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Laccases and their application in bioremediation of organic pollutants

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A B S T R A C T

Laccases are present abundantly in many white-rot fungi. They are blue multicopper oxidases, which catalyze the monoelectronic oxidation of a broad spectrum of substrates, such as ortho- and para-diphenols, polyphenols, aminophenols, aromatic, and aliphatic amines coupled with a full, four-electron reduction of O_2 to H_2O. Laccase-assisted reactions have a wide application potential. In particular, they decolorize and detoxify the industrial effluents and help in wastewater treatment. In the presence of specific mediators, they act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants. Several techniques have been developed for the immobilization of laccase to preserve their enzymatic activity. In this review, we describe the fungal source of laccases and their application in environmental protection.

Keywords: Laccases, Enzymatic Biodegradation, Micropollutants, EDC, Immobilization, Enzymes.

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Introduction

Intensive industrial and agricultural activity over the past decades led to considerable contamination of soil and water with toxic organic pollutants having detrimental effects on the health of humans, animals, plants, and microbes. Wastewaters produced by industry and municipalities contain synthetic compounds including pharmaceutical and personal care products (PPCPs), endocrine-disrupting compounds (EDCs) are increasingly discharged through wastewater treatment plants (WWTPs) [1, 2]. This organic compounds, termed micropollutants, are present at such low concentration (pg/L to ng/L) in water that most established treatment processes seldom lead to their elimination and may pose serious risks to the environment and public health [3]. Various effective mechanical, chemical, physiochemical techniques are currently available to remove pollutants but they are complex, quite expensive, may generate toxic intermediates [4-6]. Bioremediation is seen as an attractive and suitable alternative due to its reputation as a low-cost, effective and environmentally friendly approach. Recent fundamental studies demonstrated the potential of white rot basidiomycetes (WRB) and their lignin-modifying enzymes (LME) application for degradation of various aromatic compounds and treatment of soils and effluents contaminated with organic pollutants [7-10]. Especially, multi-copper phenol-oxidizing laccases (EC 1.10.3.2) secreted by many fungal species associated with the degradation of lignocellulosic material appeared to be the most promising for their environmental applications. Due to a wide range of substrate specificity, these enzymes oxidize a broad range of compounds such as phenols, polyphenols, methoxy-substituted phenols, and amines while reducing oxygen to water; however, laccase activity can be extended to non-phenolic compounds by use of mediators like HBT (1-hydroxybenzotriazole) and ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), and syringaldehyde [11, 12].

Laccase producers

Laccases are common enzymes in nature and are found widely in plants and fungi as well as in some bacteria and insects [13]. The first bacterial laccase

was detected in the plant root-associated bacterium "*Azospirillum lipoferum*"[14], where it was shown to be involved in melanin formation [15]. An atypical laccase containing six putative copper-binding sites was discovered from *Marinomonas mediterranea,* but no functional role has been assigned to this enzyme [16]. *Bacillus subtilis* produces a thermostable CotA laccase which participates in pigment production in the endospore coat [17]. Laccases have also been found in *Streptomyces cyaneus* [18] and *Streptomyces lavendulae* 19]. Laccase producing *Bacillus* were isolated from soil and the enzyme was involved in phenol degradation [20, 21]. Although there are also some other reports about laccase activity in bacteria, it does not seem probable that laccases are common enzymes from certain prokaryotic groups [22].

Laccases of fungal origins have been the most intensively studied [23]. Fungal laccases are implicated in both intra- and extra-cellular physiological processes including morphogenesis, pigmentation, pathogenesis, and delignification [24-26]. Among fungi, ascomycetes, basidiomycetes, and deuteromycetes can produce laccases, but the WRB are the most efficient lignin degraders and laccase producers [24, 27]. WRB possess a strong biochemical and ecological capacity to degrade environmental organic chemicals either by chemical modification or by influencing chemical bioavailability. Ability of these fungi to form extended mycelia networks, the low specificity of their enzymes and their ability of using pollutants as a growth substrate make them well suited for bioremediation processes. In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth in search of growth substrates [10, 28, 29]. Bacteria contain little energy, have a low bioavailability and cannot degrade substances having rare structural elements. Filamentous fungi are more advantageous where translocation of essential factors (nutrients, water, the pollutant itself) is required for the degradation of environmental chemicals by translocation resources between different parts of their mycelium. Fungi also release extracellular enzymes which allow for digestion of energy sources in their surroundings and further diffusion of these molecules through the substrate towards the fungus [30]. Owing to the broad substrate specificity of the LME and potent intracellular enzymes, in particular, cytochrome P450 monooxygenases, WRB are especially well-suited for PAH degradation compared to bacteria. Moreover, they can degrade high molecular-weight PAHs, whereas bacteria can degrade only smaller molecules. In addition, fungi are also known to produce large quantities of exudates that serve as auxiliary carbon sources for pollutant-degrading bacteria [31] delignification.

Laccases are secreted by WRB along with other ligninolytic enzymes including manganese peroxidase, lignin peroxidase, and versatile peroxidase, although the specific types of enzymes secreted may differ with the fungus [24, 32]. Many fungi contain several laccase-encoding genes, but their biological roles are mostly not well understood [33]. Pleurotus ostreatus and Trametes versicolor can be regarded as the model organisms in basic and applied laccase research. Other species of Pleurotus and Trametes genera as well as Cerrena unicolor, Coriolopsis gallica, Ganoderma lucidum, Phlebia radiata, Pycnoporus cinnabarinus, Pycnoporus sanguineus are well-known efficient laccase-producing basidiomycetes. Nevertheless, efforts are still being made with an expectation to screen naturally-occurring laccase overproducers with desired laccase yields and properties and elucidate factors regulating enzyme production [34-39].

Laccase production

The potential applications of LME in industrial and environmental technologies require huge amounts of these enzymes at low cost [27, 40]. Enhancing laccase yields is essential to lower production costs and promote industrial applications of the enzyme. Laccase production and yield are directly influenced by some parameters such as the substrate to be used, the fermentation technique, agitation, aeration, and cultivation time, among others. The production of laccase is linked straight to a complex regulation of nutrients that affects directly the expression of laccases, comprising the carbon and nitrogen sources, their respective concentration and the relationship between them, and also the concentrations and kind of inducers used [41]. However, no generalization can be made on the best nutrients or inducers and their optimal concentrations since basidiomycetes display a wide diversity in their responses to individual compounds [40, 42, 43]. Lignocellulosic wastes containing carbohydrates and inducers are often added resulting in benefits such as laccase production enhancement, lower production costs, and waste reuse [44, 45].

Literature describing laccase induction by xenobiotics, e.g., lignin breakdown products, dyestuffs

and organic pollutants evidences that the effects of organic compounds on laccase production depend on the compound structure, fungal strain, growth stage as well as the culture medium [42, 46]. Combinational induction of laccase production by metal ions and organic compounds can be either synergistic [46] or antagonistic [47].

Co-culture of laccase-producing strains with other microbes can be more advantageous and effective than chemical induction to enhance laccase production either through yield increase or induction of new isozymes. Microbial interactions with laccase inducing effects vary with the strain, but the structure of inducing metabolites and the inducing mechanism remain largely unknown [44, 48-52]. One proposed mechanism for laccase overproduction in the co-culture process is carbon source succession. Li et al. found that glycerol produced from glucose by the yeast Candida sp. is an efficient carbon source for G. lucidum upon glucose deprivation and crucial for laccase overproduction by prolonging laccase secretion time [52].

Molecular and catalytic properties of laccase

Laccases are copper-containing oxidoreductases (EC 1.10.3.2). More than 100 laccases have been isolated and characterized to various extents. Molecular structure of fungal laccases occurs often as isozymes with monomeric or dimeric protein structures. Both intracellular and extracellular isozymes may be produced from a single organism.

Laccases are glycoproteins with molecular weights 50–130 kDa. The carbohydrate portions of plant laccases constitute up to 45% of the molecule weight, whereas fungal laccases contain less carbohydrates (10–30%), that catalyse the monoelectronic oxidation of various substrates (e.g. phenols, and aromatic or aliphatic amines) to the corresponding radicals, using molecular oxygen as the final electron acceptor [53-56]. Laccases play important roles in several biometabolic steps including those involved in fungal pigmentation, plant lignification, lignin biodegradation, humus turnover and cuticle sclerotization, where in naturally occurring low-molecular-weight phenolic compounds and natural fibre polymers are utilized as substrates [56].

Laccases from different sources exhibit a wide range of redox potentials. The T1 site has a high redox potential reaching 780 to 800 mV for the *T. versicolor* and *Neurospora crassa* enzymes, whereas the plant *R. vernicifera* enzyme has a value of 420 mV [57, 58]. The redox potentials of T2 and T3 sites for the *R. vernicifera* laccase are, respectively, 390 and 460 mV at pH 7.5 [59]. The *T. versicolor* laccase T1 and T3 sites have been reported to be 785 and 782 mV, respectively [60]. In general, the T1 sites in fungal laccases are much higher than those of plant laccases and other blue copper oxidases, although significant differences in potentials also exist among fungal laccases [61]. Laccases exhibit low substrate specificity and can oxidize a range of compounds, such as diphenols, aryl diamines, and aminophenols. The Km values are generally in the range of 1–10 mM, and the change in log (kcat/Km) increases proportionally to the redox potential difference between the T1 site acceptor and the substrate donor [62, 63].

Usually, fungal laccases have an optimal pH in the acid range, and the temperature profiles for the activity of these enzymes normally do not differ from those of other extracellular ligninolytic enzymes. Purified laccase was incubated in buffers of varying pH between pH 2.0 and 8.0 for up to 180 min to determine its pH stability. Similarly, some authors showed that the purified enzyme dissolved in 0.1 M sodium acetate, pH 5.0, was incubated at varying temperatures (40–80 °C) for up to 180min to determine its thermal stability. Laccase was found to be most stable at pH 5.0 and 30°C and 5(d)). Laccase activity decreased significantly (93%) after 180 min at pH 8.0 and it was fairly stable between pH 3.0 and 7.0 for up to 180 min. While the enzyme was relatively stable below 50°C, activity decreased significantly when the temperature was 70°C or higher. In fact, laccase activity was completely lost within an hour at 80°C and 5(d) [64].

Mediators of laccases

The biotechnological importance of laccases have increased after the discovery that the enzyme substrate range could be further extended in the presence of small readily oxidizable molecules called mediators [65]. Generally, the laccase reactivity decreases with the increase of the substrate size; therefore, the limited substrate accessibility is overcome through the use of appropriate laccase mediators. As the substrate due to its size cannot enter the laccase active site, the mediator acts as a carrier of electrons between the enzyme and the substrate thereby overcoming the steric hindrances that exist

between them [66]. In this case, during the initial reaction step, the mediator is oxidized to stable intermediates with high redox potential by laccase [66]. The ideal mediator should be non-toxic, economic, and efficient, must be a good laccase substrate and reduced forms that do not inhibit the enzymatic reaction [67]. Moreover, the redox mediator should be able to continuously maintain the cyclic redox conversion. Suitable mediators are needed to maximize the potential of laccase use as a bioremediation agent. The most commonly used mediators are the ABTS and the 1-hydroxybenzotriazole (HBT) [68]. ABTS [2,2-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] is the best organic redox mediator. Its use for oxidation of non-phenolic lignin structures gave impetus to search for new laccase mediators [69]. It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines and, upon extended oxidation and abstraction of the second electron, the corresponding dictations can be obtained. These cation radical and dictation play role in the oxidation of the substrates, non-enzymatically. The redox potentials of $ABTS^{+}$ and $ABTS^{2+}$ were evaluated as 0.680 V and 1.09 V respectively [70].

1-hydroxybenzotriazole - HBT is another important organic redox mediator. HBT belongs to the N-heterocyclic compounds bearing N-OH groups mediators [69]. Consuming oxygen HBT is converted by the enzyme into the active intermediate, which is oxidized to a reactive radical (R- NO.) and HBT redox potential has been estimated as 1.1-1.2 V [70]. HBT acts as a radical mediator rather than ionic, but a severe factor limiting catalytic productivity is again deoxygenation to benztriazole. Indeed, for recalcitrant substrates in the presence of large amounts of enzyme and HBT, deoxygenation is the predominant process seen. When this occurs, rapid deactivation of the enzyme is also seen, suggesting some interaction of the oxidized mediator with the protein [71]. It has been proposed that the HBT radical forms a coupling intermediated product with lignin [72]. The intermediate product can subsequently degrade to release the reduced form of benzotriazole (BT), or form a stable complex that binds some of the HBT to lignin via covalent bonding.

2,2,6,6-tetramethyl-1-piperidinyloxyl (TMPO) is more efficient than ABTS, HBT, or the natural laccase mediator 3-hydroxyanthranilic acid. This mediator is present in the solution a stable N-oxylradical, which can perform primary modification of some high potential substrates even without laccase [73]. Laccase oxidizes TMPO to produce the oxo-ammonium ion, which reacts with the substrate. Proton removal yields the oxidized product and the reduced form of TMPO is converted to the oxidized form by laccase and then to oxo-ammonium ion. The laccase–TEMPO acts according to the ionic mechanism, whereas the HBT-mediated reactions favor the radical mechanism [73].

Despite all the associated advantages of using these mediators, there still have several drawbacks from a practical point of view: i) low catalytic efficiency, excess amounts of mediator (typically 30 mol% on substrate) are needed, some even need more than 1 equiv; ii) in some cases, laccase is inactivated by the mediator radicals, or the latter can be transformed into inactive compounds with no 10 more mediating capability (e.g. generation of benzotriazol from HBT by losing the hydroxyl group); iii) some of mediators can generate toxic derivatives; iv) long reaction time are usually required. In view of the aforementioned drawbacks of existing routes and the desire for aerobic oxidation of multi-functionalized 15 complex molecules, the development of new and highly efficient laccase-mediator system is particularly attractive [74].

Enzyme immobilization

Silicon dioxide offers a high versatility (particle size, surface area, porosity, surface chemistry, ease of synthesis, and scalability) and represents the support of choice for the enzymatic immobilization. Furthermore, silica materials are considered neutral and nontoxic and matches the natural environments.

Enzyme immobilization is one of the most promising techniques for highly efficient and economically competent biotechnological processes in the field of environmental monitoring, biotransformation, diagnostics, pharmaceutical and food industries. The immobilization of the biocatalysts onto a solid support can reduce the enzyme loss, limit the denaturation problems, and facilitate their possible reuse. Additionally, immobilization improves many properties of enzymes such as performance in organic solvents, pH tolerance, heat stability or the functional stability [13]. Laccase immobilization has been addressed as a way to intensify and stabilize biocatalytic activity [75].

The immobilization technique is based on the covalent attachment of oxidative enzymes on the nanoparticles. Different synthesis routes of nano-particulate materials exist or are under development. They comprise micro- and mini-emulsion-

based techniques, monophasic synthesis according to the method of Stoeber et al. [76] and flame spray pyrolysis (FSP). Due to the high specific surface of nontoxic nanostructured support material, fumed silica nanoparticles (fsNP) are expected to be of interest for the immobilization of laccases [77]. Furthermore, these systems are relatively cheap and are produced at industrial scale [78].

A covalent immobilization method based on glutaraldehyde and amino-functionalized SBA-15 supports has been successfully applied to covalently and stably immobilize laccase from Trametes versicolor. Aminopropyl-based laccase-SBA-15 biocatalysts displayed the best reusability properties, retaining higher activity after four repeated uses than the corresponding aminobutyl-based materials [79]. Alkyl moieties displayed higher enzyme loadings than phenyl moieties, being more adequate the larger n-butyl tethering residue likely due to its higher mobility.

Carbon materials have been long preferred as carriers for enzyme immobilization, mainly because they may provide a large surface area relative to the enzyme loading. In what concerns carbon nanotubes (CNTs), their exclusive structure and properties have been intensively studied in distinct applications ranging from energy storage, biotechnology and environmental remediation [80]. Additionally, CNT surface can be easily functionalized, tuning their properties towards specific applications and enhancing their efficiency either as supports or catalysts. Polysulfone (PSf) membranes are widely used in water applications due to their excellent heat resistance and chemical stability over a large range of pH [81].

Higher immobilization efficiency and recovered activity were obtained using CNTs oxidized with HNO_3 0.30 M (CNTox-0.30), which is related to the higher number of oxygen-containing groups available at the surface of the material. The laccase/CNTox-0.30-EN bioconjugate was successfully used for the treatment of a mixture of four phenolic compounds, the process showing similar efficiency as the analogous using the free enzyme, with the possibility of reusing the biocatalyst [82]. For improving stability of immobilized white-rot fungus to treat various effluents, some authors performed study where Phanerochaete chrysosporium cells and the combined cross-link enzyme aggregates (combi-CLEAs) prepared from Trametes versicolor were co-immobilized into the Ca-alginate gel particles in this paper [83]. The activity yields of obtained combi-CLEAs were 42.7% for lignin peroxidases (LiPs), 31.4% for manganese peroxidases (MnPs) and 40.4% for laccase (Lac), respectively. And their specific activities were 30.2 U/g as combi-CLEAs-LiPs, 9.5 U/g as combi-CLEAs-MnPs and 28.4 U/g as combi-CLEAs-Lac. The combi-CLEAs improved the adaptability of the white-rot fungal particles to adverse environmental conditions.

The experimental results indicated that magnetic graphene oxide (MGO) nanomaterials were synthesized based on covalent binding of amino Fe3O4 nanoparticles onto the graphene oxide (GO), and the prepared MGO was successfully applied as support for the immobilization of laccase from *Trametes versicolor (*commercial enzyme from Sigma-Aldrich), it effectively improved the processing efficiency and expanded the industrial application of enzymes [84]. Compared with free laccase, the MGO-laccase exhibited better pH and thermal stabilities. The optimum pH and temperature were confirmed as pH 3.0 and 35◦C. Moreover, the MGO-laccase exhibited sufficient magnetic response and satisfied reusability after being retained by magnetic separation. The MGO-laccase maintained 59.8% activity after ten uses. Compared with the free enzyme, the employment of MGO as enzyme immobilization support could efficiently enhance the availability and facilitate the application of laccase.

The operating stability of the immobilized enzyme is a very important parameter. Immobilized enzymes may be easily separated from the reaction solution and reused, which greatly decreases costs of the enzyme and increases its significance for practical application [85-87]. The immobilization of laccase from *Trametes versicolor* on the controlled porosity carrier (CPC) silica beads allowed laccase to remain stable and maintain more than 85% of its initial activity after 30 days. After immobilization, laccase degraded more than 90% of 2,4-dinitrophenol within 12 h of treatment [88].

Decrease in the activity of the immobilized laccase as a result of repeated usage may be expected due to the possibility of enzyme denaturation during the operation process. What is interesting, Wang et al. [86], observed the highest activity of commercial laccase immobilized on polyacrylonitrile or polyacrylonitrile/montmorillonite/graphene oxide (PAN/MMT/GO) at the second or third cycle. It is possible that during repeated usage, the membrane became slack and downy, contributing to more sites for the enzyme to reach the substrate.

Enzyme immobilization is the key step of bio catalytic membrane preparation which is promising in food, pharmaceutical, and water treatment

industries [89]. Polysulfone membranes containing functionalized CNTs demonstrated to be also an excellent support for the enzyme (re)-immobilization and their application in the degradation of 4-methoxyphenol. The PSf membrane with 0.1 wt% of CNTox-0.30-EN was the most efficient, presenting a comparable activity to CNTs solely and with a much lower CNT content [82].

Potential of laccases to solve ecological problems

The release of untreated industrial effluents into sewers that will empty into rivers represents a very big problem [90]. Sewage can contain many substances from industrial processes with carcinogenic, mutagenic, and teratogenic potential, including toxic effects to human beings and many fish and microorganism species. So the appropriate treatment of these wastewaters is important and can be realized by various methods, although each technique has some limitations [91]. Using of enzymatic processes in wastewater treatment is relatively new and has the advantage of decreasing the consumption of reagents and degradation of subproducts. Among the possible alternative methods can be the use of lignolytic enzymes capable of degrading many toxic and persistent substances such as dyes, solvents, inks, pesticides, fertilizers, and others [92]. Moreover, enzyme treatments are eco-friendlier and energy efficient compared to chemical treatments [93, 94].

One of the applications of laccases (together with the peroxidases) in wastewater treatment is related to the textile industry, because of the necessity of eliminating liquid residues containing textile dyes that remain in the water after the dye industrial process. Synthetic dyes often have low biodegradability, several are toxic, carcinogenic and mutagenic and it is not uncommon that their degradation products show similar properties. Furthermore, the absorption and reflection of the sunlight by dyes in surface waters interferes with bacterial and plant growth, disturbing the ecological balance [95, 96]. Therefore, decolorization of waste-waters discharged in textile processing is a major problem. In recent years it was shown that many industrial dyes could be decolorized by fungal laccases frequently immobilized on different supports, such as glass-ceramic materials, imidazole-modified silica, montmorillonite, alginate-gelatin mixed gel, hydrophobic sol-gel and green coconut fiber [97-99]. Laccases physically entrapped or immobilized by their covalent attachment to various supports are the most commonly used in decolorization processes [99]. The decolorization of waste waters by immobilized laccases is the result of enzymatic catalysis and support adsorption [98, 99]. It was shown that alginate and chitosan are biopolymers, which can be used as sorbents to remove dyes from the aquatic solutions. Although the entrapment of laccases in alginate mixed gels or hydrophobic Sol-gel decreases their activity and dye affinity, it improves their pH stability, thermostability and enables reusability of the enzymes [99]. Additionally, the immobilization of laccases on such insoluble supports limits their conformational changes that increases their operational stability and durability [99]. For example, the entrapment of laccase in alginate-gelatin, alginate-chitosan mixed gels or in hydrophobic Sol-gel matrix of trimethoxysilane and proplytetramethoxysilane led to its significant stability towards heat denaturation. However, under these conditions lower affinity of the enzyme to the substrates caused by diffusional limitations and decreased protein flexibility was observed [97-99].

In turn, Cristóvoã et al. [98] an immobilized a commercial laccase on green coconut fibers and verified the ability of the enzyme to decolorize different textile dyes. Decolorization is limited by the concentration of a mediator such as 1-hydroxybenzitriazole (HBT) and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) [97,98]. The addition of ABTS during dye decolorization by laccase immobilized on green coconut fiber significantly increased enzyme activity [98]. The same effect was observed during decolorization carried out by laccase entrapped in the hydrophobic Sol-gel [97]. The desired properties of immobilized laccases such as improved characteristics, stability and reusability obtained after they are immobilized on different carriers show the suitability of these biocatalysts for continuous treatment of different industrial effluents.

Some authors demonstrated that immobilized laccase has potential applications in dyestuff treatment. Laccase encapsulation in Cu-alginate gels was active at different conditions of pH, temperature and ionic strength, and was able to decolorize the carcinogenic dyes Trypan Blue, Bromothymol Blue and Coomassie Brilliant blue R with yields close to 90% without mediator addition [100]. The Cu-alginate derivatives retained more than 70% of catalytic activity for at least 430 h of continuous

use. The decolorization of synthetic dyes by laccase from Cotylidia pannosa grown in submerged culture and used to investigate its ability to decolorize synthetic dyes showed that the fungus as well as the crude laccase preparation decolorized the synthetic dyes such as Congo red, bromophenol blue and coomassie brilliant blue R-250 to different extent. A decolorization efficiency of 94% by fungal biomass and 40% by crude laccase was observed for congo red [101].

A laccase (Lacps1) produced by Pycnoporus sanguineus RP15 grown in wheat bran and corncob under solid-state fermentation was purified and characterized and the potential of the pure Lacps1 and the crude culture extract for synthetic dye decolorization was evaluated. The data suggested good potential for treatment of industrial dye-containing effluents [96]. The maximum decolorization percentages of bromophenol blue (BPB), remazol brilliant blue R and reactive blue 4 (RB4), at 25 or 40 ″C without redox mediators, reached 90%, 80% and 60%, respectively, using either pure and free Lacps1 or the crude extract.

The laccase from *Trametes* sp. MA-X01 is one of the promising candidates for the decolorization of textile wastewater containing various dyes, without redox mediators, the free enzyme had a good ability to degrade different kinds of dyes, including azo, heterocyclic and triphenylmethane dyes [102]. The characteristics of that laccase from the strain Trametes sp. MA-X01were studied. The dye decolorization also researched to determine the degrading capability of laccase produced from *Pleurotus eryngii*, methyl orange, tartrazine, reactive red 2 and reactive black dyes were treated with this enzyme (SSB) [103]. The highest decolorization was performed with methyl orange as 43 ± 2.8% after 5 min of treatment among them.

Purified free laccase from *Coprinus comatus* showed 49.3% decolorizing activity against remazol brilliant blue R (RBBR) and 41.6% decolorizing activity against Poly R-478 after 12 h incubation [104]. MGO-laccase were utilized in the decolorization of dye solutions and the decolorization rate of crystal violet (CV), malachite green (MG), and brilliant green (BG) reached 94.7% of CV, 95.6% of MG, and 91.4% of BG respectively. The experimental results indicated the MGO-laccase nanomaterials had a good catalysis ability to decolorize dyes in aqueous solution [105]. The immobilized enzyme in the developed scalable bioreactor system showed high efficiency in degrading various synthetic dyes under non-buffered conditions, in particular the indigoid dye Indigo Carmine [105].

Due to high efficiency and low-cost degradation of the pollutant, laccases are frequently used for the treatment of contaminated environments [85]. Given its environmental impacts, it is important to remove pesticides and herbicides compounds from wastewater [109]. Data showed that the laccase extracted from *Trametes versicolor* with an appropriate mediator promoted the degradations of five selected pesticides including atrazine, chlorothalonil, isoproturon, pyrimethanil and chlorpyrifos. Among them, the optimizing mediators were HBT for pesticide atrazine. Its degradation rate was up to 75.0% with HBT as a mediator. But with other mediators, the rates were found to be ranging from 21.7% to 38.9%. For isoproturon and pyrimethanil, the best mediators were found to be acetosyringone, ABTS, HBT and violuric acid. Syringaldehyde and vanillin were the best mediators for chlorpyrifos only. As the results shown, the mediators play important roles in the degradation of the pesticides by laccase catalysis except for chlorothalonil. The degradation of isoproturon and pyrimethanil reached above 60% in the presence of a proper mediator after 6 and 10 h incubation, respectively. However, it took 2 days for other selected pesticides to reach the similar degradation (60%).

Isoproturon belongs to type of phenylurea herbicide and produces a carcinogenic metabolates, has negatively affects to the environment [109]. The data demonstrated that the degradation rates of isoproturon and pyrimethanil were higher than other three pesticides. With violuric acid as a mediator, the decline rates of isoproturon and pyrimethanil were approximately 98% within 24h. The degradation of atrazine, chlorothalonil, and chlorpyrifos ranged from 70.4% to 91.6% (8 days) with an appropriate mediator. However, without any mediator, the degradation rate of chlorothalonil was 78.6%. Similarly, for other four pesticides, the rates were from 6.1% to 38.9% [108]. The kinetic parameters of reactions catalyzed by laccase immobilized on CMMC indicated less affinity for the substrate than that of the free enzyme. Nevertheless, the immobilized enzyme during 12 h utilized 78% and 84% of phenol and p-chlorophenol, respectively [85]. A similar high efficiency of degradation was observed by Wang et al. [86] and Xu et al. [87]. Laccase immobilized on magnetic Cu2+-chelated silica support removed pentachlorophenol with 82.9% efficiency [86],

whereas conjugation of the enzyme onto the surface of polyacrylonitrile electrospun fibrous membrane resulted in 87% efficiency of 2,4,6-trichlorophenol removal in four hours [87]. Using the aminobutyl-based laccase-SBA-15, 82%, 73%, and 55% conversion of naphthalene, phenanthrene and anthracene, respectively, were achieved after 48 h, very close to the values obtained with free laccase under the same reaction conditions [79].

Pharmaceutical compounds have shown toxic impact on aquatic ecosystems, specially related to endocrine disruption and reproductive disorders, and their bioaccumulation can also create toxic effects in humans [110]. These compounds as well as their by-products have been detected in both surface water and groundwater [110-113].

Researchers have used laccases in different forms for the degradation of a wide spectrum of contaminants particularly EOPs. The efficiency of the enzyme has been tested: (a) in a free form, (b) with co-substrates to promote the reaction and raise the redox potential as a "laccase-mediator system"(c) and in an immobilized. Most frequently types of micropollutants detected in water supplies are industrial pollutants [114] and pharmaceutical compounds such as antibiotics, analgesics and anti-inflammatory drugs [115] since WWTPs are not well-designed to remove harmful pollutants present at trace concentrations [116]. Diclofenac sodium (DFC), 5,7-Diiodo-8-hydroxyquinoline (DHQ), b -Naphthol (b-NP) and 2,4-dichlorophenol (2,4-DCP) belong to a non-steroidal anti-inflammatory drugs, prescribed as antipyretic analgesic [23] are classified as a harmful environmental pollutant because of their toxicity and biomagnification in the food chain [117]. Due to its extensive use, DCF has been found in several water supplies, even in groundwater at concentrations between 3.6 and 580 ng [118, 119]. Some authors studied the biocatalytic ability of free laccases from filtered culture supernatant of Pycnoporus sanguineus without mediators and under mild reaction conditions. This 100 U L 1 laccase cocktail removed 50% Diclofenac, 97% b-Naphthol and 71% 2,4 Dichlorophenol within 8 h of reaction and 78% for 5,7-Diiodo-8-hydroxyquinoline within 3.5 h; at initial concentrations of 10 mg and at 25˚C. Furthermore, this enzyme cocktail also removed in excess of 53% all tested compounds in a real groundwater sample from northwestern Mexico [120, 121].

The biotransformation of ritalinic acid by laccase was investigated for the first time. Free and immobilized enzymes were applied both with and without redox mediators. The results showed that free and immobilized enzymes alone did not transform ritalinic acid. The effective transformation of ritalinic acid was observed only in TEMPO-mediated systems; however, laccase activity in these systems was reduced significantly. The most effective enzyme was the free laccase of *T. versicolor* 159. Immobilization onto nanoparticles caused an increase in transformation efficiency only for the laccase of *C. unicolor* 303 [122].

Magnetic cross-linked enzyme aggregates (M-CLEAs) were prepared for Cerrena laccase and used in antibiotic treatment. Of the seven antibiotics were examined in some study, Cerrena laccase M-CLEAs were most effective in degradation of tetracycline (TC) and oxytetracycline (OTC), followed by ampicillin, sulfamethoxazole and erythromycin. The redox mediator ABTS was not able to improve efficiencies of degradation of TC and OTC. Cerrena laccase at 40 U/mL eliminated 100 μg/mL TC at pH 6 and 25 °C in 48 h in the absence of a redox mediator, with over 80% degradation occurring within the first 12 h. Laccase treatment also significantly suppressed the antimicrobial activity of TC and OTC [23]**.**

Conclusion

Because of their specific nature, laccases are receiving much attention from researchers around the globe. The interest in utilizing laccases for biotechnological applications has increased rapidly since the discovery of these enzymes potential application in emerging technologies including selective delignification in pulp bleaching, conversion of lignocellulosic materials into feed and biofuel, and treatment of environmental pollutants and toxicants generated in various industrial processes. Therefore, laccases have been widely studied for various applications, including the functionalization of lignocellulosic materials, wood fiber modification, and the remediation of soil and contaminated effluents as well as their use in biosensors. However, much more research is required to make use of laccases to protect environment and for other industrial applications.

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60 Lignin-Modified Enzymes Synthesis, Immobilization, and Application for Bioremediation.

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