycans blocks [13,19]. The generation of specific glycosyl donor and acceptor needs many protection and deprotection steps to combine high yield oligosaccharides production with high regio-and stereoselective processes. These chemical strategies allow the synthesis of bioactive oligosaccharides [19,20] such as oligogalacturonides (OGAs) [21], or xylooligosaccharides (degree of polymerization (dp) 4–10) generated by a complex blockwise synthesis approach [22]. So, in spite of the recent developments, synthesis of oligosaccharides by chemical glycosylations seems non-realistic for industrial processes because of the many protection and deprotection steps. All these oligosaccharides syntheses need a very high yielding for each step in the assembly of glycosyl donor and glycosyl acceptor.

Nevertheless, in the last decades a lot of studies have been investigated for the enantioselective production of glycosyl donor and acceptor by using biocatalysis. Enantioselectivity is one of the key qualities of enzymes making them attractive for biocatalysis processes. In fact, as many of us know, lipases and esterases are frequently used in the synthesis of optically pure compounds [23–26] and an extensive number of biocatalysis processes have already been commercialized in industry [23]. Esterases and lipases are commonly used because they can accept a broad range of substrates and often exhibit high enantioselectivity and high reactivity [23,24]. Moreover, we can now find more than 50 commercial lipases and esterases.

Concerning oligosaccharides, it is important to mention that pure regio-isomers of tetra-*O*-acetyl glycopyranoses may be used as key intermediates for the synthesis of glycoderivatives such as oligosaccharides, sugar esters, etc. [27–30]. For example, 1,2,3,6-tetra-*O*-acetyl-D-glucopyranose and related sugar compounds are very interesting due to the presence of 1,4 glycosidic bonds in natural and pharmaceutical oligosaccharides. Then, use of enzymatic catalysts such as esterases or lipases could be considered as an important tool for the preparation of tetra-acetyl donor or acceptor [27–30].

Consequently, several publications have reported the fast preparation of glycosyl intermediates for oligosaccharide synthesis [27,28] and also for the production of linear oligosaccharides by an easy monoprotective chemo-enzymatic approach [29].

In the same way, Filice et al. [30] have described an elegant chemo-enzymatic methodology to produce a library of different region-isomers of tetraacetylated glycopyranoses bearing only a free hydroxyl group. All these different deprotected monosaccharides are ideal for the fast synthesis of oligosaccharides building blocks. Nonetheless, one obstacle for biocatalysis synthesis of sugar donor and/or acceptor is to identify the best commercially available wild-type enzyme with the highest enantioselectivity for the given biotransformation. Generally, natural enzymes do not always show sufficiently high enantioselectivity and this is sometimes difficult and time consuming. Work is constantly in progress to find methods to increase enantioselectivity of lipases and esterases for the synthesis of oligosaccharides blocks [25]. These properties can be

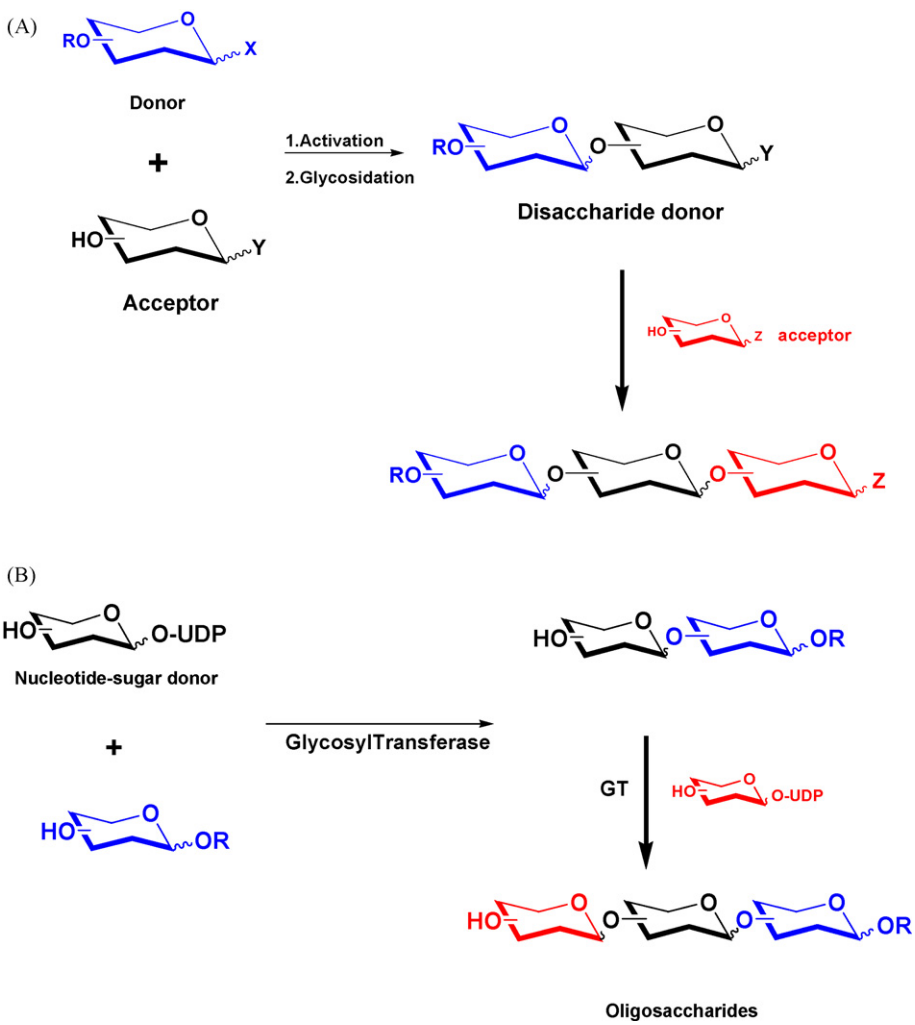


**Fig. 1.** Synthesis of oligosaccharides by glycosylation using (A) chemical process and (B) enzymatic process with glycosyltransferases.

increased and optimized by protein engineering such as cloning and directed evolution techniques [24,26].

*2.1.2. Enzymatic glycosylation in oligosaccharides production*

Specific studies of the oligosaccharide biosynthesis pathways and the use of selective enzymes such as glycosyltransferases and glycosidases allow to avoid the main disadvantages of chemical methods, since enzymes control both regio- and stereoselectivity of glycosylation without need of protection and deprotection steps during the synthesis [2,13,22]. For the preparation of complexes and highly pure oligosaccharides, methods based on the application of glycosyltransferases are currently recognized as being the most effective. Recently, Weijers et al. [31] have reviewed the potential of enzymes such as glycosyltransferases and glycosylsynthetase as efficient biotechnological tools in area of oligosaccharides synthesis. The reaction mechanisms of these enzymes are described in more detail in order to give the relation to the stereochemistry of the transfer reactions and the requirement of glycosyl nucleotide donors.

Glycosyltransferases family (EC 2.4.x.y) catalyzes transfer of sugar moieties from activated nucleotide glycosyl donors to specific glycosyl acceptors [2,13,22] allowing the specific formation of glycosidic bonds as illustrated in Fig. 1B. These highly regio- and stereoselective enzymes are commonly obtained after cloning and overexpression followed by high purification steps. Moreover, we can find other synthetase enzymes such as glycosylhydrolases (EC 3.2.1.-) which are much more readily available than glycosyltransferases but are in general less stereoselective. These specific biocatalysts traditionally used for degradation of poly- and oligosaccharides can permit glycosidic bond formation by reverse hydrolytic activities under suitable reaction conditions and the specific utilizations of nucleotide activated sugars [2,13]. Generally, oligosaccharides production yields are lower with glycosidases excepted with mutated derivates such as glycosynthases that operate more efficiently with an appropriated activated glycosyl donor such as fluorides (Fig. 2). Consequently, these last decades, the large scale productions of oligosaccharides have been largely developed, thanks to enzymatic synthesis for producing many bioactive oligosaccharides [13,22]. The new strategies to produce bioactive gangliosides and sialylated oligosaccharides have been largely described by using specific sialyltransferases [23–25]. As commonly described, these family enzymes are used to catalyze specific transfer of Neu5Ac from the sialylated sugar nucleotide donor (CMPNeu5Ac) onto terminal sugar units of glycoconjugates from cell surface [31–33].

Traditionally, these sialyl moieties (sialic acid, 5-*N*-acetylneuraminic acid) are found at the terminal positions of glycolipids and glycoproteins in various animal tissues. Sialic acids and derivates are known to play an important role as biological masks to prevent specific recognitions in cellular communication [33]. In fact, sialylated oligosaccharides participate in protein–carbohydrate interaction and that is the way, sialylated glycoconjugates are spe-

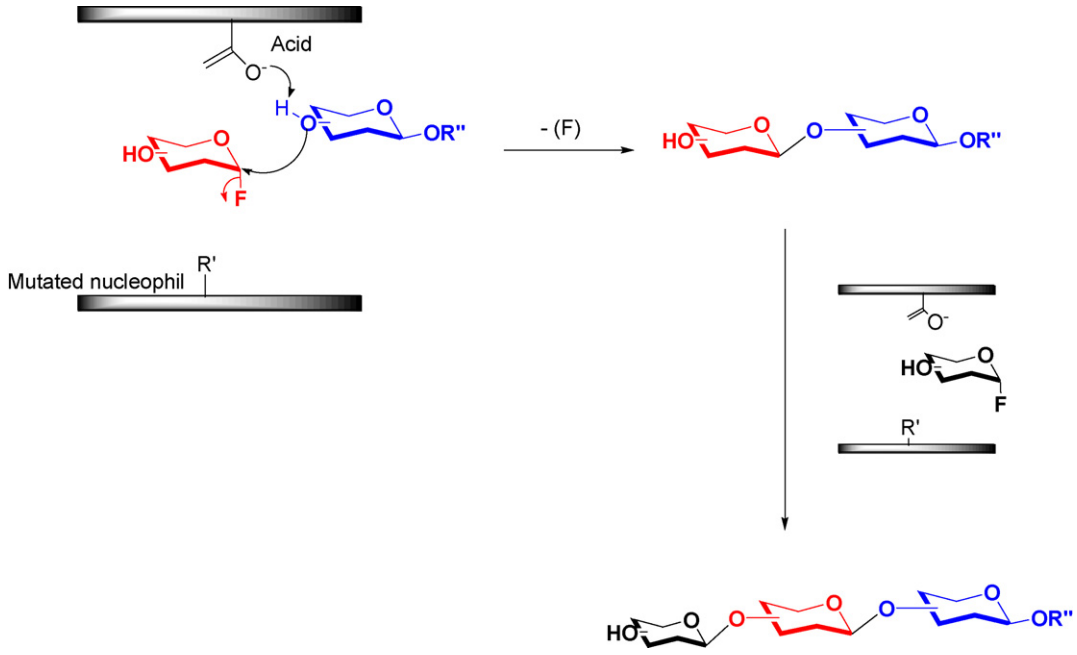


**Fig. 2.** Reaction mechanism of the synthesis of oligosaccharides with glycosynthases (R, R′′: oside).

cific ligands for microbial adhesions (attachment to a host cell), for microbial toxins, and also for lectins (cell–cell adhesion) [1,2,32,33].

Finally, there are prospects to produce many other attractive oligosaccharides such as gentiooligosaccharides from glucose derivates by transglucosylation reaction [34], galactosyl–oligosaccharides from lactose using β-galactosidases [35], fructo-oligosaccharides from sucrose using fructosyltransferases [36], and cyclic non-reducing-end maltoligosaccharides named cyclodextrins (CDs) [37] by using bacterial cyclodextrin glucosyltransferases (CGTase). These original products were used in pharmacology for their inclusion complex properties with drugs [38]. All these biotechnological processes constitute a very good alternative synthesis route to the promising automatic synthesis of therapeutics oligosaccharides [39].

### 2.1.3. *Automated solid-phase synthesis of oligosaccharides*

Thanks to the development of oligosaccharides synthesis, new ways have been investigated for the large automated production of therapeutic oligosaccharides and glycoconjugates [40].

In fact, solution-phase oligosaccharides synthesis leads to very slow process due to the need for iterative coupling and deprotection steps, with high purification processes at each step. Consequently, solid-phase synthesis has largely demonstrated efficiencies for assembly of oligosaccharides and other glycoconjugates [41]. In recent years, the Seeberger team has largely contributed to the development of bioactive glycoconjugates by using different approaches and concepts in phase oligosaccharide synthesis [40–42] including: (*i*) the solid support matrix; (*ii*) the protected glycosylating agents and (*iii*) the choice of synthetic way. That is the way, the first automated solid-phase oligosaccharides synthesizer has been developed and introduced on the basis of a modified peptide synthesizer [43]. Typically, by using glycosyl trichloroacetimidates and five different glycosyl phosphates as building blocks, an automatic solid-phase synthesis of very interesting bioactive glycoconjugates is possible reducing significantly the synthesis time [44]. The currently available automated synthesizer developed by Seeberger et al. has considerably accelerated access to several oligosaccharides/glycoconjugates and opens the way to the development of other oligosaccharides synthetizer [40–44].

Finally, it is important to mention that in the near future, oligosaccharide building blocks will become commercially available for the production of complex bioactive oligosaccharide structures. The most time-consuming step for the procurement of pure carbohydrates is the synthesis of sufficient quantities of building blocks. Nevertheless, improvement in the synthesis methodologies to prepare all possible specific glycosidic linkages with the faster protection/deprotection steps in oligosaccharides synthetic way including robotic systems development is still needed.

Moreover, as mentioned in Section 2.1.1 it appears very clear that automatic synthesis of oligosaccharides will be improved by using lipases and esterases in order to prepare very specific sugar donor and/or sugar acceptor for the very fast synthesis of building blocks oligosaccharides.

## 2.2. *Polysaccharides depolymerization*

Lot of processes have been investigated to produce large amount of oligosaccharides by depolymerization of polysaccharide [2]. That is the way, chemical treatments using acid and radical hydrolysis or physical treatments using γ-irradiations, thermal, microwaves, and ultrasonication degradations have been developed [2].

All these physical techniques have been related as environment friendly and easily methods unlike chemical or thermal degradations. Note that gamma, UV and other radiation depolymerizations deliver high energy able to cleave glycosides linkages with short reactions times [2]. Nevertheless, oligosaccharides were rarely isolated and a more long exposure time could cause nefast degradation of osidic units. Moreover, free radical can induce inter- and intramolecular recombination reactions. Globally, the structures cleavage was low yielded even with prolonged irradiations. It was a non-random process and the depolymerization decrease with low molecular weight fractions implicating the non-formation of oligosaccharides [2].

In addition, the impact of intensive thermic treatment on oligosaccharide structures has not been really treated in literature. In fact, the residual amounts of protein and/or peptides in polysaccharide could induct the Maillard reaction at high temperatures.

If these reactions have been well investigated on monosaccharides, where Amadori compounds form deoxyhexosuloses which are responsible for the formation of volatile substances and melanoidins, the browning reactions of oligosaccharides is poorly understood [2]. As for example, the thermo-mechanical depolymerization of dextran by jet-cooking with high pressure steam and extrusion have shown that intensive thermic treatment could induce dark-colored side products during polysaccharide depolymerization [2].

Therefore, enzymatic depolymerization of polysaccharides is the main approach currently used to prepare large amounts of oligomers. In these cases, microbiotechnological procedures are investigated to depolymerize various polysaccharides (from plant, algae, microorganisms, etc.) by using regio- and stereospecific microbial enzymes such as polysaccharide lyases and polysaccharide hydrolases [2,13,45]. Commonly, polysaccharide hydrolases (EC 3.2.1.-) allow to catalyze hydrolysis of polysaccharides glycosidic bonds *via* a general acid catalysis requiring a proton donor and a nucleophile base [45]. This hydrolysis occurs *via* two main mechanisms giving rise to either an overall retention or an inversion of anomeric configuration. Concerning polysaccharide lyases (EC 4.2.2.-), they constitute a specific group of enzymes which generate cleavage of polysaccharides chains *via* a β-elimination resulting in the formation of a double bond at the newly formed non-reducing end [2,45]. All these selective enzymes have been largely exploited and developed from long time to prepare a majority of bioactive oligosaccharides [2,13,45]. For example, several commercial oligosaccharides with biological properties were prepared [34] such as malto-oligosaccharides from starch using α-amylases, fructo-oligosaccharides from enzymatic hydrolysis of inulin, isomalto-oligosaccharides with action of α,β-amylases and α-glucosidases and xylo-oligosaccharides from xylan cleavage with β-(1,4) xylanases. Moreover, therapeutical oligosaccharides as oligogalacturonides have been synthesized by cleavage of pectin using pectate lyases, pectinases, and other polygalacturonases [46]. Finally, other bioactive oligosaccharides from algal polysaccharides can be produced from enzymatic depolymerization of seaweed alginate polysaccharides with alginate lyases [9,47–49] and oligo-κ-carrageenans from enzymatic digestion of sulfated galactan with κ-carrageenases [50].

Large scale production of oligosaccharides by using enzymatic degradation allows a better understanding and constitutes new approaches in therapeutic and agrochemical developments. However, regarding to the high structural variability of polysaccharides from various biotopes, these bioenzymatic methodologies impose many microorganisms as specific enzymatic sources [45]. Moreover, these strategies lead to lot of inherent disadvantages such as: long reaction times and difficulties to reuse biocatalyst because of enzyme inactivation to stop degradation. Consequently, modern enzymatic processes require development of industrial enzymatic reactor.

## 3. Enzymatic reactor

In the recent years, enzymatic microreactors have been developed to facilitate routine works in biochemical analysis, and in biocatalysis. So, most enzymes have to be immobilized for their application, and that may have special interest of the enzyme properties are improved (e.g., stability, selectivity or activity) [51–58]. Generally, immobilized enzymes find applications in affinity chromatography, biotransformations, bioprocessing and bioanalysis [51–53].

In modern biotechnology, reactors with immobilized enzymes possess many advantages such as: reusability of catalytic activities, high stability, an the easy recovery of the bioactive oligosaccharides and the most important advance is the opportunity to work in continuous system for longer period process without any lost of activity in a majority of industrial scale up processes [54,55]. A number of studies were already investigated on the immobilization of hydrolytic enzymes [56,57] and its development as bioreactor in continuous industrial processes, could offer significant economical impacts in biotechnologies processes [54,55].

As a rule, it is largely described that a proper immobilization may improve enzyme stability (multi point covalent attachment), a bad one may even decrease enzyme stability [54–57]. This fact is to be controlled to improve the scale up industrial application of enzyme reactor [54,55]. Applied to the large scale production of bioactive oligosaccharides from enzymatic polysaccharides degradation, it is a very promising industrial tool. In immobilization step of enzyme degrading polysaccharides, numerous supports such as support binding, entrapment, or cross-linking have been utilized but particle-based supports seems to be the most common [58]. Currently, entrapment processes consist in inclusion of specific enzymes into a matrix as it was largely investigated with carrageenan, agarose or more especially alginate beads [59]. Other new methods continue to be developed such as entrapment in polysaccharide-silica nanocomposites support [60] and different matrices are activated like biopolymers, inorganic supports or synthetic resins [61]. During immobilization processes, the type of support used, the ligand utilization and mass transfer regime are the key considerations for efficient practical application of enzymatic reactor tools. In fact, it is well known that mass transfer between the mobile phase and the specific stationary phase has a prominent effect on the binding capacity and the enzyme activity kinetics [61,62]. Therefore, the use of specific enzymes in industrial scale up processes for lower added values can limit the selection of matrices supports and the immobilization methodologies. However, in this kind of enzymatic reactor, there are large diffusion problems [62] and the mass transfer is primordial to obtain a good reproducibility.

Recently, several supports with efficient hydrodynamic characteristics were developed and introduced for the designing of enzymatic microreactor. Consequently, new monolith materials have been described in designing of enzyme reactors.

## 4. Monolith technology

All these years, a new polymeric macroporous material based on radical co-polymerization of glycidyl methacrylate and ethylene glycol dimethacrylate, was introduced in biochromatographic processes under the trademark CIM® (Convective Interaction Media) [63–67].

Monolithic matrices have been considered as efficient chromatographic supports where mass transfer is faster, thanks to the specific convective flow that consequently becomes a dominant transport mechanism attributable to the almost complete lack of diffusion resistance during the mass transfer [63–67]. In fact, contrary to other usual chromatographic supports consisting of macroporous particles, where the void volume between individual porous particles is unavoidable, CIM disk technology is a monolithic macroporous material where there are predominantly convective transports of the liquid within the pores and the molecules transport and transfer to active sites is not limited by diffusion [63–67]. These CIM supports consist of a single monolith with open channels. In this manner, all molecules to be separated are transported by convection into the pores of matrix, resulting in very fast separation times due to the low mass transfer resistance (convection rather than diffusion) of the CIM disk [63–67].

CIM monolithic supports represent a novel generation of stationary phase used for liquid chromatography and bioconversion. Some of these methacrylate-based monolith matrices are more and more developed and used for the fast bioactive molecules

separations. For example, CIM disk supports were profitably used for affinity chromatography separations of viruses, DNA, proteins, organic acids and smaller molecules like peptides, oligonucleotides, and oligosaccharides [68–71].

Recently, the potential of CIM disk columns as enzymatic reactors have been investigated. This macroporous support is a perfect model for the immobilization of enzymes and the fast conversion of lot of important substrates allowing to propose CIM disk as efficient enzymatic bioreactors technology [68,72–76]. Therefore, CIM disk monolith were used as support for immobilization of many important enzymes such as: malate dehydrogenase, deoxyribonuclease, citrate lyase, ribonuclease, isocitrate dehydrogenase, lactate dehydrogenase, acetylcholine esterase and glucose oxidase [74,76–79].

## 5. New monolith technology at the frontier of glycomic

### 5.1. Monolith microreactor for depolymerization of polysaccharides

CIM disk support is mostly adapted to the immobilization of enzyme and more particularly of polysaccharide lyases and hydrolases due to their high porosity (1 μm) and low back pressure. That's the way, we can maintain very fast mass transfers with negligible diffusion resistance, even with high viscous solution made of polysaccharides as substrate. The most interesting challenge was to envisage CIM disk as support for the development of a novel generation of enzymatic reactor. Recently, the first monolith enzymatic microreactor was proposed by Delattre et al. [80] for the large scale production of bioactive oligogalacturonides. After a simple immobilization of pectin lyase onto CIM epoxy disk, it is possible to manage obtention of large amounts of bioactive oligogalacturonides from enzymatic depolymerization of polygalacturonic acids in a very short time. Covalent binding immobilization offers the advantage to form strong and stable linkages between the activated support and specific enzyme. Epoxy groups are reactive groups that can form covalent linkages with nucleophilic groups such as primary and secondary amines, sulfhydryl groups or hydroxyl groups, in function of the pH of the immobilization step. In 70s Turkova et al. [81] had already proposed that proteins may react with methacrylate support with epoxy-groups.

Immobilization on epoxy groups follows a two step mechanism, incubation at alkaline pH values is necessary to have a multipoint covalent attachment [70,73,82–84]. Note to mention that these two steps mechanism of immobilization onto epoxy-matrix, a first physical adsorption followed by a limited covalent attachment has been proposed and largely described by Wheatley et al. [82–84]. In fact, in their works, authors discussed the use of high salt concentrations such as ammonium phosphate or ammonium sulfate to immobilize peptides, proteins or oligonucleotides to epoxy-activated silica and polymer supports. Generally, at low salt concentration, the affinity matrix result a lower reactivity with protein leading to low immobilization. These modified supports have been proposed in affinity applications such as affinity chromatography or immunoassays. Authors proposed a kinetic model between protein in solution and noncovalent protein–affinity matrix complexes. It was described that nucleophilic charges of proteins of the complexes react in a slow step with the epoxy-groups on the affinity phase [82]**.** Therefore, Wheatley et al. [82–84] interpreted the salt-concentration dependence of the immobilization of proteins as induction of specific hydrophobic interaction on the equilibrium formation of the "noncovalent protein–affinity support complexes".

Moreover, the fact that incubation needs alkaline pH condition to improve the enzyme reactivity has been largely explained and described by Mateo et al. [85–87]. Generally, if the matrix and affinity ligand are stable at high pH conditions, the addition of affinity ligand is realized under basic conditions. Therefore, when amino group is the nucleophilic group, the pH of the immobilization step is usually above nine [88]. In the case of ligand containing sulfhydryl groups, immobilization is essentially conducted at neutral pH [88].

In the last decade, Mateo et al. [85–87] have developed and proposed new epoxy matrices for industrial enzymes immobilization and stabilization based on the multipoint covalent attachment. According to authors, these supports are very robust and suitable for industrial scale up, thanks to 3 steps immobilization/stabilization/blokage procedures, the enzyme is more stable in this kind of sophisticated supports. Consequently, the best immobilization procedure described by Mateo et al. [85–87] is as following: (*i*) enzyme is first covalently immobilized under mild experimental conditions at neutral pH and room temperature; (ii) the already immobilized enzyme was further incubated under higher pH condition for long incubation time in order to make possible the formation of new covalent linkages between the immobilized enzyme molecule and the support; (iii) finally, the remaining epoxy groups of the support were blocked with very hydrophilic compounds to stop any additional interaction between the enzyme and the support.

In resume, the fast production and purification of α-(1,4)-oligogalacturonides was investigated using a new enzymatic reactor composed of CIM disk monolithic matrix [80]. The online one step production and purification of bioactive oligogalacturonides were investigated by association of a CIM disk with immobilized pectin lyase from *Aspergillus japonicus* and a CIM disk DEAE. It was shown that optimum temperature for free enzyme and immobilized pectin lyase onto CIM disk monolithic support is 30 °C, and optimum pH is 5. With the online association of the pectinlyase immobilized disk and a DEAE CIM disk it was clearly shown the high separation level of oligogalacturonans directly after the degradation of the polysaccharide [80]. This efficient enzymatic bioreactor production of uronic oligosaccharides from polygalacturonic acid (PGA) constitutes an original fast process to engender bioactive oligouronides with degrees of polymerization (dp) up to 7 for industrial scale up applications.

On this basis, other monolith enzymatic microreactors have been developed (Fig. 3) for the production of a large variety of therapeutic oligosaccharides such as oligoglucuronans from polyglucuronic acid [89] and oligoglycosaminoglycans from heparan sulfate and hyaluronic acid. In fact, depolymerization of anionic polysaccharides has been largely studied since acidic oligosaccharides have a great potential as biological activators [2,13,45]. Consequently, anionic oligosaccharides generated by these enzymes have been investigated for their biological properties. For example, a enzymatic microreactor for the large depolymerization of Glucuronan have been realized [89]. Glucuronan is a (1 → 4)-β-D-polyglucuropyranosyluronic acid excreted by the *Sinorhizobium meliloti* M5N1CS mutant strain (NCIMB 40472) [90]. Large amount of oligoglucuronans from its depolymerization has been described for its specific immunostimulating properties (production of Il-1, Il-6 and TNF-α cytokines) and applications on elicitation of plant natural defences [91,92]. Nevertheless, a specific biological activity is generally implicated by a specific degree of polymerization (dp) [1,2,4,13,37]. Degradation strategies are still investigated to generate specific dp reducing purification steps. Consequently, a new glucuronan lyase (EC 4.2.2.14) from *Trichoderma* sp. GL2 [93] was immobilized on a monolithic CIM® epoxy disk [89]. The immobilization yield is around 30% of the initial activity and 35% of the initial protein amount. Enzymatic depolymerization of 3 glucuronans with diverse *O*-acetylation degrees were investigated and compared with degradations using free enzymes [94]. This first immobilization of a glucuronan lyase represents a new tool to the fast production of bioactive oligoglucuronans. $^{1}$H NMR analyses were used to study the *O*-acetylation degree of oligoglucuronans and showed that the average degrees of

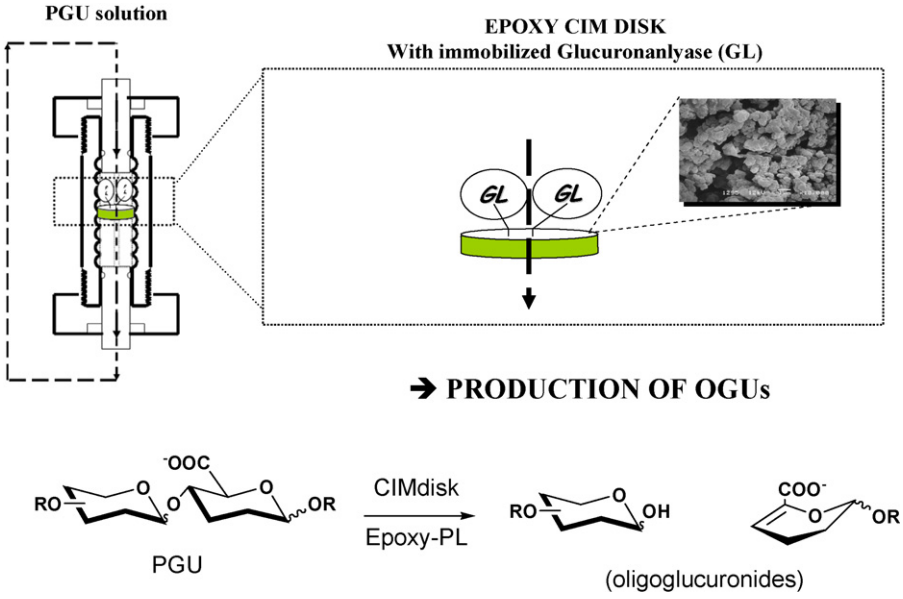


**Fig. 3.** Monolith enzymatic microreactor strategy for the large scale up production of oligosaccharides.

polymerization were between 4 and 13 after 24 h of degradation. In fact, $^{1}$H NMR spectra revealed specific signals from unsaturated oligoglucuronan generated by β-elimination such as a doublet at 5.8 ppm characteristic of the H-4 of an unsaturated unit (H-4Δ). By comparison between: H-1 signal integration of β-Δ- (4,5)-glucuronic acid (H-1Δ), all H-1 signals integrations (H-1Δ and H-1) and protons of acetates from oligosaccharide mixture, the average degree of polymerization (dp) and acetylation degree of the three products were then easily estimated. Note to mention that we have an immobilized material that can be reused on several occasions. It allows preparing oligoglucuronan pools that have higher dp comparatively to the free enzyme process where oligoglucuronans up to dp 8 with a majority of dp 2 to 4 were obtained [94]. So, the use of the CIM disk immobilized glucuronan lyase will make it possible to increase range of dp available for biological tests, in particular for dp higher than 6. Then, all these interesting results demonstrated that monolithic matrices are available for the treatment of very viscous solutions by immobilized enzymes.

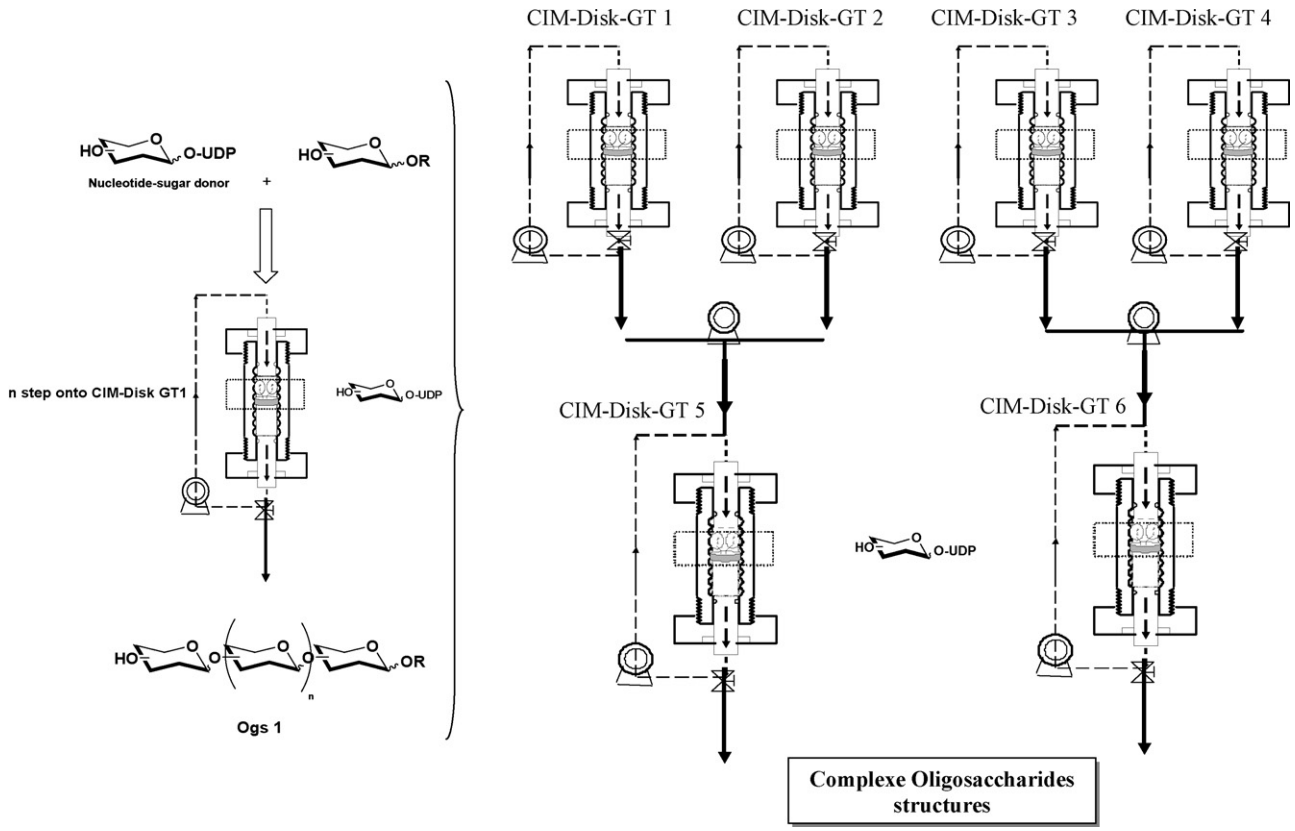


**Fig. 4.** Solid state monolith microbioreactor process for the automated synthesis of oligosaccharides (Ogs) by using glycosyltransferases (GT).

Finally, hyaluronidases have been immobilized onto CIM epoxy disk and CIM–EDA disk according to the previous immobilization described by Delattre et al. [80]. Consequently, it was possible to produce various bioactive oligoglycosaminoglycans in large microscale up. In fact, in recent years, a large amount of studies were investigated by using glycosaminoglycans and derivates in biological processus [2,95,96]. Glycosaminoglycans (GAGs) such as: chondroitin sulfate, dermatan sulfate, heparan sulfate, hyaluronan, etc. are polymers known to be correlated to their osidic composition, and in some cases to substituents linked on the carbohydrates at specific positions [95,96]. Since long time, GAGs and their oligosaccharides (sulfated or *N*-acetylated) have been described for their high biological activity in the growth stimulation of specific human cells [1,2,45]. Moreover, the specific functionalization (*N*-linkage, acetylation, phosphorylation, sulfatation, fluorescent labeling, ...) should be developed to produce and study the biological activities of glycopeptides. If new manufacturing biotechnologies have significantly increased development of functional oligosaccharides and glycoconjugates, the main drawback limiting their applications is the difficulty to engender specific glycosidic structures. In this context, the recognition of oligosaccharides as therapeutic agent and target biomolecules in different cellular processes has led to worldwide research in the area of oligosaccharides production. From then on, the market for industrial exploitation of oligosaccharide structure is already substantial and continues to expand gradually. Meanwhile, even if the potentialities of oligosaccharides are real, in most cases the lack of oligosaccharides production processes is the main drawback limiting their applications. For this, the development of oligosaccharides engineering strategies arouses currently a great importance. Research and development of novel generation of oligosaccharides with more physiological functional properties is now continuing. However, some problems remain to be solved in the development of more highly complex oligosaccharides. The most important problem is the need to achieve continuous and efficient industrial scale up production of oligosaccharides and to maintain a low cost in high-purity oligosaccharides.

### 5.2. Prospects toward monolith glycosyltransferase microreactors

In order to envisage the development of new therapeutic carbohydrate drugs, access to pure oligosaccharides becomes more and more important. Satisfactory quantities of pure bioactive molecules are the main key for biochemical, biophysical and biological studies. Given the high complexity of bioactive oligosaccharides, chemical or enzymatic synthesis needs the development of new generations of enzymatic reactors leading to the high regio- and stereoselective glycosylation reactions for the specific assemblage of oligosaccharides.

At present, works are in progress to develop new monolith glycosyltransferase microreactors for the fast automated scale up production of simple or complex oligosaccharide structures (Fig. 4). That is the way, we have investigated the synthesis in large scale up of oligoglycosaminoglycans biomimetic largely described for their main impact in cellular biological phenomena (data not shown). With these biotechnological strategies, these oligosaccharide structures will be tested for their putatives bioactivities by using DNA Array and transcriptomic technology in order to find new therapeutic drugs.

## 6. Conclusion

It is now well established that carbohydrates and more particularly oligosaccharides are strongly implicated as modulators in cell activities on bacteria, fungi, plant, *algae* and mammals. The need to improve biological and therapeutical applications allowed to develop specific functionalizations of oligosaccharides. Consequently, it appears evident that the next generation of therapeutic drug will be supported by synthetic sugars. Monolithic CIM disk is considered as an appropriate polymeric support for the efficient immobilization of enzymes such as: polysaccharide hydrolases, polysaccharide lyases and glycosyltransferases. At present, studies provide facts that the immobilization of pectin lyase or glucuronan lyase on activated CIM disk can be of great interest for the fast production and purification of oligouronides. The immobilized enzyme on the CIM disk is stable, exhibits good reproducibility; provides optimum conditions for enzymatic reactions similar to free enzymes processes and is still active after different runs. Moreover, a new generation of solid state enzymatic reactor have been developed with monolith technology by immobilization of glycosyltransferases for the automated synthesis of simple or highly complex oligosaccharides. This model appears as a very promising miniaturized system for screening and developing preparative scale methods. Nevertheless, supplementary studies on the development of new and improved immobilization technologies to increase the activity, selectivity or stability of the enzymes involved are still needed to improve the efficiency of this system for practical and industrial scale up applications in glycomics fields.

## References

[1] A. Varki, Glycobiology 2 (1993) 97.
[2] C. Delattre, P. Michaud, B. Courtois, J. Courtois, Minerva Biotechnol. 17 (2005) 107.
[3] O. Klarzynski, B. Plesse, J.M. Joubert, J.C. Yvin, M. Kopp, B. Kloareg, F. Bernard, Plant. Physiol. 124 (2000) 1027.
[4] B.L. Ridley, M.A. O'Neill, D. Mohnen, Phytochemistry 57 (2001) 929.
[5] G.F. Pineo, R.D. Hull, Annu. Rev. Med. 48 (1997) 79.
[6] J. Liu, Z. Shriver, R.M. Pope, S.C. Thorp, M.B. Duncan, R.J. Copeland, C.S. Raska, K. Yoshida, R.J. Eisenberg, G. Cohen, R.J. Linhardt, R. Sasisekharan, J. Biol. Chem. 277 (2002) 33456.
[7] S. Ghatak, S. Misra, B.P. Toole, J. Biol. Chem. 277 (2002) 38013.
[8] M. Otterlei, K. Østgaard, G. Skjåk-Bræk, O. Smidsrød, P. Soon-Shiong, T. Espevik, J. Immunother. 10 (1991) 286.
[9] O. Berteau, B. Mulloy, Glycobiology 13 (2003) 29.
[10] W.R.L. Farias, A.P. Valente, M.S. Pereira, P.A.S. Mourao, J. Biol. Chem. 275 (2000) 29299.
[11] T.Y. Wong, L.A. Preston, N.L. Schiller, Annu. Rev. Microbiol. 54 (2000) 289.
[12] Y. Iwamoto, X. Xu, T. Tamura, T. Oda, T. Muramatsu, Biosci. Biotechnol. Biochem. 67 (2003) 258.
[13] H. Barreteau, C. Delattre, P. Michaud, Food Technol. Biotechnol. 44 (2006) 333.
[14] M.A. Nugent, Proc. Natl. Acad. Sci. USA 97 (19) (2000) 10301–10303.
[15] R.M. Lauder, T.N. Huckerby, I.A. Nieduszynski, Glycobiology 10 (2000) 393–401.
[16] K.M. Koeller, C.H. Wong, Glycobiology 10 (2000) 1169.
[17] A. Hölemann, P.H. Seeberger, Curr. Opin. Biotechnol. 15 (2004) 622.
[18] A.V. Pukin, C.A.G.M. Weijers, B. van Lagen, R. Wechselberger, B. Sun, M. Gilbert, Carbohydr. Res. 343 (2008) 650.
[19] G.J. Boons, DDT 8 (1996) 331–342.
[20] G.J. Boons, Tetrahedron 52 (1996) 1095–1121.
[21] D. Magaud, C. Grandjean, A. Doutheau, D. Anker, V. Shevchik, N. Cotte-Pattat, J. Robert-Baudouy, Carbohydr. Res. 314 (1998) 199.
[22] K. Takeo, Y. Ohguchi, R. Hasegawa, S. Kitamura, Carbohydr. Res. 278 (1995) 313.
[23] U.T. Bornscheuer, in: H.J. Rehm, G. Reed, A. Pühler, P.J.W. Stadler, D.R. Kelly (Eds.), Biotechnology Series, vol. 8b, Wiley-VCH, Weinheim, 2000, pp. 277–294.
[24] U.T. Bornscheuer, M. Pohl, Curr. Opin. Chem. Biol. 5 (2001) 137–142.
[25] U.T. Bornscheuer, Curr. Opin. Biotechnol. 13 (2002) 543–547.
[26] C.A. Miller, Informatics 11 (2000) 489–495.
[27] A. Bastida, R. Ferndndez-Lafuente, G. Fernandez-Lorente, J.M. Guisdn, Bioorg. Med. Chem. Lett. 9 (1999) 633–636.
[28] O. Kirk, M.W. Christensen, F. Beck, T. Damhus, Biocatal. Biotransform. 12 (1995) 91.
[29] M. Filice, J.M. Palomob, P. Bonomi, T. Bavaro, R.F. Lafuente, J.M. Guisan, M. Terreni, Tetrahedron 64 (2008) 9286–9292.
[30] M. Filice, T. Bavaro, R. Fernandez-Lafuente, M. Pregnolato, J.M. Guisan, J.M. Palomo, M. Terren, Catal. Today 140 (2009) 11–18.
[31] A.G.M. Weijers, C.R. Franssen Maurice, M. Visser, Biotech. Adv. 26 (5) (2008) 436–456.
[32] C.P.C. Chiu, L.L. Lairson, M. Gilbert, W.W. Wakarchuk, S.G. Withers, N.J.C. Strynadka, Biochemistry 46 (2007) 7204.
[33] S.W. Chung, H.S. Joo, K.S. Jang, H.J. Lee, S.G. Lee, B.G. Kim, Enz. Microb. Technol. 39 (2006) 66.
[34] M.J. Playne, R. Crittenden, Bull. IDF 313 (1996) 22.

[35] P. Czermak, M. Ebrahimi, K. Grau, S. Netz, G. Sawatzki, P.H. Pfromm, J. Membr. Sci. 232 (2004) 91.
[36] P.T. Sangeetha, M.N. Ramesh, S.G. Prapulla, Proc. Biochem. 39 (2004) 758.
[37] P. Nigam, D. Singh, Enz. Microb. Technol. 17 (1995) 770.
[38] D.O. Thompson, Crit. Rev. Ther. Drug 14 (1997) 104.
[39] P. Sears, C.Y. Wong, Science 291 (2001) 2344–2350.
[40] P.H. Seeberger, D.B. Werz, Nat. Rev. 4 (2005) 751.
[41] P.H. Seeberger, W.C. Haase, Chem. Rev. 100 (2000) 4349.
[42] P.H. Seeberger (Ed.), Solid Support Oligosaccharide Synthesis and Combinatorial Carbohydrate Libraries, 2001.
[43] P.H. Seeberger, Chem. Commun. (2003) 1115.
[44] K.R. Love, P.H. Seeberger, Angew. Chem. Int. Ed. 43 (2004) 602.
[45] P. Michaud, A. Da Costa, B. Courtois, J. Courtois, Crit. Rev. Biochem. Mol. 23 (2003) 266.
[46] S. Aldington, S.C. Fry, Adv. Bot. Res. 19 (1993) 101.
[47] Y. Iwamoto, X. Xu, T. Tamura, T. Oda, T. Muramatsu, Biosci. Biotechnol. Biochem. 67 (2003) 263.
[48] T.Y. Wong, L.A. Preston, N.L. Schiller, Annu. Rev. Microbiol. 54 (2000) 340.
[49] A. Kawada, N. Hiura, S. Tajima, H. Takahara, Arch. Dermatol. Res. 291 (1999) 547.
[50] A. Antonopoulos, P. Favetta, W. Helbert, M. Lafosse, Carbohydr. Res. 339 (2004) 1309.
[51] J.M. Guisan, A. Bastida, A.C. Cuuesta, R. Fernandez-Lafuente, C.M. Rosell, Biotechnol. Bioeng. 39 (1991) 78.
[52] M.N. Gupta, B. Mattiasson, in: C. Seulter (Ed.), Bioanalytical Applications of Enzymes, Wiley, New York, 1992, p. 36.
[53] W. Tischer, V. Kasche, Trends Biotechnol. 17 (1999) 326.
[54] F. Vaillant, A. Millan, P. Millan, M. Dornier, M. Decloux, M. Reynes, Proc. Biochem. 35 (9) (2000) 989.
[55] K. Sarioflu, N. Demir, J. Acar, M. Mutlu, J. Food Eng. 47 (2001) 271.
[56] W.H. Hanisch, P.D. Rickard, S. Nyo, Biotechnol. Bioeng. 20 (1) (1978) 95.
[57] P.G. PiVeri, M. Tramontini, A. Malacarne, Biotechnol. Bioeng. 33 (1989) 1258.
[58] R.A. Sheldon, Adv. Synth. Catal. 349 (2007) 1307.
[59] M.D. Busto, K.E. Garcia-Tramontin, N. Ortega, M. Perez-Mateos, Bioresour. Technol. 97 (2006) 1783.
[60] Y.A. Schipunov, Y.V. Burtseva, T.Y. Karpenko, N.M. Shevchenko, T.N. Zvyagintseva, J. Mol. Catal. B. Enz. s40 (2006) 23.
[61] C. Temporini, E. Calleri, D. Campèse, K. Cabrera, G. Félix, G. Massolini, J. Sep. Sci. 30 (2007) 3076.
[62] M. Kminkova, J. Kucera, Enz. Microbiol. Technol. 5 (3) (1983) 204.
[63] T. Tennikova, J. Reusch, J. Chromatogr. A 1065 (2005) 13.
[64] T.B. Tennikova, B.G. Belenkii, F. Svec, J. Liq. Chromatogr. 473 (1990) 63.
[65] A. Strancar, P. Koselj, H. Schwinn, D. Josic, Anal. Chem. 68 (1996) 3483.
[66] A. Strancar, M. Barut, A. Podgornik, P. Koselj, D. Josic, A. Buchacher, LC–GC Int. 10 (1998) 660.
[67] H. Abou-Rebyeh, F. Koerber, K. Schubert-Rehberg, J. Reusch, D. Josic, J. Chromatogr. 566 (1991) 341.
[68] D. Josic, H. Schwinn, A. Strancar, A. Podgornik, M. Barut, Y.-P. Lim, M. Vodopivec, J. Chromatogr. A 803 (1998) 61.
[69] M. Rucevic, J.G. Clifton, F. Huang, L. Xuesong, H. Callanan, D.C. Hixson, D. Josic, J. Chromatogr. A 1123 (2006) 204.
[70] J. Champagne, C. Delattre, C. Shanthi, B. Satheesh, L. Duverneuil, M.A. Vijayalakshmi, Chromatographia 65 (2007) 648.
[71] C. Delattre, A.S. Kamalanathan, P. Michaud, M.A. Vijayalakshmi, J. Chromatogr. B 861 (2008) 185.
[72] J. Hagedorn, C. Kasper, R. Freitag, T.B. Tennikova, J. Biotechnol. 69 (1999) 1.
[73] A. Podgornik, M. Vodopivec, H. Podgornik, M. Barut, A. Strancar, in: A. Ballesteros, F.J. Plou, J.L. Iborra, P.J. Halling (Eds.), Stability and Stabilization of Biocatalysts, Elsevier Science, Amsterdam, 1998, p. 541.
[74] M. Vodopivec, M. Berovic, J. Jancar, A. Podgornik, A. Strancar, Anal. Chim. Acta 407 (2000) 105.
[75] H. Podgornik, A. Podgornik, Enz. Microb. Technol. 31 (2002) 855.
[76] M. Vodopivec, A. Podgornik, M. Berovic, A. Strancar, J. Chromatogr. B 795 (2003) 105.
[77] M. Benčina, K. Bencina, A. Strancar, A. Podgornik, J. Chromatogr. A 1065 (2005) 91.
[78] M. Benčina, J. Babič, A. Podgornik, J. Chromatogr. A 1144 (2007) 142.
[79] M. Bartolini, V. Cavrini, V. Andrisano, J. Chromatogr. A 1065 (2005) 134.
[80] C. Delattre, P. Michaud, M.A. Vijayalakshmi, J. Chromatogr. B 861 (2008) 208.
[81] J. Turkova, K. Blaha, M. Malanikova, Biochim. Biophys. Acta 524 (1) (1978) 162–169.
[82] J.B. Wheatley, D.E. Schmidt Jr., J. Chromatogr. A 849 (1) (1999) 1–12.
[83] J.B. Wheatley, D.E. Schmidt Jr., J. Chromatogr. 644 (1) (1993) 11–16.
[84] J.B. Wheatley, M.H. Lyttle, M.D. Hocker, D.E. Schmidt Jr., J. Chromatogr. A 726 (1996) 77–90.
[85] C. Mateo, V. Grazu, B.C.C. Pessela, T. Montes, J.M. Palomo, R. Torres, F. Lopez-Gallego, R. Fernandez-Lafuente, J.M. Guisan, Biochem. Soc. T 35 (6) (2007) 1593–1601.
[86] C. Mateo, O. Abian, G. Fernandez-Lorente, J. Pedroche, R. Fernandez-Lafuente, J.M. Guisan, A. Tam, M. Daminati, Biotechnol. Prog. 18 (3) (2002) 629–634.
[87] C. Mateo, O. Abian, R. Fernandez-Lafuente, J.M. Guisan, Enz. Microb. Technol. 26 (7) (2000) 509–515.
[88] G.T. Hermanson, A.K. Mallia, P.K. Smith, Immobilized Affinity Ligand Techniques, Academic Press, San Diego, C.A., 1992, p. 119.
[89] M.L. Tavernier, E. Petit, C. Delattre, B. Courtois, J. Courtois, A. Strancar, P. Michaud, Carbohydr. Res. 343 (2008) 2687.
[90] J. Courtois-Sambourg, B. Courtois, A. Heyraud, P. Colin-Morel, M. Rinaudo-Duhem, WO 9318,174 (1993).
[91] J. Courtois-Sambourg, B. Courtois, FR 27,81,673 (2000).
[92] Y. Lienart, A. Heyraud, O. Sevenou, FR 27,95,289 (1999).
[93] C. Delattre, P. Michaud, C. Keller, R. Elboutachfaiti, L. Beven, B. Courtois, J. Courtois, Appl. Microb. Biotechnol. 70 (2006) 437.
[94] C. Delattre, P. Michaud, J.M. Lion, J. Courtois, B. Courtois, J. Biotechnol. 118 (2005) 448.
[95] E. Petit, C. Delattre, D. Papy-Garcia, P. Michaud, Adv. Pharmacol. 53 (2006) 167.
[96] N. Volpi, J. Pharm. Sci. 96 (2007) 3168.