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ADVANCES IN LACCASE IMMOBILIZATION

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Abstract

Improvements in current strategies for carrier-based immobilization have been developed using hetero-functionalised supports that enhance the binding efficacy and stability through multipoint attachment. New commercial resins (Sepabeads) exhibit improved protein binding capacity. Novel methods of enzyme self-immobilization have been developed (CLEC, CLEA, Spherezyme), as well as carrier materials (Dendrispheres), encapsulation (PEI Microspheres), and entrapment. Apart from retention, recovery and stabilisation, other advantages to enzyme immobilization have emerged, such as enhanced enzyme activity, modification of substrate selectivity and enantioselectivity, and multi-enzyme reactions. These advances promise to enhance the roles of immobilization laccase enzymes in industry, while opening the door for novel applications.

#General Article

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Introduction

Biocatalytic process economics can be enhanced by enzyme reuse and the improvement in enzyme stability afforded by immobilization. The capacity to retain or recover enzymes also allows biocatalyst separation from product, thereby permitting continuous processes, and prevents carry-through of protein or activity to subsequent process steps (Polizzi et al. 2007). Immobilization can also improve enzyme performance under optimal process reaction conditions (e.g. acidity, alkalinity, organic solvents, and elevated temperatures), a requirement that has often retarded enzyme application in industrial chemical synthesis (Bommarius and Riebel 2004). In spite of the long history and obvious advantages of enzyme immobilization (Katchalski-Katzir and Kraemer 2000), Straathof et al. (2002) estimated that only 20% of biocatalytic processes involve immobilised enzymes. However, over the last few years a number of interesting new developments have been reported in the literature and patent applications (Spahn and Minteer 2008), indicating that enzyme immobilization has entered an exciting new phase.

Laccases

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belong to the group of blue oxidases and represent the largest subgroup of multicopper oxidases. These enzymes have been studied since the nineteenth century due to their ability to oxidize phenolic compounds, and their applications in several industrial sectors have been intensively studied as of late (Giardina et al., 2010; Loera et al., 2006; Madhavi and Lele, 2009; Morozova et al., 2007b).

Occurrence

Laccase was first discovered in the Japanese lacquer tree Rhus vernicifera (Giardina et al., 2010; Morozova et al., 2007b). Since then, these enzymes have been found in various plant species, insects and bacteria (Loera et al., 2006; Madhavi and Lele, 2009). However, the majority of laccases described in the literature have been isolated from higher fungi. These laccases occur in the fungal causative agents of soft rots, in most bracket fungi causing white rot, in soil saprotrophs, in plant pathogens and in many agarics, including cultivated edible fungi, e.g., champignon, *Pleurotus* and the medicinal shiitake Lentinula edodes (Morozova et al., 2007b). However, the most common laccase producers are nearly all wood-rotting fungi, such as *Trametes versicolor,Trametes hirsuta, Trametes ochracea, Trametes villosa, Trametes gallica, Cerrena maxima, Coriolopsis polyzona, Lentinus tigrinus and Pleurotus eryngii* (Madhavi and Lele, 2009; Morozova et al., 2007a).

Catalysis

Laccases have activity toward ortho- and para-diphenol groups, although their affinity is usually higher towards the latter group. These enzymes are characterized by their remarkably wide substrate specificity and a variable range of oxidizable substrates that depends on the organism producing them (Madhavi and Lele, 2009). Laccases catalyze the oxidation of a wide variety of substrates, including mono-, di-, and

polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate, with the concomitant four-electron reduction of oxygen to water (Giardina et al., 2010; Madhavi and Lele, 2009). These enzymes couple the four single-electron oxidations of the reducing substrate to the four-electron reductive cleavage of the dioxygen bond with four Cu atoms (Giardina et al., 2010). These copper atoms are classified into three groups depending on the characteristics obtained by UV/visible and electron paramagnetic resonance (EPR) spectroscopy. The type I copper (T1) is responsible for the intense blue color of the enzyme, has a strong electronic absorption approximately 600 nm and is EPR detectable. The type II copper (T2) is colorless but EPR detectable and the type III copper (T3) consists of a pair of copper atoms that give a weak absorbance near the UV spectrum and no EPR signal. The T2 and T3 copper atoms form a trinuclear cluster where the binding and multielectron reduction of dioxygen takes place (Durán et al., 2002; Madhavi and Lele, 2009).

The catalytic mechanism of the laccase enzyme starts with the donation of an electron to the substrate by the T1 copper site, followed by an internal electron transfer from the reduced T1 to the T2 and T3 copper site. The T3 copper functions as a two-electron acceptor in the aerobic oxidation process, in which the presence of the T2 copper is necessary. The reduction of oxygen to water takes place at the T2 and T3 cluster and passes through a peroxide intermediate (Durán et al., 2002; Madhavi and Lele, 2009; Morozova et al., 2007a). Substrates with a high redox potential cannot be directly oxidized by laccases, and thus, the role of laccases in lignin biodegradation is restricted to the phenolic moieties. Laccase mediator systems (LMSs) have led to a dramatic increase in the range of laccaseoxidizable compounds (Madhavi and Lele, 2009; Morozova et al., 2007b). The so-called mediator compounds act as intermediate substrates and enable laccase to indirectly oxidize large molecules and even non-phenolic substrates (Giardina et al., 2010). An ideal redox mediator should be a good laccase substrate with stable oxidized and reduced forms and should not inhibit the enzymatic reaction.

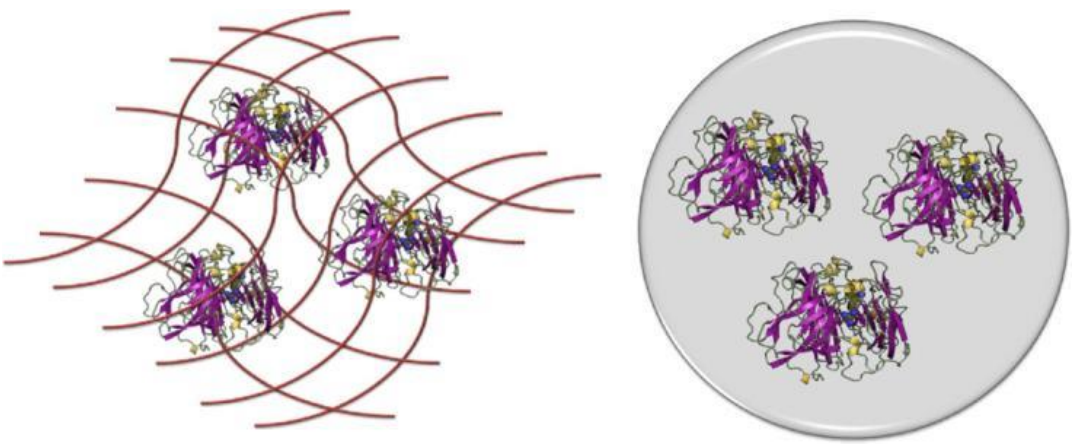
Methods for Laccase Immobilization

Entrapment and Encapsulation

Enzyme entrapment (Fig. 1a) is typically achieved using a polymer network such as an organic polymer or sol–gel and is usually performed in situ (Sheldon 2007a). Entrapment protects enzymes by preventing direct contact with the environment, thereby minimising the effects of gas bubbles, mechanical sheer and hydrophobic solvents, but has the drawback of mass transfer limitations and low enzyme loading (Lalonde and Margolin 2002). A common method of entrapment is through use of silica sol–gel matrices formed by hydrolytic polymerisation. Reetz and Jaeger (1998) used alkylsilane precursors ($RSi(OCH_3)_3$) or combinations of $RSi(OCH_3)_3$ and $Si(OCH_3)_4$ to provide heterogeneous biocatalysts with a sol–gel entrapped *Pseudomonasaeruginosa* lipase. By adjusting the polymerisation conditions the polymer porosity, network structure, surface functionalities, and particle size can all be modified. In particular, the method of drying, the solvent surface tension and polymer composition of the sol–gel allows for modulation of porosity. These gels are referred to, in order of decreasing density, as xerogels (air dried), ambigels (more hydrophobic, and hence dry with attenuated capillary stress and limited shrinkage),

and aerogels (supercritical drying, with negligible shrinkage) (Pierre 2004). Santos et al. (2008a, b) have investigated polysiloxane (POS)–polyvinyl alcohol (PVA) hybrid matrices for *Candidaantarctica* lipase B (CaL-B) immobilization, and demonstrated that the percentage of PVA in the sol–gel can significantly influence the physical properties of the particle, such as hardness and surface area. Bruns and Tiller (2005) entrapped horseradish peroxidise and chloroperoxidase in a nanophase separated amphiphilic network consisting of a co-polymer of poly(2-hydroxyethyl acrylate) (PHEA) and bifunctional poly(dimethylsiloxane) (PDMS). Initial enzyme loading occurred in aqueous media, where the hydrophilic polymer (PHEA) network swelled to allow uptake of the enzyme. The polymer was then when placed in organic medium (n-heptane) in which the hydrophilic network shrank and the separate but interpenetrating hydrophobic polymer network expanded, effectively trapping the enzyme. This particle was therefore suitable for biocatalytic application in organic solvents. Not all entrapment polymers are silicon-based. For example, Lee and Huang (2008) used epoxide activated hydrogels to immobilise trypsin, using co-polymers N-isopropylacrylamide (NIPAAm), glycidyl methacrylate (GMA) and N,N-dimethyl acrylamide (DMA; while Temino et al. (2005) used PVA to immobilise and stabilise a dehydrogenase for use in organic solvents.

Similar to entrapment, encapsulation protects the enzyme from the external environment but has limited application for the biocatalysis of large substrates as they are prone to mass transfer limitations (Lalonde and Margolin 2002). Zhang et al. (2008, 2009) have recently developed an elegant method of layered enzyme entrapment and encapsulation in which b-glucuronidase was mixed with carboxymethyl cellulose and $CaCl_2$. The solution was subsequently extruded through a needle into a 1% (w/v) alginate solution. The resultant soft capsules provided an enzyme-compatible environment. The capsules were then reacted with protamine (a small arginine-rich protein) which, being too large to enter the capsules, ionically associated with the surface alginate. The protamine was then used to precipitate silicates at the surface to form a hard silicate shell for the particle, preventing compression or swelling.



(a) **(b)**

Fig.: 1. Immobilization of Enzymes by Physical Interactions. (A) Entrapment of Enzymes Into A Porous Solid Matrix. (B) Encapsulation of Enzymes

Support Based Immobilization

Immobilization to a prefabricated support can provide rigidity there by enabling the use of various reactor configurations for biocatalysis, such as fixed-bed reactors (Kunamneni et al. 2008). However, this method suffers from dilution of volumetric and specific activity as carriers can account for 90–99% of the mass or volume of the catalyst (Sheldon 2007a; Lalonde and Margolin 2002). Adsorption is relatively simple and inexpensive method of immobilization, and does not chemically modify the enzyme, but it has as limitations the enzyme tends to leach out, especially in aqueous solvents. This can result in difficulties in process design and downstream processing. Hence the method is best suited to immobilization of lipases for use in organic solvents, such as commercial preparations of immobilised Candida antarctica lipase B (CaL-B), which include Novozyme 435 (Novozymes) and Chirazyme (Roche Molecular Biochemicals).

Macroporus acrylic polymer resins such as Amberlite XAD-7 (Takac¸ and Bakkal 2007) can be used for enzyme adsorption, while CaL-B immobilised on VP OC1600 (Bayer) is widely used for the production of speciality chemicals (Miletic´ et al. 2009). Alternatively, silica-based materials such as modified aerogels (Gao et al. 2009) or Celite can be used. Chaplin et al. (2002) immobilised *Pseudomonasfluorescens* lipase on Celite for the resolution of menthol from an eight diastereomer mix in an organic solvent based reaction. Adsorption is regularly used in large scale processes, particularly where the enzyme is inexpensive (Lalonde and Margolin 2002).

Ionic binding is another simple non-covalent immobilization technique.(Fig-2) Enzymes can be bound to polysaccharide biopolymers such as, dextran, agarose and chitosan. These polymer supports may be functionalised with a variety of chemical groups to achieve ionic interaction, including quaternary ammonium, diethylaminoethyl and carboxymethyl derivatives. This method has been applied commercially for glucose isomerase production of high fructose syrup (Lalonde and Margolin 2002). Alternatively functionalised macroporus acrylic polymer resins such as Amberlite FPC3500 (cationic) or FPA54 (anionic) can be used. Binding is reversible, and although advantageous for re-use of the support, protein leaching is a potential problem. The most interesting recent developments are in the area of immobilization through covalent binding. Here the e-amino group of lysine is typically (but not exclusively) used as the point of covalent attachment.

Lysine is a relatively common amino acid in proteins, frequently located on the protein surface, is of above average reactivity, and provides good bond stability (Krenkova and Foret 2004). Epoxide groups are typically used on the support for linkage as they are relatively stable, can bind lysine, and react with protein under very mild conditions (Mateo et al. 2007c). After immobilization the residual groups are quenched with a primary amine containing chemicals such as tris(hydroxymethyl)aminomethane (Tris) to prevent further non-specific reactions (Krenkova and Foret 2004). The optimal immobilization support would often have short spacer arms and a high density of reactive groups required for multi-point attachment, thereby providing rigidity to the enzyme (Mateo et al. 2007b, c). The commercial support Eupergit (Evonik, previously Degussa), a macroporus sphere (170 μm average diameter) activated with epoxides and made of a co-polymer of N,N0 -methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and

methacrylamide, has long been used for enzyme covalent immobilization, including commercial applications (Katchalski-Katzir and Kraemer 2000).
Sepabead EP-Cu (wherein a proportion of epoxy groups are modified with iminodiacetic acid and $CuSO_4$) gave full immobilization and good activity (70%), while dextran modified glyoxylagarose beads gave 95% activity towards styrene oxide (Mateo et al. 2007a). Although short, linking groups assist in stability through increased rigidity, it is not always optimal for activity as it can result in steric hindrance. Inclusion of hydrophobic spacer arms (1,6 diaminohexane) during glutaraldehyde immobilization on a silica support improved lipase activity (Ozyilmaz 2009). To enhance binding, resins with multiple reactive functional groups have been developed (Mateo et al. 2000), such as Sepabeads EC-HFA with epoxy groups on an ethylenediamine layer (Mateo et al. 2003, 2007c). The rationale for inclusion of the amine groups is that they rapidly bind the protein through ionic interaction with protein carboxylic acids. This aligns the protein for subsequent kinetically enhanced (through closer proximity) covalent bond formation between epoxide groups and lysine residues.

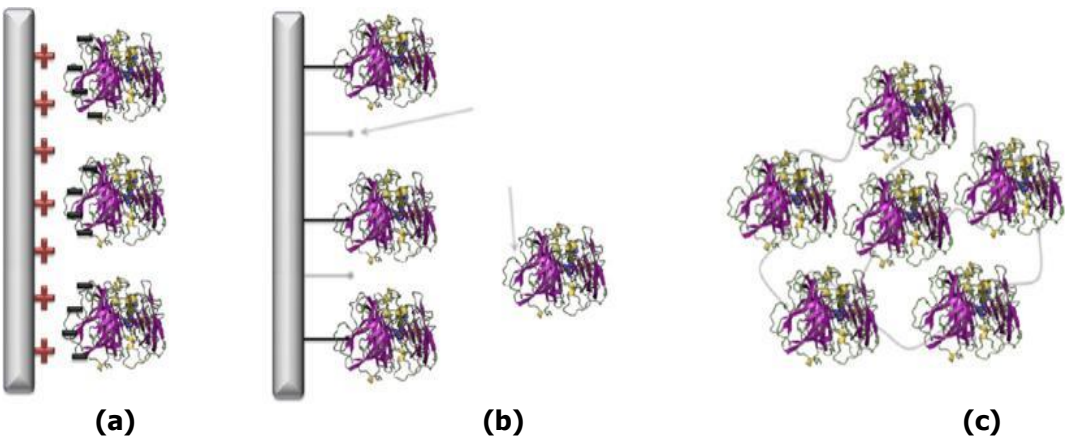


Fig.: 2. Chemical Interactions for Enzyme Immobilization (A) Adsorption of Enzymes onto a Support by Ionic Forces (B) Covalent Binding Between the Nucleophilic Groups of the Enzyme and the Support. (C) Self-Immobilization: Model of Cross-Linked Enzyme Aggregates (CLEAS)

Self-Immobilization

The use of solid supports for enzyme immobilization may reduce the specific and volumetric activity of the biocatalyst. Carrier-free enzyme immobilization is possible with the use of bifunctional cross-linkers (Brady and Jordaan, 2009).These cross-linkers include dialdehydes, di-imino-esthers, di-isocyanates and diamines activated by carbodiimide (Arroyo, 1998). Two different procedures based on this principle are described below. Cross-linked enzyme crystals (CLECs) exhibit excellent activities and operational stability, but their major drawback is the high purity required for the crystallization of the enzyme. The formation of cross-linked enzyme aggregates (CLEAs) involves the precipitation of the laccase, combining the purification and immobilization into a single operation (Arroyo, 1998; Brady and Jordaan, 2009; Cabana et al., 2007; Matijošyte et al., 2010).

As mentioned above the use of solid supports for enzyme immobilization can reduce the specific and volumetric activity of the biocatalyst by a factor of 10 or more. Carrier-free enzyme immobilization is possible using bifunctional cross-linkers, such as glutaraldehyde, to bind enzymes to each other without resorting to a support. Physically stronger biocatalysts can be produced by cross-linking when the enzymes are in close proximity, such as protein crystals. Cross-linked Enzyme Crystals (CLEC; St. Clair and Navia 1992;) were commercialised by Altus Biologics (Margolin 1996). Particle size varied from 1 to 100 μm and had high mechanical stability (partly attributable to the innate stability provided by crystallisation), and could function in organic solvents (Roy and Abraham 2004). As the crystals contain only one enzyme, this method ensures that no contaminating activities were present. Unfortunately CLEC formation requires extensive protein purification and method development and, although broadly applicable, it only works for crystallisable enzymes.

The crystal formation also means only one enzyme type can be incorporated into the particle. Although they functioned well, the drawback was cost (Brady et al. 2004). However, there is still interest in this area (Abraham and Bindhu 2009) and future advances in biotechnology may yet allow for renewed commercial application. A less-expensive method of enhancing enzyme proximity for cross-linking is by simply precipitating the protein and cross-linking the aggregates to form particles of about 50–100 μm diameter (LopezSerrano et al. 2002; Kaul et al. 2007).

Examples of enzymes immobilised this way include nitrile hydratase (Kuba´cˇ et al. 2008) lipase (Lo´pez-Serrano et al. 2002), nitrilase (Kaul et al. 2007), penicillin acylase (Pchelintsev et al. 2009), amino acylase (Bode et al. 2003) and others (Sheldon 2007b). Through subtle modification of the cross-linking conditions the properties of a CLEA can be adjusted significantly. Cross-linkers (such as glutaraldehyde, glutaraldehyde-ethylene diamine polymers, or dextran aldehyde) may be selected for optimal activity of a specific enzyme (Kaul et al. 2007). Pchelintsev et al. (2009) found that variation in the duration of the precipitation step prior to cross-linking influenced the activity and microstructure of penicillin acylase CLEA.

Wilson et al. (2009) discovered that excess cross-linking agent reduced the enzyme conversion yield, productivity andstability, while Majumder et al. (2008) also noted that the degree of crosslinking influenced enantioselectivity. Multimeric enzymes can disassociate which would lead to leaching problems and loss of activity with carrier-based immobilization where, perhaps, only one of the monomers was bound. Wilson et al., (2004) demonstrated that multimeric enzymes, such as tetrameric catalases, can be immobilised using the CLEA method and retain significant activity with negligible loss of protein under denaturing conditions of surfactant (SDS) and temperature. Incorporation of PEI into the CLEA (as a cross-linker) also appears to reduce oxygen related enzyme inactivation in sensitive enzymes such as nitrilase (Mateo et al. 2006). CLEAs may require physical support to increase rigidity for some applications. Wilson et al. (2002) immobilised CLEAs by entrapment within rigid Lentikats (polyvinyl alcohol hydrogel).

Applications of Enzyme Immobilization

Treatment of Textile Wastewaters

Dyes are chemicals with very diverse chemical structures. More than 100,000 dyes are available, and most of them are resistant to light exposure, water or chemical substances. The textile industry is responsible for a large part of the dyes market, and the effluents of this industry are controlled by governments due to environmental concerns. Therefore, the development of processes based on laccases is of interest because of their potential to degrade different types of dyes (Rodríguez Couto and Toca Herrera, 2006). However, there are still many constraints to the industrial application of laccases for the decolorization of dyes, including the non-reusability of the enzyme (Russo et al., 2008). Consequently, the immobilization of laccase could be utilized in dye decolorization, as the system should be easier to operate, the enzyme could be reused and the cost of the process would be reduced (Peralta-Zamora et al., 2003).Two interesting examples of decolorization using alumina as an immobilization support for laccase are described below.
The first example (Kandelbauer et al., 2004) details the decolorization of several dyes with *Trametes modesta* laccase immobilized by covalent binding to aluminum oxide pellets. The alumina was previously silanized with 3-aminopropyltriethoxysilane (APTES) and activated with glutaric dialdehyde. The decolorization was followed online via spectroscopic sensors immersed in the employed reactor. Several anthraquinonic (Lanaset Blue 2R and Terasil Pink 2GLA) and azo (indigo carmine and the triphenylmethane dye crystal violet) dyes were efficiently decolorized, demonstrating that this enzymatic remediation system is not limited to a certain structural group of dyes. However, azo dyes containing hydroxy groups in the ortho or para position relative to the azo bond were preferentially oxidized. Similarly, alumina was also employed in the immobilization of laccase from *T. hirsuta* and then used for the decolorization of methyl green (MG) and Remazol Brilliant Blue R (RBBR). Alumina pellets were silanized with APTES and then activated with GLU to covalently immobilize laccase. The enzyme was subsequently coated with polyallylamine hydrochloride (PAH) and polysodium4-styrenesulfonate (PSS) according to the LbL technique. As a result, 68% enzyme loading was achieved, and the coated laccase exhibited higher activity. Using this immobilized laccase, MG was decolorized to a higher extent than RBBR (Rodríguez Couto et al., 2007).

RBBR was also decolorized by using laccase that was covalently immobilized in pre-silanized controlledporosity-carrier beads (CPC-silica) activated by GLU. The decolorization of RBBR was related to the enzymatic activity instead of the adsorption of the dye onto the carrier. Moreover, the stability of the laccase was improved (Champagne and Ramsay, 2007). The entrapment of laccase has also been used as an immobilization approach for environmental applications. For this application, Lu et al. (2007)chose an alginate–chitosan complex membrane. Chitosan can interact with alginate by electrostatic interactions, enhancing the stability of the alginate beads. The optimal conditions for immobilization were 2% $CaCl_2$, 0.3% chitosan and a 1:8 volume ratio of enzyme:alginate, resulting in a loading efficiency and immobilized yield of 88.12% and 46.93%, respectively. The immobilized laccase exhibited lower activity and substrate affinity but improved stability to temperature and pH denaturation. In fact, this stability enhancement resulted in an advantage in Alizarin Red decolorization with or without the addition of a laccase

mediator. Alginate entrapment has been applied in several works and is considered a common method for the application of immobilized laccase. Using this method, *Polyborus rubidus* laccase degraded 80% of a dye in 5 days under batch conditions (Dayaram and Dasgupta, 2008). Similarly, a commercial laccase from Denilite II S was also immobilized in an alginate–gelatin matrix with polyethylene glycol (PEG) to effectively decolorize Reactive Red B-3BF.

Thus, Zhao et al. (2008) used magnetic particles of Fe_3O_4 covered by alginate that was modified with acyl chloride groups on the surface. The laccase immobilized by this method showed less affinity for 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), but the thermal and reuse stability were improved. After 12 h of treatment, 39% and 22% of Congo red dye was oxidized by immobilized and free laccase, respectively. In the same way, entrapment in hydrogel structures has also been studied as a new and interesting method for laccase immobilization. For instance, poly(acrylamide-N-isopropylacrylamide) (P(AAm-NIPA)) with semi-interpenetrating networks (semi-IPNs) of alginate was used to immobilize T. versicolor laccase, resulting in low substrate affinity but improvements in storage stability and in the decolorization of Acid Orange 52 (Yamak et al., 2009). Later, Makas et al. (2010) used semi-IPNs prepared from κ-carrageenan with either poly (acrylamide-acrylic acido-poly(acrylamideitaconic acid). At the end of 42 days of storage, the immobilized enzyme in the above-mentioned hydrogel systems retained more than 80% of its original activity, while 50% of its activity was retained after ten uses in a batch system. Methyl orange was selected as the target dye, resulting in 35% decolorization after 6 h of treatment for both systems, which increased to 70% if ABTS was included in the reaction medium as a mediator. The functionalization of supports by including epoxy groups on the surface has been used several times for covalent immobilization. This methodology was applied for the immobilization of laccase from *Pleurotus ostreatus* onto Eupergit 250 L (Russo et al., 2008).

The epoxy groups of the support reacted with the amino, thiol and carboxyl residues of the enzyme. Only 7% immobilization was achieved, but the stability and conversion kinetics during RBBR degradation demonstrated the possibility of textile effluent remediation. The epoxy modification was also involved in the covalent immobilization of laccase from the commercial product Denilite II S using Sepabeads EC-EP3 and Dilbeads Nk as supports (Kunamneni et al., 2008). The results showed the improvement of stability toward pH, temperature and storage, but not toward organic solvents. The degradation of Reactive Black 5, Acid Blue 25, methyl orange, RBBR, MG and Acid Green 27 was also performed with or without the redox mediator 1-hydroxybenzotriazole, resulting in different levels of decolorization for each dye. Other functionalization methods have also been employed for the covalent immobilization of laccase on different supports for dye decolorization. The non-porous polymer poly(glycidyl methacrylate/ethyleneglycol dimethacrylate) (poly(GMA/EGDMA)) with the spacer arm 1,6-diaminohexane (DAH) was activated by GLU and covalently bound to the *R. vernicifera* laccase. The produced spheres recovered 88% of laccase activity, and although the catalytic efficiency was lower, the immobilized laccase exhibited increased stability.

One should note that very few examples of laccase adsorption are presented in the literature. For instance, *T. versicolor* laccase was adsorbed on magnetic beads

modified with poly(4-vinylpyridine) or chelated with Cu ions to decolorize Reactive Green 19, Reactive Red 2 and Reactive Brown 10 (Bayramoglu et al., 2010b).

Degradation of Xenobiotics and Treatment of Industrial Effluents

It is generally agreed today that xenobiotic substances are becoming an increasingly large problem in water treatment because they are new substances that are frequently resistant to degradation by chemical and biological methods. For this reason, laccases could be considered as an alternative bioremediation treatment because they can oxidize a wide variety of xenobiotic compounds. The immobilization of laccase results in the greater operational stability and durability of the enzyme and, in some cases, leads to the possibility of its use in a continuous process, allowing the biocatalysts to be used at an industrial scale (Dodor et al., 2004; Fernando Bautista et al., 2010) Laccase from *T. versicolor* was immobilized in kaolinite that was functionalized by APTES and GLU and was then tested for its ability to degrade polycyclic aromatic hydrocarbons (PAHs). In the presence of the mediator ABTS, 80% of anthracene and benzo[a]pyrene (BaP) was oxidized (Dodor et al., 2004). Later, the same authors used kaolinite and nanoparticles of mesoporous silica (SBA-15) functionalized by the above-discussed method for the immobilization of *T. versicolor* laccase, achieving high pH and thermal stability and the effective oxidation of BaP, demonstrating again the potential of laccase for PAHs remediation (Hu et al., 2007).

The degradation of phenolic compounds by immobilized laccases has also been demonstrated. *Coriolus versicolor* laccase was immobilized, first by adsorption on activated carbon and then by entrapment into calcium alginate gels, for the degradation of 2,4-dichlorophenol. The immobilized laccase presented better thermal and pH stability than the free enzyme and achieved a de-chlorination efficiency of 99.5% during eight repeated batch reactions (Zhang et al., 2006).

Textile and Pulp and Paper Industry

Laccases have been accepted as potential biocatalysts for several applications in the textile as well as pulp and paper industries. The degradation and detoxification of textile wastewater has already been discussed, but several applications of laccase immobilization related to the textile industry aside from wastewater treatment have been suggested. Ibrahim et al. (2007) immobilized the laccase from Denilite II S (Novozymes) onto cotton fabrics. The cotton was previously ester cross-linked and Cu-chelated to enhance the adsorption of the enzyme. The modified fabrics presented antimicrobial properties against gram-positive and gram-negative bacteria as well as filamentous and non-filamentous fungi. Furthermore, this ability remained after several washing cycles.

Conclusion and Future Outlook

The immobilization of laccase can be performed via many differentmethods using a large number of supports. However, the recoveryof enzymatic activity after the immobilization process is not alwayssatisfactory, although improvements are frequently obtained concerningthe stability of the enzyme to temperature, pH, organic solvents,storage and operation.Similar to the free enzyme, immobilized laccase can be applied ina huge number of industrial processes, especially in environmentalapplications

and in electro biochemistry processes. The employmentof laccases for the design of biological fuel cells and biosensorsopens up new possibilities, from industrial to healthcare applications.The search for inexpensive supports and the recovery of activityduring the immobilization process should be improved to increasethe potential application of laccase immobilized systems.

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