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Review

Recent Trends in Biomaterials for Immobilization of Lipases for Application in Non-Conventional Media

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Abstract: The utilization of biomaterials as novel carrier materials for lipase immobilization has been investigated by many research groups over recent years. Biomaterials such as agarose, starch, chitin, chitosan, cellulose, and their derivatives have been extensively studied since they are non-toxic materials, can be obtained from a wide range of sources and are easy to modify, due to the high variety of functional groups on their surfaces. However, although many lipases have been immobilized on biomaterials and have shown potential for application in biocatalysis, special features are required when the biocatalyst is used in non-conventional media, for example, in organic solvents, which are required for most reactions in organic synthesis. In this article, we discuss the use of biomaterials for lipase immobilization, highlighting recent developments in the synthesis and functionalization of biomaterials using different methods. Examples of effective strategies designed to result in improved activity and stability and drawbacks of the different immobilization protocols are discussed. Furthermore, the versatility of different biocatalysts for the production of compounds of interest in organic synthesis is also described.

Keywords: biomaterials; immobilization; lipases; biocatalysis; non-conventional media

1. Introduction

Enzymes are proteins that catalyze reactions with high specificity and stereoselectivity and are widely used in industrial bioprocesses. The use of these biocatalysts has increased in recent years, mainly in the fields of organic synthesis [1,2], food modification, and biofuel development [3,4]. In organic synthesis, lipases are the most widely used class of enzymes [1]. In nature, these enzymes act on ester groups, but they are also able to catalyze hydrolysis or synthesis reactions with high chemo-, regio- and stereoselectivity with a wide variety of unnatural substrates [5], allowing their application to obtain different classes of organic compounds (Figure 1). Currently, molecular biology and protein engineering strategies have been allied with reliable high-throughput-screening methods to obtain lipases with novel catalytic properties that have potential to compete with commercial lipases. Examples of recent successful approaches are the screening of novel enzymes from metagenomic libraries [6–11] and the combined protein engineering and enzyme immobilization (immobilized biocatalyst engineering) that was recently reviewed by Bernal et al. [12]. These strategies can be

integrated to obtain immobilized lipases that have improved activity and stability in non-conventional media, properties that are required if lipases are to compete with chemical routes commonly used in industry [12]. Indeed, non-conventional media, especially organic media, are commonly used to produce manufactured products in biocatalysis [1]. In these systems, the water content is limited (i.e., they are aquo-restricted media), which favors synthesis reactions and increases the productivity of various processes, especially those involving hydrophobic substrates. Immobilization has an important role in the viability of these processes since lipases do not perform well in such systems in their free forms.

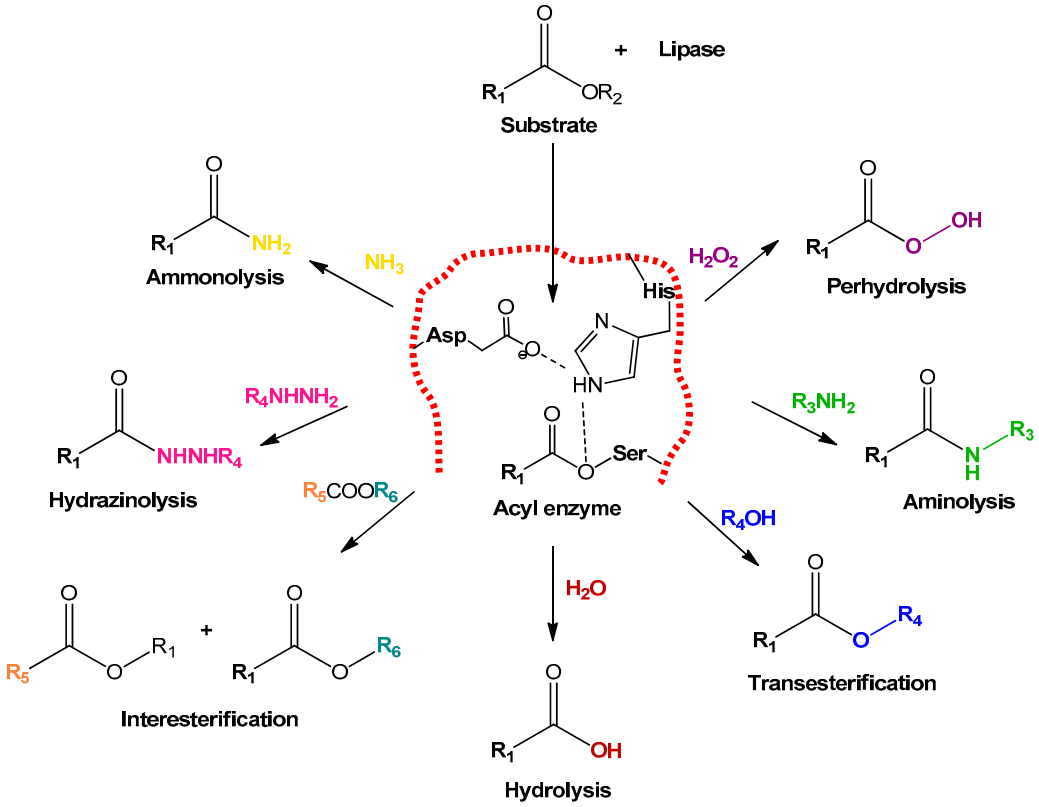


Figure 1. Examples of reactions catalyzed by lipases with different substrates [13].

Research into new materials has contributed in recent years to the development of a multitude of new supports for the immobilization of lipases, with better physical and mechanical properties. These new materials include magnetic nanoparticles [14], carbon nanotubes [15] synthetic polymers [16], mesoporous and electrospun materials [17], and biomaterials. They bring interesting features, including greater surface areas available for immobilization, which allow an effective enzyme loading and improve mass transfer during the reaction.

In this review, we discuss the use of biomaterials and their derivatives as supports for the immobilization of lipases as effective strategies to improve activity and stability. We review experimental studies with a focus on the methods of immobilization. For each biomaterial (natural polysaccharide), we briefly discuss its major properties before turning to the immobilization protocols used. However, before addressing the biomaterials, we briefly describe the general features of lipases, since they can affect the selection of the support and the method of immobilization. We also briefly describe the main methods used to immobilize lipases.

2. Lipases: General Features and Classical Strategy of Immobilization

In nature, lipases hydrolyze triacylglycerols, releasing di- or monoacylglycerols, fatty acids, and glycerol [18]. However, in practice, the definition and classification of lipases is somewhat

controversial, mainly due to the impossibility of strictly differentiating lipases from esterases. Traditionally, lipases (EC 3.1.1.3) have been defined as enzymes that hydrolyze long-chain triacylglycerols, which are water-insoluble substrates, whereas esterases (EC 3.1.1.1) hydrolyze small esters that are partially soluble in water, with both types of enzyme belonging to the sub-subclass of carboxylic ester hydrolases (EC 3.1.1) [3,5,18]. Later, Ali et al. [19] suggested classifying carboxylic ester hydrolases differently, dividing them into lipolytic esterases (EC 3.1 L) and non-lipolytic esterases (EC 3.1 NL). More recently still, Bracco et al. [20] suggested classifying lipases and esterases based on their ability to act in organic solvents that are immiscible in water (e.g., toluene), with low water activity (a_w). In this review, we use the definition whereby lipases hydrolyze triacylglycerols or acyl esters with acyl chains of 10 carbons or more.

Lipases have a common tertiary structure, the standard α/β hydrolase fold (Figure 2A) [18]. In lipases, this structure has a central nucleus composed of parallel β-strands surrounded by α-helices and contains a catalytic triad composed of the amino acids Ser (which acts as a nucleophile during catalysis), His, and either Asp or Glu, with these amino acids being located at canonical positions in the α/β hydrolase fold [5,18]. Given that the natural substrates of lipases are hydrophobic, the active site usually has a hydrophobic nature. Typically, it also has a mobile subdomain called the lid. The lid can assume two conformations, closed and open. In the closed conformation, the lid blocks the active site, preventing entry of the substrate. In the open conformation, the substrate has free access to the active site (Figure 2B). In aqueous solution, these two conformations are in equilibrium, with the closed conformation being favored. In the presence of hydrophobic substrates dispersed in water, the lipases are adsorbed at the hydrophobic interface and the equilibrium is shifted from the "closed" form of the enzyme to the "open" form, exposing the catalytic site, allowing substrate molecules to enter and suffer catalysis [5,18]. The conformational change of lipases at nonpolar-aqueous interfaces is known as interfacial activation and generally is associated with increased activity in the presence of hydrophobic interfaces [21,22]. However, although the presence of the lid is commonly correlated with the phenomenon of interfacial activation, lipases have been described that have a lid but do not present interfacial activation, for example, lipases from *Burkholderia glumae* and *Pseudomonas aeruginosa* [23,24]. Recently, Khan et al. [25] classified lipases into three different groups according to the structure of the lid domain: (1) lipases without a lid, (2) lipases with a lid formed by a single α-helix and (3) lipases with two or more α-helices forming the lid. The authors concluded that the lid domain also has a close relationship with the substrate specificity and thermostability of lipases [25].

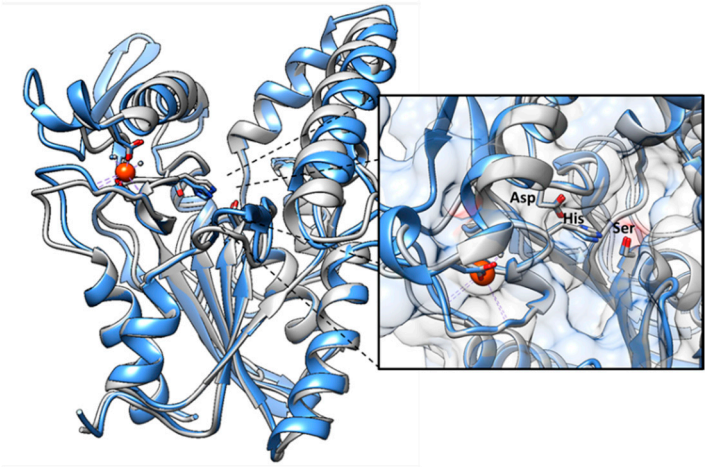
Various strategies have been used over recent years to immobilize lipases: adsorption, the production of crosslinked enzyme crystals or aggregates, covalent binding, encapsulation and entrapment [26–29]. Among these strategies, physical adsorption is the most used, since, unlike other methods of immobilization, it does not cause large modifications in the native conformation of the enzyme or loss of catalytic activity [30]. Generally, the method consists of hydrophobic adsorption of the enzyme onto porous or non-porous supports and has been used as a standard technique in the purification, immobilization, and stabilization of lipases in a single-step process [21,22].

Physical adsorption onto hydrophobic supports fixes the lipase predominantly in its "open" form, producing more active and selective biocatalysts, especially when applied to the catalysis of complex reactions, such as kinetic resolution and regioselective hydrolyses or syntheses [31–33]. However, the hydrophobic part of lipases may favor the formation of dimers or aggregates with other hydrophobic proteins present in a crude extract, which may negatively affect immobilization [34,35]. In addition, the adsorption of lipases onto hydrophobic supports is reversible, meaning that the lipase can leach from the solid support, especially in the presence of detergents, additives or organic solvents [30], or even in the presence of the substrates and products of the reaction [36], which may limit the application of the biocatalyst.

Lipase B from *Candida antarctica* (CALB) immobilized on polymeric beads (Lewatit VP OC 1600), named Novozym 435, is the commercially available immobilized lipase that is most used in biocatalysis, being applied in a wide variety of hydrolysis or synthesis reactions of interest to industry [1,37].

However, Novozym 435 has various problems that may limit its industrial application, such as enzyme leaching from the support, the fragility of the particles of the support under stirring, and dissolution of the support in polar solvents, like ethanol and methanol [37]. Therefore, it is necessary to search for new supports and to develop new immobilization protocols.

(**A**)



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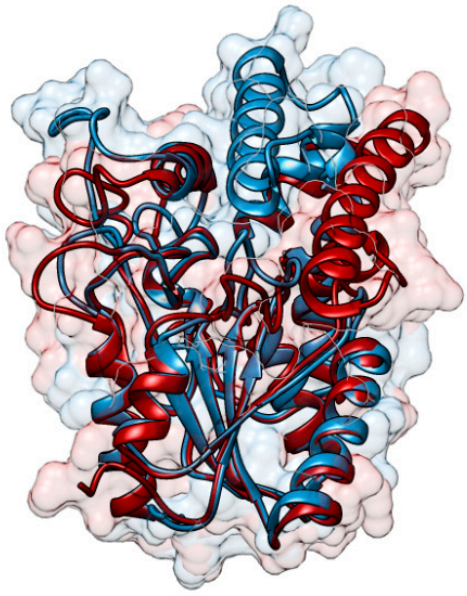

Figure 2. (**A**) Standard α/β hydrolase fold of the superposed structures of the lipases from *Burkholderia cepacia* (blue) (PDB:1YS1) and *Pseudomonas aeruginosa* (gray) (PDB:1EX9). Zoom-in of the modeled catalytic site and the calcium ion (in red); (**B**) molecular model of the metagenomic lipase LipC12 (PDB:6CL4) with the structure superposed in the "open" (red) and "closed" (blue) form.

In the following sections, we describe recent strategies of immobilization using biomaterials as supports, focusing on strategies that improve the activity and stability of the lipases, especially when they are used in reactions in organic solvents. Hereafter, when the term "conversion" appears, it represents the number of mols of ester produced as a percentage of the number of mols of ester that could potentially have been produced considering the reagents.

3. Agarose

Agarose is a linear heteropolysaccharide composed of monomeric units of agarobiose (4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactose) (Figure 3). It is the principal component of agar, a linear galactan extracted mainly from red seaweed (*Phylum rhodophyta*). Agarose has interesting features, such as mechanical resistance, high hydrophilicity, pore and particle sizes that can be varied [38], and primary hydroxyl groups that can be chemically modified. These features have enabled agarose to find many applications in molecular biology and biochemistry, where it is utilized as a matrix for electrophoretic analysis, purification, and immobilization of proteins [39]. The main advantage of using agarose as a matrix for the immobilization of enzymes is the presence of a large number of hydroxyl groups that allow crosslinking with different agents, as well as functionalization with different groups [39]. For example, glyoxyl and epoxy agarose are often used to immobilize and stabilize enzymes via multipoint covalent attachment. In the case of lipases, octyl-agarose (octyl-sepharose) is the most-used commercial support, as it allows the lipase to be immobilized in the "open" form. However, the adsorbed lipase can leach from the support in the presence of high concentrations of organic solvents or surfactants. This problem can be overcome by treatment with glutaraldehyde or polymers like dextran or polyethyleneimine after immobilization, aiming to cross-link the lipase to the support [40–42]. However, this type of treatment can result in a loss of enzyme activity and is costly.

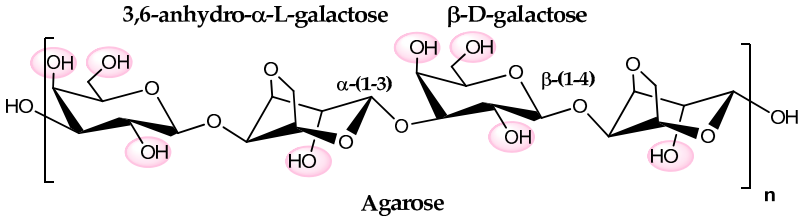

Figure 3. Schematic representation of the backbone structure of agarose: β-D-galactose and 3,6-anhydro-α-L-galactose, linked by glycosidic bonds β-(1–4) and α-(1–3). The light pink circles randomly highlight the hydroxyl groups that can be chemically modified.

Another strategy for avoiding desorption of the enzyme is to use a heterofunctional support. These supports are produced through the oxidation of commercial octyl-agarose (glyoxyl-octyl agarose) [43] or by bi-functionalization of the agarose (Figure 4A) with hydrophobic and aldehyde groups (alkyl-aldehyde agarose) [44]. These heterofunctional supports allow the immobilization of lipases by adsorption onto a dense layer of hydrophobic moieties at neutral pH, followed by multipoint covalent attachment between the adsorbed enzyme and the aldehyde groups. The main advantage of the alkyl-aldehyde support is that the numbers of aldehyde and alkyl groups on the surface of the support can be controlled. Furthermore, alkyl groups with different chain sizes may be used to allow better control of the immobilization process, producing biocatalysts with a wider range of properties than are available with commercial supports.

Different lipases have been immobilized on glyoxyl-octyl and alkyl-aldehyde agarose supports; they showed improvements in activity (a phenomenon known as hyperactivation), stability at high temperatures and high concentrations of organic solvents [43–50]. However, although these novel strategies are useful for immobilization of lipases, they have some limitations:

(i) alkaline pH values (around pH 10) are necessary to increase the reactivity of the nucleophilic residues exposed at the protein surface, especially Lys residues (pKa 10.5). Some enzymes are intrinsically unstable under alkaline conditions, for example, the lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL) [46,51];

(ii) the additional step of reduction that is necessary to establish multiple covalent linkages between the support and the enzyme may partially inactivate the enzyme;

(iii) the enzyme must have at least one amino acid residue that can react with the aldehyde groups of the support after the adsorption.

Novel strategies have been proposed to improve these protocols, ranging from chemical modification using different agents by solid-phase chemistry [40,52] to the preparation of heterofunctional supports. For example, the support octyl-glutamic agarose was proposed; it is obtained by derivatizing glyoxyl-octyl agarose with glutamic acid [48]. In this case, the immobilization relies on ionic interactions between the lipase and the support after physical adsorption with interfacial activation, allowing stabilization of the enzyme without incubation at alkaline pH [48]. However, for the formation of these ionic interactions, the protocol requires the immobilized derivative to be incubated at pH values around 4.0 or 5.0, which may lead to complete loss of activity of some lipases [48].

Melo et al. [53] proposed a novel heterofunctional agarose support containing different groups (hydrophobic or carboxylic/metal) for the adsorption of the enzyme via different regions, followed by covalent attachment with glutaraldehyde groups (Figure 4B). The main advantage of this support is that it allows a single step immobilization at neutral pH and can, therefore, be used with lipases that are intrinsically unstable at alkaline pH. Metagenomic lipase LipC12 and CRL were successfully immobilized on this support, presenting high activity and stability after immobilization [53].

The use of lipases immobilized on agarose supports in organic media is limited, especially in synthesis reactions, where the highly hydrophilic agarose matrix retains a significant amount of water that may displace the equilibrium of the reaction. This can occur even in functionalized agaroses, since steric hindrance means that "internal" hydroxyls react poorly with added reagents, meaning that it is difficult to achieve complete functionalization of these groups [38]. In addition, hydrophilic substrates or products of the reaction, such as ethanol and glycerol, may be retained in the agarose matrix, which may lead to loss of enzyme activity or mass transfer problems.

Indeed, there are relatively few reports of the use of lipases immobilized on agarose in pure organic solvents (100%) or solvent-free systems (i.e., systems in which the initial reaction medium contains only the substrates). Godoy [54] immobilized lipases from *Geobacillus thermocatenulatus*, *Thermomyces lanuginosus* and *Candida antarctica* on glyoxyl agarose combined with a new amination strategy that used different additives (dithiotreitol, anthranilic acid, methyl anthranilate, and aniline), followed by drying with *tert*-butanol. The dried biocatalysts were used in the ethanolysis of palm olein in a solvent-free system, with the maximum conversion obtained being 72% in 14 h for the biocatalyst prepared with *T. lanuginosus* lipase. This conversion was 5-fold higher than that obtained for the non-dry biocatalyst (14% conversion in 14 h), showing the positive effect of the drying procedure.

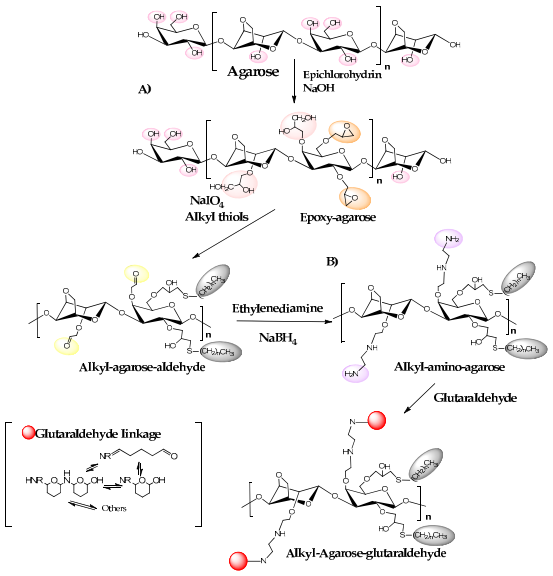

Figure 4. Schematic representation of the preparation of heterofunctional supports. (**A**) Preparation of new tailor-made alkyl-aldehyde supports; (**B**) glutaraldehyde-activation of alkyl-aldehyde support.

Although few applications in aquo-restricted media have been reported, it is important to note that agarose-based supports have been used as a proof of concept of different strategies, allowing an improvement of activity, stability, and selectivity of different lipases of interest in biocatalysis.

4. Chitosan

Chitosan-based materials have been used as matrices for the development of products in biomedicine and biotechnology. Chitosan is a natural biopolymer derived from chitin. It is non-toxic, bio-compatible, and bio-degradable. Chitin is the second most abundant natural polysaccharide after cellulose and can be obtained from natural sources, like exoskeletons of arthropods, such as insects and crustaceans [55,56].

Chitosan is composed of monomeric units of β-(1,4)-2-amino-2-deoxy-D-glucose, obtained through the partial deacetylation of chitin (β-(1,4)-2-acetamide-2-deoxy-D-glucose) in an alkaline medium or through enzymatic hydrolysis catalyzed by chitin deacetylase (Figure 5) [55,56]. The great advantage of immobilizing enzymes on chitosan is the low cost and the availability of different physical forms (powders, scales, hydrogels, membranes, fibers, and microspheres). It has reactive amino ($–NH_2$) and hydroxyl (–OH) groups that can be chemically modified, allowing the introduction of functional groups that can react with amino acid residues at the protein surface. Furthermore, the high density of amine groups in chitosan allows the preparation of reticulated matrices, in which the structural units are crosslinked using reagents such as epichlorohydrin, glycidol, polyaldehydes, genipin, citric acid, sodium triphosphate and glutaraldehyde [55–58]. Among these crosslinking agents, genipin (Figure 6) has gained prominence for being less toxic than glutaraldehyde [59], the classical crosslinking agent for chitosan reticulation.

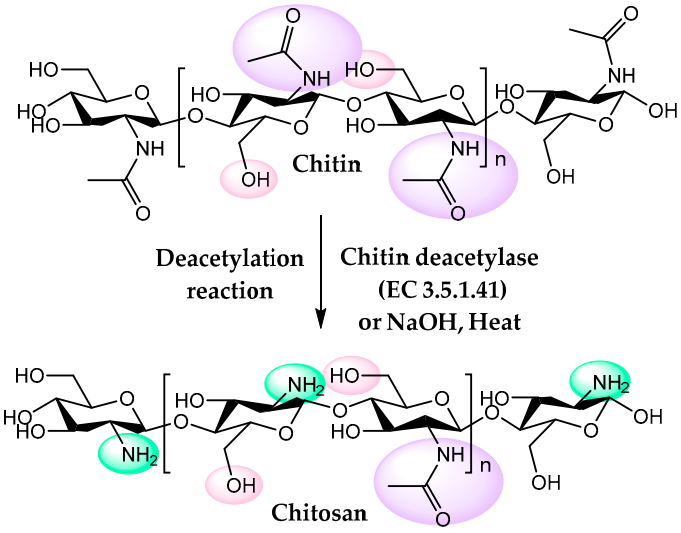

Figure 5. Chemical structure of chitosan. Chitin can be deacetylated by chitin deacetylases or by treatment in hot alkaline media. The backbone of chitosan is a linear homopolymer of β-(1,4)-2-amino-2-deoxy-D-glucose. The highlighted groups (aqua = amino, light pink = hydroxyl and mauve = *N*-acetyl) represent the sites that can be chemically modified.

Some important points should be considered in the use of chitosan for the immobilization of lipases:

(i) chitosan microspheres, beads, and fibers show poor mechanical stability, which may result in fragmentation of these structures [60,61]. Reticulation of the chitosan is important to produce a mechanically resistant matrix and also to avoid its dissolution under acidic conditions.

(ii) different agents containing reactive groups can be used to control solubility, anionic properties, and hydrophobicity of chitosan. One example is glutaraldehyde; the bonds formed between

the polymer and this agent render the chitosan-matrix quite hydrophobic [62], which may be advantageous for lipase immobilization;

(iii) immobilization may be affected by reactive groups in the chemical agent used to activate the chitosan, such as glutaraldehyde or ethylenediamine, and also by amino, hydroxyl and acetyl amine groups present in chitosan itself. Charged functional groups in the support may cause non-specific binding of the enzyme to the support, which can lead to loss of enzyme activity. In some cases, it may be necessary to block the remaining reactive groups in the support;

(iv) similarly, reactive groups present in chitosan may react with substrates or products of the reaction, depending on the reaction conditions used. For example, the amino groups may react with aldehyde and ketone groups, forming Schiff bases that affect the solubility of the polymer

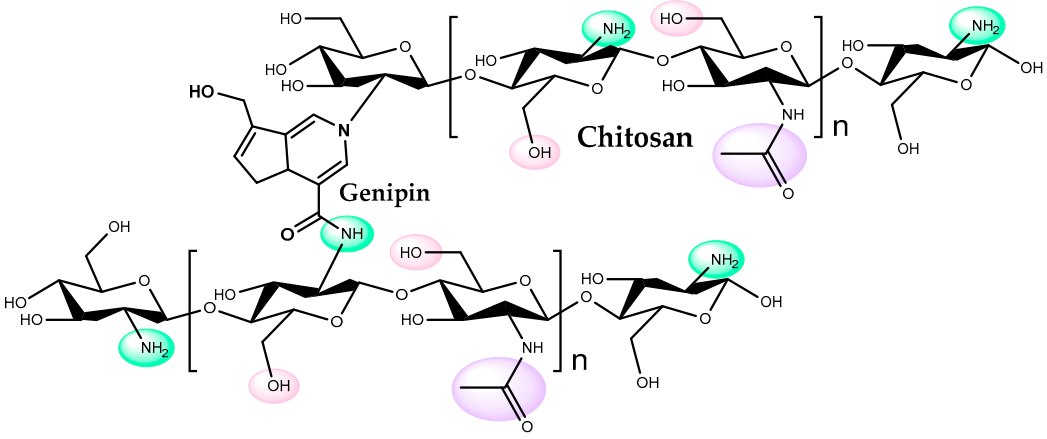

Figure 6. Schematic representation of the structure of chitosan crosslinked with genipin according to Butler et al. [63].

Chitosan is classically activated by derivatizing the amine groups with glutaraldehyde (Figure 7A). The exposed reactive group of the glutaraldehyde then forms covalent bonds with exposed amino, thiol, phenol or imidazole groups of the enzyme, conferring high stability against leaching. Glutaraldehyde-activated chitosan has been used to immobilize several lipases. For example, chitosan sequentially activated with glycidol, ethylenediamine (EDA), and glutaraldehyde (Figure 7B) was used to immobilize CALB (CALB-CH) [64]. CALB was also immobilized on glyoxyl agarose (CALB-GX) and octyl agarose (CALB-OC). CALB-CH was more stable at 65 °C and pH 7.0 ($t_{\frac{1}{2}}$ = 23 min) and in 70% dioxane ($t_{\frac{1}{2}}$ = 8 min) than was soluble CALB ($t_{\frac{1}{2}}$ = 3 min and $t_{\frac{1}{2}}$ = 1.5 min, respectively). It was also much more stable in dioxane than were CALB-GX ($t_{\frac{1}{2}}$ = 0.28 min) and CALB-OC ($t_{\frac{1}{2}}$ = 0.15 min). CALB-CH was used to synthesize ethyl oleate in near-critical CO_2 (100 bar and 29.9 °C), with 46% conversion in 6.5 h [64]. However, the preparation lost more than 90% of its activity after four cycles of reuse [64]. The authors did not investigate the effect of supercritical CO_2 on the stability of CALB-CH or soluble CALB in the absence of the reaction species, nor the effects of the conditions on the integrity of the support.

In another study, chitosan was activated with ethylenediamine (EDA) and glutaraldehyde and used to immobilize the lipase from *Cercospora kikuchiim* (a fungal plant pathogen) using a fluidized-bed dryer system [65]. The immobilized preparation had a high lipase activity and low water activity (a_w = 0.08), suitable features for biodiesel synthesis. It was used in the ethanolysis of coconut oil (*Cocos nucifera* oil). Conversions remained above 95% over five successive ethanolysis cycles. The fluidized-bed drying was advantageous: lipases are generally immobilized in aqueous media and sorbed water needs to be removed before use in organic media, to prevent it from affecting the reaction equilibrium or interfering with the diffusion of hydrophobic substrates. Other drying methods, such as lyophilization, may cause loss of enzyme activity, which was not observed using fluidized-bed drying.

Another advantage of the fluidized-bed drying process was that immobilization and drying were achieved in a single step, which would be advantageous for industrial applications [65].

Recent works have used chitosan for the preparation of magnetic composite materials. Such materials are of great interest because magnetic separation is simple and more effective than filtration or centrifugation. The coating of magnetic nanoparticles with biopolymers like chitosan protects them against erosion and provides appropriate functional groups [14]. For example, magnetic Fe_3O_4 nanoparticles were prepared by the hydrothermal method and coated with chitosan activated with glutaraldehyde and then used to immobilize CRL [66]. The biocatalyst was used to hydrolyze castor oil, with a degree of hydrolysis of 46% being obtained in 60 h at 34 °C. The activity was not stable: in the fourth hydrolysis cycle, the degree of hydrolysis was only 30%. The authors suggested that the loss of activity was due to "irreversible" adsorption of substrate and product on the support, since they washed the biocatalyst with tertiary butyl alcohol and PBS (phosphate-buffered saline 0.02 M, pH 7.0) to remove any substrate or product that had been retained on the support [66].

A similar strategy was used to prepare magnetic nanoparticles coated with chitosan; the preparation involved in situ crosslinking using citric acid, followed by activation with glutaraldehyde [67]. CALB was immobilized covalently on this support and the immobilized derivative was used to esterify itaconic anhydride with different polyols (Figure 8). It was stable in the reaction medium (a mixture of toluene:Tetrahydrofuran (THF) (2:1)), retaining 80% of the initial activity after 5 reaction cycles, at 25 °C for 72 h [67]. The chitosan-coated nanoparticle matrix maintained its shape and its physicochemical properties to prevent lipase leaching. Therefore, the loss of activity appears to be due to denaturation of the enzyme itself, not decomposition of the support. In a similar manner, squaric acid (3,4-dihydroxy-3-cyclobutene-1,2-dione) was used as a crosslinking agent in the preparation of a co-polymer coat formed by collagen and chitosan [68]. The chitosan-collagen composite was used to coat magnetic Fe_3O_4 nanoparticles. The carboxylic groups of the CRL were firstly activated with (Dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) and then reacted with NH_2 groups of the coat. The activity of this immobilized preparation was 2-fold higher (52 U mg^{-1}) than that of nanoparticles prepared with traditional crosslinking agent glutaraldehyde (26 U mg^{-1}). However, curiously, the amount of immobilized CRL was lower for nanoparticles prepared with squaric acid as a crosslinking agent. Nevertheless, both biocatalysts prepared with squaric acid and glutaraldehyde retained almost 70% of residual activity after 10 cycles of the hydrolysis of the olive oil at 37 °C, pH 7.4, for 30 min. Unfortunately, the ability of the biocatalysts to catalyze synthesis reactions in organic medium was not evaluated.

Magnetic chitosan nanocomposites were also prepared using different functional ionic liquids. Several imidazole-based ionic liquids with different functional groups (carboxyl, alkyl, hydroxyl, and amino) were used to modify the chitosan [69]. The functionalized chitosan was used to encapsulate magnetic Fe_3O_4 nanoparticles. This support was used to immobilize porcine pancreatic lipase (PPL). In related works, mesoporous silica was functionalized using ionic liquids, resulting in a support with carboxyl-functionalized ionic liquids grafted onto the material [70,71]. These groups were then used to couple the mesoporous silica with chitosan, with the composite then being used to immobilize PPL. The immobilized PPL showed high hydrolytic activity in aqueous medium and better temperature stability than the free enzyme, although this depended on the functional group and the carbonic chain of the ionic liquid in the support. Unfortunately, the activity and stability of the biocatalysts in the organic media were not evaluated.

New strategies for the functionalization of chitosan microspheres have been reported recently. For example, chitosan and chitin nanowhiskers were crosslinked using tannic acid [72]. This strategy resulted in a hybrid polymer composite with better mechanical strength than that of pure chitosan. The support was used to immobilize RML using activation with EDC and NHS. The biocatalyst was used in the synthesis of eugenyl benzoate from eugenol and benzoic acid. A conversion of 53% was obtained in 5 h at 50 °C. The reuse of the biocatalyst was not studied.

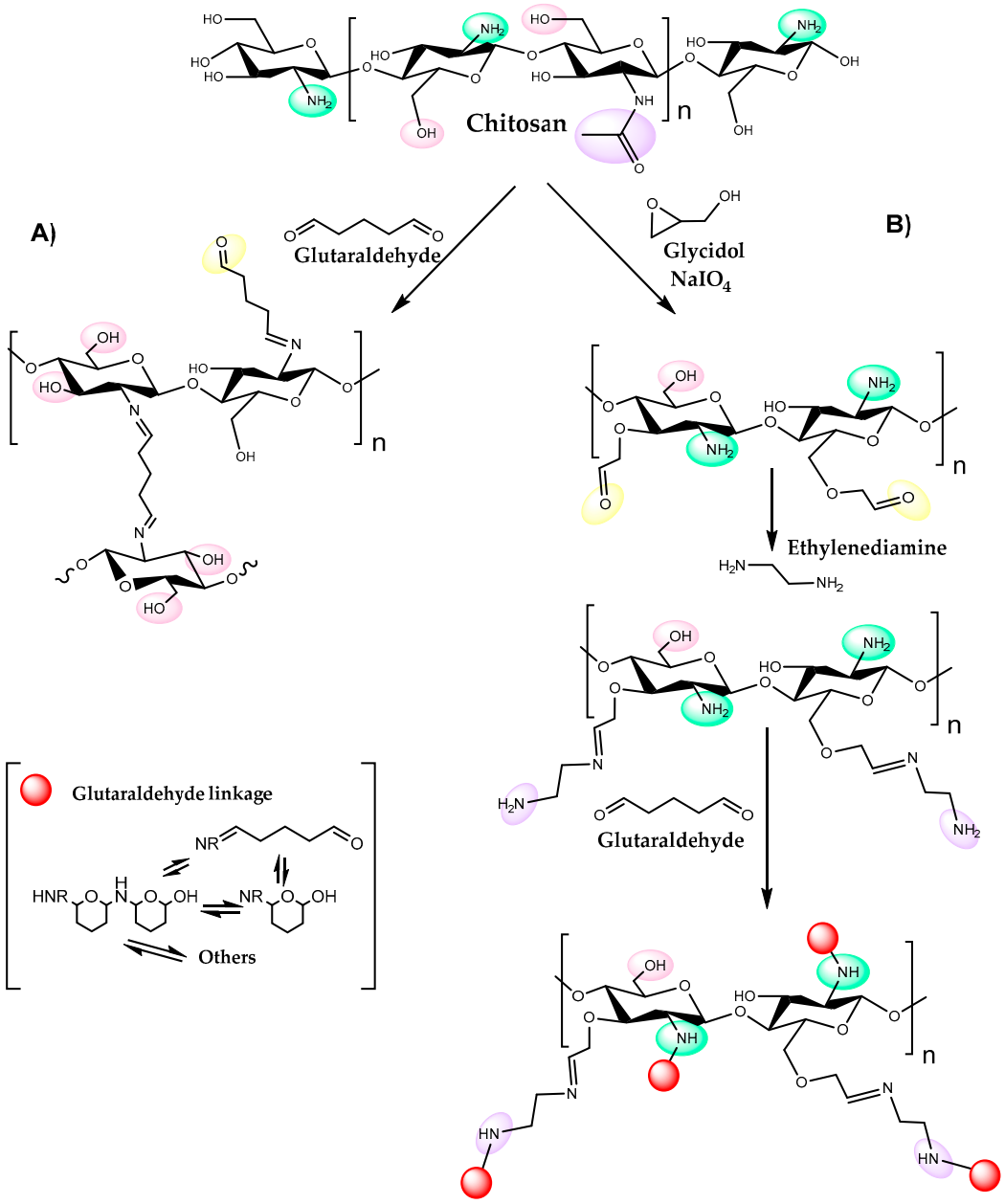

Figure 7. Activation of chitosan using the bifunctional reagent glutaraldehyde. (**A**) Classical methodology for derivatizing the primary amino groups of chitosan using glutaraldehyde as a crosslinking agent. (**B**) Preparation of supports based on chitosan activated with glycidol, ethylenediamine, and glutaraldehyde [64].

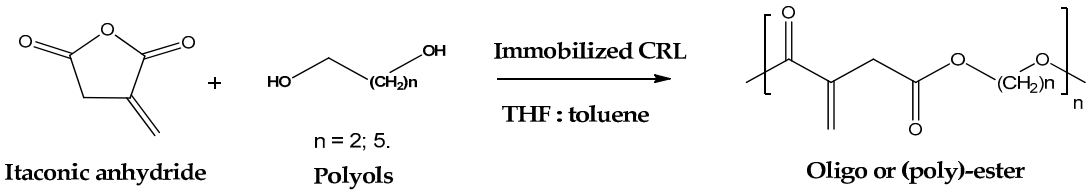

Figure 8. Esterification of itaconic anhydride with polyols catalyzed by *Candida rugosa* (CRL) immobilized on magnetic chitosan nanoparticles [65].

Recently, CALB was physically encapsulated by oppositely charged polyions in a mixture of sodium polyphosphate and chitosan [73]. The prepared biocatalyst showed high stability at 65 °C

($t\frac{1}{2}$ = 366 min), at pH 4.0. It was applied in the synthesis of benzyl acetate from benzyl alcohol and vinyl acetate, with a maximum conversion of 98% in 24 h at 30 °C [73]. Pinheiro et al. [74] prepared a new support for enzyme immobilization, divinyl sulfone-activated chitosan (Figure 9). Divinyl sulfone (DVS) is a versatile agent that can be used in alkaline media, for example at pH 10.0, while glutaraldehyde, which is the agent that is most commonly used to activate chitosan-based materials, is unstable under these conditions. Chitosan was activated with DVS at pH 10.0, 12.5 and 14.0. DVS activation was an effective strategy for immobilization of CALB, which showed higher stability in different conditions of pH and temperature than did CALB immobilized on chitosan activated with glutaraldehyde [74]. Nevertheless, an important drawback of DVS supports is that the lengthy spacer arm may reduce the rigidity induced by the multipoint covalent attachment [38,75] Additionally, it is necessary to block the remaining DVS groups of the support after immobilization and this blocking step can alter the catalytic properties of the lipase [38].

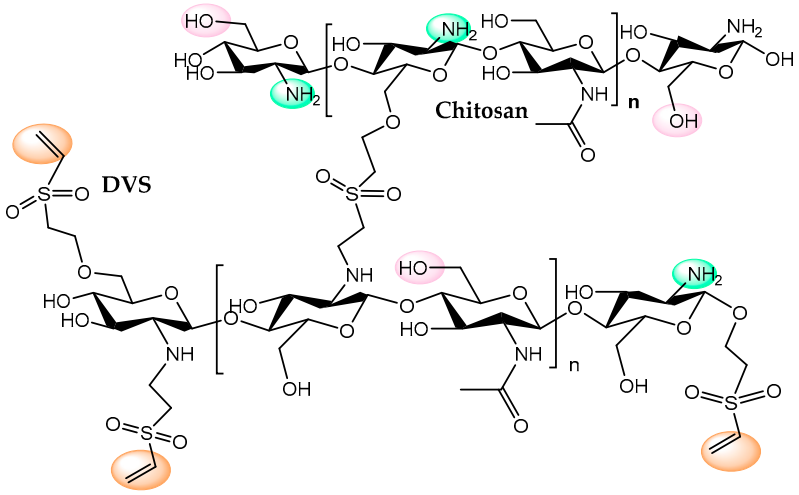

Figure 9. Schematic representation of the crosslinking reaction of chitosan with divinyl sulfone and the different functional groups on the activated chitosan surface.

The modification of chitosan with hydrophobic agents changes its hydrophilic character to a more hydrophobic one. Some studies have explored this type of modification, aiming to prepare hydrophobic supports that allow the immobilization of lipases by adsorption. For example, hydrophobic chitosan microspheres were prepared by reductive amination (using dodecyl aldehyde) for the immobilization of RML [76]. The activity of the biocatalyst was 3-fold higher than that of the free lipase. It was used to convert sunflower oil into a structured lipid containing 49% of stearic acid. The biocatalyst was used in successive cycles of transesterification, maintaining 70% of residual activity after the tenth cycle [76]. Likewise, Urrutia et al. [77] modified chitosan microspheres with different alkyl chains (C4, C8, and C12) (Figure 10) and used them to immobilize CALB and RML. The hydrophobicity obtained with C8 was greater than that obtained with C4, but there was no further increase in hydrophobicity when C12 was used. The biocatalysts were applied in the selective hydrolysis of fish oil to obtain polyunsaturated fatty acids. The length of the alkyl chains linked to chitosan affected the activity, stability, and selectivity of the immobilized enzyme [77].

As demonstrated, chitosan has been used successfully as a support for lipase immobilization in recent years. The different strategies for the functionalization of this polymer provide a wide variety of novel immobilization protocols. However, chitosan polymers have different molecular weights, degrees of polymerization and degrees of acetylation, depending mainly on the natural source and the way that the chitosan was obtained [78]. These properties may affect the characteristics of the matrix used in the immobilization process and also the properties of the biocatalyst, which may lead to problems of reproducibility.

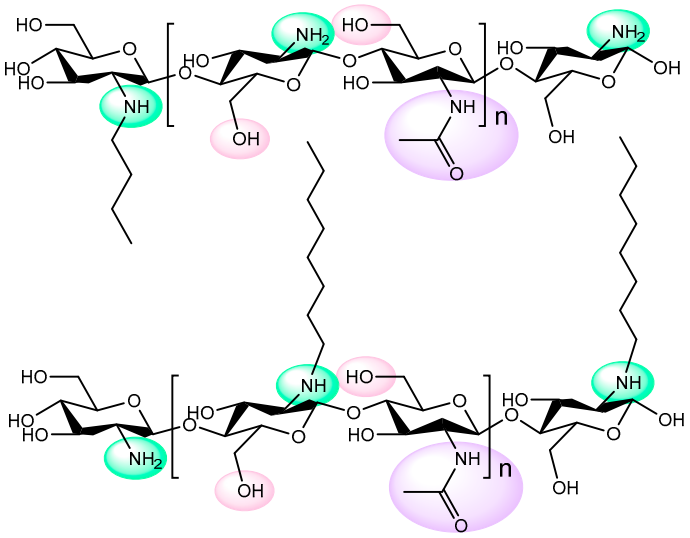

Figure 10. Schematic representation of chitosan activated with different alkyl chains.

5. Cellulose

Cellulose is the most abundant renewable biopolymer on Earth. It is a linear polymer composed of D-glucose residues linked by β-(1,4) glycosidic bonds (Figure 11). Some advantages in the utilization of cellulose as a matrix for enzyme immobilization are biocompatibility, high specific surface area, exceptional mechanical properties, and low cost [79]. Cellulose can be used in microcrystalline, nanocrystalline, and chemically modified forms, or even associated with other materials, such as nanocomposites or nanoparticles [79,80].

Despite the great potential of cellulose to immobilize enzymes, there are few reports of its use for immobilization of lipases. Karra-Châabouni et al. [81] used oxidized cellulose fibers to immobilize *Rhizopus oryzae* lipase (ROL) by physical adsorption. The cellulose fibers (a commercial microcrystalline cellulose, TECHNOCEL-150DM) were prepared by chemical oxidation of cellulose to generate carboxylic groups on the cellulose surface (Figure 12A). The content of carboxylic groups influenced the adsorption of the lipase on the support, confirming that the interactions of the enzyme with the cellulose were mainly electrostatic. The immobilized derivative was used in the synthesis of butyl oleate in *n*-hexane, giving a conversion of 80% at 37 °C [81].

Koga et al. [82] functionalized cellulose paper with 3-(trimethoxysilyl)propyl methacrylate and then immobilized a lipase from *Burkholderia cepacia* by hydrophobic adsorption (Figure 12B). The immobilized derivative was evaluated in the transesterification of (*R*,*S*)-1-phenylethanol with vinyl acetate, using isopropyl ether as the solvent. The conversion to the ester (*R*)-1-phenylethylacetate was 50% in 5 h, at 23 °C.

Cellulose was modified by using 3-aminopropyl-triethoxysilane (APTES) to introduce amino groups onto the cellulose matrix [83]. Next, glutaraldehyde was used to introduce aldehyde groups for the immobilization of CALB (Figure 13). The immobilized enzyme was used to esterify oleic acid with ethanol in *n*-heptane, giving 97% conversion in 1 h at 60 °C. It was also used in the kinetic resolution of (*R*,*S*)-1-phenylethanol in cyclohexane, with vinyl acetate as the acyl donor. The conversion of (*R*)-1-phenylethanol was 42% after 5 h at 60 °C, with an enantiomeric excess of product of 99% [83].

Cellulose can also be used to coat magnetic nanoparticles. Singh and Mukhopadhyay [84] coated Fe_2O_3 nanoparticles with cellulose acetate and then generated aldehyde groups through oxidization with HIO_4. These aldehyde groups were then used to immobilize CALB covalently. The biocatalyst was applied in the synthesis of mono- and diglycerides from olive oil. The best conversions in 5 h were obtained at 50 °C, using *tert*-butanol as the solvent. In addition, the biocatalyst was used in 11 successive reaction cycles, giving a conversion to monoglycerides of 94% of the theoretical maximum

(based on the starting substrate) [84], showing the high activity and stability of this preparation in *tert*-butanol.

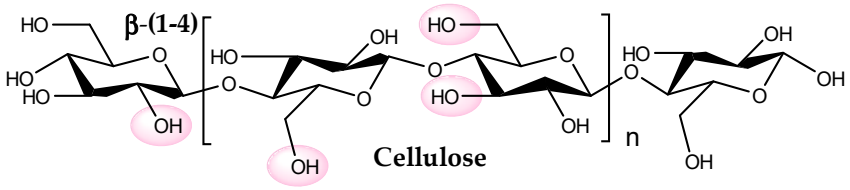

Figure 11. Schematic representation of cellulose structure. The backbone is a linear homopolymer of β-(1–4)-linked D-glucose residues. The light pink circles randomly highlight the hydroxyl groups that can be chemically modified.

Nanocellulose has also been used to prepare nanocomposite conjugates with other polymers such as chitosan, with the intention of achieving a support with good mechanical stability. Elias et al. [85] prepared beads of a nanocomposite of chitosan and nanocellulose (denominated CS-NC). CRL was immobilized on CS-NC using glutaraldehyde as the crosslinking agent. The biocatalyst was used in the synthesis of butyl butyrate, with conversions greater than 80% being obtained in four successive cycles of reaction. In addition, Raman spectroscopy and optical fluorescence microscopy showed the existence of strong hydrogen bonds between the chitosan and the nanocellulose. The lipase molecules were linked to the surface of the CS-NC through imine bonds, which were formed through a Schiff base mechanism [85].

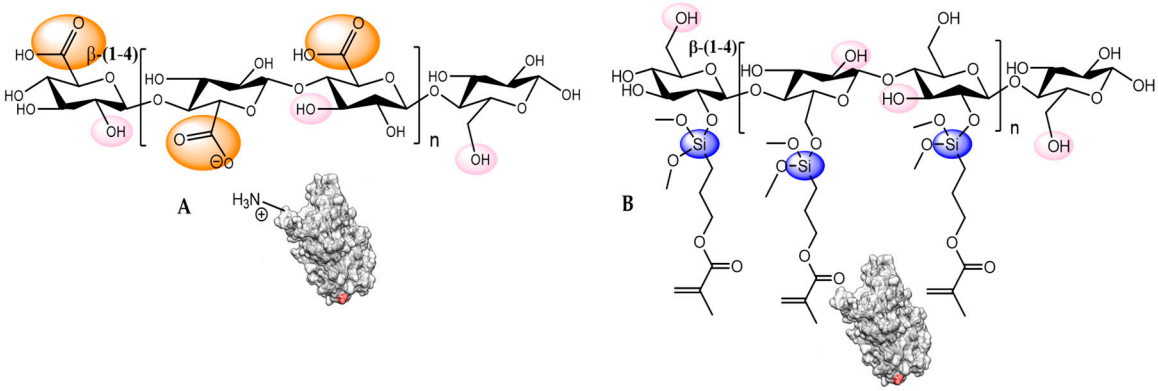

Figure 12. (**A**) Schematic representation of the structure of carboxyl-cellulose. (**B**) Schematic representation of the structure of cellulose modified with 3-(trimethoxysilyl)propyl methacrylate.

Magnetic cellulose nanocrystals (MCNCs) were prepared by a precipitation crosslinking method and used to immobilize *Pseudomonas cepacia* lipase (PCL) [80]. The biocatalyst was applied in the asymmetric hydrolysis of (*R*,*S*)-ketoprofen ethyl ester, where the yield of (*R*)-ketoprofen was 43%, with an enantiomeric excess of product of 85%. In addition, the biocatalyst was used in six successive reaction cycles, retaining over 66% of its initial activity in the last cycle [80].

Bacterial cellulose is an alternative source of cellulose. At the most basic level, the chemical composition of bacterial cellulose is identical to that of plant cellulose, with an ordered crystalline structure of high purity. Recent works have explored the use of bacterial cellulose for immobilization of lipases by physical adsorption and covalent binding [79,86,87]. Interesting properties were reported for the biocatalysts in these works, such as high activity and better stability than the free enzyme at pH and temperature values typically used in biocatalytic processes. However, the properties of the biocatalysts were not evaluated in organic media.

Semi-synthetic polymers, such as hydroxypropyl methylcellulose (HMC), have also been used to immobilize lipases. Badgujar et al. [88] immobilized four commercial lipases in HMC by entrapment.

The best results were obtained using the immobilized *Pseudomonas fluorescens* lipase, with yields of over 90% for the synthesis of 21 different *β*-amino ester compounds, via the aza-Michael addition reaction, with remarkable chemoselectivity (>94%) in the carbon-nitrogen bond formation [88].

Cellulose is a viable alternative for immobilizing enzymes and various forms of cellulose have been used to immobilize lipases: cellulose fibers, cellulose paper, cellulose acetate, nanocellulose, hydroxypropyl methylcellulose and bacterial cellulose. In addition, functionalization strategies that are used for other supports can be applied, including oxidation to generate carboxylic groups and addition of methacryloxy, amino, epoxy and aldehyde groups. Cellulose has also been used to form composites with other materials such as chitosan and magnetic nanoparticles.

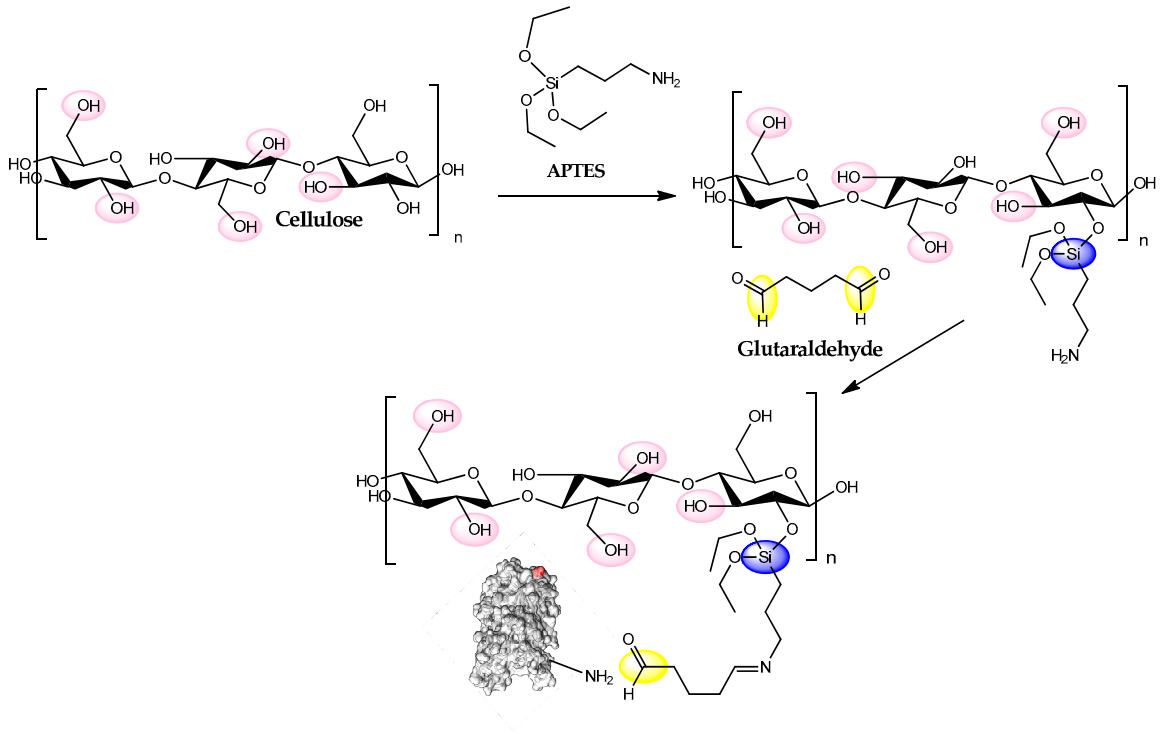

Figure 13. Schematic representation of the structure of cellulose functionalized with-aminopropyl-triethoxysilane (APTES) and glutaraldehyde.

Various methods have been used to immobilize lipases on cellulosic supports, including electrostatic adsorption, hydrophobic adsorption, covalent bonding, entrapment and precipitation-cross-linking, giving immobilization efficiencies from 10.5% to 95%. The highest immobilization efficiency (95%) was achieved using cellulose paper functionalized with methacryloxy groups, which increase in the hydrophobicity of the support and favor interaction with lipases [82]. Good immobilization efficiencies for lipases have also been achieved with hydroxypropyl methylcellulose via entrapment (>91%), using a protocol without washing or decanting [88], and with cellulose-acetate-coated Fe_2O_3 nanoparticles via covalent bonding (85%) [84]. In the latter case, the surface layer of the support has hydrophobic groups that may have enabled greater interaction of the enzyme with the support [84]. It should be noted that immobilization efficiency is not the only criterion for evaluating the effectiveness of a support. For example, CALB was covalently immobilized on cellulose functionalized with amino groups, with an immobilization efficiency of only 10.5% [83]. However, compared to Novozyme 435 (CALB immobilized on an acrylic resin), the esterification activity for synthesis of ethyl oleate was similar, and the kinetic resolution of (*R*,*S*)-α-phenylethanol was better.

Relatively few reuse cycles have been used with lipases immobilized on cellulosic supports. After 3 cycles, *Rhizopus oryzae* lipase immobilized on modified cellulose fibers gave only 25% of the

initial esterification activity [81]. After 8 cycles, CRL immobilized on a nanocomposite of chitosan and nanocellulose had lost around 50% of its initial activity in the synthesis of butyl butyrate [85]. Finally, after 11 glycerolysis cycles, *Candida antarctica* lipase immobilized onto cellulose acetate-coated Fe_2O_3 nanoparticles retained 94% of its initial activity [84].

6. Starch

Starch is a natural polymer composed of amylose, a linear polysaccharide containing α-(1,4)-linked anhydroglucose units (AGU), and amylopectin, which has α-(1,4)-AGU chains joined at branch-points by α-(1,6)-linkages (Figure 14) [89].

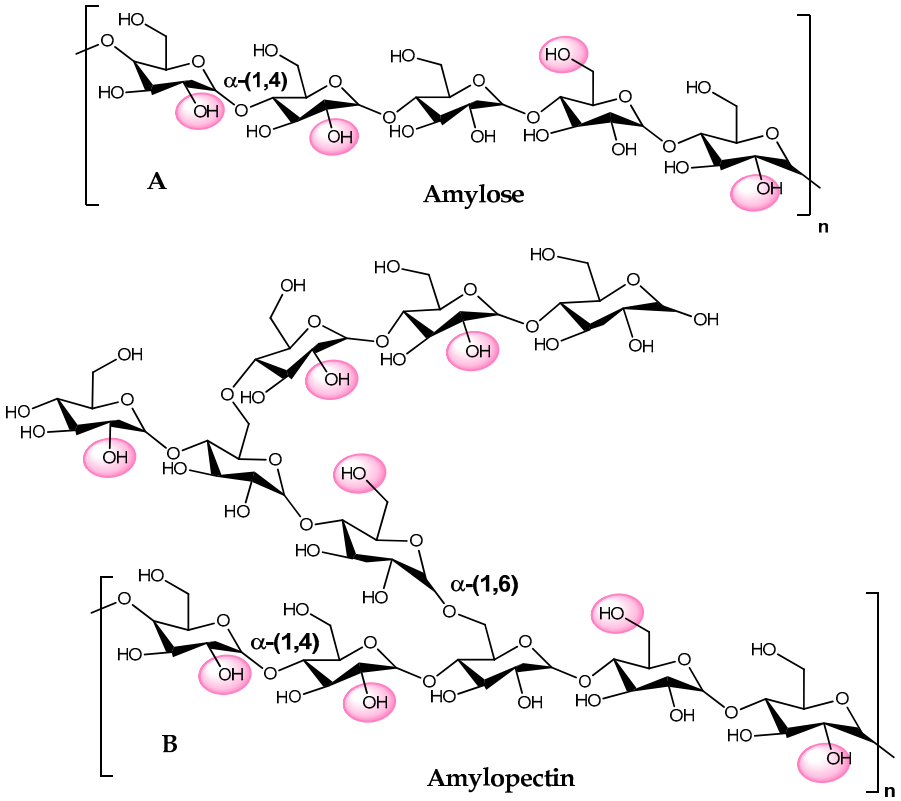

Figure 14. Schematic representation of the structure of starch. Two different polysaccharides are present in starch: (**A**) linear (1,4)-linked α-D-glucose (amylose) and (**B**) highly branched α-D-glucose (amylopectin). The light pink circles randomly highlight the hydroxyl groups that can be chemically modified.

Starch films have many advantages, such as low cost, renewability, biodegradability, and ease of production, and have been used to immobilize several lipases [90]. The matrix of the starch gel allows the entrapment of the enzyme and, after gel drying, the resulting biocatalyst can be used in different reaction media. For example, the lipase from *Burkholderia cepacia* (BCL) was immobilized by entrapment in a starch film, and successfully applied in the synthesis of aroma esters (1-octyl acetate and 1-pentyl octanoate) in media containing ionic liquids mixed with organic solvents [91]. After optimization of the reaction conditions, the conversion was greater than 99% after 24 h at 25 °C for two different reactions in different media: (i) the synthesis of 1-octyl acetate using neat methyl tert-butyl ether (MTBE) and also using diethyl ether/[BMIM][PF_6] (9:1), and (ii) the synthesis of 1-pentyl octanoate using diethyl ether/[BMIM][Cl] (9:1) [89]. Corn starch films were also prepared and used to immobilize CRL by entrapment [92]. The biocatalyst was applied in the enantioselective resolution of (*R*,*S*)-menthol using vinyl acetate as the acyl donor (Figure 15), with toluene as the solvent at 35°C. The conversion of (*R*)-menthol to (*R*)-menthyl acetate ester was 33%, with an enantiomeric excess of product of greater than 99% after 48 h [92].

Some structural properties of starch can be modified to meet specific requirements [93]. One example is oxidation with sodium periodate in an acidic medium, which leads to the formation of a polyaldehyde chain (Figure 16) [94]. The aldehyde groups make this derivative suitable for immobilization by multipoint covalent attachment with amino groups of the enzyme.

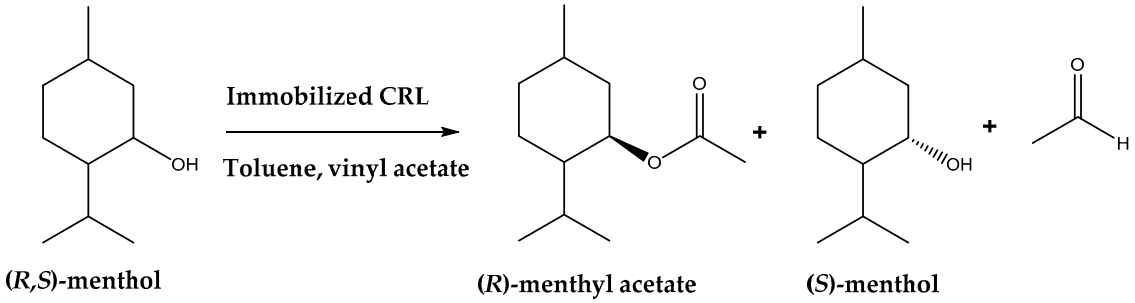

Figure 15. Kinetic resolution of (*R*,*S*)-menthol with vinyl acetate catalyzed by CRL immobilized in starch films by entrapment.

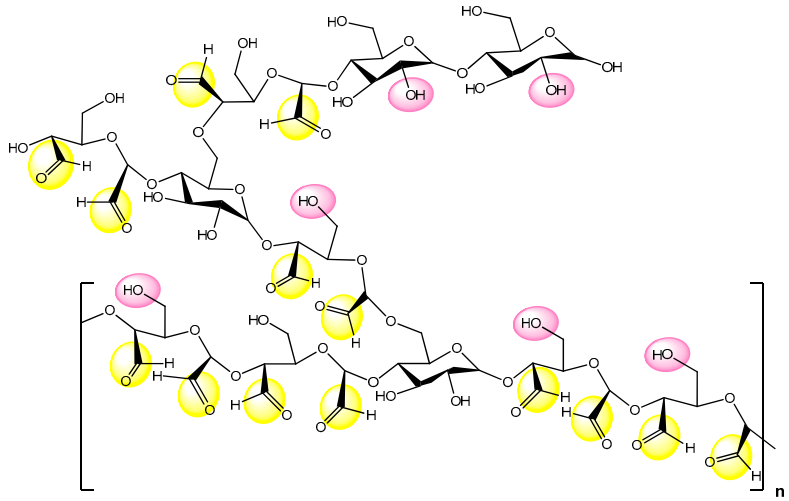

Figure 16. Schematic representation of the chemical structure of polyaldehyde starch.

This strategy was used to immobilize a lipase from *Rhizopus* sp. on magnetic nanoparticles coated with two different crosslinkers (i) polyaldehyde starch (MDSNIL) and (ii) glutaraldehyde (MNGCL) [95]. The immobilized enzyme tolerated moderately acidic environments, pH 6.0–6.5. At pH 9.0, MNGCL maintained 53% of initial activity, while MDSNIL maintained 65%, indicating a higher tolerance of the lipase in these preparations than is obtained with the glutaraldehyde crosslinked lipase. The biocatalyst showed stability at 60 °C, with relative activities of 55% and 60% being obtained, after 2 h of incubation, for MNGCL and MDSNIL, respectively. The residual activity of MDSNIL in *n*-hexane was 52.1% after incubation for 2 h. The reuse experiments showed that MNGCL retained 46.8% residual activity after 6 reuses, while MDSNIL retained 53.6% of initial lipolytic activity under the same conditions. Together with the great advantage of the immobilized biocatalyst being easily separated from the reaction medium through the application of an external magnetic field, the results of the work highlight the potential application of polyaldehyde starch as the crosslinking agent.

7. Alginate

Alginate is an anionic linear polysaccharide present in the cell walls of brown algae, including species of *Laminaria* and *Ascophyllum*. Alginates are composed of different amounts of (1–4) linked β-D-mannuronic acid and α-L-guluronic acid units (Figure 17) [96]. They are low-cost, non-toxic, and

biocompatible. Alginates are soluble but, for enzyme immobilization, they are co-precipitated with the enzyme using cations that act as ionic bridges between different molecules of alginate. The most commonly used cation is Ca^{2+} [97].

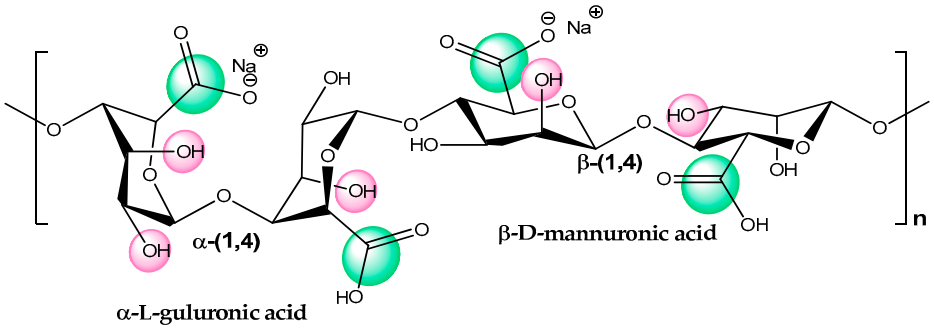

Figure 17. Schematic representation of the chemical structure of sodium alginate. The highlighted groups (aqua = carboxyl and light pink = hydroxyl) represent the sites that can be chemically modified.

As is the case with starch, alginate in gel form is used to immobilize lipases by entrapment or encapsulation. For example, a lipase from *Aspergillus niger* was entrapped in calcium alginate beads and used in the chemo-enzymatic epoxidation of α-pinene (Figure 18) in ethyl acetate as the solvent [96]. The conversion of α-pinene to α-pinene oxide was 65% in 24 h. The biocatalyst was used in 10 successive reactions, with a loss of activity of 36% after the tenth reaction [98].

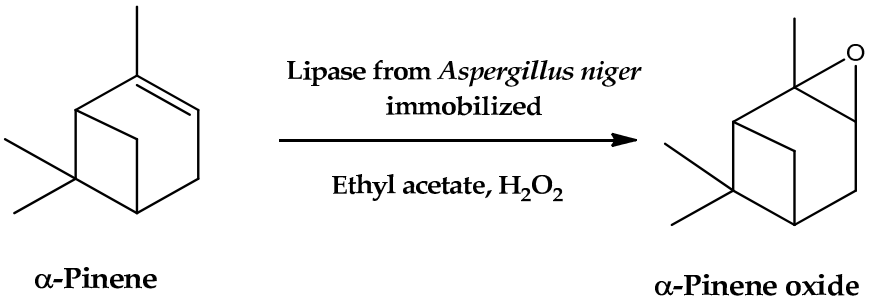

Figure 18. Chemo-enzymatic epoxidation of α-pinene catalyzed by the lipase from *Aspergillus niger* immobilized in calcium alginate beads.

The pores of the reticulated alginate network are large enough that small molecular weight molecules (<20 kDa) can diffuse into and out of the alginate beads [99]. A disadvantage of this support is that small proteins may leach into the bulk medium. For example, a crude extract containing lipases of *Yarrowia lipolytica* was immobilized by microencapsulation in alginate [100]. The immobilization itself was successful, with the immobilized biocatalyst initially having high hydrolytic activity against *p*-nitrophenyl laurate; however, 50% of the activity had leached out of the alginate beads by the end of the reaction [100]. The optimization of immobilization parameters can reduce the leaching of small proteins, for example, by controlling the amount of alginate and the concentration of Ca^{2+} to fine tune the gel properties [101].

Another strategy to avoid leaching is to use the alginate in combination with other polymers, supports or nanoparticles. For example, the lipase from oleaginous seeds of *Pachira aquatica* was immobilized by entrapment using a mixture of alginate and polyvinyl alcohol (PVA). The thermal stability increased, with the lipase keeping 60% of its original activity after 4 h at 50 °C, whereas no activity was detected after incubation of the free enzyme under these conditions. Nevertheless, this procedure did not prevent the loss of activity of the biocatalyst, given that its activity decreased by 50% after five successive cycles of hydrolysis of *p*-nitrophenyl palmitate [102].

Alginate has also been used to prepare gels conjugated with carrageenan, a sulfated galactan that occurs in numerous species of red marine algae [103]. For example, the lipase from *Burkholderia cepacia* was first crosslinked with glutaraldehyde, and then it was entrapped in gel beads containing equal amounts of calcium alginate and κ-carrageenan. The immobilized lipase was used to hydrolyze olive oil in *n*-hexane at 40 °C; after 10 cycles of reaction, the immobilized lipase had a residual activity of 75% [103]. A similar strategy was used to immobilize the lipase from *Tsukamurella tyrosinosolvents* E105 [104]. The lipase was encapsulated within a mixture of sodium alginate and carrageenan, and then crosslinked with glutaraldehyde. The biocatalyst was used to catalyse the resolution of racemic (*R*,*S*)-ethyl 2-(2-oxopyrrolidin-1-yl) butyrate to (*S*)-ethyl-2-(2-oxopyrrolidin-1-yl) butyric acid (Figure 19), an important precursor of an antiepileptic drug. The hydrolysis was performed in Tris-HCl buffer (pH 7.5) at 35 °C for 72 h, with a conversion of 46% and an enantiomeric excess of product of 98.5%. The biocatalyst was used in seven successive cycles of reaction and maintained 64% of its initial activity.

Recently, a system was described in which lipase M from *Mucor javanicus* was first adsorbed onto colloidal cationic lignin nanoparticles and then coated with calcium alginate. The biocatalyst was used in the synthesis of butyl butyrate in a biphasic system (10% *n*-hexane/90% water). Both 1-butanol and butyric acid are suitable substrates for evaluating esterification in aqueous media, because the butyl butyrate produced is insoluble in water, and is extracted into the organic phase containing *n*-hexane. The conversion to *n*-butyl butyrate was 77% in 96 h at 40 °C [105].

Chemical modification of alginates is used to improve their properties, for example, the oxidative cleavage of the uronic acid units with sodium periodate in an acid medium, which results in a polyaldehyde chain [106]. Hou et al. [107] used a carrier based on Fe_3O_4 magnetic nanoparticles modified with polydopamine and alginate polyaldehyde for the immobilization of CRL. Compared to the free lipase, the immobilized derivative had improved thermal stability, maintaining 60% of initial activity in the range of 20 to 60 °C after 30 min of incubation. The biocatalyst maintained a residual activity of 77% after 12 consecutive reuses. However, the performance of the biocatalyst in organic media was not evaluated.

Although the use of alginate is traditional in the immobilization of lipases by entrapment, some drawbacks are associated with the use of this biomaterial: (i) high viscosity of the alginate solution, which makes it difficult to form a uniform gel; (ii) low mechanical strength, which means that the beads are susceptible to breakage when subjected to shear forces, liberating the enzyme from the interior of the bead into the bulk reaction medium and (iii) restricted diffusion of substrates and products in the alginate matrix, resulting in low activities [61].

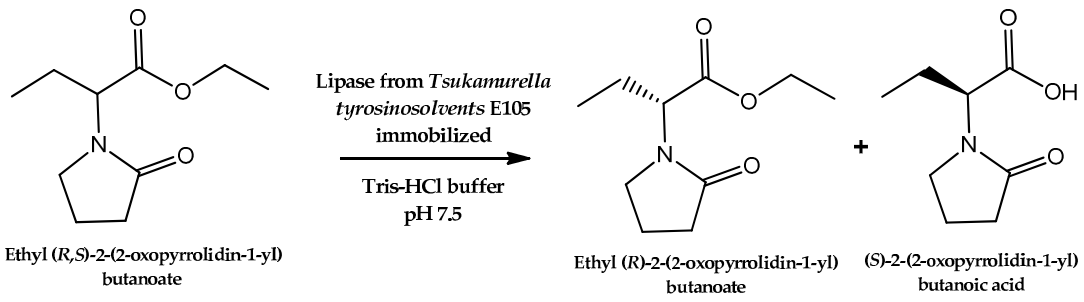

Figure 19. Enantioselective hydrolysis of (*R*,*S*)-ethyl 2-(2-oxopyrrolidin-1-yl) butyrate catalyzed by the lipase from *Tsukamurella tyrosinosolvents* E105 immobilized in calcium alginate/carrageenan beads.

8. Other Biomaterials

Other polysaccharides have also been explored as supports for enzyme immobilization, such as gelatin, dextran, xanthan and pectin, as was recently reviewed by Bilal et al. [61]. Poly-hydroxybutyrate (PHB), an energy reserve polymer produced by several microorganisms, stands out [108]. It is hydrophobic and insoluble in organic solvents frequently used in synthesis reactions. Different lipases

were immobilized on PHB and the immobilized derivatives were successfully used to produce esters by esterification and transesterification [108–111].

Another very useful polysaccharide is dextran, a complex branched glucan made by polymerization of α-D-glucopyranosyl units (Figure 20A) [112]. It has a large number of hydroxyl groups that can be chemically modified, for example by etherification and oxidation. Functionalized dextrins have been applied successfully to enhance activity and stability of various enzymes [113,114]. For example, aldehyde dextran has been used to modify free or immobilized lipases by intramolecular covalent crosslinking, and to derivatize supports for immobilization [115,116]. Polyaldehyde dextran was used to derivatize a macroporous resin containing amino groups for immobilization of CRL. The biocatalyst was applied in the esterification of oleic acid with oleic alcohol, in a solvent-free system. The best conversion was 92% in 12 h, at 40 °C. The biocatalyst was used in eight successive reaction cycles, maintaining over 86% conversion in the last cycle [117]. Tahir et al. [118] functionalized dextran using 5-chloro-1-pentyne to obtain *O*-pentinyl dextran (Figure 20B). This biomaterial was used as a support for the immobilization of the lipase from *Rhizopus arrhizus* by hydrophobic adsorption and the immobilized enzyme was applied in the esterification of octanoic acid with geraniol in *n*-hexane. The maximum conversion was 90% in 160 h at 21 °C [118].

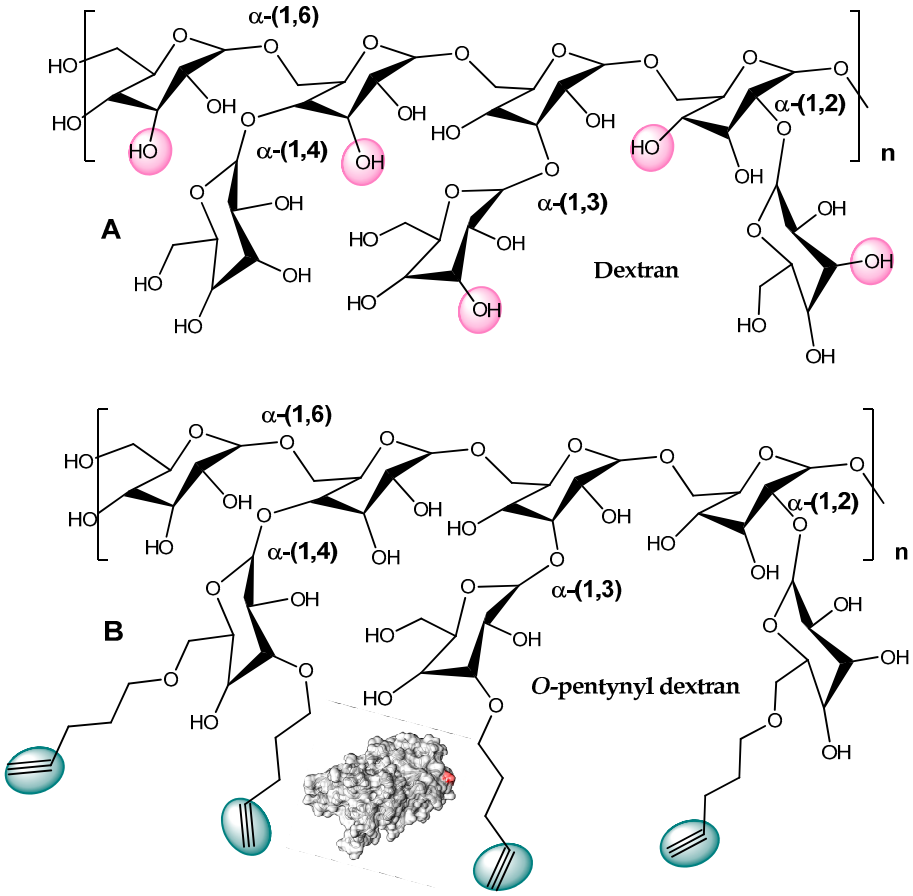

Figure 20. (**A**) Schematic representation of the chemical structure of the dextran (α-D-glucopyranosyl units). (**B**) Schematic representation of the chemical structure of the *O*-pentynyl dextran. The light pink circles randomly highlight the hydroxyl groups that can be chemically modified.

Recently, the use of derivatized pullulan has increased in the pharmaceutical and biotechnological fields [119]. Pullulan is a fungal polysaccharide consisting of maltotriose units (three glucose units linked by α-1,4 glycosidic bonds) connected by α-(1,6) glycosidic bonds. Xu et al. [120] immobilized a lipase from *Burkholderia cepacia* by ionic adsorption onto spherical particles of pullulan acetate

modified with succinic anhydride (Figure 21). The immobilized lipase was applied in the resolution of (*R*,*S*)-1-phenylethanol in *n*-heptane, with a conversion to (*R*)-1-phenylethyl acetate of 50%, in 2 h at 45 °C, with an enantiomeric excess of the substrate of 99%. The immobilized lipase also had good operational stability; after being used for 10 cycles, it still retained over 80% of its original activity [120].

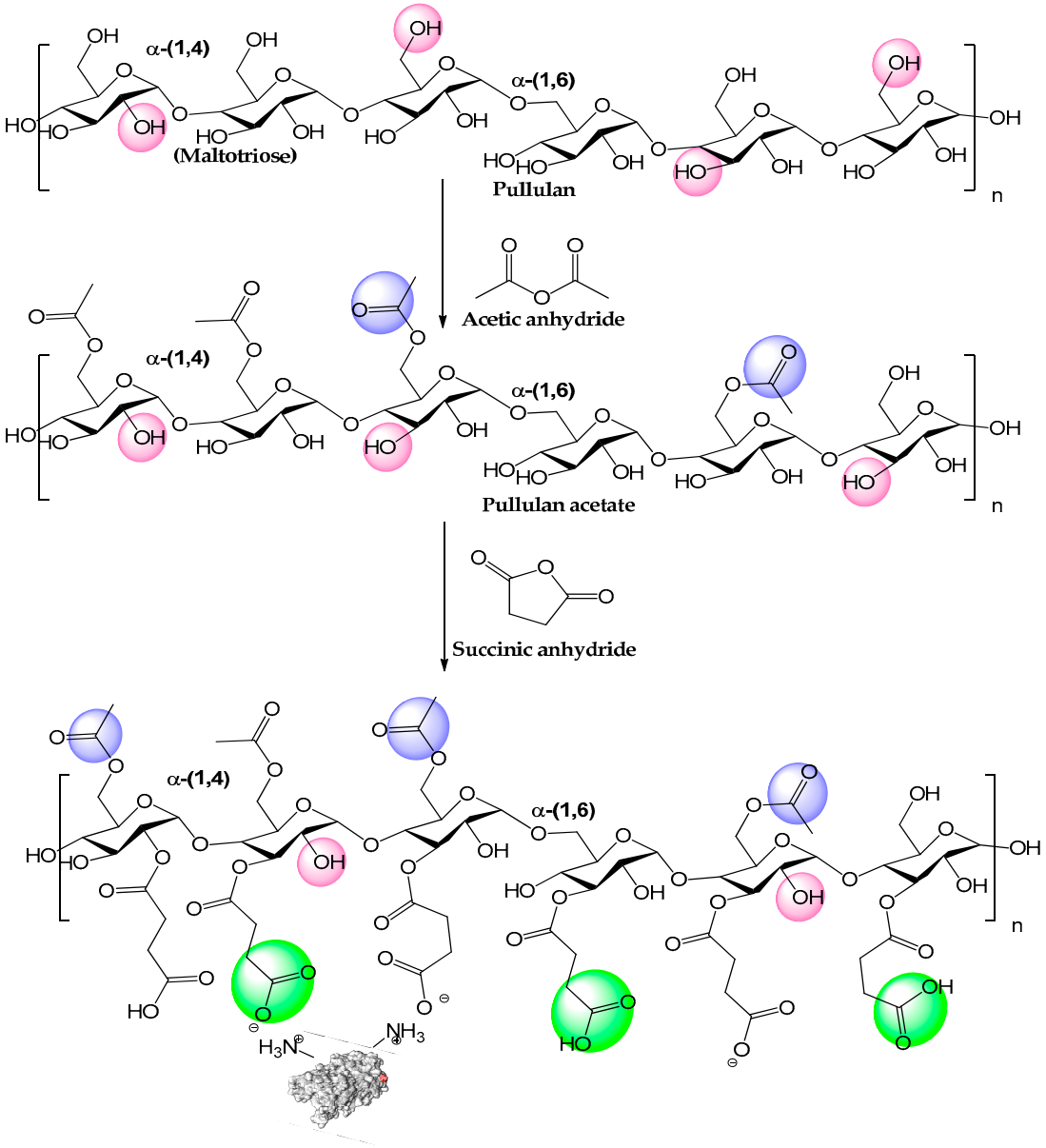

Figure 21. Schematic representation of the chemical structure of succinylated pullulan acetate. The backbone of pullulan is a linear polymer of α-(1,6)-maltotriose.

Natural materials that are generally classified as agro-industrial wastes have also been used to immobilize lipases. For example, sugarcane bagasse has been used as a support for in situ immobilization of lipases produced in solid-state fermentation, as recently reviewed by Krieger et al. [121]. Corn cobs, rice husks, banana stalks, coconut husks [122], and loofah sponges have also been used. For example, rice husk (RH) was oxidized with $NaIO_4$, functionalized with the spacer hexamethylenediamine (HMDA), and activated with glutaraldehyde, then CALB was immobilized covalently [123]. The resulting CALB-RH was used to catalyze the polycondensation of dimethylitaconate and 1,4-butanediol in solvent-free medium. The best conversion of dimethylitaconate

to polyester was 92% in 72 h at 50 °C and 70 mbar [123]. This enzyme-based polymerization is undertaken at low temperatures and, therefore, maintains the vinyl groups intact, making them available for subsequent modifications; chemical polymerization is undertaken at temperatures above 150 °C, in which the vinyl group of the itaconic acid reacts, leading to undesirable radical isomerization and crosslinking [123]. As another example, loofah sponge was used to immobilize BCL by adsorption and used in the synthesis of 1-octyl acetate, with acetonitrile as the solvent; the conversion was 94% in 24 h at room temperature [91].

9. Pros and Cons of Using Biopolymers to Immobilize Lipases

The main advantages of using biopolymers like agarose, chitin, chitosan, cellulose and starch to immobilize lipases are that they are sustainable and abundant resources, non-toxic and biocompatible. They are also easy to modify, due to the abundance of reactive groups on their surfaces. They can be used in a variety of forms, including gels, beads, and films, and can be used with a range of immobilization methods, such as entrapment, adsorption, and covalent bonding. Consequently, it is possible to produce biocatalysts with a range of different shapes and properties.

Biopolymers also have some drawbacks as supports for the immobilization of lipases, although many of these drawbacks are common to other types of support. The gels, beads and films that are produced with these biopolymers often have poor mechanical stabilities that make them unsuitable for industrial application. For instance, the shear stress caused by agitation in batch bioreactors used for biodiesel synthesis may disrupt the support [124]. Additionally, in the case of immobilization by entrapment or by simple adsorption, the lipase may leach from the matrix, leading to a loss of activity and to poor reusability of the immobilized preparation. Some biopolymers, such as chitosan, have low chemical stability. In fact, the poor long-term stability of chitosan is still a major impediment to its use in large-scale applications.

Since lipases are typically immobilized to allow their reuse, these drawbacks limit the industrial application of lipases immobilized on biopolymers. The reusability of immobilized lipases is normally determined in terms of residual activity, but the reasons for the loss of activity strongly depends on the support activation, immobilization mechanism and reaction media. In aqueous media, activity will typically be lost by the lipase leaching from the support if it is immobilized by ionic adsorption but in case of hydrophobic adsorption the trend of the lipase is stilling adsorbed on activated hydrophobic supports. In water-restricted media, such as organic solvents, lipases adsorbed on activated hydrophobic supports can be desorbed but not in case of ionic adsorption. The leakage of the lipases from the support has to be added to a distortion mechanism capable to inactivate the immobilized lipase. In fact, in case of supports capable to immobilize through covalent linkages, this is the only mechanism for the deactivation of the catalyst. Additionally, part of the immobilized preparation can be lost during the recovery step between reaction cycles.

The problems of enzyme leaching and poor mechanical stabilities of biomaterials have been addressed through the use of cross-linking agents such as glutaraldehyde [41,58] and epichlorohydrin [38]. Another strategy to increase the mechanical resistance is to use composite materials, which may contain different biopolymers [125–127], inorganic materials or magnetic nanoparticles [14].

The use of biopolymers to immobilize lipases needs to be carefully thought out, since many biopolymers, in their original form, are highly hydrophilic, while lipases tend to perform better when immobilized on hydrophobic surfaces. For this reason, biomaterials used for the immobilization of lipases are often modified by the addition of groups that lead to more hydrophobic preparations. This is the case of cellulose paper functionalized with methacryloxy groups [82], and hydrophobic chitosan microspheres prepared by reductive amination using dodecyl aldehyde [76]. An additional consideration is that hydrophilic biomaterials can favor partitioning, leading to a microenvironment that can negatively influence the success of the immobilization and the performance of the lipase [128,129]. For example, in the production of biodiesel by esterification or transesterification, the alcohol substrate,

and the water that is generated in the reaction are highly hydrophilic as is the product glycerol, in the case of transesterification. The alcohol can partition onto hydrophilic supports [128,129], leading to a high concentration in the microenvironment of the lipase causing competitive inhibition or even denaturation [124,130] Glycerol can also partition onto hydrophilic supports, where it forms a layer that hinders mass transfer. Finally, interactions between hydrophilic supports and the medium may lead to different substrate concentrations in the bulk solution and in the microenvironment of the enzyme, possibly promoting phase separation and making it difficult to describe the reaction kinetics [131,132]. Relatively little attention has been given to the microenvironment of lipases immobilized on hydrophilic supports. The use of advanced analytic strategies, such as opto-chemical sensing [133], could allow better characterization of the behavior of lipases in these microenvironments.

10. Conclusions

In this review, we have summarized and discussed the latest advances in the methods and strategies involved in the immobilization of lipases on biomaterials. There are many possibilities in the use of this type of material to immobilize and stabilize lipases of industrial interest, following simple protocols. Furthermore, the use of renewable materials provides an opportunity to develop processes that are more sustainable and economical, making the biocatalysts more competitive for use in industry.

However, there are still some issues to be addressed before biocatalysts based on these biomaterials can be applied at large scale, especially in non-conventional media. For example, although many papers have reported lipase immobilization in biomaterials, in most cases the biocatalysts have only been applied in synthesis reactions at laboratory scale. Thus, there is a need for studies to evaluate the behavior of the processes at large scale. Issues such as the effect of shear stress on the integrity of the immobilized preparation and the regeneration of the support matrix after use need to be addressed, so that the support can be reused in many cycles. It is important to realize that, for the biocatalyst to be competitive in industry, it is not sufficient for it to last for 5 or 10 cycles (the typical duration of laboratory studies into reuse), it must last much, much longer. Additionally, it would be desirable to have a better understanding of the phenomena occurring during reactions with immobilized lipases in complex non-conventional media, including the sorption of solvents, reagents, products, and enzymes on the support matrix.

Finally, a continuing challenge with respect to the use of new biopolymeric matrices lies in the identification of new biopolymers that have properties superior to those of existing biomaterials. For example, the number of polysaccharides available on the market is limited when compared to the large number of polysaccharides whose structures have been published or patented.

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