

## Enzyme immobilization onto renewable polymeric matrixes: Past, present, and future trends

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**ABSTRACT:** In this review, we present an overview of the different renewable polymers that are currently being used as matrixes for enzyme immobilization and their properties and of new developments in biocatalysts preparation and applications. Polymers obtained from renewable resources have attracted much attention in recent years because they are environmentally friendly and available in large quantities from natural sources. Different methods for the immobilization of enzymes with these matrixes are reviewed, in particular: (1) binding to a prefabricated biopolymer, (2) entrapment, and (3) crosslinking of enzyme molecules. Emphasis is given to relatively recent developments, such as the use of novel supports, novel entrapment methods and protocols of polymer derivatization, and the crosslinking of enzymes. © 2015 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2015**, *132*, 42125.

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### INTRODUCTION

In this review, we discuss the use of renewable biopolymers as a solution for immobilizing enzymes, pointing out the advantages and drawbacks of the different immobilization protocols. Unfortunately, there is no universal strategy that will solve all enzyme limitations for a wide range of industrial processes conditions. Therefore, for better understanding, we start by describing the general benefits that immobilization may provide if properly used. We then highlight the main positive and negative properties of some biopolymers that may be used for immobilization and some activation possibilities. Last but not least, examples of industrial (or potential) applications are pointed out.

Enzyme immobilization is one of the techniques that have been used to achieve enzyme reuse or retention (e.g., in continuous fixed-bed reactors) and, therefore, the facilitation of large-scale and economic formulation of biotechnological industries.<sup>1–3</sup> The use of immobilized enzymes also minimizes protein contamination of the product because an ease separation of the biocatalyst from the product may be achieved.<sup>2,4</sup> Apart from these well-known advantages, enzyme immobilization, if properly used, may also promote positive effects on the enzyme activity and enzyme stabilization. Through the selection of the optimal immobilization strategy, enzyme limitations may be overcome; as a result, the enzyme performance is improved,<sup>5</sup> and other benefits emerge, such as enhanced enzyme activity and stability, the modulation

of enzyme selectivity and specificity, the reduction of inhibition problems, and uses in multienzyme and chemoenzyme processes.<sup>3,5</sup> Stabilization, for instance, may be achieved by the prevention of intermolecular interactions, generation of artificial favorable microenvironments around the enzyme, prevention of subunit dissociation (in the case of multimeric enzymes), enzyme reactivation after partial deactivation, and rigidification via multi-point covalent attachment.<sup>5</sup> In fact, several methods and factors that influence the performance of immobilized enzymes are described in recent reviews, which are available in the literature.<sup>1,5–9</sup> Fundamentally, the technologies available to immobilize enzymes can be divided into three traditional methods:<sup>3,10</sup> (1) entrapment (within an inert support), (2) carrier bonding (by physical adsorption or covalent coupling), and (3) crosslinking (the formation of insoluble proteins; see Figure 1). It is also important to notice that enzyme immobilization protocols may induce conformational modifications that may cause a partial (or total) loss of activity.<sup>9</sup> Indeed, immobilized enzymes may be even less stable than the free enzyme if the immobilization protocol is not well planned.<sup>8</sup>

In this review, *entrapment* is defined as the inclusion of enzymes in a polymer network, membrane, or microcapsule.<sup>3,9</sup> So, the insoluble biocatalyst is produced by the promotion of the synthesis of the polymeric support in the presence of the enzyme, and immobilization occurs within the inert support.<sup>2,10</sup> This

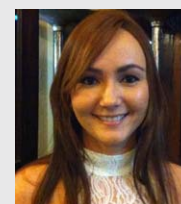
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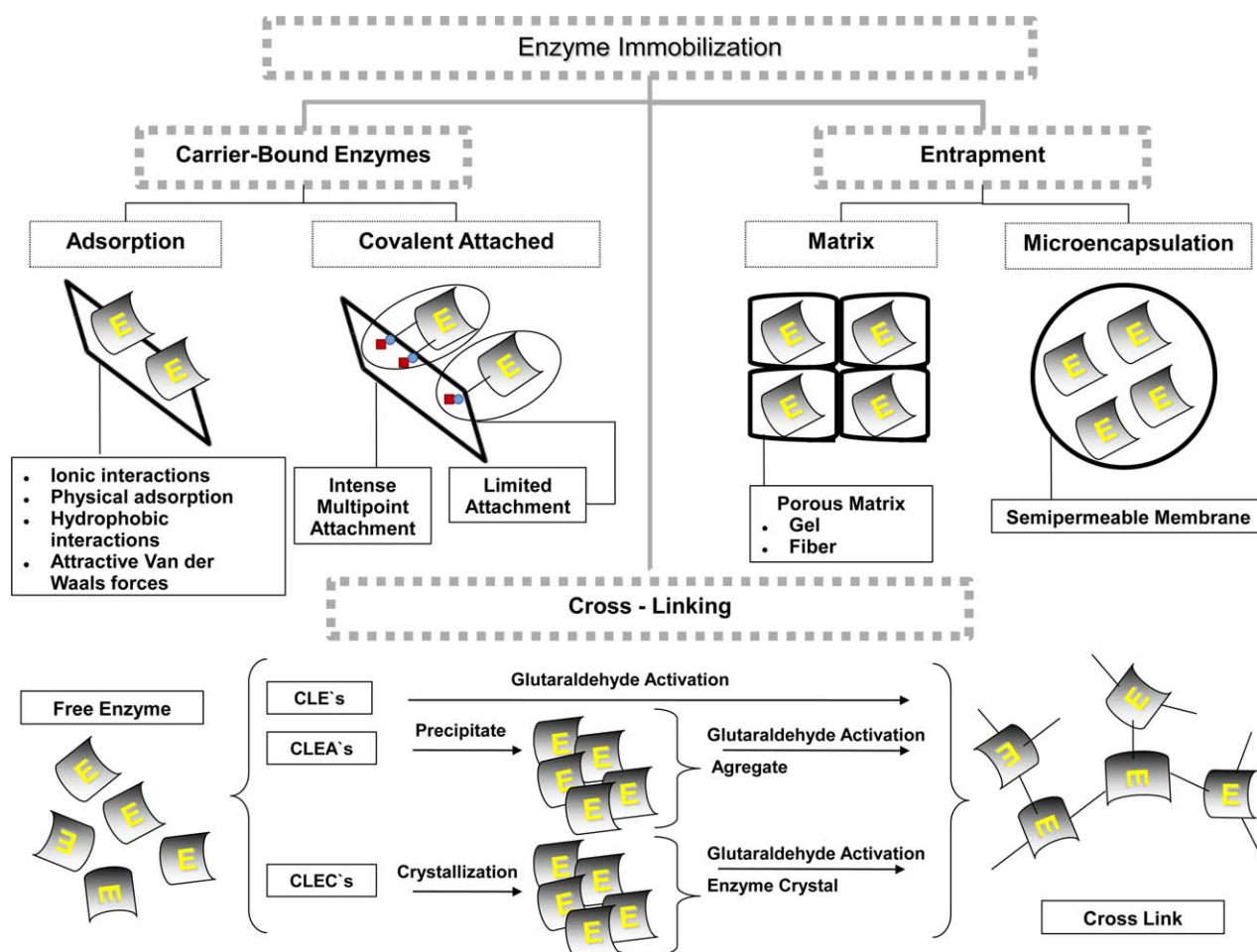


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physical immobilization method has been applied mostly with natural polymers, such as collagen, chitosan, cellulose, and  $\kappa$ -carrageenan,<sup>1,11</sup> probably because they are biodegradable, biocompatible, and nonantigenic supports.<sup>12</sup> Furthermore, this immobilization method can be achieved by a relatively simple operation, which does not cause modification to the structure of the enzyme.<sup>13</sup> Because entrapment prevents the direct contact of the enzyme with the environment, some inactivation causes are prevented, for instance, the effects of enzyme–gas bubble interactions, mechanical shear, and hydrophobic solvents.<sup>4,14</sup> Nevertheless, some disadvantages include the following: (1) the large pore sizes could cause enzyme leakage, (2) small pore sizes could prevent the diffusion of large substrate molecules into the gel, (3) conditions during polymerization can destroy the enzymes, and (4) there may be low enzyme loading.<sup>4,15</sup> Although this strategy is more suitable for the immobilization of whole cells,<sup>9</sup> the use of natural polymers as supports in this immobilization technique is discussed later because efficient examples of industrial (or potential) applications have been described in the literature.

Another immobilization method that will be reviewed is binding to a support, which can be physical, ionic, or covalent in nature.<sup>3</sup> Independent of the protocol, enzyme immobilization inside a porous support alone will prevent some of the inactivation causes mentioned before, and if the enzyme is supported on a solid surface, no autolysis or proteolysis is possible.<sup>5</sup> Further advantages of support-based immobilization (with porous or nonporous matrixes) have been reported; these include rigidity, which enables the use of the biocatalyst in different reactor configurations.<sup>4</sup> Nevertheless, this method causes the dilution of volumetric and specific activity because the support (noncatalytic) accounts for 90–99% of the total biocatalyst, and this results in lower space–time yields and productivities.<sup>3,4</sup> Physical adsorption is the simplest, fastest, and cheapest method of preparing immobilized enzymes with high catalytic activity.<sup>5,16</sup> In this case, an insoluble biocatalyst is formed by enzyme adsorption onto a solid support, mainly by weak interactions, such as van der Waal's, electrostatic, and hydrophobic forces.<sup>10</sup> This method also has the advantage of allowing the reuse of expensive supports after the inactivation of the immobilized enzyme.



**Figure 1.** Main methods used for enzyme immobilization.<sup>1,5–9</sup> E = enzyme. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

Nevertheless, the physical forces involved in adsorption are generally not very strong, and some of the adsorbed proteins may desorb from the support during industrial operation (high substrate and product concentrations, high ionic strength, etc.).<sup>2,9</sup> Cellulose membranes,<sup>17</sup> pectin,<sup>18</sup> and chitosan particles,<sup>19,20</sup> for instance, are renewable supports used for immobilization by adsorption. Covalent attachment is one of the most widely used methods for enzyme immobilization because the bonds formed between the enzyme and support are more stable; this prevents the release of the enzyme into the environment.<sup>3,9</sup> Robust biocatalysts may be produced, and they can be used in aqueous, multiphase, and viscous media.<sup>9</sup> Chitosan,<sup>16,19,21–24</sup> agarose,<sup>24–26</sup> gelatin,<sup>27</sup> and pectin<sup>18</sup> are some of the often used renewable polymeric matrices for covalent immobilization. With regard to enzyme stabilization, the most interesting and powerful strategy is multipoint covalent immobilization on a prefabricated support.<sup>4,5,10</sup> To achieve that stabilization, the proper choice of support, reactive group, and immobilization conditions are imperative.<sup>5</sup> Some examples that can render optimal results are epoxide,<sup>28</sup> amino groups activated with glutaraldehyde,<sup>29</sup> and glyoxyl supports.<sup>25</sup> In this review, the possibility of achieving enzyme stabilization with biopolymers as supports is also addressed.

It is important to mention that the use of prefabricated supports can be advantageous because it is possible to select the carrier based on the desired properties.<sup>5</sup> The choice of the support, though, is a difficult task because it plays an important role in the performance of the biocatalyst and in the type of reactor used. Some characteristics are desirable, such as the presence, distribution, and density of functional groups; inertness, high porosity, and large surface area; physical strength; stability; resistance to microbial attack; regenerability; and the ability to increase enzyme specificity/activity and reduce product inhibition.<sup>1,5,30</sup>

Supports can be classified as inorganic or organic, which are divided into naturally occurring and synthetic organic carriers.<sup>6</sup> Among inorganic carriers, porous silica and mesoporous silicates appear to be promising supports for enzyme immobilization.<sup>31,32</sup> Several natural polymers (agarose, chitosan, alginate, dextran, and carrageenan) and synthetic polymers (polyamides, polystyrene, and polyacrylamide) are usually used as carrier matrices for enzyme immobilization.<sup>4,9,30</sup> Synthetic polymers investigated for enzyme immobilization include a variety of materials, such as acrylic, methacrylic, and styrene resins.<sup>9</sup> Nevertheless, this group of polymers is made from

**Table I.** Main Advantages and Disadvantages of the Use of Immobilized Enzymes<sup>1,6,7,16,21,23,30,116,159</sup>

Advantage	Disadvantage
Easier reactor operation	Diffusional limitation
Easy separation of the enzyme from the product	No general method that is applicable for all enzymes
Wider choice of reactors	Additional cost (support and chemicals)
Facilitates a large scale	Loss of enzyme activity on immobilization
Reuse	Alterations in kinetic properties (unfavorable)
High selectivity	
Use of small sample volumes	
Robust biocatalysts with increased lifetimes; stability against different temperatures, organic solvents, and pH variations; and high operational and storage stability	

nonrenewable petroleum resources;<sup>33</sup> this method has been criticized because of the fossil fuel depletion problem. Natural polymers, on the other hand, can be found widely in renewable sources,<sup>30</sup> are available in large quantities, and are cheap starting materials for the production of support materials.<sup>6</sup> They also represent an attractive alternative from an economic point of view. Because the main goal of this article is to discuss the use of renewable matrixes, results achieved with natural polymers as supports for enzyme immobilization are reported. The different supports, along with the existing techniques used for enzyme immobilization and insights into recent developments for each of them, are addressed.

Another strategy that has gained attention in recent years is immobilization through the crosslinking of enzyme molecules. Water-insoluble particles can be obtained by the extensive crosslinking of NH<sub>2</sub> groups at the surface of the enzyme with a bifunctional reagent; this may lead to low production costs because of the exclusion of an additional and sometimes expensive support.<sup>3,9</sup> Other advantages include high stability and concentrated enzyme activity in the catalyst.<sup>3</sup> Some main strategies have been studied to prepare insoluble biocatalysts, such as crosslinking over the soluble enzyme [crosslinked enzyme (CLE)], over crystallized enzyme proteins [crosslinked enzyme crystals (CLECs)], and over enzyme aggregates [crosslinked enzyme aggregates (CLEAs)].<sup>34</sup> A disadvantage of CLECs is the need to crystallize the enzyme; this is often a laborious procedure that requires an enzyme of high purity. CLEAs, on the other hand, can be easily prepared in a two-step protocol: (1) first, precipitation as physical aggregates of protein molecules are promoted by methodologies often used to purify enzymes, and (2) then, the aggregates are stabilized by crosslinking, with glutaraldehyde being the most common agent, because it is inexpensive and readily available in commercial quantities.<sup>3,34</sup> An aspect that will be discussed later in this article is that CLEA can be modified by crosslinking in the presence of a second protein containing multiple lysine residues, such as bovine serum albumin (BSA), an important biopolymer. Some authors have reported that CLEAs may be obtained from crude enzyme preparations, for instance, crude cell-free extracts obtained from fermentation broth, because they combine purification and immobilization into a single-unit operation.<sup>2</sup> Nevertheless, high levels of purification

should not be expected because the precipitant agent (e.g., ammonium sulfate) may promote the precipitation of most of the proteins present in the sample and not only the target enzyme. Some purification may be achieved, though, if the precipitation conditions of the target protein (enzyme) is milder compared to those of the contaminant proteins (Table I).

An important point that should be considered in the selection of a biocatalyst for industrial applications is if it is indeed competitive from the economical point of view; as a result, immobilized enzymes should be produced at affordable costs and, in case the immobilization strategy involves the use of a support or a feeder (very common in the preparation of CLEAs), the material should be also available in reasonable amounts.<sup>9</sup> With this in mind, natural biopolymers appear as alternative materials from an economic point of view. For each application of an immobilized enzyme, it is necessary to choose the simpler and cheaper protocol that results in a biocatalyst with expected technological advantages (e.g., high activity retention and operational stability).<sup>3,9,21</sup> Some key parameters in the selection of an immobilization method can be found in the literature,<sup>5</sup> and in this review, the use of renewable biopolymers as supports or additives in the immobilization of enzymes is discussed.

## RENEWABLE POLYMERIC MATRIXES USED FOR ENZYME IMMOBILIZATION

Structural and globular proteins and carbohydrates are natural organic polymers<sup>6</sup> that may be used for enzyme immobilization. In that sense, (bio)polymers generally recognized as safe, such as carbohydrates, are of special interest.<sup>15</sup> Some examples of renewable polymeric matrixes often used for enzyme immobilization are discussed later. Over the past decades, different methods for the immobilization of enzymes on renewable polymers have been used. Table II shows some examples of those polymeric supports with their respective immobilization protocol.

### Proteins

Different intermolecular interactions may result from the interaction between the amino acid residues that are present in proteins, and this offers a wide possibility of chemical functionalities and functional properties<sup>33</sup> that may be explored to enzyme immobilization. It is important to mention the use



**Table II.** Some Common Renewable Organic Polymers Used as Supports for Enzyme Immobilization

Support	Enzyme	Immobilization technique	Reference
BSA	Dehydrogenase	CLEAs	46
SF	Glucose oxidase	Entrapment	160
Silk fibers	$\beta$ -gal	Covalent binding	161
Calcium alginate	Peroxidase	Entrapment	96,99,100,102
	Tannase	Adsorption	
Agar	$\alpha$ -Amylase	Entrapment	56
$\kappa$ -Carrageenan	Lipase	Entrapment	102
Chitosan	Lipase	Crosslinking	19,116,162
	$\beta$ -gal	Covalent binding	
Cellulose membranes	Lipase	Crosslinking	17
Poly(ether sulfone)		Adsorption	
DEAE-cellulose	$\alpha$ -Amylase	Ionic binding	56
Agarose	Chymotrypsin	Covalent binding	26,86,162
	$\beta$ -gal	Adsorption	
	Penicillin G acylase	Covalent binding	
	Lipase	Covalent binding	
	Glutaryl-7-aminocephalosporanic acid acylase	Covalent binding	
Polyacrylamide-gelatin	Invertase	Covalent binding	163
Gelatin	Urease		54,56-58
	$\alpha$ -Amylase	Covalent binding	
	Lipase	Organogel	
	Palatase		
Pectin	Lipase	Covalent binding	18
		Adsorption	
Starch	PAL	CLEAs	154

of immobilized lipases for the selective adsorption of other lipases,<sup>35-37</sup> which has opened some new ideas for the immobilization and purification of those enzymes.

**BSA.** BSA is a globular protein (~66 kDa)<sup>38</sup> that is used in different biochemical applications, such as the stabilization of enzymes during storage and for enzymatic reactions.<sup>39</sup> With regard to enzyme immobilization, BSA is often used as an additive<sup>40,41</sup> rather than a support because it has a stabilizing effect on enzymes.<sup>3</sup> Furthermore, in the preparation of CLEAs from solutions containing low concentrations of enzymes or when dealing with a low-amine-group-content enzyme, BSA can be used as a proteic feeder; this facilitates the formation of the aggregates.<sup>42-45</sup> Other strategies have been described to involve the enzyme in the crosslinking in the literature, but the use of a protein, such as BSA, may have some advantages: lower interaction among the protein and the enzyme, lower alteration of the enzyme environment, and higher stabilization of the enzyme because of the rigidity of the protein in comparison to other species used for the same purpose.<sup>45</sup>

In fact, Kim *et al.*<sup>46</sup> observed that the addition of BSA increased the recovery activity of the CLEAs of formate dehydrogenase

from *Candida boidinii* by 130%; this means that BSA was beneficial in reducing the extensive crosslinking of the enzyme by glutaraldehyde. Stable CLEAs of phenylalanine ammonia lyase (PAL) were also prepared in the presence of BSA.<sup>47</sup> The product is referred to as BSA-CLEAs, and they exhibit a high thermostability and storage stability in comparison with free PAL and PAL-CLEAs. According to the authors, the number of amine groups of PAL was not great enough to achieve efficient crosslinking and allow enzyme molecules to be released from the aggregates under high temperatures when

CLEAs were prepared in the absence of BSA.

**Collagen and Gelatin.** Collagen is found in connective tissue and possesses characteristics such as a low toxicity, elasticity, good mechanical strength, and low immune response. Because of these characteristics and the demand for new biomaterials, collagen has been described in biotechnological applications.<sup>48-50</sup> Wang and Vieth,<sup>51</sup> for instance, prepared collagen/enzyme membranes for the construction of biocatalytic reactors that were used in a recirculation system for the conversion of substrates. The biocatalytic reactors showed initial decreases in the activity to stable limits, which were maintained over a large

number of reactor volume replacements. The stable limits corresponded to approximately 35% of the initial activities for lysozyme and invertase, 25% for urease, and 15% for glucose oxidase. The immobilization of glucose oxidase enzyme in collagen has also been reported in the literature, where the optimum conditions of pH and temperature for the immobilized enzyme were the same as those of the soluble enzyme, but the immobilized enzyme was more active than the soluble form at high temperature and pH.<sup>52</sup> Nevertheless, it has not been used lately for the immobilization of enzymes; the main applications are in the biomedical field.

Gelatin, with a high molecular weight and formed by three polypeptide molecules arranged in a helix, is a protein obtained by the partial hydrolysis of collagen. According to Rivero *et al.*,<sup>53</sup> the helical formation of the gelatin molecule is important for gel formation. Among the main reactive groups of gelatin, hydroxyl, carboxyl, and amino groups should be mentioned. Enzyme immobilization in this type of material can occur, for example, through the covalent interaction of the enzyme molecule with the free amino groups of gelatin. Compared to other materials used for enzyme immobilization, such as alginate acid and polyacrylamide, the cost of this support is low.<sup>54,55</sup> According to Ewadh and Al-Khafaji,<sup>54</sup> the immobilization of enzymes onto gelatin primarily occurs via crosslinking between the free amino groups of the carrier and the enzyme molecule with crosslinking agents, which form a covalent bond. Kikani *et al.*,<sup>56</sup> for instance, studied the covalent coupling of  $\alpha$ -amylase from *Bacillus amyloliquifaciens* TSWK1-1 with gelatin, using glutaraldehyde as a crosslinker. The authors observed that the thermal and pH stabilities were significantly increased after immobilization. The half-life of the free  $\alpha$ -amylase was 31.5 h in 20 mM phosphate buffer at pH 7 at 60°C, whereas it increased to 62 h upon immobilization; that is, the immobilized enzyme was almost two times more thermally stable than the free enzyme. The immobilized enzyme also presented a reasonable operational stability because it was reused repeatedly for 20 successive cycles at its optimum temperature and pH, retaining 83% of its original activity.

Nowadays, gelatin is being used to prepare hydrogel formulations. For instance, the efficacy of organogel supports for the immobilization of lipases was studied.<sup>57,58</sup> It was reported that the gel maintained its physical appearance during the experiment, and the enzyme remained active after 15 cycles with a yield in the range 65–70%.<sup>58</sup> Queiroz<sup>59</sup> also observed that the organogel system was effective for the immobilization of enzymes and their use in organic synthesis. The authors studied the efficacy of organogel supports for the immobilization of palatase M and achieved satisfactory results in the physical integrity of organogel heptane (for 70 days) and the catalytic activity palatase M, which remained active after 10 cycles of reaction (the yield of the reaction remained in the range 84–88%). This proved the possibility of reuse. Zhang *et al.*<sup>57</sup> observed that the lipase immobilized in microemulsion-based organogels maintained a high catalytic activity in the presence of organic solvents, whereas the free enzyme was easily inactivated in polar solvents. Furthermore, the immobilized enzyme maintained 70% of its original activity after 16 cycles.

**Hydrophobins.** Hydrophobins are fungal proteins, rich in cysteine, with a molecular mass of around 10 kDa and a high content of hydrophobic amino acids.<sup>60</sup> They are capable of self-assembling at hydrophilic–hydrophobic interfaces into amphipathic films,<sup>61</sup> which can be used to introduce hydrophobic moieties on the surface of hydrophilic supports. For that purpose, hydrophobins were attached by covalent binding to a hydrophilic matrix (agarose) to construct a support for the noncovalent immobilization and activation of lipases.<sup>37,62</sup> The authors claim that this immobilization strategy may be used to attempt the physical modification of the lipase active center without the introduction of chemical modifications on the lipase itself.<sup>35</sup> Other authors<sup>63</sup> studied the immobilization of two redox enzymes, glucose oxidase from *Aspergillus niger* and horseradish peroxidase, by physisorption on glassy carbon electrodes coated with *Schizophyllum commune* hydrophobin. They proposed an easy protocol to produce stable, enzyme-based catalytic surfaces for applications in biosensing.

**Open Lipases.** Lipases present an interfacial activation mechanism, which allows the substrate to have access to the active site of the active enzyme conformation (open form).<sup>64</sup> Furthermore, they have a natural tendency to form bimolecular aggregates via interactions between hydrophobic surfaces near the active site.<sup>65</sup> Aware of that, Palomo *et al.*<sup>35</sup> proposed a new tool for purifying and immobilizing lipases. They immobilized lipases from *Pseudomonas fluorescens* (PFL) on a support (glyoxyl–agarose) with their active center exposed to the medium. Next, they allowed the adsorption of other lipase molecules via a similar mechanism to the one that yields bimolecular aggregates. Afterward, the enzymes were easily and fully desorbed from the support with this detergent, and several adsorption–desorption cycles could be performed. This methodology is very useful for the efficient purification of crude enzyme extracts because these enzymes may contain other proteins and enzymes, such as esterases.<sup>36</sup> Further studies of the same group<sup>36</sup> proved that the selective adsorption of lipases can be achieved by the preparation of supports with other lipases in addition to PFL; this shows that this simple method could be very useful in the separation and purification of new lipases.

**Silk Fibers and Silk Fibroin (SF).** Widely used in the textile industry, SF is a fibrous protein that has great potential for use as a biomaterial because of its high mechanical strength, thermal stability, biocompatibility, and biodegradability.<sup>66</sup> SF, as do all fibrous proteins, has a high concentration of hydrophobic amino acid residues, and this makes it insoluble in water.<sup>67</sup> This rather exotic support has been reported in the literature, for instance, in the immobilization of glucose oxidase by entrapment into SF membranes without glutaraldehyde treatment<sup>68</sup> and horseradish peroxidase on magnetic SF nanoparticles.<sup>69</sup> Silk fibers were investigated as a support for the immobilization of  $\beta$ -galactosidase ( $\beta$ -gal);<sup>69</sup> alkaline phosphatase and aspartate aminotransferase on methylated silk and on silk by the azide method, respectively,<sup>70</sup> and lipase from *Candida* sp. 99e125 on two silk fabrics with different hydrophilic/hydrophobic properties.<sup>71</sup> Nevertheless, to reduce the cost of the support, the use of waste silk fibers has appeared as an alternative, which also provides a responsible and reasonable use of this bioresource.<sup>72</sup>

## Carbohydrates

Usually, the hydroxyl groups of sugar-based matrixes can be exploited for the covalent immobilization of proteins; cellulose and agarose are examples of polymers that can be activated with this strategy.<sup>9</sup> Biopolymers with amino functionalities are also an interesting choice of matrix; for example, chitosan has been used for enzyme immobilization because of its primary amino groups, which can be preactivated with bifunctional reagents, such as glutaraldehyde.<sup>9,73</sup> Next, we present an overview of the different carbohydrates that are currently being used as matrixes for enzyme immobilization and their properties and applications.

**Agar and Agarose.** Agar is obtained from red algae, and its main components are virtually neutral agarose and charged agaropectin.<sup>74,75</sup> Agarose, a neutral linear polymer free of sulfates, is the predominant component, and it is formed by agarobiose repeating units, a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose. Agaropectin, a sulfated polysaccharide (3–10% sulfate), has a similar structure, but with a lower content of 3,6 anhydro-L-galactose, being L-galactose-6-sulfate and D-galactose-4-sulfate, the major sulfate residues.<sup>74,76,77</sup> The polysaccharide gelation occurs through hydrogen bonds between the agar molecules,<sup>78</sup> in which agarose is fundamental to this gelation process.

Few articles have reported the immobilization of enzymes in agar;<sup>56</sup> for instance, agar was used to immobilize  $\alpha$ -amylase by entrapment. The temperature and pH profiles broadened, whereas thermostability and pH stability were enhanced after immobilization. Nevertheless, after 20 cycles of use, the biocatalyst lost 29% of its original activity and was less stable than the other immobilized enzymes (with different supports) investigated by the authors.

Agarose gels are easily handled and activated because of the presence of a very large density of surface hydroxyl groups; therefore, they are often used for enzyme immobilization in aqueous media. The hydrophilic characteristics, facile derivatization, absence of charged groups (extremely inert supports), and availability are some advantages of the use agarose as a matrix.<sup>79,80</sup> Studies have reported these gels to have properties, such as large surface area and porosity, that are suitable for enzyme immobilization.<sup>66,81–83</sup> Covalent immobilization on agarose has been reported in the literature since the 1980s, for instance, protocols developed<sup>84</sup> to immobilized trypsin,<sup>84,85</sup> penicillin G acylase,<sup>82</sup> and chymotrypsin<sup>86</sup> by multipoint covalent attachment on glyoxyl–sepharose. In this strategy, enzymes, via their amino groups, react with aldehyde groups on the support surface. According to those studies, the proper combination of support, reactive groups, and conditions of immobilization allows the achievement of a very intense multipoint covalent attachment, and it is probably the most powerful way to achieve enzyme stabilization.<sup>5</sup> After that, several examples of enzymes immobilized on glyoxyl–agarose, prepared by the etherification of agarose (6%) with glycidol and further oxidation with sodium periodate, have been reported.<sup>24,26,79,83,87</sup> This support has gained attention because of the good prospects of getting multipoint covalent attachment of glyoxyl–agarose. Apart from

glyoxyl–agarose, other agarose supports are commercially available and have been used in many instances to immobilize many different enzymes. Some examples are tosyl chloride, cyanogen bromide, diethylaminoethyl, glutaraldehyde activated supports, and Q-sepharose.<sup>25,88</sup> Hydrophobic agaroses, for instance, octyl–agarose, have also been used to immobilize lipases.<sup>89</sup> Because a universally applicable method of enzyme immobilization is not available,<sup>31</sup> modified agarose supports may be used until a biocatalyst with the desired target properties is obtained. This is because enzyme features (e.g., selectivity, specificity, activity) may be changed by the orientation of the immobilized enzyme;<sup>90,91</sup> in other words, different immobilization protocols may produce biocatalysts with different characteristics. For example,  $\beta$ -gal from *Kluyveromyces fragilis* was immobilized on agarose by different protocols, such as physical adsorption and covalent attachment.<sup>24</sup> Glyoxyl–agarose and glutaraldehyde–agarose were used for the covalent attachment of  $\beta$ -gal. Monoaminoethyl-N-ethyl agarose (MANAE-agarose) was used in the physical adsorption of the enzyme. After physical adsorption, crosslinking postimmobilization techniques were evaluated with different concentrations of poly(aldehyde dextran) or glutaraldehyde. In this particular case, covalent attachment to agarose did not produce active biocatalysts, and the authors attributed this result to intense crosslinking that could distort the native structure of  $\beta$ -gal. The physical adsorption on MANAE-agarose at low ionic force rendered excellent immobilization yield and recovery activity results; nevertheless, it did not have any significant effect on the enzyme stability. Although it was reported in the literature that treatments with crosslinker agents, such as glutaraldehyde or polyaldehyde dextran, exerted a positive effect on the stability of different enzymes,<sup>29,92</sup> the use of this postimmobilization strategy has presented a destructive effect on the catalytic activity of enzyme previously adsorbed on MANAE-agarose. It is important to mention that MANAE-agarose was described for protein immobilization with very mild carbodiimide activation of carboxyl groups.<sup>93</sup> In that article, the authors evaluated the possibility of transforming the ionic adsorption into a covalent attachment. They observed that the addition of small concentrations of carbodiimide to the adsorbed enzyme on low-pK activated supports was enough to attain covalent immobilization with a negligible loss of catalytic activity.

Glutaraldehyde-activated agarose should also be mentioned because of the possibility of producing heterofunctional supports, which are defined as support that possess several distinct functionalities on their surface that are able to interact with proteins.<sup>8</sup> Their matrix has primary amino groups and a hydrophobic glutaraldehyde chain and can covalently react with primary amino groups of the enzyme. So, the first event of the immobilization may be promoted by different causes, and different positions of the enzyme surface, depending on the activation degree and immobilization conditions, are possible. Immobilization is usually performed at neutral pH values because of the low stability of the glutaraldehyde groups at alkaline pH values, and under those conditions, the most reactive amino group in the protein tends to be the terminal amino group. Nevertheless, after the first immobilization, other nucleophiles groups of protein may interact with the support; this allows new covalent enzyme-

support bonds to be formed. This feature should be considered in the design of the experimental protocol in the use of highly glutaraldehyde activated supports, or some problems may arise, and misleading results may be obtained.

**Alginate.** Produced by brown algae and microorganism, such as *Pseudomonas* and *Azotobacter*, alginates are linear copolymers formed essentially by  $\alpha$ -L-guluronic and  $\beta$ -D-mannuronic acids. This polymer shows a wide variation in the proportions of mannuronic and guluronic acids. It has been reported in the literature that alginates with a high content of guluronic acid in its structure forms more resistant gels compared to ones that have a greater amount of mannuronic acid.<sup>78,94,95</sup>

Widely used in the food, textile, and paper industry, alginate possesses characteristics that arouse great interest as a biomaterial for the formation of heterogeneous catalysts. Recently, alginate has been used for nanoparticle production for the release of different materials.<sup>95</sup> This biopolymer is often used for the encapsulation of enzymes. For instance, tannase (Tan410) was entrapped in calcium alginate beads and evaluated for the removal of tannins from green tea infusion. The beads were used for six successive runs; this resulted in the overall hydrolysis of 56% of the tannins.<sup>96</sup> However, a very common strategy observed nowadays is the use of alginate as a copolymer to prepare more stable supports. This strategy was investigated for the immobilization of lipases, for instance, by Silva *et al.*<sup>97</sup> and Mendes *et al.*<sup>98</sup> In both works, the immobilization of lipases on epoxy-chitosan/alginate was considered an attractive tool for obtaining biocatalysts with the potential for commercial use in both aqueous and anhydrous aqueous media. Matto and Husain<sup>99</sup> also studied enzyme immobilization in a hybrid support prepared with calcium alginate-starch. The authors immobilized peroxidase by entrapment and observed that the biocatalyst obtained was more stable when exposed to denaturants compared to the ones obtained by adsorption.<sup>100</sup>

Alginate is also used as support in an industrial biocatalyst that is used by DuPont for the regioselective hydration of adiponitrile to 5-cyanovaleamide, an intermediate in the manufacture of an herbicide.<sup>9</sup> Calcium alginate was used to entrap whole cells containing nitrile hydratase (enzyme), and the immobilized biocatalyst was recycled several times and generated more than 3000 kg of product/kg of catalyst.

**Carrageenan.** Extracted from red seaweed, *carrageenan* is a generic name for a family of gel polysaccharides formed mainly by sodium, calcium, magnesium, copolymers of 3,6-anhydrogalactose, potassium, and sulfate esters of galactose. There are three commercially important carrageenans, and  $\iota$ - and  $\kappa$ -carrageenans are gel-forming carrageenans.  $\kappa$ -Carrageenan is the main type of carrageenan and possesses in its structure sulfate groups, one per repeating dyad; this carrageenan presents a good water-retention capacity and allows this compound to be widely used, especially in the food industry.<sup>15,78,101</sup> Generally, gels produced with  $\iota$ -carrageenan are soft and weak but freeze-thaw stable. Gels produced with  $\kappa$ -carrageenan, on the other hand, are hard, strong, and brittle but freeze-thaw unstable. Both types of gels will melt when heated and form a gel again after cooling because they are thermoreversible gels.<sup>15</sup> Indeed,

Jegannathan *et al.*<sup>102</sup> used  $\kappa$ -carrageenan to immobilize lipases by encapsulation, and they observed that the resulting biocatalyst could not be heated beyond 50°C because it dissolved beyond this temperature. However, the authors obtained good results of stability against various alcohols and alkenes for 10 days at room temperature without significant changes in activity. Datta *et al.*<sup>1</sup> reported studies in which carrageenan was an excellent enzyme support for the entrapment of  $\alpha$ -galactosidase enzyme and a cheap and highly durable material. Nevertheless, the immobilization of enzymes into carrageenan was applied to fewer examples than in whole-cell encapsulation.<sup>15</sup>

**Cellulose.** Cellulose is a linear, long-chain polymer present mainly in the cell walls of plants, along with lignin, hemicellulose, and pectin. It presents a crystal structure and in normal conditions is insoluble in water.<sup>103</sup> According to Vroman and Tighzert,<sup>33</sup> because of its characteristics, cellulose should be modified to be used. Cellulose has many hydroxyl groups,<sup>104</sup> so it can be activated in a similar way as agarose. Although its binding capacity for enzymes is generally lower when compared to agarose, it is inexpensive and commercially available.<sup>9</sup> One of the major cellulose derivatives is cellulose acetate,<sup>33</sup> which is one of the most important esters of cellulose and was used, for example, by Mocolini *et al.*<sup>105</sup> to immobilize laccase with the aim of determining methyl dopa. The authors achieved satisfactory results compared with the spectrometry method. Another common modified cellulose derivative used as a support for enzyme immobilization is diethylaminoethyl cellulose (DEAE-cellulose).<sup>1</sup> The resolution of racemic amino acids for the production of L-amino acids was conducted with aminoacylase from *Aspergillus oryzae* immobilized by ionic adsorption on diethylaminoethyl (DAE)-Sephadex (modified cellulose); this is known as the Tanabe process.<sup>2</sup> It is important to mention that the process has been used in industry since 1969, and both the enzyme and the support were reported to be very stable.<sup>106</sup> In another example,  $\alpha$ -amylase from *Bacillus amyloliquefaciens* TSWK1-1 was also immobilized by ionic binding in DEAE-cellulose and was considered to be the most effective support with regard to biocatalytic properties and stability among the ones investigated by the authors.<sup>56</sup> The biocatalyst presented a specific activity of 6500 U/mg, a maximum velocity ( $V_{\max}$ ) of 2186 mol mL<sup>-1</sup> min<sup>-1</sup>, and a Michaelis-Menten constant ( $K_m$ ) of 0.8 mg/mL with starch as substrate. The authors also report that the thermostability was significantly increased after immobilization; the half-life of the immobilized enzyme was 86.5 h, which was 4.7 times higher than the free enzyme half life. The biocatalyst also presented pH stability, which was higher than that for the free form. The authors attributed this gain in stability to two factors: (1) a molecular rigidity introduced by attachment to a rigid support and the creation of a protected microenvironment and (2) the prevention of subunit dissociation (multimeric enzymes) by intersubunit crosslinking immobilization through the reduction of the conformational inactivation. Last but not least, with regard to the operational stability,  $\alpha$ -amylase immobilized in DEAE-cellulose was reused in 20 consequent cycles with a marginal loss of 4% in its original activity.

Novel supports, such nanocomposites<sup>107</sup> and bacterial cellulose (BC),<sup>108</sup> have also been reported in the literature. Studies have



focused on the development of new supports and strategies of protein bounding. In that sense, Incani *et al.*<sup>107</sup> proposed the immobilization of glucose oxidase onto nanocrystalline cellulose nanocomposites through a robust amide bond between the amine groups of a lysine residue in enzymes and a thiol-modified nanocomposite. According to the authors, this strategy enhanced the enzyme stability and yet allowed the enzyme to function as if it was free in solution but prevented aggregation of the protein molecules.

The use of BC for enzyme immobilization is yet less popular than plant-derived cellulose. BC has gained attention because of its unique physical properties, including its mechanical strength, ultrafine fiber, biodegradability, and high crystallinity.<sup>108</sup> The pellet type of BC has potential in enzyme immobilization and was used by Wu and Lia<sup>108</sup> to immobilize glucoamylase. In this study, BC was produced by the strain of *Acetobacter xylinum* in a shaking flask with baffles, but the authors suggested the use of an airlift reactor to a large-scale production of the pellet form. Different types of BC beads and some activated methods were investigated, and the epoxy method with glutaraldehyde coupling was the best one. Furthermore, the immobilized enzyme presented higher thermal and pH stabilities when compared to the free enzyme.

**Chitosan and Chitin.** Chitin is a renewable polysaccharide mainly found in the exoskeleton of crustaceans, insects, marine crabs, and so on; it contains 2-deoxy-2-amino glucose units linked by  $\beta$ -1,4 linkages.<sup>52,109</sup> Indeed, it is obtained as a byproduct of the fishing industry, which often causes negative environmental impacts. Therefore, some attempts have been made to use it as a support for enzyme immobilization;<sup>110</sup> chitosan, the deacetylated form of chitin, is much more popular. Porous spherical chitosan particles are commercially available (Chito-pearl, Fuji Spinning) and have been used for enzyme immobilization.<sup>6,16,20,111</sup>

Chitosan has a similar molecular structure to cellulose, differing only in its functional groups. Like in cellulose, hydroxyl groups are present in the general structure of chitosan, but the presence of amino groups appears as the main difference between them.<sup>16,21,112</sup> The presence of this amino group and its protonation-generating  $\text{NH}_3^+$  ions render the special properties that differentiate it from vegetable fibers.<sup>21</sup> The positive charges on these amino groups, along its linear chains, allow the development of electrostatic interactions with molecules containing negatively charged groups.<sup>113</sup> Chitosan is widely used in the cosmetic, pharmaceutical, and food industries and in the development of biomaterials, and this is due to the characteristics of this type of material, including antimicrobial properties, biocompatibility, biodegradability, and low toxicity.<sup>114,115</sup> For the immobilization of enzymes, hydrogels and chitosan membranes are widely used because of its physical characteristics, such as the pore diameter of the support, which are suitable for the process of immobilization.<sup>21</sup> Furthermore, the presence of functional groups on chitosan chains, such as  $-\text{OH}$  and  $-\text{NH}_2$ , allows chemical modifications.<sup>113</sup> Different protocols may be used to achieve enzyme immobilization, such as adsorption, encapsulation, and covalent bounding.<sup>24,116</sup> A common method

of protein immobilization in chitosan is the covalent linkage of the enzyme to the polymeric material with bifunctional cross-linking reagents, such as glutaraldehyde, which establishes intermolecular bonds between the amino groups of the protein and those of the polymer. The linkage yield may be enhanced if the less reactive hydroxyl groups of the chitosan molecule are activated to react with amine groups of the enzyme.<sup>97</sup> Indeed, Hung *et al.*<sup>73</sup> named this method of immobilization, involving the amino and hydroxyl groups of chitosan, *binary immobilization*.

Several researchers have reported the enzyme immobilization on chitosan,<sup>23,24,81,107,116–118</sup> with glutaraldehyde being the most common crosslinking reagent. For instance, chitosan activated with glutaraldehyde was described for the immobilization of *Kluyveromyces lactis* NRRL  $\beta$ -gal Y1564, and the authors observed that it is a suitable alternative low-cost support for  $\beta$ -gal immobilization because high operational and storage stabilities in comparison with the soluble enzyme were achieved.<sup>23</sup> Nevertheless, in another study, chitosan was derivatized by two methodologies, with glutaraldehyde and epichlorohydrin for crosslinking and activation of the support, and the effects on its physicochemical characteristics and its applicability as a carrier for *Bacillus circulans*  $\beta$ -gal immobilization were investigated. The authors concluded that chitosan is a polymer that if properly derivatized can be used to prepare high-quality supports for the generation of robust biocatalysts<sup>116</sup> because it is the best compromise between the biocatalyst activity and thermal stability achieved when chitosan particles were prepared with epichlorohydrin in a two-step process. Other authors<sup>17</sup> also investigated the immobilization of  $\beta$ -gal, but from *K. fragilis*, on chitosan-based supports by different protocols; these included the use of glutaraldehyde, glycidol, and epichlorohydrin as activating agents and the use of hybrid hydrogels chitosan–alginate and chitosan– $\kappa$ -carrageenan beads as supports. According to the authors, the immobilization of  $\beta$ -gal by covalent attachment on chitosan coagulated at 50°C in 500 mM KOH and activated with a low concentration of glutaraldehyde produced the best immobilized biocatalyst.

It is important to mention that chitosan and other biopolymers, such as alginate and carrageen, may be used to prepare hybrid supports for enzyme immobilization. The preparation of various hybrid polymers may be achieved with chitosan, which forms polyelectrolyte complex products with natural polyanions. The strong electrostatic interaction of the amine groups of chitosan with the carboxyl groups of other polymers, such as sodium alginate, the sodium salt of polyuronic acid containing variable proportions of 1–4-linked  $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid, lead to the formation of a chitosan–alginate hybrid gel. This complex is a stronger than pure chitosan; consequently, it has a higher activity under drastic conditions of mechanical stirring and temperature.<sup>119</sup> The binary immobilization of CALB on chitosan and chitosan–alginate complexes has been explored in the literature<sup>97</sup> with glutaraldehyde, glycidol (2,3-epoxy-1-propanol), and ethylene diamine as activating agents. According to the authors, the presence of ethylene diamine in the support increased the distance between the enzyme and the support, and this increased lipase immobilization yield

because of a higher enzyme access. Indeed, chitosan–alginate activated with 2% glutaraldehyde was the most stable derivative prepared in this study with regard to the thermal stability (at 60°C) and operational stability, as assayed in butyl oleate synthesis (12 h each).

Some novel strategies in support preparation have been described, such as the use of ultrasound<sup>19</sup> and nanotechnology.<sup>120</sup> De Mello *et al.*<sup>19</sup> proposed the use of ultrasound sonication to produce the fragmentation of the chitosan polymer to enhance its surface area and make it more accessible to the lipases. The authors showed that ultrasound-treated chitosan was an effective support and enabled them to obtain active immobilized lipase systems with higher immobilization efficiencies when compared to the ones produced with the conventional chitosan beads as supports. The authors suggested that the enzyme was initially adsorbed by ionic exchange and then immobilized covalently. In addition, the activation of the support with glutaraldehyde promotes increased the hydrophobicity of the support surface and increased the yield of the immobilized enzyme and the stability because of the multipoint covalent attachment formed between the enzyme and the activated support.<sup>19</sup>

Currently, nanotechnology has received a lot of attention for application in enzyme immobilization<sup>120</sup> because of the nano-materials' small size and large surface-area-to-volume ratio.<sup>121</sup> Studies have shown that nanoparticles of chitosan are suitable supports for enzyme immobilization and confer a strong stability to the biocatalyst. Furthermore the separation of the enzyme–support complex is easy from the reaction mixture at the end of the biochemistry process.<sup>121–123</sup> Indeed, chitosan nanoparticles are widely used as supports for the immobilization of enzymes.<sup>122,124–127</sup> This polymer can be used alone for the immobilization of enzymes<sup>128</sup> to colloiddally stabilize polymer nanoparticles<sup>129</sup> and coated magnetic nanoparticles.<sup>130</sup>

**Dextrans.** Dextrans are polysaccharides produced by microorganisms that possess units of  $\alpha$ -D-glucose linked primarily by 1–6-glycosidic linkages. Studies have reported that dextran-producing microorganisms mainly belong to the *Lactobacillaceae* family, genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, and *Rizopus*.<sup>131–133</sup> Widely used in the pharmaceutical and biomedical industry, new areas of application of dextrans and derivatives are currently being looked for and this is due to the characteristics of the material, such as their hydrophobicity, stability, and ability to form clear and stable solutions, and the fact that they are derived from renewable resources and degraded by ecological systems.

Recent studies have shown the use of dextrans in the modification of *Candida rugosa* lipase,<sup>134</sup> as a coating of immobilized enzymes to prevent its inactivation,<sup>135,136</sup> as long and hydrophilic spacer arms for enzyme immobilization,<sup>80</sup> in the stabilization of multimeric enzymes by chemical crosslinking<sup>137,138</sup> and as a crosslinker in the preparation of CLEAs.<sup>139,140</sup> Some authors have reported that the use of crosslinking reagents of greater size, such as dextran aldehyde (100 a 200 kDa), can improve the stability and increase the particle size of CLEAs.<sup>139</sup> Another interesting application (coating) was described by

Betancor *et al.*,<sup>135</sup> who used dextran aldehyde to prevent inactivation (by gas bubbles) of glucose oxidase immobilized on magnetic nanoparticles, a nonporous support. The use of dextran also proved to be useful to immobilized enzymes and proteins acting on macromolecular substrates.<sup>80</sup> For instance, by promoting the attachment of aldehyde–dextrans on very poorly activated amino–agarose gels, dextran–agarose composites were produced.<sup>80</sup> The authors attributed this behavior to the fact that dextran is very inert and presents flexible arms, which do not promote side interactions with proteins. Furthermore, they are long enough to prevent steric hindrances promoted by the proximity of the support surface to the enzyme, when compared to the conventional short aliphatic arms often used for enzyme immobilization.

**Pectin.** Pectin is a branched polysaccharide composed primarily of polymers of galacturonic acid, rhamnose, arabinose, and galactose. The percentage of D-galacturonic acid esterified with methanol is what tells us the degree of esterification.<sup>141–143</sup> The repeating segment of the pectin molecule have some functional groups, including (1) carboxyl, (2) ester, and (3) amide groups.<sup>144</sup> Furthermore, a high availability of secondary hydroxyl groups are present in the structure of pectin.<sup>18</sup> It has been reported in the literature that pectins that have a low degree of esterification and have an excellent ability to form gels through interaction with divalent cations.<sup>145</sup>

Pectins are widely used mainly in the pharmaceutical, cosmetic, and food industries but currently there have been studies exploring the biotechnological potential of this material, such as in the production of supports for enzyme immobilization, for example, lipases.<sup>18,146</sup> The use of pectin as support for covalent enzyme immobilization was also reported by Jadhav and Singh<sup>147</sup> for the coimmobilization of  $\alpha$ -amylase and glucoamylase. Hybrid supports [pectin/poly(vinyl alcohol)] were also described for the encapsulation of enzyme  $\alpha$ -amylase.<sup>148</sup> The combination of different materials for the production of supports has been extensively studied because it is possible to assemble the main characteristics of different materials to obtain more specific supports for enzyme immobilization.<sup>97,100</sup>

The use of pectin as a support for lipase immobilization was reported.<sup>18</sup> The authors immobilized the enzyme by adsorption and covalent binding. The adsorption of the enzyme onto pectin resulted in a system with lower catalytic activity compared to that of biocatalysts obtained by covalent immobilization. A possible explanation is that pectin–lipase interactions occurring in the adsorption process were random in nature, and this may have led to an immobilized lipase with active site inaccessible to the substrate.<sup>146</sup> In the covalent attachment, on the other hand, specific groups in the lateral chain of lipase, which were probably far from the active site, were attached to the support and so resulted in a system without steric hindrance. To allow covalent immobilization, the authors evaluated the use of sodium metaperiodate and glutaraldehyde as crosslinking agents. According to their results, it was possible to achieve better results with the first agent because of the presence of vicinal hydroxyl groups in the pectin structure. The reactive carbonyl group, which is present in the metaperiodate pectin derivative,

**Table III.** Industrial and Potential Industrial Applications of Immobilized Enzymes with Renewable Supports

Support	Enzyme	Application	Reference
Calcium alginate	$\alpha$ -Amylase	Food industry: starch hydrolysis	164
Carrageenan	Penicillin G acylase	Antibiotics production: penicillin G	165
Chitosan	$\beta$ -gal	Food industry: lactose hydrolysis	166
Pectin	Lipase	Food, pharmaceutical, fine chemical, and energy industries: fat interesterification	18
DEAE-cellulose	Glucose isomerase	Food industry	155

is especially reactive to imidazole or amino groups, for example, those present at the lateral chain of arginine and lysine residues in the proteic structure of lipase. The immobilization conditions used by the authors favored the reaction between the nitrogen from imidazole or amino groups and the oxygen from the carbonyl group.<sup>146</sup> Furthermore, these authors also attributed the low yield of covalent immobilization achieved with glutaraldehyde to the low content of amino groups in pectin.

**Starch.** Structurally, starch consists essentially of two molecular components: amylose and branched amylopectin. Starch is a biopolymer and is nontoxic, renewable, and cheap. This is why it has been used, alone or in combination with other polymers, for the production of bioplastics and for the preparation of nanoparticles.<sup>149–152</sup>

Starch may be used as a thickener, a carrier, and a slow-release agent in the pharmaceutical, cosmetics, and food packaging industries. In biotechnology, it has been reported that starch films are excellent immobilization supports for various kinds of lipases.<sup>153</sup> These systems have been successfully used for the enantioselective resolution of (*R,S*)-1-phenylethanol under mild reaction conditions. Nevertheless, there have not been many examples of starch being used as a support for enzyme immobilization.

Another possible application of starch for enzyme immobilization, one that is yet not very popular, is its use as a coaggregated additive for the preparation of CLEAs. This coaggregation strategy was studied in the literature,<sup>154</sup> and the effect of starch addition on the crosslinking efficiency of CLEAs of PAL from *Rhodotorula glutinis* was evaluated. However, the addition of starch had slight effects on the crosslinking process of CLEA formation when compared to BSA. To the best of our knowledge, this was the only article that reported the use of starch as a coadditive in the production of CLEAs.

### INDUSTRIAL BIOCATALYSTS AND FUTURE TRENDS

Although there is a vast literature reporting methods for enzyme immobilization with biopolymers, there have been few reports of biocatalysts (immobilized enzymes) that are available commercially or are successfully used on a large scale. Table III shows some examples of possible large-scale use of biocatalysts. It is important to note that chemical companies may produce and immobilize their own enzymes in parallel with process development.<sup>155</sup> Furthermore, it is also possible to purchase a soluble form of the enzyme and prepare an immobilized form of the

enzyme in an industrial facility. For this reason, the portfolio of commercial immobilized enzymes may be apparently smaller.

The extensive effort required to develop a competitive biocatalyst and process have prevented the large use of biocatalysis in industries.<sup>156</sup> For the industrial application of biocatalytic processes, some drawbacks should be overcome. With regard to immobilization technologies, some factors should be taken into consideration: (1) the protocols must be robust, reproducible, and scalable; (2) the enzyme stability should remain high during the intermediate steps; (3) the environmental and societal impacts of the process should be low because the handling of crosslinking chemicals and dust-producing materials are often required, and (4) pilot-scale correlation should be evaluated to consider future developments.<sup>156–158</sup> It is also important to have in mind that independent of which technology is used, routes with the highest chances of success are the ones with few steps and low environmental impacts.<sup>158</sup>

Not all industrial enzymes are expensive, so the cost is often only a minor component in the overall process economics; this makes the additional costs associated with enzyme immobilization difficult to justify. Indeed, it is important to estimate the cost contribution of an immobilized enzyme by determining the total productivity on a kilogram of product per kilogram of biocatalyst basis. This can be indirectly measured by the determination of the number of times the enzyme is reused. Therefore, it is imperative to better understand the process: what are the factors involved in the implementation of an immobilized enzyme process? This question should be answered to further integrate the biocatalyst into the large-scale process. In that sense, the choice of a support, for instance, may not be based only on its cost but should also be accounted for the opportunity it will give to the selection of optimal operating conditions or to decide upon the feasibility of different process options.

The need to develop more sustainable processes based on green chemistry favors the use of immobilized enzymes and renewable materials, for instance, as supports or additives during immobilization. A wide range of opportunities (potential processes) are described in the academic literature, but the commercial success is difficult to estimate.<sup>157</sup> In this sense, the investment of research in the process design and development is mandatory.

Last but not least, nanotechnology has opened a new frontier in the development of potential supports for enzyme immobilization because it use may allow the movement of immobilized enzyme products into other areas of application, such as

biosensors, intelligent materials, and processes dealing with insoluble and macromolecular substrates.

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