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Ligninolytic Fungal Laccases and Their Biotechnological Applications

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Abstract Lignin is amorphous in nature, lacks stereoregularity, and is not susceptible to hydrolytic attack. Despite its resistant nature, it is however degraded by various microorganisms, particularly, white-rot fungi. Such fungi are capable of extracellular production of lignin peroxidase, manganese peroxidase, and laccase, the three major enzymes associated with ligninolysis. Though all white-rot fungi do not produce all the three enzymes, laccase occupies an important place in ligninolysis. Laccase belongs to a diverse group of enzymes called oxidoreductases and is also known as benzenediol: oxygen oxidoreductase. They have low substrate specificity. The copper-containing enzyme laccase has been detected in a variety of organisms such as bacteria, fungi, plants, and insects. Mostly, these are extracellular proteins, although intracellular laccases have also been detected in some fungi and insects. Fungal laccases are believed to play a variety of roles, such as, morphogenesis, pathogenesis, and lignin degradation. As an oxidase, laccase is used in many agricultural, industrial, and medicinal applications. Current investigations are focused on laccase-based biooxidation, biotransformation, biosensor, and enzymatic synthesis of organic compounds. By enhancing laccase production using different physiochemical parameters, better understanding of the mechanism for the reactions of interest, and optimizing the catalytic activity of laccase, it can be used in a better way in diverse fields of biotechnology.

Keywords Bioremediation · Laccase · Lignin · Lignocellulosics · White-rot fungi

Introduction

Lignin is a complex oxyphenyl propanoid polymer, found in all vascular plants including herbaceous species, which provides rigidity, support, and protection to the plants [1]. It is synthesized by one-electron oxidation of the precursors; *p*-coumaryl alcohol, coniferyl

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alcohol, and sinapyl alcohol, generating phenoxy radicals which then undergo nonenzymatic polymerization. These unspecific reactions create a high-molecular-weight, heterogeneous, three-dimensional polymer. Lignin polymer comprises of a variety of monomers connected by various C–C and C–O–C nonhydrolyzable bonds with irregular arrangement of successive monomeric and intermonomeric bonds (Fig. 1) [2]. Ether bonds between propyl side chains and aromatic nuclei (arylglycerol-β-aryl or β-O-4 ether) constitute the major parts. Carbon–carbon bonds which occur primarily between aromatic nuclei and propyl side chains (diaryl propane or β-1 bond) are less frequent.

The degradation of wood in nature is mainly caused by fungi. The better degradative efficiency of fungi is due to their hyphal organization, which imparts them penetration capacity. Depending upon their mode of attack, the fungi are classified into three main categories [3].

(a) *Soft-rot fungi*: wood decay by soft-rot fungi results in softening of the tissues [4]. Biochemical studies have shown that soft-rot decay results in lower methoxy content of wood lignin, thus making it more soluble [5]. The ligninolytic system of soft-rot fungi does not have oxidative potential to attack the recalcitrant guaiacyl lignin but they can oxidize and mineralize syringyl lignin [6]. These fungi degrade wood by forming microscopic cavities within the secondary cell wall [7].

(b) *Brown-rot fungi*: the fungi belonging to this group preferentially degrade cellulose and hemicellulose of wood and modify lignin only to a limited extent [8]. Logs decomposed in this manner fall apart into brown powder consisting mainly of enzymatically liberated lignin. Colonization by such fungi is usually confined to the

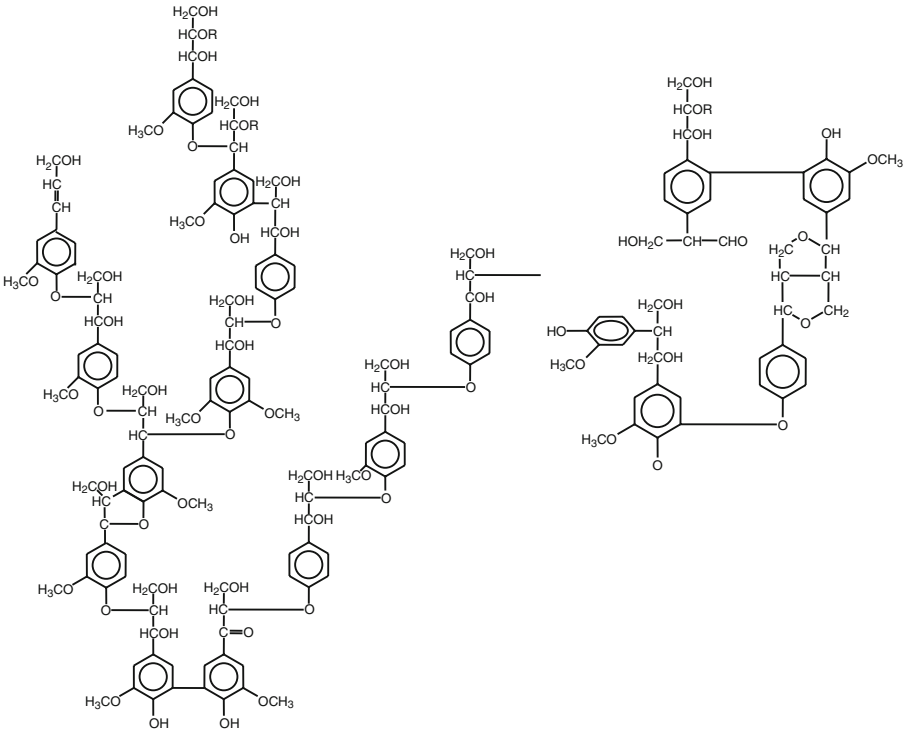

Fig. 1 Schematic structural formula of lignin adapted from Alder [2]

less lignified layers of the secondary cell wall. It was observed that erosion and thinning of cell wall pattern was similar to that caused by white-rot fungi. In brown-rotted lignin, its methoxy content and aliphatic hydroxyl content decrease, while the carboxyl and phenolic hydroxyl contents greatly increase [9]. Certain brown-rot fungi have been reported to possess intracellular laccase activity, suggested to be responsible for the generation of phenoxy radicals by *Gloeophyllum trabeum* during lignin degradation [10].

(c) *White-rot fungi*: these are the most efficient lignin degraders and leave behind a white powdery fibrous material. So far, these are the only organisms known to completely mineralize lignin to CO_2 and H_2O but they cannot use it as a sole carbon and energy source [11]. These are also capable of degrading cellulose which serves as the energy-providing cosubstrate. White-rot fungi oxidize side chains and cleave the aromatic nuclei, leaving aliphatic carboxyl-rich fragments in the polymer [9].

The capability of white-rot fungi to degrade lignin is dependent on the production of extracellular ligninolytic enzyme system, which is primarily comprised of laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and hydrogen peroxide generating oxidases [12]. The ligninolytic system of white-rot fungi is not homogenous. Different white-rot fungi have been shown to possess one or more enzymes. Nerud and Misurcova [13] divided white-rot fungi into four groups on the basis of enzymes present, i.e., LiP–MnP–laccase-producing fungi; MnP–laccase-producing fungi; LiP–MnP-producing fungi; laccase-producing fungi.

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) represents a family of copper-containing polyphenol oxidases and usually called multicopper oxidases (Fig. 2). Laccases catalyze monoelectronic oxidation of substrate molecules to corresponding reactive radicals with the assistance of four copper atoms that form catalytic core of the enzyme, accompanied with the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals [14]. However, all substrates cannot be directly oxidized by laccases, either because of their large size which restricts their penetration into the enzyme active site or because of their particular high redox potential. To overcome this hindrance, suitable chemical mediators are used which act as intermediate substrate for laccase, whose oxidized radical formed are then able to interact with high redox potential substrate targets (Fig. 3) [14].

Laccases exhibit broad substrate range which varies from one laccase to another. Although it is known to be diphenol oxidase, monophenols like 2,6-dimethoxy phenol or guaiacol are better substrates than phenols (e.g., catechol or hydroquinone) [15].

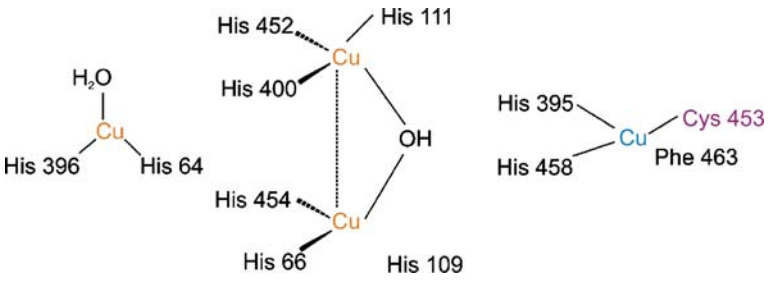

Fig. 2 Model of catalytic cluster of laccase made of four copper atoms. Type 1 (T1) copper confers the typical blue color to the protein and is the site where substrate oxidation takes place. Type 2 (T2) and type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place

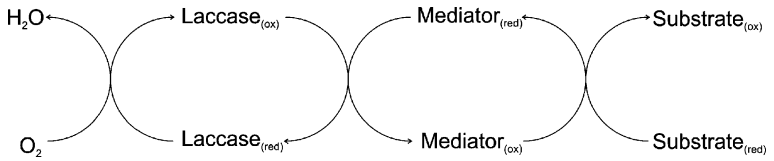

Fig. 3 Schematic representation of laccase-catalyzed redox cycle for substrate oxidation in the presence of chemical mediator

Syringaldazine is considered to be a unique laccase substrate [16], as long as hydrogen peroxide is avoided in the reaction, as this compound is frequently oxidized by peroxidases. Thus, laccases fall under the category of oxidases which can oxidize polyphenols, methoxy-substituted phenols, aromatic diamines, and a range of other compounds. Laccases do not require addition or synthesis of a low-molecular-weight cofactor like hydrogen peroxide, as its cosubstrate, oxygen, is present in the environment. Laccase has its characteristic blue color due to the presence of copper atoms in its catalytic center [17, 18]. However, those which lack T1-type copper atoms are not blue in color and therefore called "yellow" or "white" laccases. Yellow laccase from *Pleurotus ostreatus* has been purified and characterized [19]. It was also discovered in straw cultures of *Panus tigrinus* and *Phlebia radiata* and seems to have changed oxidation state of copper in active center, probably caused by integration of aromatic lignin degradation products [20]. A "white laccase" has also been reported from *P. ostreatus* [21] and an unidentified basidiomycete [22].

If the comparison between plant and fungal laccases is taken up, the former takes part in radical-based polymerization of lignin [23] whereas fungal laccase contributes to lignin biodegradation due to which it has gained considerable significance in green technology. Fungal laccases have isoelectric points (pI) ranging from 3 to 7 whereas plant laccases pI ranges from 3 to 9. Fungal laccases often have lower molecular mass than the plant laccases.

Laccase-Producing Organisms

Laccase was first detected in exudates of Japanese lacquer tree *Rhus vernicifera*. Among fungi, Basidiomycetes, Ascomycetes, and Deuteromycetes can produce laccase and white-rot fungi have been found to be the most efficient laccase producers (Table 1). A number of brown-rot fungi have also been known to produce laccase. D'Souza et al. [24] for the first time showed the presence of laccase gene-specific sequence as well as laccase activity in brown-rot fungi *G. trabeum*, comparable to a number of white-rot fungi [25, 26]. A number of other brown-rot fungi such as *Postia placenta*, *Antrodia vaillantii*, *Fomitopsis pinicola*, and *Coniophora puteana* have been shown to produce laccase to variable levels [24]. There are some records of low laccase-like activity in some yeast species isolated from decayed wood [27]. Laccase was isolated and purified only from *Cryptococcus neoformans*. This basidiomycetous yeast produces a true laccase capable of oxidation of phenols and aminophenols and unable to oxidize tyrosine [28]. Laccase activity is almost exclusively associated with cell walls in white-rot fungi *Irpex lacteus* [29], in the yeast *C. neoformans* [30], and in spores of *Trichoderma* spp. [31]. Laccase production has not been demonstrated in lower fungi, i.e., Zygomycetes and Chytridiomycetes. However, these groups have not yet been studied in detail.

Extensive studies were made to detect ligninolytic enzymes, including laccase in ectomycorrhizal (ECM) fungi [32, 33]. Gene fragments similar to laccase of some wood-rotting fungi have been reported from several isolates of ECM species including *Amanita*,

Table 1 Some laccase-producing fungi.

Organisms		References
White-rot fungi		
1	*Agaricus bisporus*	[182]
2	*Armillaria mellea*	[95]
3	*Armillaria ostoyae*	[95]
4	*Bjerkandera adusta*	[183]
5	*Ceriporiopsis subvermispora*	[184]
6	*Coprinus cinereus*	[185]
7	*Coprinus friesii*	[186]
8	*Coriolopsis polyzona*	[187]
9	*Cryptococcus neoformans*	[89]
10	*Cyathus bulleri*	[188]
11	*Cyathus stercoreus*	[112]
12	*Daedalea flavida*	[113]
13	*Daedalea quercina*	[15]
14	*Dichomitus squalens*	[189]
15	*Fomes annosus*	[190]
16	*Ganoderma australe*	[134]
17	*Ganoderma lucidum*	[145]
18	*Ganoderma valesiacum*	[191]
19	*Lentinula edodes*	[47]
20	*Marasmius quercophilus*	[192]
21	*Panaeolus papilionaceus*	[186]
22	*Panaeolus sphinctrinus*	[186]
23	*Panus tigrinus*	[17]
24	*Phellinus ribis*	[48]
25	*Phlebia brevispora*	[114]
26	*Phlebia fascicularia*	[189]
27	*Phlebia floridensis*	[189]
28	*Phlebia radiata*	[114]
29	*Phlebia tremellosa*	[193]
30	*Piloderma byssinum*	[34]
31	*Pleurotus eryngii*	[194]
32	*Pleurotus florida*	[115]
33	*Pleurotus ostreatus*	[115]
34	*Pleurotus pulmonarius*	[49]
35	*Pleurotus sajor-caju*	[195]
36	*Polyporus pinsitus*	[196]
37	*Polyporus sanguineus*	[114]
38	*Polyporus versicolor*	[69]
39	*Pycnoporus cinnabarinus*	[77]
40	*Rigidoporus lignosus*	[197]
41	*Schizophyllum commune*	[99]
42	*Sclerotium rolfsii*	[79]
43	*Trametes gibbosa*	[169]

Table 1 (continued).

Organisms		References
44	*Trametes gallica*	[198]
45	*Trametes trogii*	[109]
46	*Trametes hirsuta*	[169]
47	*Trametes villosa*	[124]
48	*Volvariella speciosa*	[199]
49	*Volvariella volvacea*	[97]
Ascomycetes		
1	*Cryphonectria parasitica*	[102]
2	*Glomerella* sp.	[200]
3	*Melanocarpus albomyces*	[201]
4	*Neurospora* sp.	[100]
5	*Podospora anserina*	[202]
6	*Xylaria polymorpha*	[196]
Imperfect fungi		
1	*Aspergillus nidulans*	[203]
2	*Botrytis cinerea*	[204]
3	*Cantharellus cibarius*	[53]
4	*Gaeumannomyces graminis*	[106]
5	*Monocillium indicum*	[205]
6	*Ophiostoma ulmi*	[103]
7	*Penicillium chrysogenum*	[81]
8	*Rhizoctonia solani*	[125]
9	*Trichoderma atroviride*	[31]
10	*Trichoderma giganteum*	[175]
Brown-rot fungi		
1	*Gleophyllum trabeum*	[24]
2	*Postia placenta*	[24]
3	*Coniophora puteana*	[206]

Cortinarius, *Hebeloma*, *Lactarius*, *Paxillus*, *Piloderma*, *Russula*, *Tylospora*, and *Xerocomus* [34, 35]. However, the gene presence does not necessarily correspond to the production of the enzyme. In *Paxillus involutus*, a species containing other putative laccase sequence, oxidation of syringaldazine has never been detected [36, 37].

Lignin biodegradation research has been mainly concentrated on white-rot fungi, yet several actinomycetes and bacterial species can also degrade lignin. Some *Streptomyces* species degrade lignin but only a few of them produce laccase [38]. Laccase in bacteria is present intracellularly and as periplasmic protoplast [39]. *Azospirillum lipoferum* was the first bacterium in which laccase was discovered [40], followed by a variety of bacteria such as *Bacillus subtilis*, *Bordetella compestris*, *Caulobacter crescentus*, *Escherichia coli*, *Mycobacterium tuberculosum*, *Pseudomonas syringae*, *Pseudomonas aeruginosa*, and *Yersinia pestis* [41, 42]. Recently, *Stenotrophomonas maltophilia* strain was found to be laccase producing, which was used to degrade synthetic dyes [43]. These bacteria produce protein sequences similar to that of fungal laccases but these proteins have not been analyzed for laccase activity.

The plants in which the laccase enzyme has been detected include lacquer, mango, mung bean, peach, pine, prune, and sycamore [44]. Techniques are also being developed to express laccase in the crop plants. Recently, laccase has been expressed in the embryo of maize seeds (*Zea mays*) [45]. The laccase enzyme has also been detected in different insects, e.g., *Bombyx*, *Calliphora*, *Diploptera*, *Drosophila*, *Lucilia*, *Manduca*, *Musca*, *Orycetes*, *Papilio*, *Phormia*, *Rhodnius*, *Sarcophaga*, *Schistocerca*, and *Tenebrio* [44].

Structural and Biochemical Characteristics

Structural and biochemical properties of fungal laccase emanate from the study of purified proteins. Until now, hundred laccases have been purified from fungi but very few have been characterized. Fungal laccases are mostly extracellular but most of the white-rot fungi produce both extracellular and intracellular laccases with isoenzymes showing similar patterns of activity involving isoelectric focusing. The intracellular and extracellular laccase activity was also detected in *Suillus granulatus* [36], *Phanerochaete chrysosporium*, and *Lentinula edodes* [46, 47]. Laccases are more stable in their extracellular role as they are often produced as highly glycosylated derivatives where the carbohydrate moieties increase their hydrophilicity.

Most fungal laccases are monomeric proteins. However, quite a few exhibit a homodimeric structure, the enzyme being composed of two identical subunits with molecular weight typical for monomeric laccases, e.g., in *Phellinus ribis* [48], *Pleurotus pulmonarius* [49], *Trametes villosa* [50], and the mycorrhizal fungus *Cantharellus cibarius* [51]. The Ascomycetes *Gaeumannomyces graminis*, *Monocillium indicum*, and *Podospora anserina* also produce oligomeric laccases.

These enzymes are copper-containing glycoproteins with molecular weight between 60 and 80 kDa and with carbohydrate content between 15% and 20%. They usually contain four copper atoms but enzymes with one, two, and three copper atoms are also known [51, 52]. Laccase sequences contain one cysteine and ten histidine residues which are involved in the binding of the copper atoms [53]. Three types of copper atoms can be distinguished by their spectroscopic and paramagnetic properties and are classified as follows: type 1 confers the greenish blue color on these proteins and is involved in the electron transfer; type 2 is also involved in the electron transfer and type 3 is responsible for the binding of oxygen [54].

Some fungal laccases have quaternary structure under nondenaturing conditions [28]. Purified laccases exhibit a characteristic blue appearance from their electronic absorption around 600 nm (due to type I Cu) and 320 nm (due to type III Cu) and catalyze four one-electron oxidations of mostly phenolic compounds like catechol, hydroquinone, 2,6-dimethoxyphenol, and syringaldazine with redox potential up to 0.8 V [55]. Laccases are able to catalyze a variety of reactions, viz. cleavage of alkyl-phenyl, Cα–Cβ bonds and phenolic lignin dimers [56], demethoxylation [57], demethylation, polymerization, and depolymerization [58].

The optimum pH for fungal laccases lies in the acidic range. Each substrate for its oxidation has its own specific pH optima. Xu [59] proposed the bell-shaped pH profile of phenolic compounds, when there exist two opposing effects. Different laccases have different catalytic preferences and accordingly they can be grouped as ortho-, meta-, para-substituted phenols. Blaich and Esser [60] taking crude laccase preparation showed that ortho-substituted compounds (guaiacol, *o*-phenylenediamine, caffeic acid, catechol, dihydroxyphenylalanine, protocatechuic acid, gallic acid, and pyrogallol) were better

substrate than para-substituted compounds (*p*-phenylenediamine, orcinol, resorcinol, and phloroglucinol). Laccase from *Ganoderma lucidum* catalyzed the oxidation of only ortho- and para-dihydroxyphenyl compounds, *p*-phenylenediamine, and polyphenols and not the meta-hydroxymethyl compounds [61].

Laccases can catalyze the oxidation of various reducing substances such as inorganic or organic metal complexes, anilines, thiols, and especially phenols with redox potentials higher than their own which are typically 0.5–0.8 mV vs normal hydrogen electrode [62]. Although laccase strongly prefers O_2 as its oxidizing substrate, it usually has low specificity toward reducing substrates. It is believed that laccase catalysis involves:

1. Reduction of the type 1 copper by reducing substrates
2. Internal electron transfer from the type 1 copper to the type 2 and type 3 copper
3. Reduction of O_2 to water at the type 2 and type 3 copper site [63]

The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the formation of a free (cation) radical which in general is unstable and may undergo further laccase-catalyzed oxidation or nonenzymatic reaction. The electron transfer from substrate to type 1 copper is probably controlled by redox potential difference [59]. The structure and action mechanism of laccases were well reviewed by Wong [64].

Study at the molecular level reveals that several species produce a variety of isoenzymes. More than one isoenzyme is produced by most of the white-rot fungi. Eight different isoenzymes are produced by *P. ostreatus*, six of which have been isolated and characterized [21]. The production of laccase isoenzymes in *P. ostreatus* is regulated by the presence of copper and the two dimeric isoenzymes have only been detected in the presence of copper [21, 65]. Many fungal species, e.g., *Coriolopsis rigida*, *Dichomitus squalens*, *Physisporinus rivulosus*, and *Trametes gallica*, produce isoenzymes that are closely related, both structurally and in their catalytic properties. In *P. chrysosporium*, production of different laccase isoenzymes was detected in cell extract and in the culture medium [46]. However, laccase gene was not found in the complete genome sequence of this fungus [66], and thus these were suggested to be multicopper oxidases rather than true laccase [67]. The molecular basis for the production of different isoenzymes is the presence of multiple laccase genes in fungi [34]. Isoenzymes of laccase with different molecular weight and pI were also detected in litter-decomposing fungus *Marasmius quercophilus* [68].

Screening and Assay Methods

Screening Methods

Different workers used different media and physiochemical parameters for isolating laccase-producing fungi. Dion [69] used various phenolic compounds for screening of laccase-producing *Polyporus versicolor* which resulted in visual color change in the presence of phenolic materials. Westermark and Eriksson [70] used agar plates containing lignin for screening laccase-producing fungi. To test for laccase activity, plates were flooded with guaiacol which gives an intense red to brown color with laccase. Arora and Sandhu [71] used lignin guaiacol agar medium for screening such fungi.

Field et al. [72] demonstrated that a polymeric dye, red poly(vinylamine) sulfonate anthrapyridone (poly R-478), could be used for screening white-rot fungi which have the ability to degrade anthracene and benzo[a]pyrene. Kiiskinen et al. [73] cultivated fungi on solid media containing indicator compounds (such as RBBR, poly R-478, guaiacol, and

tannic acid) that enabled the detection of laccases as specific color reaction. 2,2′Azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) is also suitable for screening because its one-electron oxidation product is soluble in water, stable, and intensely green.

Assay Methods

Laccase activity can be assayed quantitatively by different methods. In guaiacol method, the activity is represented as colorimetric unit per milliliter while in other described methods it is micromole product formed per minute per milliliter.

Guaiacol (o-Methoxy Phenol) Method

This was given by Hiroi and Eriksson [74] and was modified by Arora and Sandhu [71] and further used by Guillen et al. [75]. In this method, 5 ml of the reaction mixture containing sodium acetate buffer (10 mM, pH 5.0), guaiacol (2 mM), and 0.2 ml of enzyme extract is incubated at 25 °C for 2 h. The absorbance is read at 450 nm. In the blank, the guaiacol is substituted by an equal volume of buffer.

ABTS Method

Childs and Bradsley [76] were the first to determine laccase activity by monitoring the oxidation of the diammonium salt of ABTS. Eggert et al. [77] and Geng et al. [78] assayed laccase by monitoring the oxidation of 0.5 mM ABTS in sodium tartrate buffer (50 mM, pH 4.5) at 420 nm. One unit of enzyme activity is defined as the amount of enzyme required for the oxidation of 1 μmol of ABTS per minute in the reaction mixture. ABTS method has also been used by other workers with different buffers (pH 4–5) and slight modifications [15, 79].

Dimethoxyphenol Method

This method was first used by Paszczynski et al. [80]. One-milliliter reaction mixture contained 2 mM 2,6-dimethoxyphenol in sodium tartrate buffer (200 mM, pH 5.0) and 0.25 ml of culture fluid. The absorbance was read at 468 nm [81, 82].

Syringaldazine Method

This method was first used by Harkin and Obst [83]. Galliano et al. [84] measured laccase activity by following the oxidation of syringaldazine. The quinone formation was monitored at 526 nm. Three milliliters of reaction mixture contained 0.01 ml syringaldazine (1.6 mg/ml methanol), phosphate buffer (100 mM, pH 6), and the enzyme. Ullah et al. [85] also measured the laccase activity by monitoring the oxidation of catechol as well as syringaldazine.

Biological Importance of Laccase in Nature

A variety of functions have been attributed to laccase. In plants, laccase is involved in lignification. However, fungal laccases are believed to play a variety of roles, as explained further.

Morphogenesis

Laccase plays an important role in morphogenesis. Temp and Eggert [86] reported that glucose-grown culture of *Pycnoporus cinnabarinus* produces laccase which is associated with ligninolysis as well as formation of pigment, cinnabarinic acid, which imparts a characteristic orange-red color to the fruiting bodies of fungus and serves as antimicrobial agent.

Two of the several phenoloxidases of *Tribolium*, namely laccases 2Aand 2B, are required for larval, pupal, and adult cuticle sclerotization and pigmentation. Both of these isoforms are generated from a single gene, TcLac2 [87]. Pigmentation in *C. neoformans* requires fungal laccase [88, 89]. Laccase encoding gene (abr2) of *Aspergillus fumigatus* is responsible for its conidial pigmentation [90].

Two laccases were characterized in *Aspergillus nidulans*, which are involved in synthesis of conidial pigments [91, 92]. In number of fungi such as *Daldinia concentrica* and *L. edodes*, laccase activity is associated with pigment formation. Fruiting body formation may involve the laccase-catalyzed synthesis of extracellular pigments connected with oxidative polymerization of cell wall components for strengthening cell-to-cell adhesion [93]. Worrell et al. [94] described the formation of another developmental form, rhizomorphs (mycelial strands formed from large number of tightly appressed hyphae), in *Armillaria mellea* to be associated with laccase production. Laccase is considered to be responsible for polyphenolic glue that sticks the hyphae together [95]. Laccase was found in relation to fruiting body development in the mycelium of *L. edodes* on a sawdust-based substrate [96] and in *Volvariella volvacea* [97].

In some fungi, laccase level is regulated in relation to fruiting body development. Wood [98] has reported that the commercial mushroom *Agaricus bisporus* secretes abundant laccase into the medium during vegetative growth but rapid loss of extracellular laccase activity occurs during the fruiting body formation. In *Schizophyllum commune*, the dikaryotic strains that are able to develop fruiting body can secrete high level of laccase at 30 °C in continuous darkness but in the presence of light or at 24 °C and the coisogenic monokaryotic strains do not produce laccase [99]. Laccase is also involved in sexual differentiation of some fungi [100].

Pathogenesis

Extracellular laccase has also been found to play a role in pathogenesis. Laccase from *Cryphonectria parasitica*, which causes chestnut blight, is thought to be responsible for the detoxification of tannins in chestnut bark [101, 102]. It has been reported that *Ophiostoma ulmi* and *Ophiostoma novo ulmi*, both vascular wilt pathogens and causative agents of Dutch elm disease, can be differentiated by their potential to produce extracellular laccase [103]. *O. novo ulmi* is a strong pathogen on native North American and European elm [104] and produce high amount of laccase. On the contrary, *O. ulmi* is a weak pathogen and secretes very low amount of enzyme. No direct involvement of laccase in Dutch elm disease has been demonstrated so far, though it may be possible that the enzyme is capable for detoxifying endogenous phenols present in host or produced in response to infection. Laccase is a major virulence factor of the pathogenic fungus *C. neoformans* in mice brain and lung infections [105]. Three laccase genes were isolated from *G. graminis* var. *tritici* which are considered as important pathogen of wheat [106].

Laccase and Lignin Degradation

Laccase plays an important role in lignin degradation. It was proven by Ander and Eriksson [107] through their genetic studies on *Sporotrichum pulverulentum*, in which laccase-less

mutants were not able to degrade lignin. Bermek et al. [108] also reported that laccase-less mutants of *P. cinnabarinus* do not degrade kraft lignin. The role of laccase in lignin degradation is also evident from the following observations:

(a) Majority of white-rot fungi, which degrade lignin also produce laccase extracellularly.
(b) Lignin contain phenolic hydroxyl group which constitute a substrate for laccase.
(c) Lignin degradation as well as laccase catalysis is oxidative in their nature.

Laccases are able to oxidize a large range of aromatic compounds such as polyphenols, substituted phenols, etc. [59]. Garzillo et al. [109] determined oxidation rate of different substrates by laccase from *Trametes trogii* and observed that oxidation rates of phenolic and nonphenolic compounds get largely enhanced when two o- and p-oriented groups are present simultaneously.

Laccase catalyzes one-electron subtraction from phenolic hydroxyl group of lignin to give phenoxy radicals which generally undergo polymerization via radical coupling accompanied by partial depolymerization of propyl side chains via alkyl-aryl cleavage. It has been shown that lignin preparations treated with laccase are partly depolymerized but mostly polymerized. The three types of reactions mediated by laccase via phenoxy radicals of substrates include Cα–Cβ cleavage (between C_1 and C_2 carbon), alkyl-aryl cleavage (between C_1 and aryl group), and Cα (C_1) oxidation. A simple reaction catalyzed by laccase (I) and a disproportionation reaction (II), which is nonenzymatic, are shown in Fig. 4. Following the single-electron oxidation, many different reactions can occur, some of which are summarized in (Fig. 5). The initial step in the catalytic mechanism of laccase involves an electron donation to a specific Cu^{2+} site with the formation of free radical products of organic substances. The cuprous state is then oxidized to cupric state by molecular oxygen, e.g., mechanism of the catalytic action of laccase on 2,6-dimethoxyphenol as shown in Fig. 6.

Laccases can catalyze the oxidation of many compounds which have very high redox potentials such as aromatic alcohols in the presence of appropriate primary substrates called mediator compounds. It has been shown that the laccase is able to oxidatively cleave a Cα–Cβ linkage in nonphenolic β-1 lignin model dimer and many other nonphenolic lignin compounds and oxidize veratryl alcohol to veratraldehyde [54, 110]. The oxidation rate of veratryl alcohol depends on the nature and concentration of mediator.

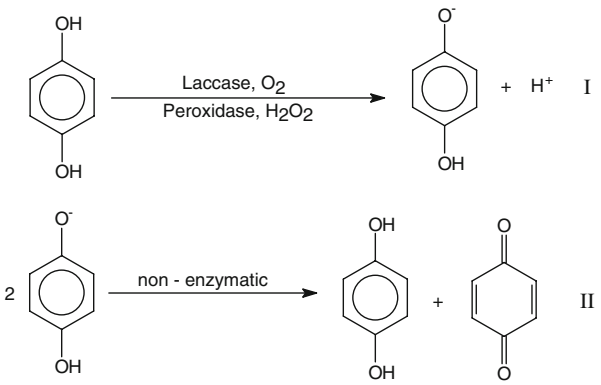

Fig. 4 Oxidation of hydroquinone by laccase or peroxidase to give phenoxy radical which disproportionate into hydroquinone and *p*-benzoquinone

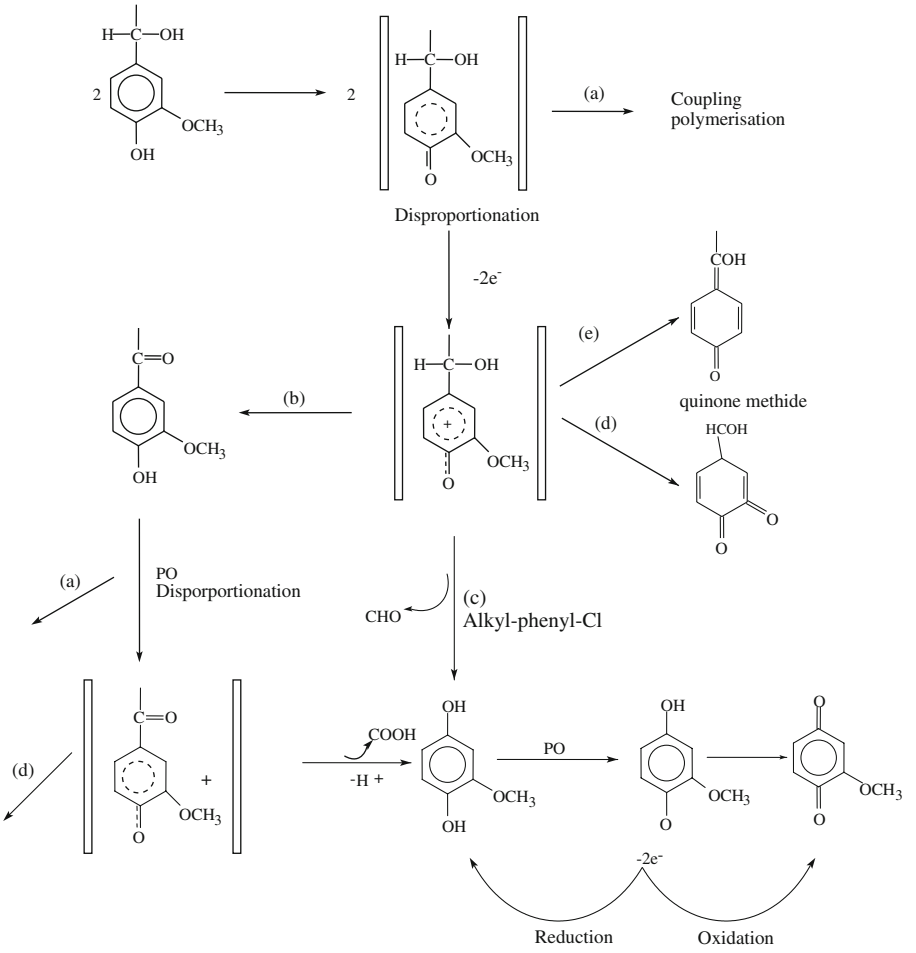

Fig. 5 Different reactions (**a**–**e**) after single electron oxidation of lignin-related phenols, catalyzed by laccase [207]. **a** Oxidation coupling and polymerization, **b** oxidation of α carbinol groups to α carbonyl structures, **c** alkyl-phenyl cleavage, **d** demethoxylation to produce methanol, **e** quinone methide formation

Microbial Physiology of Laccase Production

The production of laccase enzyme can be increased by varying different physiochemical parameters such as in the presence of rich medium like malt or yeast extract and the presence of specific enzyme substrate/s (e.g., phenolic compounds) as an adaptive response and the presence of certain inducers. *P. chrysosporium* is known to lack laccases in normal conditions but can produce them when the fungus is grown on high-nitrogen-cellulose medium [111]. Studies on laccase production by different fungi have shown that laccase levels are substantially higher in media containing sufficient nitrogen [112]. Different workers have shown malt/yeast extract to be quite effective in giving higher laccase yield [109, 113, 114]. Sugars usually repress laccase production in most of the fungi [98, 107, 113, 115] except in *Botrytis cinerea* where maximum laccase was produced in media containing 2% glucose [116]. In *Pleurotus florida*, laccase levels are enhanced in the presence of lactose [117].

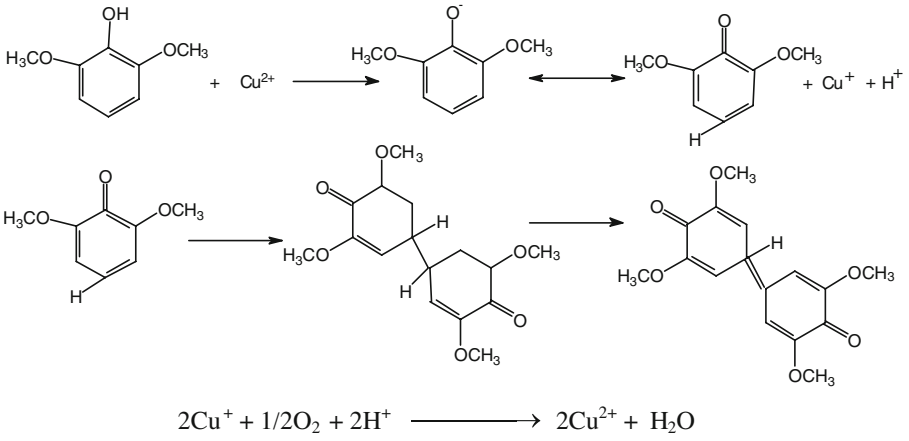

Fig. 6 Catalytic breakdown of 2,6-dimethoxyphenol by laccase

Many substances have been found to induce laccase up to different levels in various fungi. These include gallic acid, cycloheximide, 2,5-xylidine, ferulic acid, veratryl alcohol, guaiacol, indulin AT, polyfon, humic acid, substituted phenols, amines, benzoic acid, and lignosulfonate [77, 113].

Shaking has variable effects on laccase production. *Rhizoctonia practicola* gives similar laccase production in shaking and stationary cultures. In *P. cinnabarinus*, shaking increases laccase production whereas in many other white-rot fungi, it is suppressed [77, 112]. In general, optimum pH for laccase production and activity ranges from 3.0 to 4.5 [77, 109]. Optimum temperature for laccase activity ranges from 20 to 37 °C [113]. Laccase of most white-rot fungi is stable below 50 °C. Under identical conditions, thermophilic fungal laccases are more thermostable than mesophilic laccases.

Laccase can be inhibited by various reagents. Small anions such as halides (excluding iodide), azide cyanide, and hydroxide bind to the type 2 and type 3 copper, resulting in an interruption of the internal transfer and activity inhibition. Other inhibitors include metal ions (e.g., Mg^{2+}, Ca2+, Sn^{2+}, Ba^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, desferal, ethylenediaminetetraacetic acid, tropolon, L-cysteine, dithiothreitol, glutathione, thiourea, and quaternary ammonium detergents [15, 118].

Molecular Characterization of Laccase

Various methods are known for isolation of DNA from filamentous fungi but the most popular one is reported by Reader and Broda [119]. For the isolation of laccase clone, workers have shifted to complementary DNA (cDNA) clones due to the ease of amplifying first-strand cDNA using degenerate primers designed from conserved copper binding domains. This partial cDNA clone can easily be extended to full length by using rapid amplification of cDNA ends (RACE) [97]. For the study of laccase expression, cDNA clones have been used [120]. For studying the gene families and the copy number of laccase gene, genomic DNA library construction is useful. The high sequence conservation of the laccase signature sequencing has been exploited to design DNA primes, hybridization to clone, and finding one or more laccase gene from a variety of organisms. Liu et al. [121] had used degenerate primers designed from first and fourth copper binding

regions of most laccases. These primers were used to amplify a partial laccase sequence from *Fomes lignosus* cDNA which was later used to design another set of primers for RACE to get a full length of cDNA primers designed from this full-length cDNA P_{NS} and P_{CS} was used to get a 2,201 bp of genomic clone.

Laccase Gene Family

Laccases are mostly secreted as multiple isozymes [122]. Such a diversity in laccase isoenzymes was first attributed to posttranslation modifications of the same gene product, but the characterization of several laccase gene families suggested that at least a part of this biochemical diversity could be the result of the multiplicity of gene in fungal genomes [123]. Differential expression of laccase genes have been reported for a few fungi [65]; *Trametes* spp. strain I-62 1 cc1, 1 cc2, and 1 cc3 genes were observed in cultures with different carbon sources and nitrogen levels. 3,4-DMBA, 2,5-DMBA, and 3,5-DMBA produce different induction patterns on the expression of the three laccase genes from *Trametes* sp. strain I-62. The copy number of laccase gene varies among fungi. A laccase gene family in which the gene encoding two of five laccase was located on the same chromosome was found in *T. villosa* [124] and the three laccase genes were found to be clustered within approximately 11 kb of each another in the plant pathogen, *Rhizoctonia solani* [125]. Many reports permit to conclude that fungi are able to produce several laccases and the proportion of the enzymes produced depends upon the culture composition and operational conditions employed [126]. Gene family probably produce closely related proteins that are subtly different in their activities, allowing the transformation of a wider range of substrates or showing differential regulation [127]. Moreover, until transcripts for all the laccase genes are not detected, the possibility that some of the nonexpressed laccases genes are pseudogenes or expression under different physiological conditions is not ruled out.

Biotechnological Applications of Laccase

Laccase can oxidize/polymerize/cleave a wide variety of biological and synthetic phenolic and nonphenolic compounds. Enzymatic treatments have potential in variety of industrial fields including pulp and paper, textile, and food industries. These systems are advantageous over physical and chemical treatments as enzymes are biodegradable catalyst and specific in action, and enzymatic reactions are carried out in mild conditions.

Delignification of Lignocellulosics

Separation of lignin from cellulose fibers is an important step in processing of wood for manufacturing of paper pulp. Conventional methods involve chlorine-, sulfite-, or oxygen-based chemical oxidants which impose serious drawbacks of disposal of chlorinated and sulfide by-products or loss of cellulose fiber strength. To overcome these drawbacks, microbial or enzyme-based delignification systems can be used. Laccase is capable of degrading natural or synthetic lignin polymers. Oxidation by laccase results in breakage of aromatic and aliphatic C–C bonds and depolymerization of lignin [77].

Presence of lignin in the forage used as ruminant feed affects its digestibility. Biodelignification of such agricultural lignocellulosics not only enhances the digestibility of the feed but also improves their nutritional value [128, 129]. Some white-rot fungi,

notably *L. edodes*, produce edible fruit bodies and can be used to convert lignocellulose directly to food for humans. As these fungi delignify these substrates while growing, the material remaining after mushroom harvest can also be used as animal feed [130].

Fermentation of lignocellulosics to generate fuels such as ethanol or butane diol also requires delignification [131]. Lignocellulosic hydrolysates such as furan derivatives and organic acids may inhibit the microbial fermentation of agricultural residues to desirable fuel products [132]. Purified laccase from *Trametes versicolor* has also been used for such delignification, resulting in better productivity.

Biopulping and Biobleaching

Cellulose is the major polymer of primary interest to paper manufacturers. Different woody substrates like eucalyptus, bamboo, and agricultural residues are used for manufacturing paper depending on the type and quality of paper. Pulping and bleaching of these substrates are the two main steps for paper manufacturing. During pulping, the raw materials are reduced to the fibrous state using mechanical and chemical methods. Pulping is followed by bleaching which involves the consumption of enormous amounts of chemicals, thus leading to environmental pollution hazards. To cope up with this problem, biological processes have been explored to provide environmentally safe technology which primarily involves the use of laccase [133]. The use of white-rot fungi for the treatment of wood chips prior to mechanical or chemical pulping is called biopulping. Laccases from white-rot fungi can be applied as biopulping agents to wood chips before pulping as they partially degrade lignin and loosen lignin structures [134]. This not only improves strength of the paper but also reduces the kappa number in the pulp which is a measure of residual lignin [135, 136]. Biopulping saves electrical energy, improves strength properties, and is environmentally compatible due to reduced effluent toxicity [137]. It also results in the reduction of resins, which are responsible for sticky deposits on the paper machine leading to serious problems in the production process.

Conventional bleaching uses chlorination to remove the residual lignin while Laccase Mediator System has been shown to possess the feasibility to substitute chlorine-containing reagents thus reducing the pollution load caused by chloroorganics. Laccase can also be applied as biobleaching agent as it degrades the residual lignin in pulp and decolorize it [138]. It is an ideal oxidative enzyme for pulp bleaching because it is readily available and use atmospheric oxygen as its electron acceptor. Laccase produced by *T. versicolor* has been studied widely for biobleaching of paper pulp, treatment of effluents, and various other industrial applications [139].

Textile Dye Transformation

Early research into dye decoloration by white-rot fungi was largely focused on *P. chrysosporium* and was mainly attributed to LiP and MnP. In recent years, laccase has been well studied in relation to dye degradation [140]. Swamy and Ramsay [141] investigated the production of LiP, MnP, and laccase by *T. versicolor* during sequential dye decoloration. Extracellular LiP was not detected during decoloration of the azo dye; thus, it was concluded that both MnP and laccase were potentially involved in dye decoloration. Kirby et al. [142] reported that laccase from *Phlebia tremellosa* decolorized eight synthetic textile dyes added to culture under stationary conditions. Cell-free enzyme extracts obtained from various white-rot fungi including *Phlebia* species have been successfully employed for biodecolorization of many synthetic and industrial dyes [143, 144]. Malachite green was successfully transformed by laccase from *G. lucidum* [145].

Laccase has been reported to prevent back staining of dyed or printed textiles. As part of the washing solution, laccase could quickly bleach released dye stuff, thus resulting in the reduction of processing time, energy, and water needed to achieve satisfactory quality of the textile. Laccase-catalyzed textile dye bleaching may also be useful in giving finishing touches to dyed cotton fabric. Replacing conventional chemical oxidants (e.g., hypochlorite), a laccase-based system has been shown to be capable of bleaching indigo dye in denim and achieving various bleached appearances on the fabric. The utilization of laccase with the combination of mediators and cellobiose dehydrogenase for the decolorization of azo textile dyes proved to be a valid alternative to more expensive and less environmentally friendly chemical treatments of textile dye wastes [146].

Removal of Phenolics from Must and Wine

Color and taste of must and wine are dependent on particular phenolic compounds present in different kinds of wine. Many groups of phenolic compounds are found in wine: cinnamic acid derivatives and catechins are present in different amounts in all wines, while red and rose wines are characterized by the presence of anthocyanins. All phenolics are subjected to various fates during shelf-life of wine and some problems can arise from their modifications, which are then involved in various chemical reactions.

Many innovative treatments, such as enzyme inhibitors, complexing agents, and sulfate compounds, have been proposed for the removal of phenolics responsible for discoloration, haze, and flavor changes but the possibility of using enzymatic laccase treatments as a specific and mild technology for stabilizing beverages against discoloration and clouding represents an attractive alternative [147]. Since such an enzyme is not yet allowed as a food additive, the use of immobilized laccase might be a suitable method to overcome such legal barriers as in this form it may be classified as technological aid. So laccase could find application in preparation of must and wine and in fruit juice stabilization [148, 149].

Waste Effluent Treatment

Various aromatic xenobiotics and pollutants generated by pulp and paper, coal conversion, petrochemical, alcohol distilleries, dyeing, and textile industries are responsible for imparting color to the wastewater and toxicity to mammals and fishes. White-rot fungi that produce lignin-degrading enzymes are reported to be the most efficient in detoxification and decolorization of such effluents [150]. Decolorization of the effluents by a marine fungal isolate was also reported [151, 152]. A major source of phenolic wastes is alkaline extraction stage effluent arising during bleaching of wood or pulp, which contains over 50% of color load [138]. Conventional treatment methods, such as aerated lagoons and activated sludge plants, are ineffective in removing this color. Moreover, chemical and physical treatments methods, including ultrafiltration, ion exchange, and lime precipitation, are expensive, so alternative biotreatment processes are now being considered. Laccase and peroxidase oxidize phenolics to aryl-oxy radical insoluble complexes. Enzyme-mediated bioremediation processes include polymerization among pollutants themselves or copolymerization with other nontoxic substances such as humic materials, thus facilitating easy removal by such means as adsorption, sedimentation, or filtration [5, 153]. Laccase mediator system was also successfully applied in the treatment of paper mill effluent [154] and detoxification of olive mill residue and its wastewater effluents [155, 156].

Fossil Fuel Desulfurization

Fossil fuels emit harmful sulfur-containing compounds during postcombustion or precombustion processes. The emission of such harmful chemicals can be reduced by various chemical and physical desulfurization methods but they require extreme conditions like high temperature and pressure and high maintenance costs. On the contrary, desulfurization by enzymes such as laccase can be done under milder conditions and these methods remove organic sulfur more efficiently [157, 158].

Biosolubilization of Coal

Coal solubilization offers the possibility of converting coal to a wide range of organic chemicals which include low-grade ores and liquid fuels. However, the irregular complex structure and insoluble nature of coal make it much less susceptible to bioconversion [159]. In the recent years, many research efforts have been made to find microbial biocatalysts capable of depolymerizing coal into low-molecular-weight substances. Certain basidiomycetes have the potential ability to depolymerize coal humic substances and several studies indicate that ligninolytic enzymes are involved in the process [160]. Laccase which uses only molecular oxygen as a cofactor may prove to be economical. *T. versicolor* is able to decolorize coal-derived humic acids under conditions where laccase is active and peroxidase activity is zero or negligible [161].

Degradation of Herbicides; the Potential Soil Pollutants

Phenyl-urea-based herbicides are widely used for control of grasses and broad-leaved weeds in winter cereal crops. These herbicides are potential pollutants of soil and cause oxidation reaction in soil, plant, and microbial system, including N-dealkylation, aryl, or ring substituent hydroxylation [162]. As a result of oxidation process, hydroxylated metabolites are formed in soil, which appear as phenolic compounds and behaves like substrates of laccase. Jolivalt et al. [163] reported that laccase from white-rot fungus *T. versicolor* has the ability to transform the herbicide *N*,*N*′-dimethyl- *N*-(hydroxyphenyl) urea into insoluble purple phenolic compounds, *p*-benzoquinone, at pH 3, which can be easily metabolized by other fungi. It has been observed that pH also determines the nature of reaction products. The lower pH is favorable for transformation process, as, at pH 3, *p*-benzoquinone (easily metabolized product) has been formed, whereas at pH 6 mixture of *N*,*N*′-dimethyl-*N*-[(2,5-cyclohexadiene-1-one)-4-xylidene] urea (difficult to metabolize) has been formed. White-rot fungus *Phlebia brevispora* can degrade commercially produced 2,4,6-trichlorophenyl *p*-nitrophenyl ether herbicide [164].

Polycyclic aromatic hydrocarbons (PAHs) together with other xenobiotics are a major source of contamination in soil. Laccase can be used to degrade such recalcitrant molecules. [165]. Thus, laccases were able to mediate the coupling of reduced 2,4,6-trinitrotoulene metabolites to an organic soil matrix, which resulted in detoxification of munition residue [166]. Moreover, PAHs, which arise from natural oil deposits and utilization of fossil fuels, were also found to be degraded by laccases [167].

Food Treatment

Laccase activity has been suspected to play an undesirable role in the processing of certain foods. Excessive or uncontrolled laccase activity along with the action of other phenol

oxidases could cause browning of canned foods or vegetables or participate in various fruit pathogenesis. But recently, Minussi et al. [148] have explained the potential applications of laccase in different aspects of food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar–beet pectin gelation, baking, etc. They can be applied to certain processes that can enhance or modify color appearance of food and beverage. It is mainly involved in baking due to its ability to cross-link biopolymer [168]. Selinheimo et al. [169, 170] explained that a laccase from white-rot fungi *Trametes hirsuta* increased the maximum resistance of dough extensibility in both flour and gluten dough and in wheat bread making. Laccase is also applicable in elimination of undesirable phenolics, responsible for browning, haze formation, and turbidity development in clear fruit juices, beer, and wine. One interesting case involves ripe olive processing in which laccase replaces the conventional lye solution and oxidatively polymerizes various phenolics (such as oleuropein) in the olive, resulting in color darkening and debittering.

Medicinal Applications

Laccase has been reported to synthesize products of pharmaceutical importance. Poison ivy produces toxic catechol, urushiol, which causes poison ivy dermatitis. Laccase oxidizes and detoxifies urushiol, thus rendering it ineffective. Laccase can also oxidize iodide to produce iodine [59], a reagent widely used as disinfectant. The synthesis of actinocin from 4-methyl-3-hydroxyanthranilic gave the chromophore of actinomycin antibiotics, suggesting a route to synthesis of cytotoxic compounds with actinomycin-related structures. The coupling activity of the laccase is used in the synthesis of novel compounds and materials [171, 172]. Laccase catalysis can be used to synthesize flavonoids, textile dyes, cosmetic pigments, aromatic aldehydes, pesticides, and heterocyclic compounds. Laccase-based hair dyes are less irritant than those commonly available since laccase replaces hydrogen peroxide as an oxidizing agent in the dye formulation [173]. Recently, proteins for skin lightening have also been developed [174]. Wang et al. [175] carried out the assay for HIV reverse-transcriptase inhibitory activity using *Tricholoma giganteum* laccase purified from its fruiting body. The capability of laccase to synthesize new compounds might also be used to generate new therapeutic compounds for treatment of microbiol infection or cancer.

Biosensor and Immunochemical Applications

A biosensor is a device that detects, transmits, and records information regarding a physiological or biochemical change. Laccase-containing biosensors for detecting O_2, glucose, aromatic amines, phenolic compounds, and a wide variety of reducing substrates have been developed [176]. Two types of laccase-based O_2 sensors are widely used. One type monitors visible spectral changes (at 600 nm) of laccase resulting from the reoxidation of the type 1 copper in laccase by O_2. Another type monitors current or voltage change from a modified oxygen electrode on which O_2 reduction is enhanced under the electrocatalysis of immobilized laccase. For detecting phenols, anilines, or other reducing substrates, three types of laccase-based sensors have been reported [177]. One type detects the photometric change resulting from the oxidation of a chromogenic substrate; the second type monitors the O_2 concentration change that is coupled to the substrate oxidation, and the third type uses an electrode that replace O_2 as the acceptor for the electrons drawn from the substrate (through laccase). Laccase-based biosensors can be used for different electrochemical measurements [178].

Laccase catalysis can be used to assay other enzymes. In these assays, either the enzyme of interest catalyzes the production of a compound whose subsequent oxidation by laccase generates detectable physical change or a product from laccase catalysis (whose production is accompanied by a detectable physical change) is quenched by the activity of the enzyme of interest. The strategy has been applied to assay various enzymes including amylase (alanine, cysteine, or leucine specific), amino-peptidases, alkaline phosphatase, γ-glutamyl transpeptidase, arylamidase, cellobiose oxidase, chymotrypsin, glucosidase, kallikrein, plasmin, and thrombin [179]. The laccase- and tyrosinase-based biosensors can also be used to monitor a wide range of compounds [180].

Laccase that is covalently conjugated to an antibody or antigen can be used as a marker enzyme for immunochemical assay. In this application, binding of the antibody (or antigen) to its immunological counterpart is detected by localized laccase activity on a gel or a blot membrane, much like the conventional peroxidase or phosphatase-assisted immunochemical assays [177]. Under certain conditions, the antibody–antigen binding impairs the function of conjugated laccase, thus allowing immunochemical detection through modulation of laccase activity [181].

Future Prospects

Laccase is used in many industrial, agricultural, and medicinal applications where oxidation or oxidative derivatization is involved. Investigations are being carried out on laccase-based enzymatic synthesis, biooxidation, biotransformation, and biosensor development. Emerging research areas include the tailoring of lignocellulosic materials by laccase-assisted biografting of phenols and other compounds and the use of laccase for adhesion enhancement in binderless wood boards. Different laccase applications have been successfully carried out in either laboratory or pilot plant scale. However, the major drawbacks in large-scale applications are lack of sufficient enzyme stocks and the cost of redox mediators. To overcome this drawback, efforts are under way to achieve cheap overproduction of laccase by further modifications.

Protein engineering techniques could be used to optimize laccase catalysis in various applications. Production of recombinant laccases has been reported, which have similar molecular and biochemical properties to those of common native enzymes. This demonstrates not only the feasibility of laccase expression in alternative hosts but also the potential for its large-scale production.

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