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Research review paper

Recent developments and applications of immobilized laccase

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ABSTRACT

Laccase is a promising biocatalyst with many possible applications, including bioremediation, chemical synthesis, biobleaching of paper pulp, biosensing, textile finishing and wine stabilization. The immobilization of enzymes offers several improvements for enzyme applications because the storage and operational stabilities are frequently enhanced. Moreover, the reusability of immobilized enzymes represents a great advantage compared with free enzymes. In this work, we discuss the different methodologies of enzyme immobilization that have been reported for laccases, such as adsorption, entrapment, encapsulation, covalent binding and self-immobilization. The applications of laccase immobilized by the aforementioned methodologies are presented, paying special attention to recent approaches regarding environmental applications and electrobiochemistry.

Contents

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Abbreviations: (EPR), Electron paramagnetic resonance; (LMSs), Laccase mediator systems; (ITO), Indium tin oxide; (GLU), Glutaraldehyde; (NHS), N-hydroxysuccinimide; (NPG), Nanoporous gold; (EDC), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; (PHEMAH), Poly(hydroxymethylmethacrylate-n-methacryloly-(l)-histidine-methylester); (LDH), Layered double hydroxide; (SAM), Self-assembled monolayer; (RR), Resonance Raman; (SERS), Surface-enhanced Raman scattering; (LbL), Layer by layer; (MCM), Mobil composition of matter; (CNS), Cyano-modified silica; (SBA-15), Santa Barbara amorphous; (PS), Polystyrene particles; (AAEM), β-Diketone groups; (PVAs), Poly(vinyl alcohol) cryogel particles; (CLECs), Cross-linked enzyme crystals; (CLEAs), Cross-linked enzyme aggregates; (HFBs), Hydrophobins; (PEI), Poly(ethyleneimine); (SGZ), Syringaldazine; (DMP), 2,6-Dimethoxyphenol; (PPD), *para*-phenylenediamine; (APTES), 3-Aminopropyltriethoxysilane; (MG), Methyl green; (RBBR), Remazol brilliant blue R; (PAH), Polyallylamine hydrochloride; (PSS), Polysodium 4-styrenesulfonate; (CPC-silica), Controlled-porosity carrier beads; (PEG), Polyethylene glycol; (ABTS), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); (semi-IPNs), Semi-interpenetrating networks; (MCFs), Mesostructured siliceous cellular foams; (PAHs), Polycyclic aromatic hydrocarbons; (BaP), Benzo[a]pyrene; (BPA), Bisphenol A; (DCP), Dichlorophenol; (TCP), 2,4,6-trichlorophenol; (CPs), Chlorophenols; (OMW), Olive oil mill wastewater; (TCS), Triclosan; (OMMs), Ordered mesoporous materials; (MWL), Milled wood lignin; (RKL), Residual kraft lignin; (CCE), Carbon ceramic electron; (GC), Glassy carbon electrode; (LDG), Low density graphite electrode; (DET), Direct electron transfer; (NTA), Nitriloacetic acid; (1-AP), 1-aminopyrene; (Pt), Platinum; (CC), Cyanuric chloride; (HQ), Hydroquinone; (HGA), Homogentisic acid; (CEPEI), Cetyl ethyl poly(ethyleneimine); (MB), Methylene blue; (AuNPs), Gold nanoparticles; (SPEs), Screen printed electrodes; (POMs), Polyoxometalates.

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1. 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).

1.1. 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). Additionally, laccases occur in saprophytic ascomycetes such as *Myceliophthora thermophila* and *Chaetomium thermophile*, which are involved in the humification of composts (Morozova et al., 2007b).

The best-known fungal laccases are extracellular proteins, but intracellular laccases have also been described. There are essentially three possible roles of fungal laccases: pigment formation, lignin degradation and detoxification (Loera et al., 2006).

1.2. 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 laccase-oxidizable 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. Mediator oxidation by laccase produces a high redox potential intermediate able to oxidize non-phenolic substrates. This intermediate compound is then reduced to restore its initial form and close the redox cycle (Morozova et al., 2007b). The LMSs have been successfully applied in different fields of biotechnology, such as paper pulp bleaching and delignification (Moldes et al., 2010), the degradation of polycyclic aromatic hydrocarbons (Gómez et al., 2006) and the decolorization of textile dyes (Moldes and Sanromán, 2006).

1.3. Applications

Laccases have great biotechnological potential due to their ability to oxidize a broad range of substrates that are employed in several industrial sectors. Their capacity to degrade phenolic compounds makes them appropriate for dye decolorization or the degradation of xenobiotics in the treatment of wastewaters. Electrobiochemistry has received increased attention during the last two decades. In this field, laccases have been employed for the design of biosensors, the detection of phenols in wastewaters or in food industry applications and the development of biofuel cells. Laccases have also been used in the pulp and paper industry for bleaching, delignification and for the production of novel paper products. Some potential applications are summarized in Table 1. Additionally, excellent reviews regarding laccase applications can be found in the literature (Riva, 2006; Rodríguez Couto and Toca Herrera, 2006).

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Table 1
Several recent methods for laccase immobilization.

Laccase source	Support	Substrate	Application	Reference
Entrapment				
Rhus vernicifera	Microporous polypropylene hollow fiber membranes		Xenobiotics degradation	(Moeder et al., 2004)
Trametes villosa and *Aspergillus niger*	Polyaniline matrix		Biosensor	(Timur et al., 2004)
Trametes villosa	Polymers. Cetyl ethyl poly(ethyleneimine) and Nafion	Pyrocatechol	Biosensor	(Yaropolov et al., 2005)
Trametes villosa	Cu or Ca–alginate	4-methoxybenzylalcohol		(Brandi et al., 2006)
Trametes versicolor	Alginate–carbon beads	ABTS		(Khani et al., 2006)
Cerrena unicolor	Hydrophilic tetramethoxysilane film on graphite particles	ABTS and syringaldazine	Biofuel cell	(Nogala et al., 2006)
Coriolus versicolor	Ca–alginate		Xenobiotics degradation	(Zhang et al., 2006)
Novozymes	Alginate–chitosan microcapsules	ABTS	Dyes decolorization	(Lu et al., 2007)
Trametes versicolor	Porous carbon tubes	ABTS	Biofuel cell	(Servat et al., 2007)
Polyborus rubidus	Alginate beads		Dyes decolorization	(Dayaram and Dasgupta, 2008)
Trametes versicolor	Polypyrrole matrix	ABTS	Biofuel cell	(Merle et al., 2008)
Streptomyces psammoticus	Ca and Cu–alginate beads	Phenols	Phenol degradation	(Niladevi and Prema, 2008)
Myceliophthora thermophila (Denilite II S)	Alginate/gelatin with PEG		Dyes decolorization	(Wang et al., 2008b)
Cerrena unicolor	Poly(N-isopropylacrylamide) gel in ITO	ABTS		(Klis et al., 2009)
Trametes versicolor	Sol–gel matrix of diglycerysilane	Phenols	Biosensor	(Montereali et al., 2010)
Lentinus polychrous	Cu, Zn and Ca–alginate beads	ABTS	Dyes decolorization	(Phetsom et al., 2009)
Trametes versicolor	Hydrogel structures and semi-IPNs.	ABTS	Dyes decolorization	(Yamak et al., 2009)
	Semi-IPNs of κ-carrageenan		Dyes decolorization	(Makas et al., 2010)
Encapsulation				
Trametes hirsuta	Alumina spherical pellets with self-assembled LbL	Catechol	Dyes decolorization	(Rodríguez Couto et al., 2007)
Unspecified	Polymer. Poly(ethyleneimine)	ABTS and p-phenylenediamine	Biofuel cell	(Rochefort et al., 2008)
Trametes versicolor	Sol–gel silica	2,6-dichlorophenol		(Mohidem and Mat, 2009)
Trametes trogii	Gold disc electrodes with self-assembled LbL	ABTS		(Szamocki et al., 2009)
Trametes versicolor	Alumina pellets with self-assembled LbL	ABTS	Paper industry	(Crestini et al., 2010)
Unspecified	Sol–gel silica	ABTS	Xenobiotics degradation	(Qiu and Huang, 2010)
Cerrena unicolor	Sol–gel silica. Glass and polymethyl-2-methylpropenoate (Plexiglass®)		Biofuel cell	(Nogala et al., 2010)
Trametes versicolor	Poly(ethyleneimine) (PEI) microcapsules			(Zhang and Rochefort, 2010)
Adsorption				
Pycnoporus sanguineus	Magnetic chitosan microspheres	ABTS		(Jiang et al., 2005a; Jiang et al., 2005b)
Trametes versicolor	Graphite electrodes	Catechol	Biosensor	(Portaccio et al., 2006)
Trametes hirsuta	Graphite electrodes	Catechol	Biosensor	(Shleev et al., 2006)
Coriolus versicolor	Activated carbon		Xenobiotics degradation	(Zhang et al., 2006)
Trametes villosa	Aluminum hydroxide			(Ahn et al., 2007)
Myceliophthora thermophila (Denilite II S)	Cotton fabrics		Textile industry	(Ibrahim et al., 2007)
Cerrena unicolor	Indium-doped tin oxide films, polycrystalline gold, glass covered by gold and silver and gold electrodes	Syringaldazine		(Mazur et al., 2007)
Trametes versicolor	Glassy carbon electrode and colloidal suspension	ABTS and catechol	Biosensor	(Mousty et al., 2007)
Trametes versicolor	Sonogel-carbon electrodes	Phenols	Biosensor	(ElKaoutit et al., 2007; ElKaoutit et al., 2008)
Trametes versicolor	Nanoporous gold on glassy carbon electrodes	2,6-dimethoxyphenol	Biofuel cell	(Qiu et al., 2008)
	Mesoporous silica: MCM-41			(Wang et al., 2008c)
Pycnoporus sanguineus	Magnetic Cu2 + chlated particles	Catechol		(Wang et al., 2008a)
Cerrena unicolor	Granocel	ABTS and syringaldazine		(Rekuć et al., 2008)
Trametes versicolor	Poly(hydroxyethylmethacrylate) films-glycidymethacrylate: PHEMA-g-GMA	Syringaldazine	Xenobiotics degradation	(Bayramoglu and Arica, 2009)
Myceliophthora thermophila (Denilite II Base)	Crystals of Mg/Al layered double hydroxide on nanoporous gold	Syringaldazine		(Córdova et al., 2009)
	Magnetic chitosan nanoparticles	ABTS		(Fang et al., 2009)
Trametes versicolor	Nanoporous gold	2,6-dimethoxyphenol		(Huajun et al., 2009)
Trametes versicolor	Mesoporous silica: MCM-41	Catechol	Biosensor	(Xu et al., 2009)
Trametes versicolor	Poly(hydroxyethylmethacrylate-n-methacryloly-(L)-histidinemethylester): PHEMA nanospheres	ABTS		(Çorman et al., 2010)
Trametes hirsuta and *Myceliophthora thermophila*	Mesoporous silica particles: MCM, CNS and SBA-15	ABTS		(Forde et al., 2010)
Trametes versicolor	Magnetic beads		Dyes decolorization	(Bayramoglu et al., 2010b)
Trametes versicolor	Mesoporous silica: SBA-15		Xenobiotics degradation	(Fernando Bautista et al., 2010)
Trametes versicolor	Carbon based electrodes. Carbon nanotubes and porous carbon tubes	ABTS	Biofuel cell	(Rubenwolf et al., 2010)
Covalent binding				
Trametes versicolor	Kaolinite	ABTS	Xenobiotics degradation	(Dodor et al., 2004)
Trametes modesta	Y-aluminum oxide pellets	ABTS	Dyes decolorization	(Kandelbauer et al., 2004)

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Table 1 (*continued*)

Laccase source	Support	Substrate	Application	Reference
Rhus vernicifera	Nylon membrane grafted with glycidyl methacrylate	Quinol		(Grano et al., 2004)
Myceliophthora thermophila (Denilite)	Glassy carbon electrodes and platinum electrode		Biosensor	(Quan and Shin, 2004a; Quan and Shin, 2004b; Quan and Shin, 2004c; Quan et al., 2004a; Quan et al., 2004b)
Rigidoporus lignosus	Self assembled monolayer of 3-mercaptopropionic acid on gold surface	ABTS and syringaldazine	Biosensor	(Vianello et al., 2004)
	Mercury thin film electrode (MTFE) + gelatin	Catechol	Biosensor	(Kirgöz et al., 2005)
Coriolus hirsutus	Gold surface	Catechol	Biosensor	(Solná and Skládal, 2005)
Trametes villosa	Eupergit C and activated carbon	4-methoxybenzylalcohol		(Brandi et al., 2006)
Coriolus versicolor	Chitosan			(Chen et al., 2006)
Trametes versicolor	Kaolinite and mesoporous silica SBA-15	ABTS	Xenobiotics degradation	(Hu et al., 2007)
	Silver and gold electrodes	Syringaldazine		(Michota-Kaminska et al., 2006)
Trametes versicolor	Graphite electrodes	Catechol	Biosensor	(Portaccio et al., 2006)
Trametes versicolor	Composite magnetic particles: poly(styrene-co-actoacetoxyethyl-methacrylate) + maghemite	ABTS		(Pich et al., 2006)
Trametes hirsuta	Aminated porous glass beads	Catechol	Biosensor	(Shleev et al., 2006)
Coriolus versicolor	Indium tin oxide electrode (TIO)		Biosensor	(Tang et al., 2006)
Rigidoporus lignosus	ECH-Sepharose (resin)	Phenols	Biosensor	(Vianello et al., 2006)
Pycnoporus sanguineus	Magnetic nanoparticles: Copper or Zinc tetra-aminophthalocyanine-Fe_3O_4			(Huang et al., 2006; Huang et al., 2007)
Pycnoporus coccineus	Eupergit C and Eupergit C 250 L		Xenobiotics degradation	(Berrio et al., 2007)
Cerrena unicolor	Copolymer of butyl acrylate and ethylene glycol dimethacrylate	Syringaldazine		(Bryjak et al., 2007)
Trametes versicolor	CPC-silica beads (controlled porosity carrier)	ABTS	Dyes decolorization	(Champagne and Ramsay, 2007)
Trametes versicolor	Vitroceramic supports, pyrolytic graphite and carbon fiber electrode	Syringaldazine	Xenobiotics degradation. Biosensor	(Cordi et al., 2007)
Trametes versicolor	Nylon membranes grafted with glycidyl methacrylate	Bisphenol A	Xenobiotics degradation	(Manco et al., 2007)
Cerrena unicolor	Gold electrode	ABTS		(Klis et al., 2007)
Trametes hirsuta	Woven polyamide 6.6 (nylon)	ABTS		(Silva et al., 2007)
Fluka	Carbon electrode with magnetic core-shell nanoparticles Fe_3O_4–SiO_2		Biosensor	(Zhang et al., 2007a)
Fluka carbon electrode	Magnetic nanoparticles (Fe_3O_4–SiO_2) on Catechol	Biosensor	(Tang et al., 2008; Zhang et al., 2007b)	
Trametes sp.	Gold electrodes		Biofuel cell	(Balland et al., 2008)
Rhus vernicifera	Polypropylene membrane	Phenol		(Georgieva et al., 2008)
Myceliophthora thermophila (Denilite II S)	Sepabeads EC-EP3 and Dilabeads NK	ABTS	Dyes decolorization	(Kunamneni et al., 2008)
				(Liu et al., 2008)
	Silanized magnetic silica nanoparticles			(Liu et al., 2008)
Trametes versicolor	Porous carbon tubes grafted with aminopolypyrrole	ABTS	Biofuel cell	(Merle et al., 2008)
Rhus vernicifera	Dendrymer on gold nanoparticles. Glassy carbon electrode	Catechin	Biosensor	(Rahman et al., 2008)
Pleurotus ostreatus	Eupergit 250 L	ABTS	Dyes degradation	(Russo et al., 2008)
Trametes hirsuta	Low density graphite electrodes (LDG)	ABTS and catechol	Biofuel cell	(Vaz-Dominguez et al., 2008)
	Magnetic particles Fe_3O_4–alginate	ABTS	Dyes decolorization	(Zhao et al., 2008)
Coriolus versicolor (Fluka)	Chitosan		Xenobiotics degradation	(Zhang et al., 2008)
Cerrena unicolor	Granocel	ABTS and syringaldazine		(Rekuć et al., 2008; Rekuć et al., 2009b)
Rhus vernicifera	Non-porous poly(glycidyl methacrylate/ethyleneglycol dimetacrylate): (poly(GMA/EGDMA))	Syringaldazine	Dyes degradation	(Arica et al., 2009)
Pleurotus sajor-caju	Polyamide 6.6 membranes	ABTS	Xenobiotics degradation	(Rasera et al., 2009)
Cerrena unicolor	Mesostructures siliceous cellular foams (MCFs)	ABTS	Dyes decolorization	(Rekuć et al., 2009a)
Trametes versicolor and *Pycnoporus cinnabarinus*	Magnetic macroporous beads cellulose	ABTS and syringaldazine	Dyes decolorization	(Rotková et al., 2009)
Agaricus bisporus	Ceramic-chitosan	ABTS		(Shang et al., 2009a; Shang et al., 2009b)
Pleurotus sajor-caju	Mesoporous silica: SBA-15	Syringaldazine	Xenobiotics degradation	(Salis et al., 2009)
Trametes trogii	Gold disc electrodes	ABTS		(Szamocki et al., 2009)
				(Tan et al., 2009)
Trametes versicolor	Chitosan-multiwalled carbon nanotubes on glassy carbon electrode	ABTS	Biofuel cell and biosensor	(Tan et al., 2009)
Laccase Roglyr Lite 1540	Poly(vynil alcohol) cryogel particles (PVA)	ABTS		(Stanescu et al., 2010)
Trametes versicolor	Inorganic ceramic supports in honeycomb estructure	ABTS	Dyes decolorization	(Plagemann et al., 2011)
Unspecified	Macroporous exchange resins cross-linked with GLU		Dyes decolorization	(Zhang et al., 2010)
Trametes versicolor	Polypropylene membranes	ABTS	Biofuel cell	(Georgieva et al., 2010)
Unspecified	Glassy carbon electrode. Poly aryl amide and multiwalled carbon nanotubes		Biofuel cell	(Zeng et al., 2010)
Trametes versicolor	Chitosan activated by EDC.	ABTS	Xenobiotics degradation	(Cabana et al., 2011)
Bacillus subtilis	Glassy carbon electrode. Functionalized by aminophenyl monodiazonium salts	ABTS	Biofuel cell	(Beneyton et al., 2011)
Coriolopsis gallica	Graphite electrode + 4-2-aminoethyl benzoic acid hydrochloride (AEBA)	ABTS	Biofuel cell	(Martinez-Ortiz et al., 2011)

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Table 1 (*continued*)

Laccase source	Support	Substrate	Application	Reference
Self-immobilization				
Coriolopsis polyzona	CLEAs with polyethylene glycol and GLU	ABTS	Xenobiotics degradation	(Cabana et al., 2007)
Pleurotus ostreatus	Crystaline silicon surface + hydrophobins: HBF monolayer	2,6-dimetoxyphenol		(De Stefano et al., 2009)
Myceliophthora thermophila (Denilite II base and Denilite II assist)	Spherezymes. Cross-linked with GLU and ethylenediamine	ABTS		(Jordaan et al., 2009)
Trametes versicolor and *Trametes villosa*	CLEAs with polyethylene glycol and GLU or N-oxy radical TEMPO	Catechol	Xenobiotics degradation	(Matijošyte et al., 2010)

2. Methods of laccase immobilization

The immobilization of an enzyme is defined as its attachment to an insoluble support (Arica et al., 2009). Many recent studies on enzyme immobilization have focused on laccase, but the most recent review of laccase immobilization methods and their possible applications was published in 2002 (Durán et al., 2002). Several recent methods of laccase immobilization are summarized in Table 1.

As previously mentioned, laccases are excellent biocatalysts for biotechnological and environmental applications because of their high activity, selectivity and specificity, which permit them to perform complex chemical processes under experimental and natural conditions (Mateo et al., 2007). However, the use of these enzymes for practical applications is still limited due to their low stability and high production costs (Hu et al., 2007; Rekuć et al., 2009b).

The immobilization of laccases can overcome some of the aforementioned limitations by improving some of the properties of the enzyme (Kunamneni et al., 2008). The major advantages of laccase immobilization are the increase in the thermostability of the enzyme and its resistance to extreme conditions and chemical reagents. In addition, immobilized laccases may be easily separated from the reaction products, allowing the enzymes to be employed in continuous bioreactor operations (Arica et al., 2009; Arroyo, 1998; Georgieva et al., 2008). However, the immobilization processes could result in conformational alterations of the enzyme, the heterogeneity of the enzyme on the support and a slight loss of activity (Arroyo, 1998).

Conceptually, there are two basic methods for enzyme immobilization, as the enzyme-support link may take place by physical or chemical interactions. These different types of links involve several types of immobilization methodologies. Physical coupling methods include the entrapment of the enzyme in a tridimensional matrix or its encapsulation in an organic or inorganic polymer (membranes) (Matijošyte et al., 2010), whereas chemical coupling can occur through adsorption, covalent binding to the carrier or self-immobilization (no support required).These techniques are reviewed by Sheldon (2007). Each of these methodologies presents advantages and disadvantages.

2.1. Entrapment

Entrapment is defined as the physical retention of enzymes in a porous solid matrix, such as polyacrylamide, collagen, alginate or gelatin (Dayaram and Dasgupta, 2008; Lu et al., 2007; Moeder et al., 2004; Niladevi and Prema, 2008; Phetsom et al., 2009; Timur et al., 2004). The enzyme is first suspended in the monomer solution, and a subsequent polymerization process keeps the enzyme trapped, preventing direct contact with the environment (Fig. 1(a)). Entrapment is the easiest immobilization method and induces no structural alteration of the enzyme. However, this methodology is characterized by mass transfer limitations and low enzyme loading (Arroyo, 1998; Brady and Jordaan, 2009). Several examples of laccases immobilized by this method are presented in the literature, mainly for dye decolorization applications. For instance, *Cerrena unicolor* laccase was immobilized in a hydrogel matrix of poly(*N*-isopropylacrylamide) and attached to an indium tin oxide (ITO) film electrode. The laccase entrapped in this matrix allowed for the control of the catalytic efficiency of the film by changing the temperature (Klis et al., 2009).

2.2. Encapsulation

Similar behavior is observed in the encapsulation of laccases in comparison to the entrapment method, because the enzyme is protected from the environment and mass transfer represents a serious limitation in both of these immobilization methods (Brady and Jordaan, 2009). An alternative of this immobilization method is

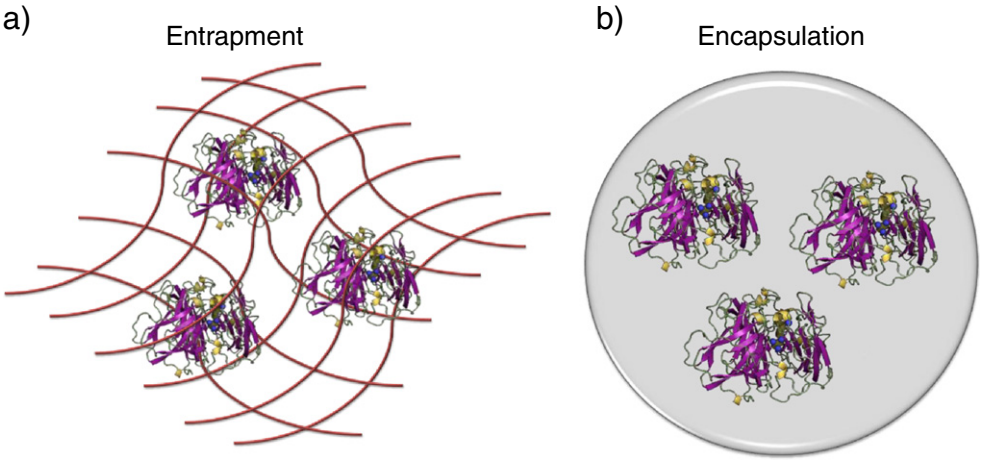

Fig. 1. Immobilization of enzymes by physical interactions. (a) Entrapment of enzymes into a porous solid matrix. (b) Encapsulation of enzymes.

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microencapsulation, in which a bioactive agent is confined in the core of micron-sized spheres made from a semipermeable material. Microencapsulated laccases are surrounded by semipermeable membranes, such as polymers (e.g., polyethyleneimine) or inorganic materials (e.g., SiO_2) (Rochefort et al., 2008) (Fig. 1(b)). Since 2002, a few studies have been performed to investigate the different methods and applications of laccase encapsulation. Two groups have recently used a sol–gel silica matrix for laccase encapsulation (Mohidem and Mat, 2009; Qiu and Huang, 2010). Mohidem and Mat (2009) demonstrated the change of the optimum pH (4–5) of laccase from *Trametes* sp., and the authors also showed that the quantity of the immobilized laccase influenced the activity. The layer-by-layer (LbL) technique is another microencapsulation method that has been employed during recent years (Crestini et al., 2010; Szamocki et al., 2009). Thin films can be formed by this method, and a wide variety of materials can be deposited, providing a simple way to control the thickness. Gold electrodes and alumina pellets have been employed as supports for this technique. For example, Szamocki et al. (2009) microencapsulated *Trametes trogii* laccase by LbL in gold electrodes that had been previously functionalized with dithiobis-*N*-succinimidyl propionate.

2.3. Adsorption

The adsorption of laccase onto a support is based on ionic and/or other weak forces of attraction (Fig. 2(a)). Adsorption is a relatively simple and inexpensive method for laccase immobilization and may therefore have a higher commercial potential than other methodologies (Bayramoglu and Arica, 2009; Brady and Jordaan, 2009). The pH and ionic strength of the medium and the hydrophobicity of the support surface must be taken into account during the immobilization process (Ahn et al., 2007; Fang et al., 2009; Forde et al., 2010; Huajun et al., 2009; Mousty et al., 2007; Qiu et al., 2008; Rekuć et al., 2008; Wang et al., 2008a; Xu et al., 2009). Several adsorption studies have focused on the immobilization of *T. versicolor* laccase. Silicate-based supports have been the main carriers for laccase adsorption.

Porous supports such as the mesoporous molecular sieve MCM-41 have been used to adsorb laccase, improving its thermal, pH and operational stability (40% of residual activity after 10 cycles) (Wang et al., 2008c). Mesoporous silicate particles, such as Mobil composition of matter (MCM), cyano-modified silica (CNS) and Santa Barbara amorphous (SBA-15), have also been used to immobilize *T. hirsuta* and *M. thermophila* laccases. The surfaces used for adsorption in these studies were previously modified by bifunctional (glutaraldehyde (GLU) and ethyleneglycol-*N*-hydroxysuccinimide (NHS)) and monofunctional (citraconic anhydride) methods. The best results were achieved by the adsorption of the *M. thermophila* laccase with GLU onto SBA-15 silica (Forde et al., 2010).

Some studies have shown that adsorption is preferable to other techniques for the immobilization of some particular laccases. Huajun et al. (2009) immobilized the laccase from *T. versicolor* by electrostatic adsorption and covalent binding. The former was conducted by treating nanoporous gold (NPG) with lipic acid and methylene blue, and the latter was accomplished by treating NPG with lipic acid and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Adsorption was demonstrated to be the best and easiest method of those tested. NPG has several advantages for laccase immobilization and its subsequent application as a support because it offers a wide range of porous diameter and may be completely cleaned with a nitric acid treatment, rendering it reusable. Other metal-based supports, such as magnetic Cu^{2+} nanoparticles, have also been used as a support for metal-chelated adsorption. The laccase from *Pycnoporus sanguineus* has been shown to recover 65% of its activity following immobilization on this new carrier and to maintain 87% of the laccase activity after ten operation cycles. In addition, the immobilization of laccase in Cu^{2+} nanoparticles improved the catalytic capacity and stability of the enzyme toward various parameters such as pH, temperature, reuse and storage time (Wang et al., 2008a). Magnetic chitosan microspheres have also been employed as a support for the laccase from *P. sanguineus* by using GLU as a cross-linker. The optimal conditions for the immobilized enzyme were shown to be different from those of the free enzyme. Additionally, the thermal, operational and storage stabilities were greatly improved (Fang et al., 2009; Jiang et al., 2005a).

An important aspect that should be noted is the general improvement in laccase activity and stability observed with all supports. However, there are some particular supports that cannot produce this effect, although they present advantages for other specific situations. For example, aluminum hydroxide has been tested as a support for *T. villosa* laccase but did not provide promising results, as the adsorbed laccase had similar activity to the free enzyme but lower resistance to thermal and proteolytic degradation. However, the immobilized laccase proved to be less sensitive to inhibition by humic acids, and no significant changes were detected in its secondary structure due to its adsorption to aluminum hydroxide. These results suggest that laccases in soil may be predominantly available in complex species with aluminum hydroxide. This study suggests that laccase could be a practical tool for soil remediation (Ahn et al., 2007).

Another adsorption technique proposed for laccase immobilization is based on the use of ion exchange resins with several functional groups and mobile ions, such as dextran, agarose and chitosan (Arroyo, 1998; Bayramoglu and Arica, 2009; Córdova et al., 2009; Çorman et al., 2010; Ibrahim et al., 2007; Jiang et al., 2005b; Mazur et al., 2007). For instance, *T. versicolor* laccase has been immobilized on poly(hydroxyethylmethacrylate-n-methacryloly-(l)-

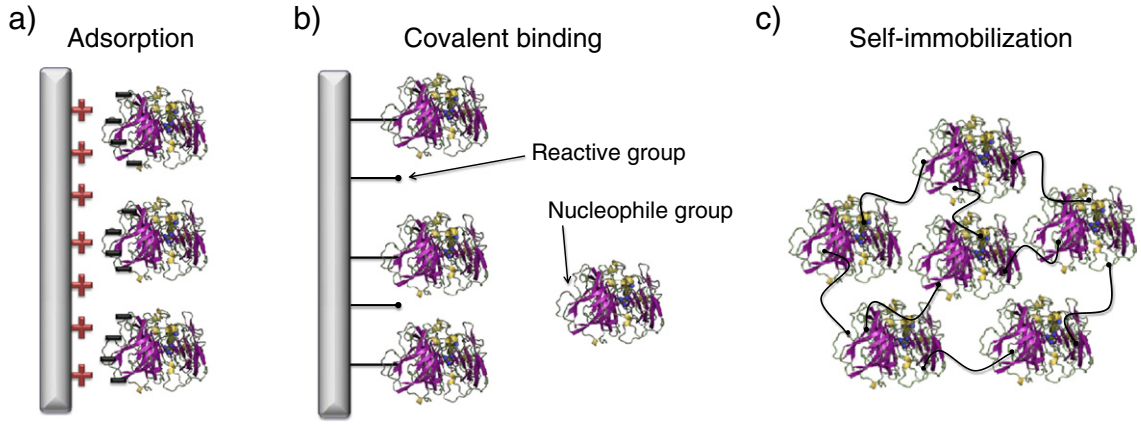

Fig. 2. Chemical interactions for enzyme immobilization. (a) Adsorption of enzymes onto a support by ionic forces. (b) Covalent binding between the nucleophilic groups of the enzyme and the support. (c) Self-immobilization: model of cross-linked enzyme aggregates (CLEAs).

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histidinemethylester) (PHEMAH) nanospheres by Cu-chelation. These histidine-containing nanospheres exhibited favorable adsorption properties, and the immobilized laccase had higher thermostability and activity than the free enzyme over a wide range of pH and temperature. Metal-based supports, such as ITO films, polycrystalline gold, glass covered by gold and silver and gold electrodes, have also been tested as ion exchange supports for laccase adsorption after treatment with $ZrOCl_2$, in which Zr^{4+} is coordinated with the carboxyl termini of proteins (via zirconium phosphonate/carboxylate). High K_m values were observed with this support, indicating conformational changes of the enzyme (Mazur et al., 2007). An ion exchange method has also been employed with a layered double hydroxide (LDH) produced by the co-precipitation of crystals of Al and Mg. The commercial laccase from Denilite II Base was adsorbed in this synthetic clay with positive electrostatic charges, resulting in the recovery of 92% of the activity (Córdova et al., 2009).

2.4. Covalent binding

The most interesting method of laccase immobilization for industrial applications is covalent binding. Therefore, covalent binding has been the most widely used method for laccase immobilization during the last decade. In this technique, chemical groups on the support surface are activated and react with nucleophilic groups on the protein (Arroyo, 1998) (Fig. 2(b)). Most enzymes are covalently attached using their lysine amino groups because of their frequent presence on the protein surface and high reactivity (Brady and Jordaan, 2009). Covalent immobilization methods are based either on inert or on commercially available active carriers (Russo et al., 2008). The optimal support for immobilization should contain short spacer arms and a high density of reactive groups. These characteristics are required for the multipoint attachment of the laccase, providing its rigidity.

Many different supports have been used for covalent laccase immobilization, including silica-based supports such as kaolinite or mesoporous silica nanoparticles (Champagne and Ramsay, 2007; Dodor et al., 2004; Hu et al., 2007; Liu et al., 2008; Salis et al., 2009). Liu et al. (2008) used silanized and GLU-activated silica nanoparticles as a support. The thermal and operational stabilities of laccase were improved, as illustrated by the retention of 61% of the residual activity after 4 h at 60 °C and the retention of 55% of the activity after 10 cycles of operation.

Epoxy-activated resins such as Eupergit and Sepabeads have been frequently employed (Berrio et al., 2007; Brandi et al., 2006; Kunamneni et al., 2008; Russo et al., 2008). For instance, Brandi et al. (2006) used this type of support for the comparison of the covalent and entrapment methods of immobilization. In this study, better results were achieved by immobilizing *T. villosa* laccase with Eupergit C than with activated carbon and entrapment on a Cu–alginate matrix.

Many different types of electrodes based on carbon, glass, gold, silver or graphite have been designed to act as suitable supports for laccase immobilization (Balland et al., 2008; Cordi et al., 2007; Klis et al., 2007; Michota-Kaminska et al., 2006; Quan et al., 2004a, 2004b; Rahman et al., 2008; Shleev et al., 2006; Solná and Skládal, 2005; Szamocki et al., 2009). Silver and gold surfaces have been employed as supports after the modification of the surface by thiol monolayers with carboxylic and amino groups (SAMs). Immobilization has also been studied using resonance Raman (RR) and surface-enhanced Raman scattering spectroscopy (SERS), both of which are surface-sensitive techniques that can detect single molecules adsorbed or covalently bonded onto rough metal surfaces (Michota-Kaminska et al., 2006). A gold electrode with organothiol monolayers has also been used for the immobilization of *C. unicolor* laccase. Covalent bonds were formed between the amino groups of the enzyme and the carboxylic groups of mercaptoundecanoic or mercaptopropionic acids, which were activated by immersing the electrode in EDC and NHS. The catalytic site of the immobilized protein was studied using SERS, along with the electrical connectivity of the enzyme with the electrode in the presence of mediators. The authors concluded that the use of smaller acids allowed for the transfer of a higher proportion of the catalytic activity to the electrode (Klis et al., 2007).

The use of magnetic supports, such as nanoparticles or beads, for covalent immobilization may offer the advantage of quick separation in a magnetic field (Huang et al., 2006; Pich et al., 2006; Rotková et al., 2009; Tang et al., 2008; Zhang et al., 2007a; Zhao et al., 2008). The most interesting examples were reported by Huang et al. (2006). This group prepared a magnetic nanoparticle composite of copper tetraaminophthalocyanine-Fe_3O_4 that was used with the laccase from *P. sanguineus* with an immobilization yield of 20%. After 1 month of storage, the immobilized laccase was found to retain 85% of the residual activity, in contrast with the free laccase, which retained only 30% of its residual activity. The operational stability was also improved, as 80% of the initial activity was retained after 5 cycles. The same authors later designed a similar composite of zinc tetraaminophthalocyanine-Fe_3O_4. The immobilization yield obtained with this new design was only 25%, but good levels of thermal, storage and operational stability were achieved (Huang et al., 2007). The magnetic support properties have been combined with polymeric particles to improve the support characteristics. In particular, polystyrene particles (PS) with reactive β-diketone groups (AAEMs), which represent the binding site for the enzyme, have been combined with maghemite nanoparticles on the surface or in the core. Better immobilization performance was obtained when the polymeric particles had the maghemite nanoparticles on the surface, most likely due to the additional chemisorption of the enzyme as a consequence of the electrostatic interactions between the iron oxide nanoparticles and the protein. However, this chemisorption effect resulted in a partial loss of the recovered activity, which was improved when the maghemite was present in the core of the PS particles (Pich et al., 2006).

Several types of fibers and polymers have also been employed for covalent immobilization by means of a previous linkage between the enzyme and the support. In addition, the support may be cross-linked (Bryjak et al., 2007; Grano et al., 2004; Manco et al., 2007; Rasera et al., 2009; Shang et al., 2009a; Silva et al., 2007; Tan et al., 2009; Zhang et al., 2008). With this approach, Bryjak et al. (2007)used the copolymer of butyl acrylate and ethylene glycol dimethacrylate to immobilize *C. unicolor* laccase. Three bifunctional agents were employed for carrier activation: GLU, divinyl sulfone and carbodiimide. GLU proved the most effective because an enhancement in operational stability was obtained using a packed bed reactor at 30 °C. Nylon was used for the immobilization of *R. vernicifera* laccase after grafting with glycidyl methacrylate (nylon-poly (GMA)-HMDA-GLU). Non-isothermal assays were conducted to demonstrate an increase in the catalytic activity under a temperature gradient (Grano et al., 2004). Silva et al. (2007) proposed that woven nylon offers several advantages over nylon membranes, as the former is inexpensive, chemically inert, non-toxic, mechanically stable, insoluble in water, readily available and can be obtained in several forms. Woven nylon was pretreated with protease for amine activation, and a spacer (1,6-hexanediamine) was included to increase the flexibility of the immobilized enzyme. Laccase has also been immobilized using chitosan. Chen et al. (2006) determined the optimal conditions for this process: 5% GLU for 8 h and 20 mg of laccase per gram of support with a 6 h reaction time. The stability and reusability of the laccase were considerably improved through this immobilization process, and 52.2% of the original activity was recovered. *A. bisporus* laccase was immobilized on a ceramic-chitosan support using GLU, resulting in a 51% immobilization yield and improvements in the thermal, operational and storage stabilities (Shang et al., 2009a; Shang et al., 2009b). Recently, another polymer, poly(vinyl alcohol) cryogel particles (PVA), was used for the covalent immobilization of the commercial laccase Roglyr Lite 1540. GLU was employed as the cross-linking agent and β-alanine as a spacer. The immobilized laccase exhibited a lower specific activity than the free enzyme, but enhancements in the stability and operational pH range could be observed (Stanescu et al., 2010).

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Alumina (Crestini et al., 2010; Kandelbauer et al., 2004; Rodríguez Couto et al., 2007) and Granocel have been commonly used as covalent supports. The latter was selected for the study of *C. unicolor* laccase immobilization after the modification of several support characteristics, including the functionalization, surface density and pore size. GLU and divinyl sulfone were employed as bifunctional agents. The least favorable results were obtained with carriers containing –OH or –OH and –COOH groups (Rekuć et al., 2008).

From these examples, it is easy to conclude that GLU is the most widely employed cross-linking agent. Several factors, including the pH, ionic strength, protein concentration and additives, may affect the enzymatic covalent link via GLU (Bryjak et al., 2007), and therefore, these factors must be taken into account when performing an immobilization reaction. In most cases, the immobilization process exhibits low laccase recovery but improvements in the operational stability and stability against denaturing agents are evident (Rekuć et al., 2009b).

The immobilization of laccase by covalent links on the above-mentioned supports allows easy manipulation. Furthermore, the quantity of the enzyme is constant, facilitating its use in continuous, packed bed, stirred tank and fluidized bed reactors. The tertiary structure of the enzyme is stabilized, offering higher resistance to denaturing agents. However, the main drawback of covalent binding may be the possible modification of the laccase structure in the active site (Arroyo, 1998).

2.5. 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, diiminoesthers, diisocyanates 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) (Fig. 2(c)). Another interesting process is the formation of self-assembled biofilms with hydrophobins that may create a bioactive substrate by binding enzymes. De Stefano et al. (2009) designed a self-assembled biofilm of hydrophobins (HFBs) from *Pleurotus* sp. that form a hydrophobic layer in a crystalline silicon surface. This HFB monolayer resulted in a bioactive substrate to bind other proteins, such as laccase, with improved stability.

Recently, a new variation of the self-immobilization method was developed, known as Spherezymes (SZs) or spherical catalytic macro-particles (Fig. 3). This innovative approach consists of macro-particles composed of the enzyme cross-linked in an oil emulsion, generating spherical particles (Brady and Jordaan, 2009; Jordaan et al., 2009). The only example of this method applied to laccase was reported by Jordaan et al. (2009). This group used this new method with GLU and ethylenediamine as cross-linking agents and then post-coated with polyethyleneimine (PEI) and ethanolamine. The stability of the laccase towards pH, temperature and some mediators, namely 2-hydroxyphtalimide, N-hydroxybenzotriazole and 2,2,6,6-tetramethylpiperidinooxy free radical, was improved.

2.6. Selection of an immobilization method

In approaching this issue, one should note that there is no universal method of enzyme immobilization nor can a universal surface for all possible applications be identified (De Stefano et al., 2009). The chosen support must be insoluble and compatible with laccase and should maintain enzyme stability in process solutions. A possible unfavorable interaction between laccase and the surface of the support should also be considered (Silva et al., 2007). Additionally, the diffusion of the substrate into the support should be easily accomplished to conduct the biocatalytic reaction. Non-porous materials have a

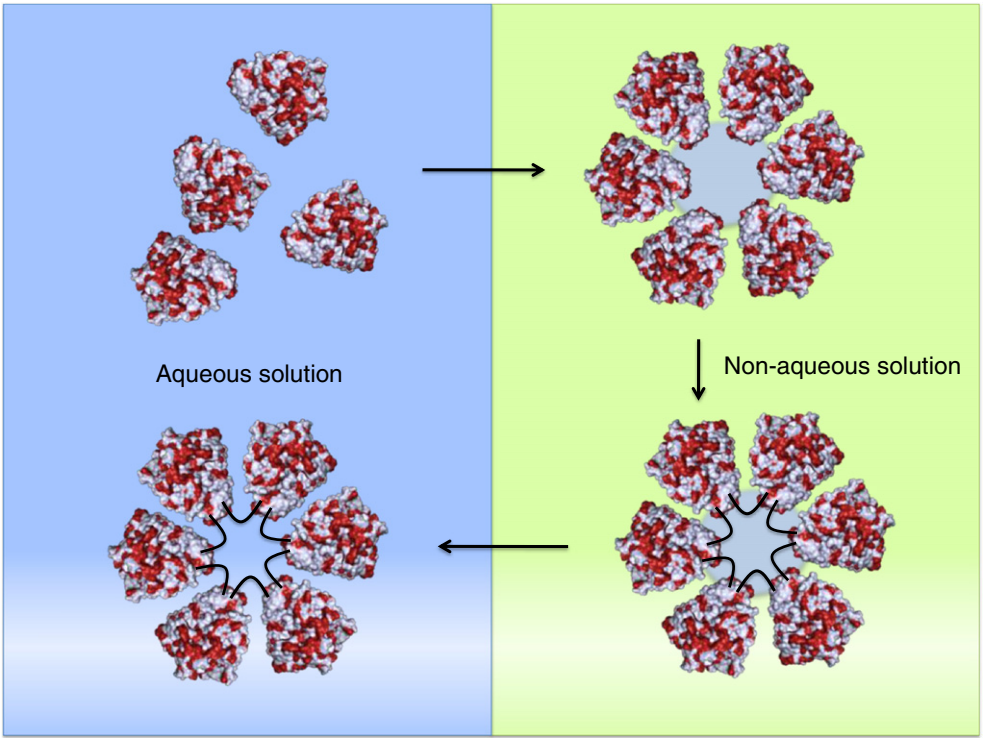

Fig. 3. Scheme of the formation of Spherezymes.

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minimal diffusion limitation but achieve limited enzyme immobilization. In contrast, porous materials can load large amounts of enzyme, but large molecular weight substrates would suffer diffusion limitations (Arica et al., 2009).

The immobilization of laccase is potentially advantageous compared to the free enzyme because such a system should be easier to operate, may offer a significant reduction of enzyme loss and may also permit its reuse (Champagne and Ramsay, 2007).

As mentioned in this paper, there are many laccase immobilization protocols, and it is necessary to summarize all available information. Table 2 compiles the most interesting results, and the kinetic and stability properties of the free and immobilized laccases are listed. This tool provides important information for the selection of the most suitable laccase/immobilization method combination.

3. Applications of immobilized laccase

3.1. Environmental applications

3.1.1. Dye removal: 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) (Fig. 4).

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 polysodium 4-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 presilanized controlled-porosity-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. The addition of PEG was shown to improve laccase stability (Wang et al., 2008b). Phetsom et al. (2009) immobilized *Lentinus polychrous* laccase in Cu, Zn and Ca alginate. The optimal reaction temperature of the immobilized laccase was increased compared with that of the free enzyme. Cu and Zn alginate bioparticles showed better stability and activity toward RBBR, methyl red, indigo carmine and bromophenol blue than free laccase. At the same time, alginate was used in decolorization studies after a previous covalent immobilization. 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 acid) [P(AAm-AA)/κ-car] or poly(acrylamide-itaconic acid) [P(AAm-IA)/κ-car]. 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

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Table 2
Kinetic parameters and stability properties of free and immobilized laccases from different sources.

Laccase source	Specific activit (U/mg)		Kinetic parameters		Thermal Stability	Storage stability	Operat. stability	Reference
			K_m	V_m				
Trametes villosa[a] (Novo Nordisk)		Free	0.18 mM	14.35 mM O_2/min	Ea: 25 kcal/mol			(Ahn et al., 2007)
		Immob.	0.15 mM	12.77 mM O_2/min	Ea: 11.8 kcal/mol			
Rhus vernicifera[c]		Free	0.27 mM	96.4 U/mg	55 °C 2 h: 39% 65 °C 2 h: 7%	4 °C 6w: 0%		(Arica et al., 2009)
		Immob.	0.47 mM	77.6 U/mg	55 °C 2 h: 64% 65 °C 2 h: 45%	4 °C 6w: 52%		
Trametes versicolor[c] (Sigma)	20	Free	10 mM	27.1 U/mg	55 °C 2 h: 59% 65 °C 2 h: 11%	4 °C 4w: 13%		(Bayramoglu and Arica, 2009)
		Immob.	23 mM	15.4 U/mg	55 °C 2 h: 76% 65 °C 2 h: 47%	4 °C 4w: 74%		
Trametes versicolor[c] (Sigma)	20	Free	9.4 μM	21.7 U/mg	55 °C 2 h: 53% 65 °C 1.5 h: 0%			(Bayramoglu et al., 2010a)
		Immob.	19.7 μM	15.6 U/mg	55 °C 2 h: 81% 65 °C 1.5 h: 58%			
Trametes versicolor[c] (Sigma)	20	Free	0.32 mM	23.1 U/mg	55 °C 2 h: 24% 65 °C 2 h: 3%	4 °C 10w: 9%		(Bayramoglu et al., 2010b)
		Immob.	0.41 mM	17.8 U/mg	55 °C 2 h: 71% 65 °C 2 h: 35%	4 °C 10w: 24%		
Cerrena unicolor[c]	55	Free	0.183 mM		60 °C 21 h: 0%			(Bryjak et al., 2007)
		Immob.	0.059 mM		60 °C 21 h: 60%			
Trametes versicolor[b] (Sigma)		Free			40 °C 12 h: 0%			(Cabana et al., 2011)
		Immob.			40 °C 12 h: 45–65%			
Coriolopsis polyzona[b]		Free	32 μM	10.2 μmol/s·mg	40 °C 24 h: 0% $t_{0.5}$: 2 h			(Cabana et al., 2007)
		Immob.	28–31.9 μM	0.02–63.9 μmol/s·mg	40 °C 24 h: 20–40% $t_{0.5}$: 7.7–19.3 h			
Trametes versicolor[b] (Sigma)		Free		$6.89 \cdot 10^{-9}$U/mg	45 °C 4 h: 26.5%			(Çorman et al., 2010)
		Immob.	> than free	$4.76 \cdot 10^{-9}$U/mg	40 °C 4 h: 66%			
Trametes versicolor[b]	34	Free	0.262 mM	5.2 mM/min·U	20 50 °C: 60–35% >50 °C: 0%			(Dodor et al., 2004)
		Immob.	0.165 mM	3.5 mM/min·U	20–50 °C: 100% >50 °C: 85%			
Pycnoporus sanguineus[b]		Free	36.8 μM	6.6 mM/min	60 °C 3.5 h: 19.4%			(Jiang et al., 2005b)
		Immob.	171.1 μM	3.5 mM/min	60 °C 3.5 h: 74%	4 °C 4w: 70%	10 cycles: 80%	
	16.2	Free						(Jordaan et al., 2009)

Myceliophthora thermophila[b] (Denilite II Base) 70 °C: ↑2.70 fold		Immob.			50 °C: ↑3.07 fold 60 °C: ↑2.73 fold			
Trametes versicolor[b]		Free Immob.	$3.4 \cdot 10^{-5}$ M $4 \cdot 10^{-5}$ M[(1)] $3.7 \cdot 10^{-5}$ M[(2)]	$2.2 \cdot 10^{-7}$ mM/min $1.9 \cdot 10^{-7}$ mM/min[(1)] $2 \cdot 10^{-7}$ mM/min[(2)]				(Khani et al., 2006)
Myceliophthora thermophila[b] (Denilite II S)		Free Immob.			75 °C: 59% 80 °C: 37% 75 °C: 72% 80 °C: 48%	4 °C 16w: 3.8%	17 cycles: 84%	(Kunamneni et al., 2008)
Trametes versicolor[c] (Fluka)	27.5	Free Immob.	$6.7 \cdot 10^{-3}$ mM $2.52 \cdot 10^{-2}$ mM[(3)] $1.08 \cdot 10^{-2}$ mM[(4)]	$1.8 \cdot 10^{-3}$ mM/min $6.8 \cdot 10^{-3}$ mM/min[(3)] $4.4 \cdot 10^{-3}$ mM/min[(4)]	30 °C 1.5 h: 90[(3)]–96[(4)]% 50 °C 1.5 h: 78[(3)]—91[(4)]%		10 cycles: 62[(3)]–52[(4)]%	(Makas et al., 2010)
Lentinus polychrous lev.[b]		Free Immob.			60 °C 0.5 h: 30[(5)], 60[(6)], 20[(7)]%			(Phetsom et al., 2009)
Trametes versicolor[b] (JenaBIOS)	3.7	Free Immob.			70 °C 1.5 h: 0% 70 °C 1.5 h: 40%			(Pich et al., 2006)
Trametes versicolor[d] (Sigma)	3.7	Free Immob.			50 °C 2 h: 6% 50 °C 2 h: 60%	4 °C 4w: 30% 4 °C 4w: 100%	8 cycles: 65%	(Qiu et al., 2008)
White rot fungi from Chinese Academy of Sciences[b]	31.8	Free Immob.			70 °C 4 h: 0% 70 °C 4 h: 82% 70 °C 24 h: 11%	25 °C 6w: 16% 25 °C 6w: 72%		(Qiu and Huang, 2010)
Trametes versicolor[e]		Free Immob.	400 μM 65 μM			4 °C 24w: 73%		(Rochefort et al., 2008)
Trametes versicolor[(b)(c)]		Free Immob.	0.0337 mM 0.0328 mM	$1.98 \cdot 10^{-4}$ mM/s 0.0106 mM/s		5–10 °C 30 d: 100%	7 cycles: 100%	(Rotková et al., 2009)
Pycnoporus cinnabarinus[(b)(c)]		Free Immob.	0.0508 mM 0.0129 mM	$7.47 \cdot 10^{-6}$ mM/s 0.0114 mM/s		5–10 °C 30 d: 100%	7 cycles: 100%	(Rotková et al., 2009)
Pleurotus sajor-caju[c]		Free Immob.	35.7 μM	0.33 μM/s	Range: 5–30 °C		10 cycles: 84% 14 cycles: 60%	(Salis et al., 2009)
Pycnoporus sanguineus[b]		Free Immob.	0.0508 mM 1.597 mM	1.128 μmol/mg·min 0.767 μmol/mg·min				(Wang et al., 2008a)
Trametes versicolor[c] (Fluka)		Free Immob.	$6.7 \cdot 10^{-3}$ mM $8.8 \cdot 10^{-2}$ mM[(8)] $5.5 \cdot 10^{-2}$ mM[(9)] $1.8 \cdot 10^{-2}$ mM[(10)]	$1.8 \cdot 10^{-3}$ mM/min $2.5 \cdot 10^{-2}$ mM/min[(8)] $1.5 \cdot 10^{-2}$ mM/min[(9)] $6.1 \cdot 10^{-3}$ mM/min[(10)]		4 °C 56 d: 42% 4 °C 56 d: 91[(8)], 79[(9)], 86[(10)]%		(Yamak et al., 2009)

Kinetic and stability parameters: V_m: maximum rate, K_m: Michaelis–Menten constant, $t_{0.5}$: half-life time, E_a: energy of activation. *Susbstrates used for laccase determination*: [(a)]oxygen consumption, [(b)]ABTS, [(c)]SGZ, [(d)]DMP, [(e)] PPD. *Time units*: s (seconds), min (minutes), h (hours), d (days), w (weeks). *Symbols*: % (residual activity), ↑ (improvement of immobilized enzyme in comparison to free one). *Supports*: [(1)]Alginate, [(2)]Alginate/carbon, [(3)]P(AAm-AA)/k-car, [(4)]P(AAm-AA)/k-car semi-INPs, [(5)]Cu–alginate, [(6)]Zn–alginate, [(7)]Ca–alginate, [(8)]P(AAm-NIPA)-L, [(9)]P(AAm)/Alg-L, [(10)]P(AAm-NIPA)/Alg-L.

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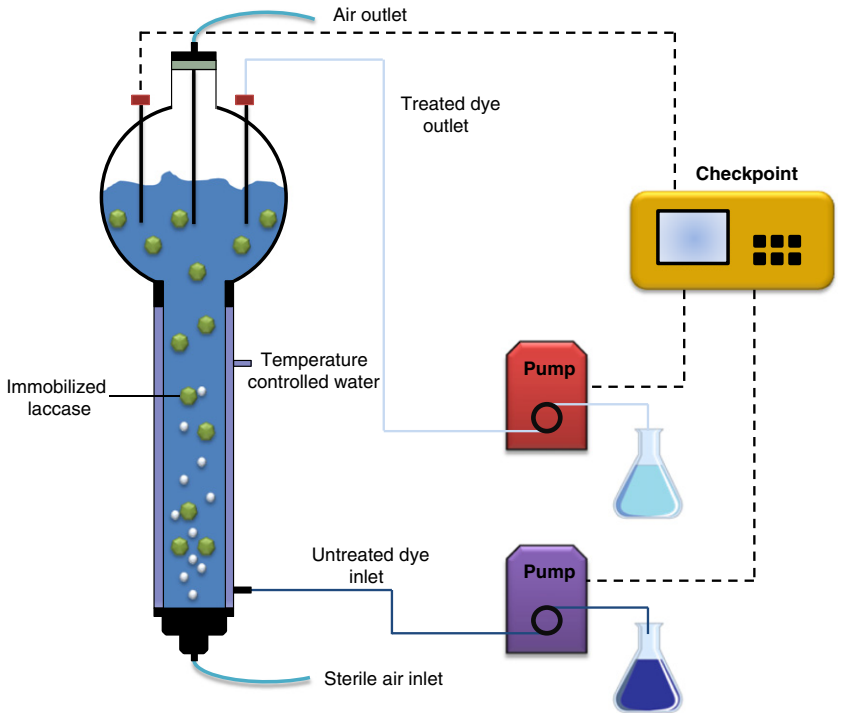

Fig. 4. Fluidized bed bioreactor operating in a continuous mode with an immobilized laccase for dye decolorization.

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. Moreover, Reactive Red 120 was efficiently degraded (Arica et al., 2009). An effective decolorization of indigo carmine was also carried out by *C. unicolor* laccase immobilized in mesostructured siliceous cellular foams (MCFs) that were previously functionalized by various organosilanes and activated with GLU (Rekuć et al., 2009a). MCFs present a more open structure, and they are significantly smaller than other cellulose-based supports. The *T. versicolor* and *Pycnoporus cinnabarinus* laccases were immobilized in magnetic macroporous cellulose beads by binding to the aldehyde groups of this support. An oriented immobilization was achieved by adding glycoproteins, allowing the enzyme to be bound by its glycosidic moieties and resulting in high activity. This laccase immobilization system showed good results in anthraquinone and azo dye decolorization (Rotková et al., 2009). Magnetic chitosan beads are another laccase immobilization support that has been tested for dye treatment. In this case, *T. versicolor* laccase was covalently bound via epichlorhydrin, improving its operational and thermal stability. The effective decolorization of Reactive Yellow 2 (80%) and Reactive Blue 4 (55%) by this laccase was achieved in batch reactors (Galliker et al., 2010). Several anthraquinonic dyes, namely RBBR, Disperse Blue 3 and the indigoid dye Acid Blue 74, were degraded faster than the azo dyes Acid Red 27 and Reactive Black 5 using laccase immobilized on CPC-silica beads via GLU. However, the decolorization of the anthraquinonic dyes increased the toxicity of the solution, while the less efficiently decolorized solutions of azo and indigoid dyes became less toxic. Therefore, the limitations of using *T. versicolor* laccase immobilized on CPC-silica beads for the decolorization and detoxification of a range of dye classes was demonstrated (Osma et al., 2010).

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).

3.1.2. 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).

To illustrate this point, some examples of the degradation of aromatic compounds by immobilized laccases are discussed as follows. The degradation of selected hydroxylated aromatic compounds was conducted by *R. vernicifera* laccase immobilized in fiber membranes. The laccase was co-trapped with horseradish peroxidase in microporous polypropylene. With the exception of 2-hydroxy-decahydronaphthalene, the effective degradation of 3,4-dimethylphenols, 4-ethylphenol, 2-hydroxy-1,2,3,4-tetrahydronaphthalene and 4-hydroxybiphenyl was achieved, ranging from 50 to 100% with 2 days of treatment (Moeder et al., 2004). Phenol degradation was studied by Georgieva et al. (2008) with laccase from *R. vernicifera* immobilized in a polypropylene membrane that was modified with chromic acid and subsequently activated by ethylenediamine and GLU. The notable results of this study were the narrower pH–activity profile of the soluble laccase compared with the immobilized laccase and the improvement in the pH and thermal stability of the insoluble enzyme.

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 same support, SBA-15, was

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later used for the immobilization of *T. versicolor* laccase by adsorption and covalent binding methods. The covalently attached laccase to aminopropyl and aminobutyl functionalized SBA-15 degraded naphthalene with 35% and 39% in 5 h, respectively (Fernando Bautista et al., 2010).

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 dechlorination efficiency of 99.5% during eight repeated batch reactions (Zhang et al., 2006). Bisphenol A (BPA), an endocrine disruptor frequently found in wastewaters, was also degraded by *T. versicolor* laccase that had been covalently bound to nylon membranes grafted with glycidyl methacrylate and phenylenediamine as a spacer. It is noticeable that low concentrations of BPA and non-isothermal conditions showed the best results. These conditions are common in polluted waters, giving the above-discussed finding special significance (Manco et al., 2007). Radioactive ^{14}C-BPA has recently been used as a substrate for the identification of the transformation products and bioconjugates formed during the enzymatic reaction of *C. polyzona* laccase; this enzyme was immobilized in silica nanoparticles produced by the Stöber method that had been previously functionalized by APTES and GLU (Pang et al., 2010). In other studies, sol–gel silica glasses were designed for the degradation of dichlorophenol (DCP) and 2,4,6-trichlorophenol (TCP). Laccase was encapsulated in the sol–gel, and its thermal, reaction and storage stability were improved. The kinetic parameters indicated that the tested laccase had higher affinity for TCP than DCP, and the maximum concentration of chlorophenols (CPs) that could be tolerated by the immobilized laccase was relatively high (Qiu and Huang, 2010).

The oxidation of phenol, *p*-chlorophenol and aniline was tested by Bayramoglu and Arica (2009) with a laccase from *T. versicolor* that had been immobilized by ionic adsorption using poly(hydroxyethylmethacrylate)-glycidylmethacrylate aminated (p(HEMA-g-GMA)) as a support, resulting in the recovery of 71% of the activity and effective phenol degradation. Likewise, the removal of phenolics from olive oil mill wastewaters (OMWs) was also studied. Both the degradation and polymerization of phenolics were induced by the immobilized laccase of *Pycnoporus coccineus*. This laccase was covalently immobilized in the epoxy-activated resins Eupergit C and Eupertit C 250 L. The latter showed the highest activity, reaching 110U/g. In addition, the thermal and pH stability of the laccase was improved (Berrio et al., 2007). Ordered mesoporous materials (OMMs) were employed to immobilize laccase for the oxidation of protocatechuic acid, caffeic acid, sinapic acid and ferulic acid from OMW (Salis et al., 2009). Through this method, SBA-15 mesoporous silica functionalized with APTES and GLU allowed for the immobilization of 220 U/g of laccase with 84% of the initial activity recovered after 17 cycles of application.

The use of CLEAs is also reported in the literature, such as the cross-linked *C. polyzona* laccase, which was used for the degradation of nonyl phenol p353NP, BPA and triclosan (TCS) (Cabana et al., 2007). CLEAs of *T. versicolor*, *T. villosa* and *Agaricus bisporus* formed with PEG and GLU have also been recently studied, mainly in the aerobic oxidation of linear aliphatic alcohols (Matijošyte et al., 2010). Chitosan was also chosen as a support for the covalent immobilization of *C. versicolor* laccase using GLU. A degradation efficiency of 65% was achieved for 2,4-dichlorophenol (2,4-DCP) after 6 cycles of operation. The activity of the immobilized laccase was lower than that of the free laccase, but the stability was clearly improved (Zhang et al., 2008). Entrapment has also been used to immobilize laccases in several works related to phenol degradation. The laccase from *Streptomyces psammoticus* was immobilized in Ca and Cu alginate. The latter resulted in the best support, retaining 61% of the activity. In addition, 50% of the activity remained after 8 cycles of degradation (Niladevi and Prema, 2008).

3.2. Electrobiochemistry

3.2.1. Biological fuel cells

Enzyme-based biological fuel cells use enzymes to catalyze the oxidation of biomass-based materials for the generation of electrical energy. Biofuel cells are capable of using naturally available biomass as fuel. As a result, they are an excellent alternative to conventional fuel cells. Dioxygen is the most common electron acceptor used in the cathodic compartment because it is readily available and a good oxidant (Servat et al., 2007). Laccase can reduce oxygen directly to water in a four-electron transfer step without the intermediate formation of hydrogen peroxide at the expense of the oxidation of a variety of mediators (Tan et al., 2009) (Fig. 5). For this reason, the application of these enzymes is a promising approach for the production of electricity. The effective immobilization of the laccase onto the

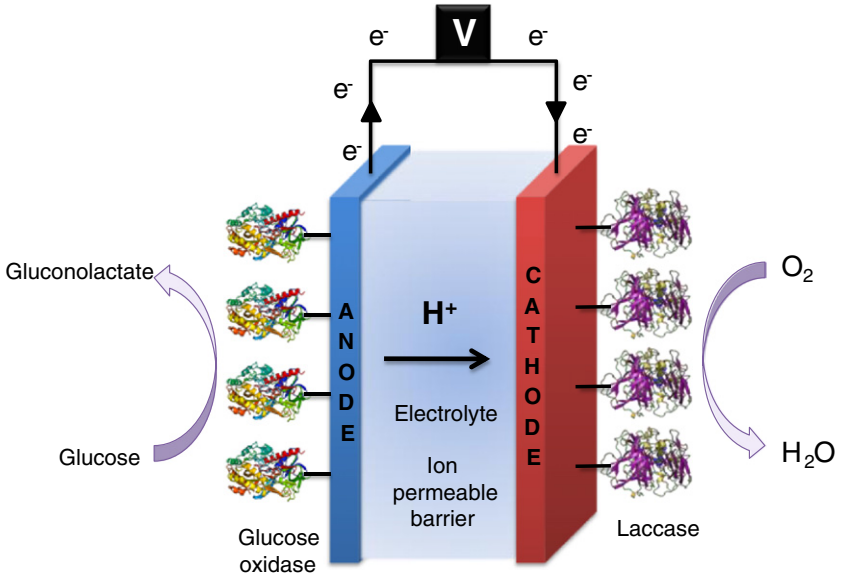

Fig. 5. Model of an enzymatic biofuel cell involving laccase and glucose oxidase.

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electrode is essential for the preparation of an efficient and stable catalytic system. To use such enzymes as efficient biological fuel cells, the laccase must be placed on the surface of the electrode. This immobilization must meet certain requirements, including enzyme retention on the electrode, enzymatic stability and rapid electron transfer (Nogala et al., 2006; Rochefort et al., 2008).

Many conducting supports and methods have been designed to immobilize laccase for use in fuel cells. Nogala et al. (2006) designed a stable voltammetric carbon ceramic electrode (CCE), and laccase from *C. unicolor* and ABTS were deposited onto its surface after enzyme and mediator encapsulation in two different silicate matrixes: hydrophilic tetramethoxysilane films and methyltrimethoxysilane. This new electrode exhibited electrocatalytic activity towards dioxygen and the stable immobilization of mediator and enzyme. Alginate/carbon beads were developed to obtain a biocompatible matrix for *T. versicolor* laccase and glucose oxidase encapsulation for application in biofuel cell technology. Two-thirds of both enzymes were irreversibly retained inside the alginate beads. This proportion increased to 80% for laccase in combined alginate/carbon. The kinetic parameters were similar to those obtained in the free enzyme, demonstrating that free diffusion was occurring through the beads (Khani et al., 2006).

Porous carbon tubes are an original conducting support for laccase and a suitable transporter of dissolved dioxygen solutions via diffusive flow through the porosity. Servat et al. (2007) immobilized *T. versicolor* laccase in polypyrrole films that provide a homogeneous, chemically stable and adherent carrier. These porous carbon tubes were used as an enzymatic support and for the transport of dioxygen. High current densities up to 280 μA/cm^2 have been reported using this system. The use of polypyrrole was also reported in another paper in which three different methods of laccase immobilization onto carbon porous tubes were performed with *T. versicolor* laccase: entrapment in a polypyrrole matrix, immobilization by recognition with avidin and biotin reagents and covalent binding to an aminopolypyrrole film. The latter showed the best efficiency, and the electrode stability was characterized (Merle et al., 2008). The microencapsulation of laccase in PEI over a glassy carbon electrode (GC) was shown to increase the enzyme stability. In addition, this system allows the immobilization of large amounts of enzyme and prevents the diffusion of inhibiting molecules. Increasing the amount of laccase during the preparation of the microcapsules resulted in a better bioelectrode response than did increasing the number of capsules on the electrode because the latter could decrease the diffusion of the substrate or mediator (Rochefort et al., 2008). *T. hirsuta* laccase was also covalently immobilized in low-density graphite electrodes (LDG) modified with 2-aminophenol and aminophenyl monolayers. Using this modified electrode, ester or amide bonds between the carboxylate groups of the protein and the hydroxyl or amino groups of the electrode surface were favored. The direct electron transfer (DET) to the carbon base was efficient, producing a stable and chloride-resistant biocathode (Vaz-Dominguez et al., 2008).

In recent years, the use of nanomaterials for electrode construction has received special attention. Gold nanoparticles are commonly used because of their biocompatibility, high specific surface area and reactivity. However, these nanoparticles tend to agglomerate, resulting in the reduction of enzymatic activity. Qiu et al. (2008) used NPG adsorbed to a GC electrode for laccase immobilization by adsorption. Better enzyme immobilization was achieved with smaller pore sizes, but the pore size must also be large enough to allow enzyme and substrate diffusion. The laccase stability was improved, and the efficient electroreduction of oxygen was conducted. Gold was also employed for the immobilization of a histidine-tagged recombinant laccase. A thiolated nitrilotriacetic acid (NTA) monolayer over the surface of the gold electrodes provided the support for the site-specific covalent immobilization of laccase. After immobilization, cyclic voltammetry was employed to investigate the enzyme activity in the presence of a diffusive one-electron redox mediator. The efficiency of the immobilization strategy could be carefully determined (Balland et al., 2008). A chitosan-multiwalled carbon nanotube/GC electrode was applied for the covalent immobilization of *T. versicolor* laccase. The resulting biofuel cell exhibited a maximum power density of 9.6 μW/cm^2, a voltage of 0.19 V and an intensity of 114 μA/cm^2 (Tan et al., 2009). Carbon nanotubes were also tested after a non-covalent functionalization with 1-aminopyrene (1-AP) and were subsequently used to immobilize laccase via GLU. The laccase immobilized on 1-AP nanotubes showed higher electrocatalytic activity and better stability than the corresponding control of laccase immobilized on pristine nanotubes (Pang et al., 2010).

3.2.2. Biosensing

Biosensors are of growing importance because of their inexpensive, rapid, accurate, sensitive and selective detection of analytes. Electrochemical biosensors are normally based on the production or consumption of electrons by enzymatic catalysis. The target analyte is involved in the reaction, which takes place on the active electrode surface, and the produced ions create a potential, which is subtracted from that of the reference electrode to give a measurable signal (Fig. 6). Laccase-based biosensors have interesting potential uses in the detection of phenolic compounds in the food industry and wastewaters as well as in biomedical and bioremediation applications. To

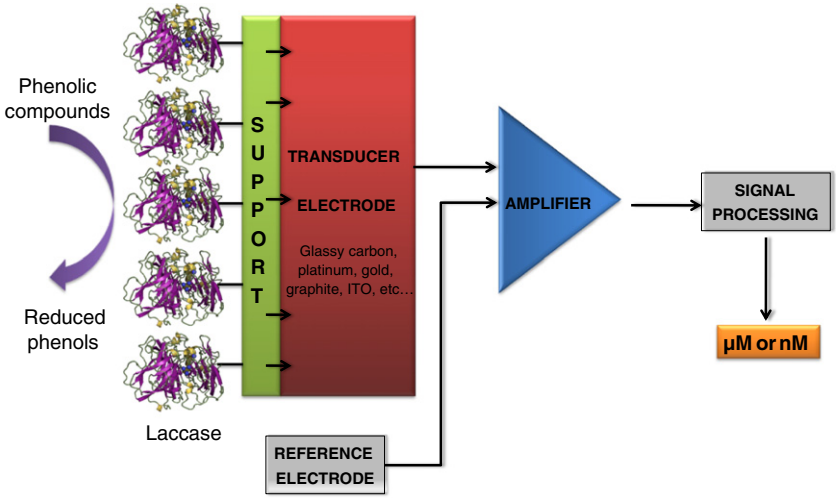

Fig. 6. Design of an electrochemical biosensor made with laccase for the detection of phenolics.

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avoid enzyme loss, the immobilized enzyme must be securely attached to the electrode and must spread over the transducer for rapid detection.

Quan and Shin designed a GC and platinum (Pt) laccase biosensor based on surface modifications. The GC electrode was modified by the electrochemical oxidation of 1,5-pentanediol or by the direct electrochemical oxidation of the electrode itself to introduce carboxylate groups. The link between laccase and the functional groups was achieved via the previous electrode activation with EDC and NHS. In the case of the Pt electrode, electrochemical oxidation was used to introduce hydroxyl groups, which were activated by cyanuric chloride (CC). Alternatively, silanization with APTES and the subsequent activation with GLU were performed (Quan and Shin, 2004c). Laccase from Denilite was also immobilized on a Pt electrode for the amperometric detection of hydroquinone (HQ) and homogentisic acid (HGA). HQ sensitivity was 280 nA/μM with a linear range between 0.2 and 35 μM and a detection limit of 50 nM. In the case of HGA, sensitivity was 53 nA/μM with a linear range of 1–50 μM and a detection limit of 0.3 μM. The fast response and the long-term stability are the principal advantages of this biosensor (Quan and Shin, 2004b). The same Pt electrode with the immobilized Denilite laccase was employed for the detection of catechol and catecholamines. The sensitivities were 210, 75, 60 and 45 nA/μM with detection limits of 0.07, 0.2, 0.3 and 0.4 μM for catechol, dopamine, norepinephrine and epinephrine, respectively (Quan and Shin, 2004a). Laccase immobilized on a silanized Pt electrode detected ABTS, *p*-phenylenediamine and *p*-aminophenol with sensitivities of 75, 330 and 385 nA/μM and linear ranges of 0.6–15, 0.14–29 and 0.12–22 μM, respectively (Quan et al., 2004a). *T. versicolor* and *Aspergillus niger* laccases were entrapped in a polyaniline matrix for the determination of phenolic compounds. The linear detection ranges of this biosensor were between 0.4 and 6 μM for phenol, 0.2–1 μM for catechol and 0.2–20 μM for L-DOPA in the case of *T. versicolor* laccase and 0.4–4 μM, 0.4–15 and 0.4–6 μM, respectively, for *A. niger* laccase (Timur et al., 2004).

Some biosensors have been designed as bi-enzymatic systems in which laccase was co-immobilized with other enzymes. Several examples of this procedure are reported in the literature. Laccase was immobilized with tyrosinase, resulting in a sensitivity increase of 70% for phenylenediamine detection (Quan et al., 2004b).

Rigidoporus lignosus laccase was covalently immobilized by carbodiimide chemistry on a self-assembled monolayer of 3-mercaptopropionic acid deposited on a gold surface with a GC electrode as the amperometric transducer. This approach resulted in phenol sensitivity of 3 nA/μM when 1,4-hydroquinone was used as the substrate and could be employed to detect a large number of phenols occurring in OMW (Vianello et al., 2004). GC electrodes were also used for the immobilization of *T. hirsuta* laccase by entrapment in different polymers, namely positively charged cetyl ethyl poly(ethyleneimine) (CEPEI) and negatively charged commercial Nafion and Eastman AQ 29D. Laccase immobilized in the negatively charged polymers exhibited the shortest response time (Yaropolov et al., 2005). A new biosensor was developed that contained a mercury thin film deposited onto GC electrodes by gelatin, which was cross-linked with laccase. Using this biosensor, catechol and phenol were detected in the concentration range of 0.5×10^{-6}–5×10^{-6} M and 2.5×10^{-6}–2×10^{-6} M, respectively (Kirgöz et al., 2005). *Coriolus hirsutus* laccase was used to design an amperometric biosensor by forming a self-assembled monolayer of 4-mercapto-1-butanol over a gold electrode that was activated with epichlorhydrin. This biosensor was more stable under flow conditions than others and presented the successful co-immobilization of different enzymes in one array (Solná and Skládal, 2005). Mousty et al. (2007) described a laccase biosensor based on the entrapment of the enzyme into redox-active LDH with a clay colloidal suspension [Zn–Cr-ABTS] on GC electrodes. ABTS intercalated within the LDH layers, playing the role of a redox mediator and performing the electrical wiring of laccase. The determination of dissolved oxygen ranged between 6×10^{-8} and 4×10^{-6} M, and very low detection limits for azide (5.5 nM), fluoride (6.9 nM) and cyanide (6.2 nM) were observed. This biosensor offers a fast and a sensitive response to the presence of dissolved oxygen, and it has been proposed for use in the detection of laccase inhibitors (Wang et al., 2008a).

All of the carbon-based anodes previously mentioned exhibit high specific capacity and superior cycling properties.

The utility of graphitized carbon as a negative (anode) electrode material is well established. Thus, Portaccio et al. (2006) immobilized the *T. versicolor* laccase by adsorption and covalent coupling to a graphite electrode. For the latter approach, graphite was treated by the difference of electric potential or with nitric acid. The coupling agents employed were hexamethylenediamine and GLU. The use of the covalent bond was demonstrated to ensure higher sensitivities compared to immobilization by adsorption. From those tested, the biosensor containing graphite modified by nitric acid and coupling mediated by GLU showed the best results. Moreover, the pH range in which the maximum response was achieved was 4.25–5.5; this range allows the direct application of this biosensor in wastewaters from agricultural and industrial activities. Graphite was also employed to design a biosensor for monitoring kraft lignin and sulfate pine lignin by means of *T. hirsuta* laccase adsorption. Some improvements over a Clark electrode were obtained using this method, such as the reproducibility, linear dynamic range and accuracy for the detection of lignin and its model compounds (Shleev et al., 2006). Cordi et al. (2007) compared the immobilization of laccase from *T. versicolor* onto different vitroceramic supports, pyrolytic graphite and carbon fiber electrodes. The coupling agents were carbodiimide and GLU. The best support for immobilization was pyrolytic graphite. The biosensor prepared with this material showed a good linear response to catechol. The same system was subsequently used for phenol removal in kraft pulp.

Magnetic material was used in several studies and combined with other carbon-based electrodes to generate different biosensors. Fe_3O_4 magnetic nanoparticles with core-shells (Fe_3O_4–SiO_2) were amino-modified, and laccase was cross-linked to these nanoparticles by GLU. The bionanoparticles obtained were then immobilized onto the surface of a GC electrode. The linear range obtained for HQ determination was 1×10^{-7} to 1.375×10^{-4} M with a detection limit of 1.5×10^{-8} M. This biosensor showed high sensitivity, a wide range and low cost in manipulation along with good stability and selectivity (Zhang et al., 2007a). Rahman et al. (2008) designed a new biosensor that allowed the study of the DET process of laccase. Synthetic branched polymers with layered architectures, called dendrimers, were encapsulated with gold nanoparticles (AuNPs) that had been previously functionalized by a conducting polymer, namely 3′,4′-diamine-2,2′,5′,2″-terthiophene (PDATT). PDATT/Den(AuNPs)/laccase was used to study DET and to produce a catechin biosensor. Again, magnetic nanoparticles of Fe_3O_4–SiO_2 with a carbon electrode were used for the catechol biosensor. Laccase from Fluka was covalently immobilized after the functionalization (APTES) and activation (GLU) of the nanoparticles. The linear range of detection was 7.5×10^{-7} to 2.75×10^{-4} M with a detection limit of 7.5×10^{-7} M. Similar results were obtained using HPLC (Tang et al., 2008; Zhang et al., 2007b).

Xu et al. (2009) used the mesoporous silica sieve MCM-41 for *T. versicolor* laccase immobilization by physical electrostatic adsorption. This support provides a satisfactory immobilization due to its large pore size, high surface area, good biocompatibility and favorable conductivity, which occurs due to the previously adsorbed conductive dye methylene blue (MB). MB improved the current and decreased the response time for catechol detection. An amperometric method was designed based on the detection of polyphenolic compounds generated from salicylic acid, which was added into the culture medium during the course of *Escherichia coli* metabolism. Laccase was

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covalently immobilized using the APTES and GLU functionalization of ITO. The biosensor showed high sensitivity for the detection of *E. coli* density with a linear range of 1.6×10^3 to 1.0×10^7 cells/mL and a detection limit of 9.7×10^2 cells/mL (Tang et al., 2006). *R. lignosus* laccase was covalently bound to ECH-Sepharose resin via carbodiimide. The detection of phenols in the nanomolar range was achieved, and the operational stability was demonstrated for more than 100 working days. This biosensor was successfully used to detect phenols in the wastewater from OMW without sample preparation (Vianello et al., 2004). A new approach was also tested with *T. versicolor* laccase, which was immobilized onto sonogel carbon electrodes by adsorption. In this case, a previous modification of the enzyme was performed by polymerization with glutaricdialdehyde and Nafion in an ultrasonic bath, forming the immobilization matrix. The biosensor obtained using this method was employed for the detection of phenolic compounds in beer to obtain a valid estimation of the classical Folin–Ciocalteu index (ElKaoutit et al., 2007). For the wine industry, an amperometric biosensor was designed with screen-printed electrodes (SPEs) that were modified with ferrocene and with the entrapment of *T. versicolor* laccase in a diglyceryl silane matrix. This method resulted in a rapid and simple immobilization process for the detection of polyphenols (Montereali et al., 2010).

The biofuel cell and biosensor concepts are related, as shown by the above-mentioned biofuel cell designed by Tan et al. (2009), which was also used as a catechol biosensor with linear detection between 0.1 and 50 μM and a detection limit of 20 nM.

3.3. 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.

Regarding the pulp and paper industry, Gamelas et al. (2007) designed a new and interesting method for kraft pulp biobleaching. This group used an enzymatic reactor for the oxidation of polyoxometalates (POMs) as mediator compounds and the subsequent separation of the enzyme and the oxidized POMs by ultrafiltration. The oxidized POMs were then used for the biodelignification of *Eucalyptus globulus* kraft pulp by means of residual lignin oxidation, and the resulting reduced POMs were then recirculated to the enzymatic reactor for POMs reutilization in a closed circuit. This system allows the generation of oxidized POMs in optimal conditions for the confined enzyme, whereas the biobleaching of pulp may be performed in different conditions to maximize both the production of POMs and the biobleaching of pulp.

With respect to the possible upgrading of lignin from the pulp industry, milled wood lignins (MWLs) and residual kraft lignins (RKLs) were employed as target compounds to be modified with immobilized laccase. The objective of such modifications is to obtain new added-value products from these renewable compounds. The laccase employed for this application was previously immobilized using the LbL technique onto alumina pellets with polyelectrolyte layers. Mediators were able to diffuse from the catalytic site to the bulk of the solution, oxidizing and depolymerizing the lignin (Crestini et al., 2010).

4. Conclusion and future outlook

The immobilization of laccase can be performed via many different methods using a large number of supports. However, the recovery of enzymatic activity after the immobilization process is not always satisfactory, although improvements are frequently obtained concerning the stability of the enzyme to temperature, pH, organic solvents, storage and operation.

Similar to the free enzyme, immobilized laccase can be applied in a huge number of industrial processes, especially in environmental applications and in electrobiochemistry processes. The employment of laccases for the design of biological fuel cells and biosensors opens up new possibilities, from industrial to healthcare applications.

The search for inexpensive supports and the recovery of activity during the immobilization process should be improved to increase the potential application of laccase immobilized systems.

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