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REVIEW PAPER

Applications and mechanisms of free and immobilized laccase in detoxification of phenolic compounds - A review

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Abstract−Phenolic compounds are present in different concentrations in the effluent from numerous industrial and agricultural activities. These compounds are harmful to living organisms due to their high toxicity, hence indicating a serious environmental concern. Although conventional methods such as chemical, physical, and physicochemical procedures have been widely used for treating phenol-contaminated wastewaters, they are not useful owing to some shortcomings. Compared to conventional procedures, much attention has been recently devoted to enzymatic methods because of high catalytic efficiency, mild operating conditions and environmentally friendly feature. Among various enzymes, laccases have demonstrated a superior potential for removing phenolic contaminants. Thus, this review summarizes the up-to-date literature on the use of free and immobilized laccases from different microbial source in the degradation and remediation of phenolic pollutants in batch processes and continuous reactors. In general, examples through the review approve that free laccases as well as immobilized laccases onto inorganic, organic (natural or synthetic) and hybrid supports show excellent performance in the remediation of phenolic compounds from wastewater. In contrast to immobilized laccases, free laccases suffer from high prices, low operative stability, and inability to recover and reuse in their native forms. Moreover, the possible mechanisms associated with oxidation of phenolic compounds by the laccase-catalyzed systems are assessed.

Keywords: Phenolic Compounds, Laccase, Detoxification, Immobilization, Biodegradation, Enzyme

INTRODUCTION

Phenol and its derivatives are aromatic organic molecules comprised of hydroxyl groups bonded to the phenyl ring. These aromatic compounds are essential for the production of resins, polycarbonates, detergents, adhesives, polyamides and various pharmaceutical drugs [1]. Furthermore, a great variety of pesticides utilized as algicides, bactericides, herbicides, fungicides, molluscicides, and insecticides contain phenolic compounds [2]. Notably, some phenolic compounds are commonly used at industrial level, for example, *p*-nitrophenol, used in the preparation of analgesics, dyes, pesticides as well as in the processing of leather; naphthol, used for the production of dyes, plastics, and rubbers; and chlorophenols, used as biocides [3-5]. Notwithstanding these applications, the presence of phenolic compounds in industrial effluents poses serious risks to water reservoirs and soils because of their toxicity and persistence in the environment [6]. Therefore, remediation of phenol-contaminated wastewaters before discharge into the environment is a critical issue [7]. In this context, there are several conventional methods for treating phenolic wastewaters such as chemical, physical, and physicochemical procedures, but they are costly and produce secondary hazardous pollutants [8-13]. Compared to conventional treatment technologies, biological treatment has attracted more attention because it has been revealed to be cost-effective and versatile, resulting in the complete mineralization of phenol [6]. In such a treatment, degradation of phenols has been conducted using bacteria, microbes, yeast, fungi, algae and/or enzymes [14-19]. Microbial treatment has been widely employed for degrading phenolic compounds to innocuous end products; however, it has shown serious shortcomings, including expensive maintenance of microbial culture, the problem with the survival of cells in the environment, completion of the indigenous population, and metabolic inhibition [20]. This discloses the necessity for the development of an alternative method for the remediation of phenolic wastewaters. Enzymatic treatment represents a powerful alternative over the conventional methods to the removal of phenolic contaminants from industrial effluents [21]. The main advantages of enzymatic treatment are: enzymes remain active at very low and high concentrations of pollutants in a wide range of pH, temperature, and salinity; do not need biomass acclimation; do not produce toxic waste [20]. Other exciting property of enzymes is that they can remove the pollutants within a very short time in comparison with microbial treatment [22]. Furthermore, enzymes are also more tolerant towards compounds and minerals, which may cause tox-

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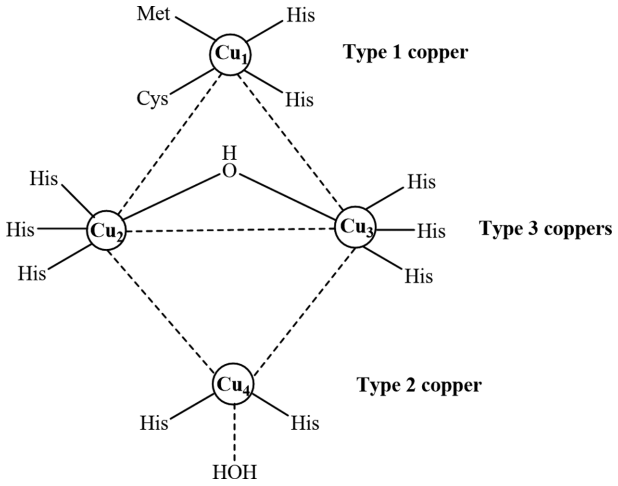
icity to the microbes [23]. Taking into account these potential advantages, several oxidases (polyphenol oxidases, tyrosinases and laccases) and peroxidase (horseradish peroxidase, manganese peroxidase and lignin peroxidase) enzymes have been applied for biodegradation and transformation of phenol derivatives [24-28]. Among various enzymes, laccases demonstrated a superior potential for removing phenolic contaminants. Taking advantage of their catalytic potentials, remarkable advances have been made in the engineering of laccases to produce suitable biocatalysts in environmental applications. Recently, some reviews have been reported on the application of laccases as biocatalyst for polymerization, elimination, mitigation or biodegradation of hazardous contaminants in the environment [29-31]. To our knowledge, there is no review focused on the application and mechanisms of free and immobilized laccase in detoxification of phenolic compounds. The main purposes of this review are to (a) summarize various advancements in utilizing free and immobilized laccases for the removal of phenolic contaminants from wastewaters, (b) evaluate the possible mechanisms associated with laccase-catalyzed detoxification of phenolic compounds, (c) evaluate challenges of the free laccases on phenols bioremediation, and (c) help in better understanding of laccases immobilization for phenols mitigation.

SOURCES AND HEALTH EFFECTS OF PHENOLIC COMPOUNDS

Phenolic compounds are classified as ubiquitous environmental pollutants which are discharged from various industrial processes including petrochemical industry, resins, synthetic rubber, plastics, paper, oil refineries and pharmaceutical industries [9,32-33]. Additionally, phenolic compounds also enter the environment from natural sources, such as plants, aquatic organisms, and animals that are considered as a secondary source of natural phenols [4]. The discharge of phenol-contaminated wastewaters to the environment without appropriate treatment is harmful to humans, plants and animals owing to their high toxicity [34]. Notably, these aromatic contaminants possess multiple toxic effects of both acute and chronic exposures. For example, chronic exposure to these compounds can cause numerous health problems such as headaches, diarrhea, skin irritation, anorexia, and gastrointestinal discomfort [9]. On the other hand, acute exposure to phenol is fatal to humans because it leads to malfunctioning of the heart, kidneys, liver and nervous system [14]. Thus, it is vital to develop an eco-friendly or green technology for the removal of phenolic compounds.

GENERAL PROPERTIES OF LACCASES

Laccases (benzenediol : oxygen oxidoreductase, EC 1.10.3.2), known as multicopper oxidases, are one of the oldest types of enzymes which were discovered by Yoshida in 1883 [35]. They are detected in higher plants, fungi, several bacteria, and insects. Laccases include four copper atoms classified using UV/visible and electronic paramagnetic resonance (EPR) spectroscopy into three types (Scheme 1): Type 1 (T_1) or blue site, Type 2 (T_2) or normal site, and Type 3 (T_3) or binuclear site. Type 1 Cu can be distinguished *via* strong EPR signals and an intense absorption at 600 nm caused by charge transfer from the cysteine sulfur to the copper atom. This charge transfer gives the blue color to the laccase. Type 2 Cu is EPR detectable but they do not indicate any absorption in the visible spectra. Type 3 coppers are comprised of a pair of Cu atoms in a binuclear conformation that is characterized by a weak absorbance at 330 nm and the absence of EPR signals [36].

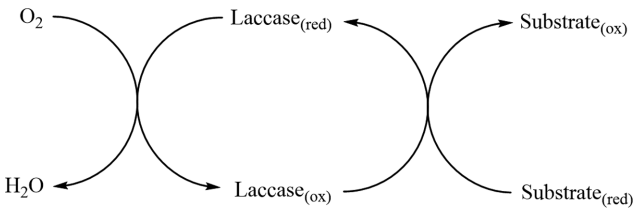


Scheme 1. Schematic representation of laccase indicating the orientation of copper atoms [36].

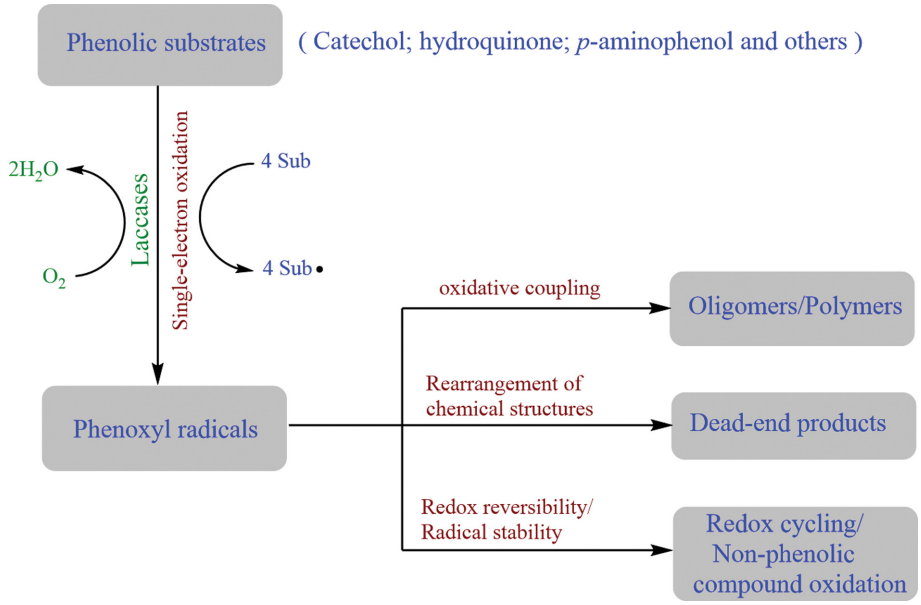
LACCASE-CATALYZED REACTIONS

Over the last few decades, the use of laccases has drawn increasing attention because of their features and advantages: laccases are easily available; they can catalyze the oxidation of numerous substrates including phenolic and non-phenolic compounds in aqueous solvent under mild reaction conditions while reducing O_2 to H_2O (Scheme 2) [37]. As a result, the capability of the aforementioned enzymes for oxidizing a wide range of phenolic compounds has stimulated the development of laccase-catalyzed processes in wastewater treatment, pulping, food preparation, textile manufacturing, bioremediation, and organic synthesis [38-44].

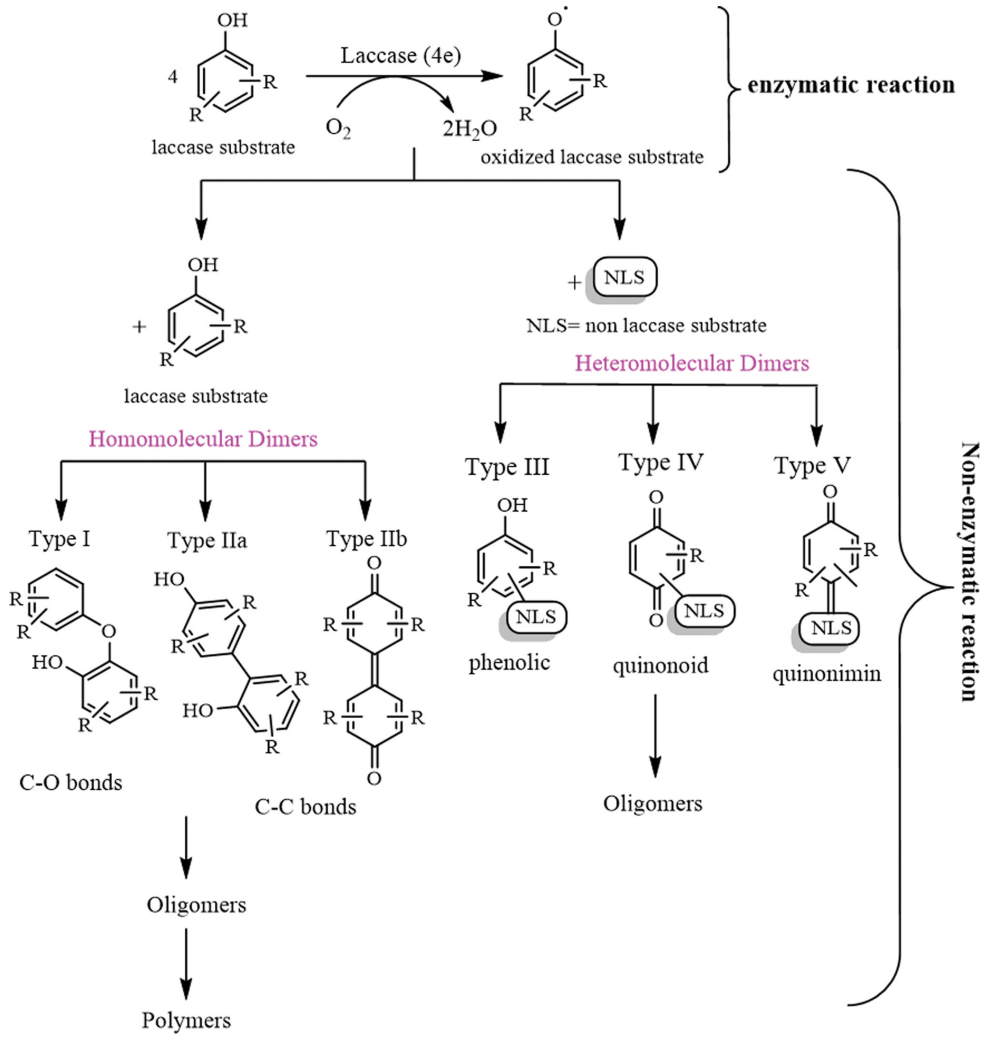
The laccase-catalyzed oxidation process involves three major steps [45]: (i) Type 1 Cu is reduced by accepting electrons from the reducing substrate; (ii) electrons are subsequently transferred from Type 1 Cu to the tri-nuclear T_2/T_3 cluster; (iii) finally, electrons reduce molecular oxygen to water at the tri-nuclear T_2/T_3 cluster.



Scheme 2. Scheme of laccase-catalyzed redox mechanism for substrate oxidation.



Scheme 3. Schematic representation of oxidation of phenolic substrates using laccases.



Scheme 4. Formation of homomolecular and heteromolecular dimers [47].

LACCASE-CATALYZED BIODEGRADATION OF PHENOLIC COMPOUNDS

Laccases have mostly been used for oxidizing substrates with redox potentials lower than their own (range, 400-800 mV); therefore, phenols due to low redox potentials are considered as the most common substrates of these enzymes. Oxidation of phenolic substrates with laccase is demonstrated in Scheme 3. As shown, laccase initially abstracts one electron and one proton and generates phenoxy radicals. Then, the resulting phenoxy radicals undergo oxidative coupling with the formation of oligomers and polymers, or promote the radical rearrangement with formation of dead-end products. Additionally, reversibility of the oxidation dependent on the phenoxyl radical stability can be also occurring [46].

On the other hand, Catherine et al. have reported that laccases can catalyze homo- or hetero-molecular coupling reactions (Scheme 4) [47]. During homomolecular coupling reactions, homomolecular dimers (C-O and C-C dimers) are obtained *via* nucleophilic attack of phenoxyl radicals with laccase substrate. Ultimately, this coupling results in the formation of oligomers or polymers. But, in hetero-molecular coupling reactions, phenoxyl radicals are coupled with non-laccase substrates, thus forming new heteromolecular dimers.

In summary, laccases promote only the formation of phenoxy radicals and further steps, including dimerization, oligomerization or polymerization are nonenzymatic reactions (Scheme 4).

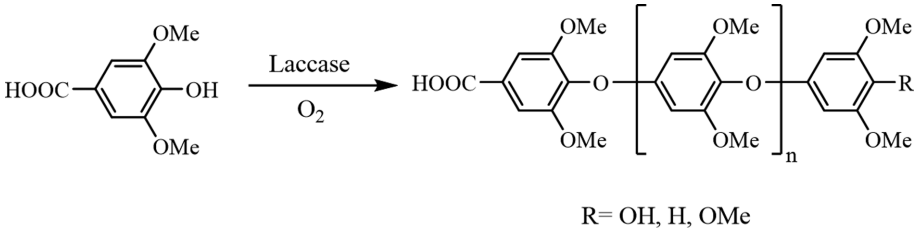
LACCASES IN FREE STATE FOR THE REMOVAL OF PHENOLIC COMPOUNDS

As stated, laccases oxidize phenolic compounds into radical intermediates, which can undergo self-coupling reactions. This leads to the formation of less toxic insoluble compounds such as dimers, oligomers, or polymers, which are easily removed by centrifugation/filtration. The following instances in this section display the homogeneous laccase-catalyzed reactions for the remediation of phenolic pollutants in batch processes and continuous reactors. A study of such was accompanied in 2006 by Vainiotalo et al. [48], who utilized laccase produced by *Trametes versicolor* for the polymerization of two phenolic compounds including syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) and 2,6-dimethylphenol. The initial concentration of the tested compounds and laccase was 2.5 mmol and 2.95 mg, respectively. After 24 h, the obtained polymers were separated *via* filtration and identified by two different mass spectrometric techniques comprising matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) MS with collision-induced dissociation (CID) experiments. Notably, typical yields were 40-50% for polymers of 2,6-dimethylphenol and 54-58% for polymers of syringic acid. Based on the data analysis of mass spectrometric techniques, the prepared polymers of syringic acid were categorized into three forms: The two main forms were the same except for the end groups: one has a hydroxyl group, but the other has hydrogen instead. Nevertheless, one other form was achieved; it contains a methoxy group instead of a hydroxyl group (Scheme 5).

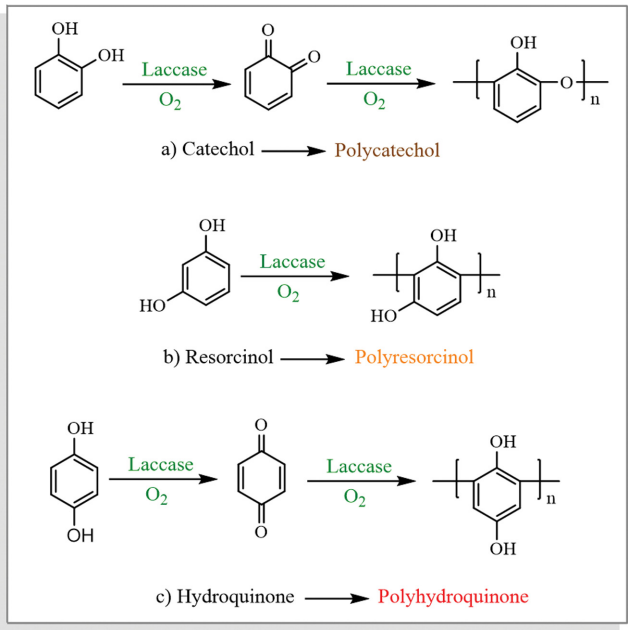
Researchers have also investigated the mechanism of laccase-catalyzed polymerization of syringic acid. This mechanism begins with the formation of phenoxy radicals, which further undergo coupling with each other to yield a quinoid-type intermediate. This proposed mechanism approves that the phenolic group is modified during the oxidation reaction.

Zelić and co-workers have reported the *Trametes versicolor* laccase-catalyzed oxidation of phenolic compounds such as catechol and L-3,4-dihydroxyphenylalanine (L-DOPA) in continuously operated microreactors [49]. Notably, the 41.3% removal of catechol was accomplished at the optimal conditions (retention time of 72 s, inlet oxygen concentration of 0.271 mmol/dm^3), but almost the same removal of L-DOPA (45.0%) was attained using a higher inlet oxygen concentration (0.544 mmol/dm^3). The researchers calculated the oxidation rates to better describe the performance of microreactor in the oxidation of phenolic compounds; catechol oxidation rates were 18~67-times faster than the case in a macroreactor. The catechol oxidation rate in microreactor experiments was also two times faster than value achieved in a cuvette. Therefore, these findings clearly verify that microreactors can be applied as efficient reaction systems for the laccase-catalyzed oxidation of phenolic compounds.

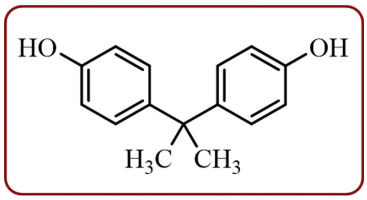
In another study, Sun et al. [50] investigated the oxidative polymerization of three phenolic compounds including catechol, resorcinol, and hydroquinone by laccase under mild conditions. This enzymatic polymerization was carried out with higher efficiency and selectivity compared to conventional methods utilizing chemical catalysts. After the polymerization, the resulting colored polyphenols were separated *via* filtration and characterized by UV-Vis and FT-IR techniques. These products are of great importance in industry for the dyeing of the fabrics made of natural or synthetic fibers. On the other hand, based on the results presented in this work, the investigators have proposed that phenolic compounds initially generate quinone-intermediates. Then, these intermediates form covalent bonds *via* further oxidation. Ultimately, catechol units and both resorcinol and hydroquinone units are linked



Scheme 5. Structures of different products achieved from syringic acid [48].



Scheme 6. Proposed mechanism and chemical structure of laccase-catalyzed polyphenols [50].

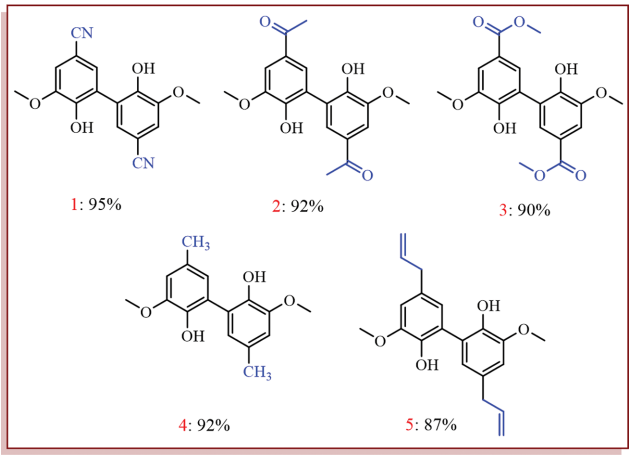


Scheme 7. The structure of bisphenol A.

together with C-O and C-C bonds, respectively (Scheme 6).

Beyond the works presented above, Asadgol et al. [51] introduced the isolated laccase from *Paraconiothyrium variabile* (PvL) as an efficient biocatalyst for elimination of phenol and bisphenol A (Scheme 7). In this study, the authors investigated the effect of different parameters including laccase activity, pH, and temperature on the elimination of the mentioned phenolic pollutants. It was found that maximum of removal percent of both phenolic contaminants (96.3% of phenol and 88.3% of bisphenol A) was achieved in the presence of PvL (5 U/mL) at pH 5 and temperature of 50 °C after 30 min treatment.

A green and easy to perform method was used by Llevot et al.

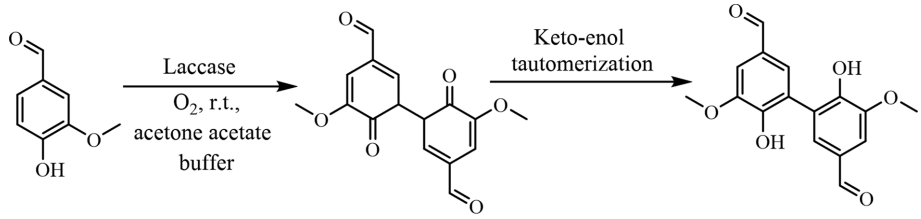


Scheme 9. The dimers obtained of laccase-catalyzed oxidative coupling of 1) 4-hydroxy-3-methoxybenzonitrile, 2) acetovanillon, 3) methylvanillate, 4) 2-methoxy-4-methyl phenol and 5) eugenol.

[52] to prepare divanillin *via* vanillin dimerization in the presence of laccase as a biocatalyst (Scheme 8). The main advantages of this process are that (i) the divanillin synthesis can be run at room ambient using molecular oxygen as an environmentally benign oxidant, (ii) the utilized (co)solvent, 10% of acetone, displays a low toxicity, (iii) divanillin was obtained in high yield (95%) by an easy work-up procedure because the solvent conditions make the vanillin soluble while the resulting divanillin precipitates, (iv) a low enzyme loading was used and it could be recycled several times, improving the process economics.

Furthermore, this dimerization procedure was applied to some phenolic substrates such as 4-hydroxy-3-methoxybenzonitrile, methyl vanillate, 4-methyl-2-methoxyphenol, 2,6-dimethoxy-phenol and eugenol (Scheme 9). It can be seen that the corresponding dimers were attained with yields over 85%, without any purification.

Bettin et al. recently evaluated the potential of *Pleurotus sajor-caju* PS-2001 and its enzymes in stirred-tank reactor (STR) and internal-loop airlift reactor (ILAR) for treatment of wastewaters containing phenolic compounds. Note that *P. sajor-caju* PS-2001 is capable of generating different oxidative enzymes such as laccases and peroxidases in growing media comprised of phenolic compounds. In a stirred-tank reactor (STR), this fungus can remove 77, 82, 92 and 36% of phenol at initial concentrations of 1.0, 2.0, 3.0 and 4.0 mmol L^{-1}, respectively. Although this fungus generates different enzymes, phenol removal is related to the activity of lac-



Scheme 8. Dimerization of vanillin (1.5 g) in acetone/acetate buffer 10/90 using laccase from *Trametes versicolor* (20 U, 12.4 mg) under O_2 at r.t. for 24 h [58].

cases. On the other hand, with an internal-loop airlift reactor (ILAR), removal of 70, 76, 82, 77 and 82% was achieved for phenol concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 mmol L^{-1}, respectively. In summary, based on the results obtained, the authors stated that the removal of phenol is the same in both bioreactors, but in ILAR, the activity of laccases is much higher and the growth is faster. ILAR also show relatively low power demand and operational costs. Therefore, the use of ILAR for growing *Pleurotus sajor-caju* PS-2001 in the treatment of phenolic effluents is more promising [53].

IMMOBILIZED LACCASE FOR THE DEGRADATION OF PHENOLIC COMPOUNDS

In the context of economic development based on bio-technology the application of enzymes in industrial processes is of very great interest. However, free enzymes have some limitations for use in non-biological applications, because the free enzymes are efficient catalysts, operating under mild conditions such as ambient temperature/environmental pressure, aquatic environment, physiological pH, and these may not be capable of maintaining stability under industrial conditions [54-56]. Therefore, for many industrial applications, enzyme immobilization technology is an ideal procedure for the preparation of effective and stable biocatalyst. The immobilization of enzymes allows the recovery of catalyst, easier product separation, recyclability, enhanced stability under harsh conditions, and ongoing use in enzymatic processes in especially in case of great volume of wastewaters, which will reduce the operational cost [57-63]. Many various carriers such as natural or synthetic, inorganic, or organic substances have been used for enzyme immobilization [64], that examples of these carriers are presented in continuance.

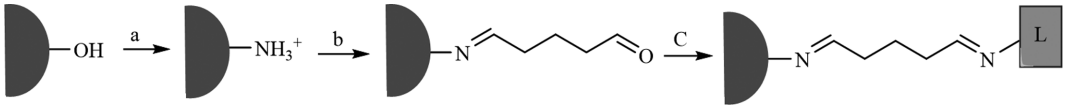
1. Immobilization of Laccases on Silica-based Supports and Detoxification of Phenolic Compounds

Silica and its derivatives are one of the most promising carrier materials for the immobilization of laccase because of its trustworthy chemical stability, biocompatibility, and reactivity with different coupling agents [65].

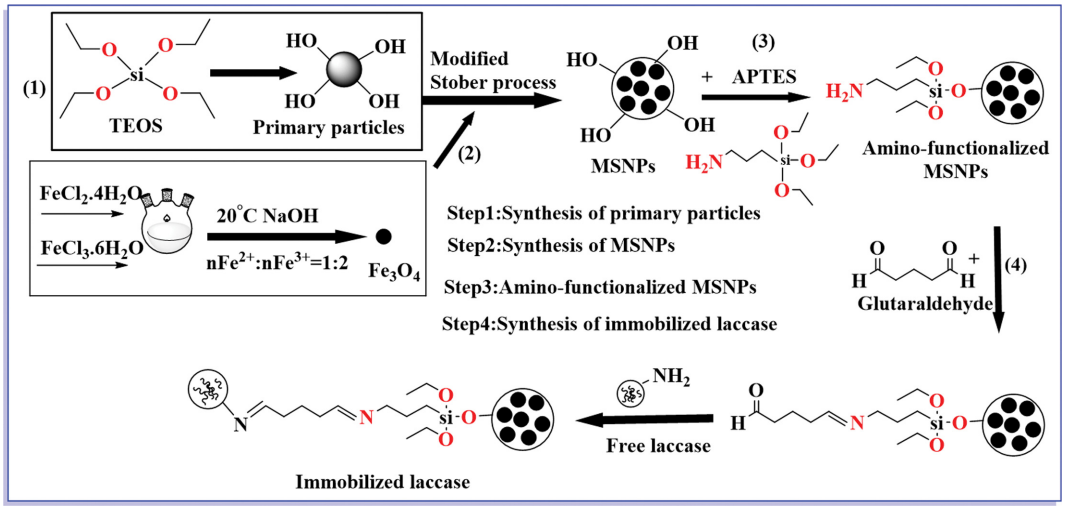
Salis and co-workers [66] immobilized laccase from *Pleurotus sajor-caju* on functionalized SBA-15 mesoporous silica *via* chemical adsorption and checked the modification through FT-IR spectroscopy, and they observed that the activity of the immobilized biocatalysts reached a maximum at L=217 kU g^{-1} SBA-15. They used the immobilized laccase for the oxidation of several phenolic compounds (protocatechuic acid, ferulic acid, sinapic acid and caffeic acid) present in olive mill wastewaters (OMWs). The biocatalyst was stable after ten reaction cycles, reaching a conversion of 84 mol%.

In another study, Shahgaldian and co-workers [67] reported a system based on silica nanoparticles chemically modified with a laccase from *Coriolopsis polyzona* (Scheme 10). The immobilized laccase has been shown to be effective for the degradation of 4,4′-isopropylidenediphenol (bisphenol A) in the aqueous buffered system. The developed procedure, based on LC-MS and GC-MS allowed the identification of the alteration products formed during the enzymatic reaction.

Guo and co-workers [68] immobilized laccase from *Aspergillus* on the core-shell structure of amino-functionalized magnetic silica nanoparticles (AF-MSNPs) *via* a chemical crosslinking method



Scheme 10. Schematic of the synthetic route to laccase-modified silica nanoparticles (a: Amino-functionalized silica nanoparticles, b: glutaraldehyde activation, c: enzyme anchoring; L: laccase) [67].



Scheme 11. Synthesis process of the immobilized laccase onto magnetic silica nanoparticles [68].

(Scheme 11). The activity recovery obtained from immobilized laccase was around 53.4±3.1% and the immobilized amount of laccase was 613.5±10.5 mg g^{-1} of settled carrier particles. Compared to the free laccase, the laccase immobilized showed better resistance to a broader pH and temperature value. Also, the application of the immobilized system for oxidation of the guaiacol as a phenolic lignin model compound and the radical polymerization between the phenoxy radicals was investigated, which displayed that using the immobilized laccase to catalyze lignin is a very promising technology for major applications in the wood industry.

Moreira and co-workers reported the immobilization of laccase from *T. versicolor* onto different types of silica-coated magnetic nanoparticles and non-magnetic nanoparticles, as well as their use as biocatalyst for removal of phenol. Several methods of enzyme immobilization were investigated based on ionic exchange and covalent bonding. The best activity yield was achieved by using laccase immobilized on silica-coated magnetic nanoparticles (2.66 U mg^{-1} NPs). Moreover, its use in the biotransformation of phenol was evaluated at different pH and about higher than 60% of phenol transformation was obtained at pH 6 for 24 h. The immobilized laccase is magnetically recoverable after four cycles of phenol oxidation [69].

Laccase from *Myceliophthora thermophila* was immobilized on epoxy-functionalized silica particles by Mohammadi and his team to remove phenol, *p*-chlorophenol and catechol. The effect of various parameters such as pH, temperature, and organic solvent on enzyme activity was determined for both immobilized and free enzymes by 2,2′-azinobis-(3-ethylbenzylthiozoline 6-sulfate) (ABTS) as substrate. V_{max} values were 10.0 and 1.6 μM min^{-1} while K_m values were 24.0 and 25.3 μM for free and immobilized laccase, respectively. The removal efficiency of catechol by immobilized laccase was about 95% after 2 h. The laccase catalyzed reactions proceed by the oxidation of phenols to the corresponding reactive radical, and phenolic radicals formed polymers by self-coupling reactions [59].

An interesting example of silica used as support for laccase immobilization is presented by Faramarzi et al. [70]. In this study, they investigated types of entrapped laccase in mesoporous silica including simply adsorbed, entrapped cross-linked enzyme (E-CLE), and cross-linked enzyme aggregate (E-CLEA) to explore their potential in phenol compounds removal (Scheme 12). The E-CLEAs displayed improved thermal and pH stability and activity maintenance in hydrophobic and hydrophilic solvents than free enzyme. Also, the immobilized biocatalyst showed good operational stability and reusability via retaining up to 79% of its initial activity after 20 cycles of successive reactions.

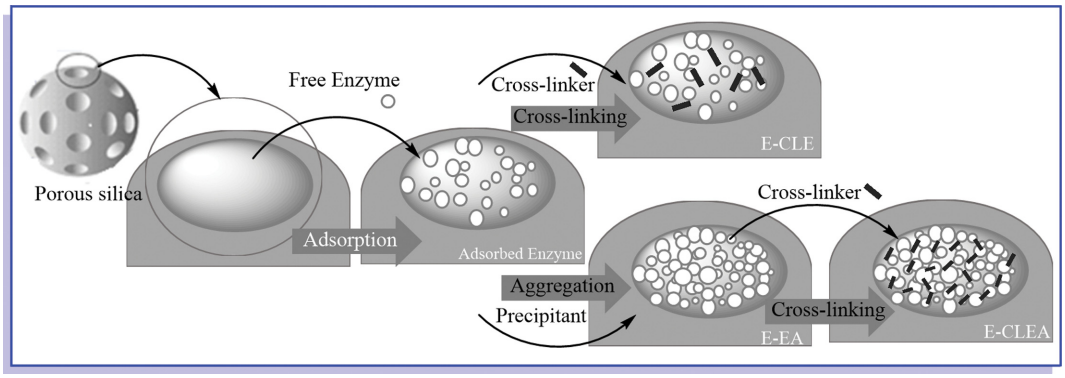
2. Natural Polymers for Laccases Immobilization and Removal of Phenolic Compounds

A wide variety of natural polymers have been used as support for laccase immobilization due to easy availability, inexpensive, and show thermal and mechanical resistance.

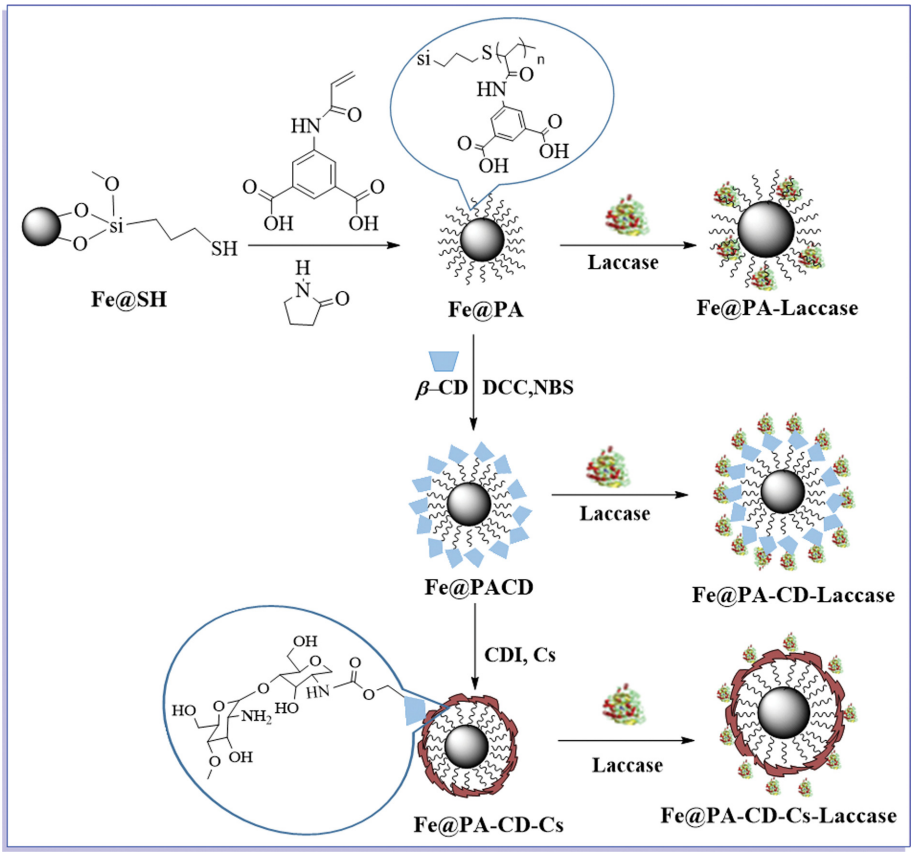
Aydemir and her group used chitosan-clay composite to immobilize laccase from *Trametes versicolor* by glutaraldehyde crosslinking for phenol removal. The immobilized enzyme displayed the maximal activity at pH 5.0. The K_m value of immobilized laccase (0.410 mM) was higher than that of free laccase (0.297 mM), which means that the immobilized laccase had a lower affinity for the substrate. The immobilized laccase was more refractory to pH changes and the maximum degradation of phenol was raised to 80% on 4 h contact time. Moreover, the initial activity of the immobilized laccase remained about 53-67% after ten cycles [71].

In another study, laccase was immobilized on Cu(II)-chelated chitosan nanoparticles through adsorption by Metin and his team, and was used for remove phenol from aqueous solution Cu (II)-chelated chitosan-graft-poly glycidyl methacrylate (PGMA) nanoparticles were prepared using poly ethylene imine (PEI), which is employed as both a spacer arm and metal chelator and used to immobilize laccase by coordination. The maximum laccase loading capacity of CHT-PGMA-PEI-Cu (II) NPs was determined as 65.75±2.51 mg g^{-1}. The immobilized enzyme showed improvement on thermal, pH and storage stability with a great performance for reusability than free enzyme, since it was able to keep 50±0.62% of its initial activity after eight cycles of continuous use. The K_m and V_{max} values of free and immobilized laccase were 0.055 mM, 0.070 mM, and 0.19 U/mg, 0.14 U/mg, respectively. More than 96% of compounds phenol was removed in aqueous samples with immobilized laccase in the presence of mediators ABTS or *N*-hydroxybenzotriazole (HBT); thus, the results showed that the immobilized laccase have great potential for industrial applications [72].

Three types of modified Fe_3O_4 magnetic nanoparticles (MNPs), including poly (amidoisophthalic acid) coated magnetite nanopar-



Scheme 12. Schematic of steps laccase immobilization in porous silica *via* E-EA, E-CLE and E-CLEA [70].



Scheme 13. Schematic representation of the preparation of Fe@PA-laccase, Fe@PA-CD-Laccase, and Fe@PA-CD-Cs-laccase [55].

ticles (Fe@PA), cyclodextrin (CD) anchored Fe@PA (Fe@PA-CD), and chitosan (Cs) coated Fe@PA-CD (Fe@PA-CD-Cs), were synthesized as support for laccase by Khoobi and co-workers (2015) [55] (Scheme 13). Laccase immobilization on these three modified magnetic nanoparticles was carried out through physical adsorption and was used for phenolic compound removal in the presence of HBT as mediator. The maximal and minimal loading capacity was obtained for Fe@PA and Fe@PA-CD-Cs, respectively. Fe@PA-CD-Cs-laccase displayed around 100% of the maximum activity at pH 4 and retained 70% of its initial activity at the temperature range of 15-55 °C, but the free laccase, Fe@PA-laccase, and Fe@PA-CD-laccase maintained 10%, 28%, and 33% of initial activity, respectively; thus Cs coated nanoparticles were more effective than non-coated. It seems that Fe@PA-CD-Cs could be a suitable support for immobilization of other enzymes in different industrial application, especially bio-removal of phenolic compounds [73].

3. Synthetic Polymer Immobilized Laccases for Mitigation of Phenolic Compounds

Several types of synthetic polymers have been employed as support for the immobilization of laccases. Arıca and co-workers immobilized laccase *via* adsorption on modified p(HEMA-g-GMA)-NH_2 films [74]. The immobilized enzyme improved temperature resistance and maintained pH stability. The amount of immobilized laccase of the fibrous polymer grafted films was determined as 139 μg/cm^2 films; also, the V_{max} and K_m values of laccase immobilized on the films were calculated to be 15.4 U/mg and 23 mM, respectively. The recovered activity of the immobilized laccase on the p(HEMA-g-GMA)-NH_2 films was about 71% compared to free enzyme. Finally, the immobilized laccase was operated for enzymatic oxidation of phenolic compounds, and about 72% of phenol, 81% of *p*-chlorophenoland and 58% of aniline were degraded in 10 h.

A wide-pore poly (vinyl alcohol) cryogel was used by Stanescu and co-workers to immobilize the laccase of *Trametes pubescens* [75]. The biocatalyst was used in the oxidation of polyhydroxy phenolic compounds (catechol, catechin, chlorogenic acids, and caffieic). The biocatalyst had been chosen for this study based on high activity toward phenols in an acid pH area (typical pH of apple juice), high stability in a wide range of temperature and good chemical and biological defiance. The amount of immobilized enzyme on the PVA cryogel was 5.2 mg g^{-1}, and, also, biocatalyst showed 40% activity after storing at 60 °C for 24 h when compared to free laccase.

Şenel et al. developed a novel synthetic polymer, which poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) [p(HEMA-coGMA)] cryogels were used for covalent immobilization of *Trametes versicolor* laccase by the substitution reaction between amino groups of the enzyme and epoxy groups of the cryogel matrix. The effect of various parameters such as temperature, pH, reaction time, and storage period on enzyme activity was inspected for both free and immobilized enzymes by model substrate (ABTS);

the cryogels were used for oxidative removal of phenols in wastewater. The K_m value for immobilized laccase (156.0 mM) was lower than that free laccase (165.1 mM), which can mean enzyme affinity for the substrate. On the other hand, the biocatalyst retained 82.5% of its original activity after six cycles of use [76].

4. Novel Carriers for Immobilization of Laccases and Phenolic Compound Removal

Modified magnetic nanoparticles and carbon nanoparticles have been used as supports for laccases and their application for oxidation of phenolic compounds have been investigated.

4-1. Modified Magnetic Nanoparticles as Support for Laccases Immobilization

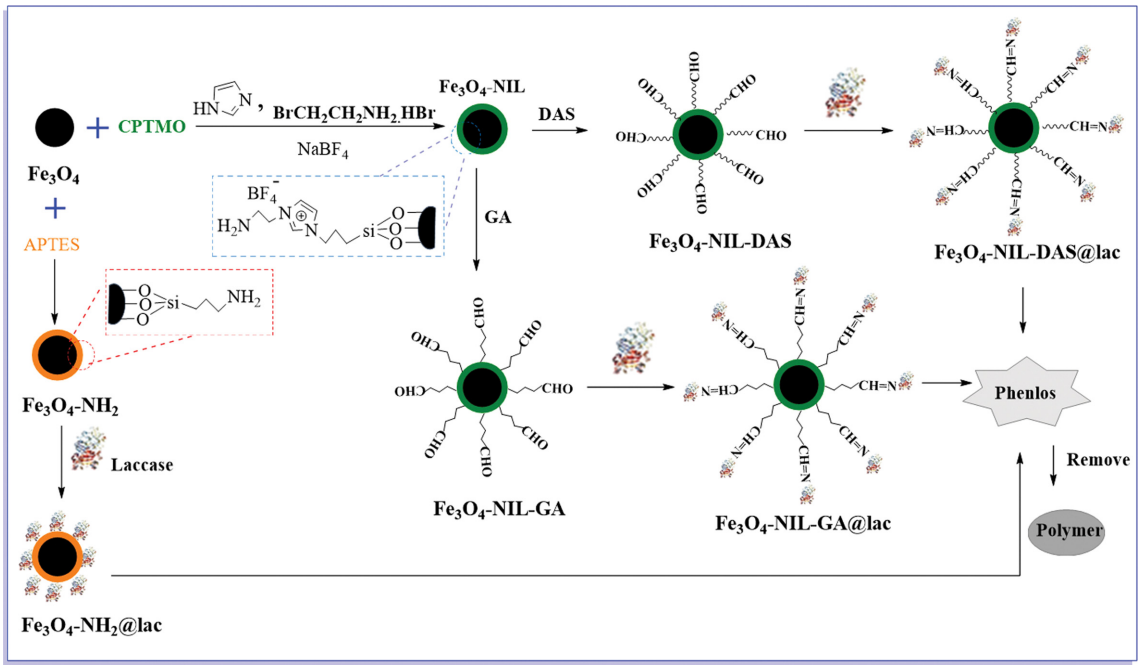
Hu et al. synthesized an efficient biocatalyst using Fe_3O_4 magnetic nanoparticles modified with the amino-functionalized ionic liquid [77]. The hybrid material (termed as Fe_3O_4-NIL) was combined with the organic polymer dialdehyde starch (DAS) *via* Schiff base reaction, and this organic macromolecule was used as a cross-linking agent to laccase immobilization (Scheme 14). Fe_3O_4-NIL-DAS and Fe_3O_4-NIL-DAS@lac were characterized by FTIR, TEM, SEM, EDS, TGA, XRD and etc. The immobilized laccase maintained 83.5% of initial activity after 30 days, also Fe_3O_4-NIL-DAS@lac displayed much better immobilization efficiency (85.8%) and storage stability. Moreover, Fe_3O_4-NIL-DAS@lac could effectively remove phenolic compounds; they observed that about 86.1% of phenol, 93.6% of 4-chlorophenol and 100% of 2,4-dichlorophenol were degraded.

Hu and co-workers immobilized laccase on materials of Institut Lavoisier frameworks (MILs) that are a series of metal-organic frameworks (MOFs) magnetic metal-organic framework (MOF), by the adsorption and covalent binding method (Scheme 15). The biocatalyst displayed satisfactory enzymatic properties, including good tolerance to high temperature and low pH, high activity recovery, organic solvent stability and storage stability. After the immobilization process, laccase was capable of retaining 89% of its initial activity after 28 days, and when the ambient temperature was 85 °C, the immobilized laccase displayed 49.1% remaining activity even after 6 h preservation. Finally, application of the biocatalyst for 2,4-dichlorophenol removal was investigated. Free radicals are created by the oxidation reaction of 2,4-dichlorophenol, which these radicals could be coupled together to form a polymer. The removal efficiency of 2,4-dichlorophenol reached 87%, which was due to the high adsorption capacity and the excellent degradation ability of Fe_3O_4-NH_2@MIL 101(Cr). After the reaction was complete, the immobilized laccase was easily separated from the solution by a magnet and was used in the next cycle. Hence, the immobilized laccase had high potential in wastewater treatment [78].

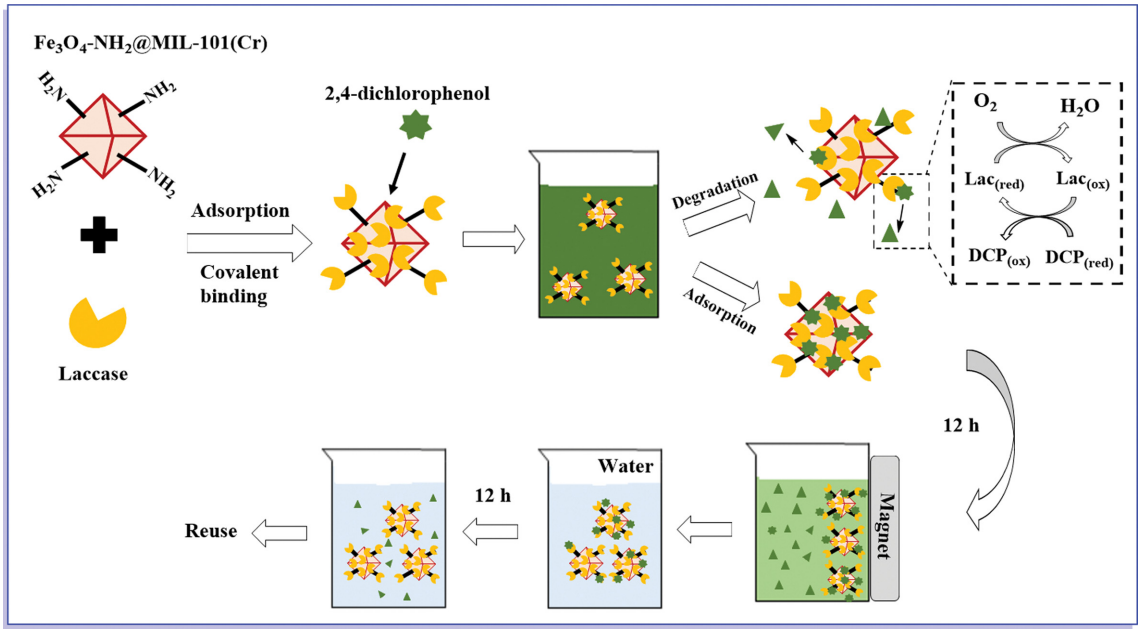
4-2. Carbon Nanomaterials as Support for Laccases Immobilization

Carbon nanoparticles have emerged as promising candidates for enzyme immobilization. In this context, Zeng and co-workers reported laccase immobilization on bimodal carbon-based mesoporous magnetic composites (CMMC) for the removal of phenol, with an investigation on the adsorption effects of the support [32]. They obtained a large adsorption capacity (491.7 mg g^{-1}) and excellent activity recovery (91.0%) of the immobilized enzymes than the free laccase. Also, thermal and pH stability was increased significantly. By employing the laccase immobilized, 78% and 84% of phenol and *p*-chlorophenol were removed at the end of the reaction, respectively.

Zhang and co-workers investigated the laccase immobilized on different carbon nanomaterials, fullerene (C_{60}), multi-walled carbon nanotubes (MWNTs), oxidized-MWNTs (O-MWNTs), and graphene oxide (GO) [75]. The loading capacity was lowest for C_{60} and highest for O-MWNTs. After immobilization, the residual activity of laccase decreased in the following order: GO>MWNTs>



Scheme 14. Schematic illustration of ionic liquid modified magnetic carriers for laccase immobilization [77].



Scheme 15. Preparation process of laccase immobilization on Fe_3O_4-NH_2@MIL-101(Cr) [78].

O-MWNTs>C_{60}. The nanoparticle-immobilized laccase was used for the removal of bisphenol and catechol substrates, that they had significantly reduced reaction rates than free laccase. These results display that carbon nanoparticle-mediated effects on substrate availability and dissemination limitation need to be carefully considered, which may lead to increased reaction times, high economic costs and low efficiency.

CONCLUSIONS

This review reveals the feasibility and effectiveness of employing laccases as biocatalyst for the treatment of effluents containing phenolic compounds. The use of laccases in this process exhibits some favorable features such as high catalytic efficiency, mild operating conditions, green and eco-friendly, and lack of secondary pollution generation. Also, laccases are capable of oxidizing phenolic compounds into radical intermediates. Then, these free radicals generate less toxic insoluble compounds, which can be simply eliminated by centrifugation/filtration. Nevertheless, laccases cannot be used at industrial level due to low operational stability and poor reusability, resulting in high prices. To overcome these drawbacks, researchers have proposed the immobilization of laccases on a solid support as a useful strategy. The enzyme immobilization technology reduces application costs by enabling recovery and recyclability of the enzyme, increasing the enzyme's utilization in industrial processes. Moreover, immobilization also improves the stability of the enzymes and develops their range of operation such as pH and temperature. In the current review, various literature surveys revealed that immobilized laccases onto various supports illustrated high catalytic performance for the detoxification of phenols-contaminated wastewater as well as elevated recovery and recyclability of the enzymes. Among the applied supports for immobilization of laccases, iron oxide magnetic nanoparticles have received particular attention due to their large surface area, controlled pore diameter, good stability in a wide range of temperature and pH values, easy surface modification for chemical attachment protocols and easy separation from the reaction mixture by an external magnet. Therefore, for future investigations, more attention must be paid to the immobilization of enzymes onto iron oxide magnetic nanoparticles to create stable biocatalysts with high enzyme loading together with easier separation and reusability for the detoxification of phenolic compounds.

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