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Review

Structure–function relationship among bacterial, fungal and plant laccases

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ABSTRACT

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2), a multicopper oxidase enzyme, widely distributed in plants, fungi and bacteria have ability to catalyze oxidation of various phenolic and non-phenolic compounds as well as many environmental pollutants. The diversified functions of laccases, including the antagonistic ones such as their involvement in lignin biosynthesis (in plants) as well as lignin degradation (in fungi and bacteria), make them an interesting enzyme for study from the point of view of their structure, function and application. Important applications of laccases include delignification, pulp bleaching and bioremediation. The ability of laccases to polymerize natural phenols helps to develop new cosmetic pigments, hair dyeing materials, deodorants, toothpastes, mouthwashes and other useful products.

Recently, the utility of enzyme has also been shown in the nanoparticle based biosensor technology as well as in medical fields. In the present review, a comparative account of the bacterial, fungal and plant laccases has been presented from these points of views. Laccases are dimeric or tetrameric glycoproteins usually containing four copper atoms per monomer. To perform catalytic function, laccase depends on Cu atoms that are distributed at the three different copper centers. These copper centers in laccases are categorized into three groups: Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers. The four copper atoms are differing in their characteristic electronic paramagnetic resonance (EPR) signals. The phylogenetic analysis reveals, laccases from these groups (viz. bacteria, fungi and plant) form independent clades, in consistent with that of taxonomical classification. From previous experimental evidence and from *in silico* studies, it is evident that despite their wide taxonomic distribution and substrate diversity, molecular architecture of laccases is common to multicopper oxidases. Three dimensional structure predictions, at monomeric level, for all laccases (bacterial, fungal and plant) suggest that they are composed of three sequentially arranged cupredoxin-like domains. Multiple alignment of primary sequences of all three modeled laccases shows that, the copper binding motives are highly conserved in all sequences. The similarities are more significant in the N- and C-terminal regions, corresponding to Domains 1 and 3, as the copper interacting motif is present in Domains 1 and 3 not in Domain 2. This structural conservation reflects a common reaction mechanism for the copper oxidation and the O_2 reduction in these enzymes. Putative binding pocket analysis depicts, larger binding cavity for bacterial laccase as compared to those for plants and fungi. An in depth analysis of copper binding site, yielded significant differences in conserved residues for laccases of bacteria, fungi and plants which provided the basis for the dual and contrasting functions of laccases.

Contents

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1. Introduction

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) belong to the superfamily of multicopper oxidases [1]. Laccases are widely distributed in many eukaryotes e.g. fungi, plants [2] as well as in prokaryotes e.g. bacteria [3] and exhibit various functions, depending on their source organism, physiological and pathological conditions. Range of functions exhibited by laccases is broadly divided into three categories: (1) cross-linking of monomers, (2) degradation of polymers, and (3) ring cleavage of aromatic compounds [4]. Thus, the various functions carried out by laccases include lignification, wound healing and iron oxidation (in plants), delignification, pigmentation, fruiting body formation as well as pathogenesis (in fungi) and melanin formation, endospore coat protein synthesis (in bacteria) [5–10]. The localization of plant and fungal laccase is extracellular, while in bacteria most of the laccases are intracellularly localized [11]. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic function, laccases depend on Cu atoms that are distributed at the three different copper centers viz. Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers, differing in their characteristic electronic paramagnetic resonance (EPR) signals [12,13]. At three dimensional structure level, laccases (bacterial, fungal and plant) have been suggested to have three sequentially arranged cupredoxin-like domains. From wet lab analysis as well as from *in silico* studies, it is evident that despite their wide taxonomic distribution and substrate diversity, molecular architecture of catalytic site of laccases exhibits commonality with that of multicopper oxidases. The ability of laccases to catalyze the oxidation of various phenolic as well as non-phenolic compounds, coupled to the reduction of molecular oxygen to water, makes them valuable from the standpoint of their commercial application [14].

This review presents a comparative analysis of laccases form bacteria, fungi and plant from the point of view of their structure and function. Furthermore, the potential of laccases as a promising enzyme to replace the conventional chemical and mechanical processes in several industries such as the pulp and paper, textile, pharmaceutical, and nanoparticle based biosensor has also been presented.

2. Broad categories of laccases

2.1. Bacterial laccases

The first bacterial laccase was found in plant-root associated bacterium *Azospirrullum lipoferum* [15], which was involved in melanin formation [16]. *Azospirillum* bacteria were prevalently found in soil and in the rhizosphere of a variety of grasses and cereals. Cultivated plant inoculated with these bacteria shows significant growth improvements [16]. Laccase activity was also reported in a heterocystous cyanobacterium, *Anabaena azollae* [17]. In *Bacillus subtilis* a thermostable cot A laccase, involved in production of brown spore pigment in endospore coat, was reported [18]. These laccases could help in the protection of spore coat against UV light and hydrogen peroxide. Laccases in *Streptomyces cyaneus* [19] and *Streptomyces lavendulae* [20] were also reported. Most of the bacterial laccases are intracellular for example, *A. lipoferum* [11], *Marinomonas mediterranea* [21] and *B. subtilis* [18] laccases. In contrast to fungal laccases, bacterial laccases are highly active and much more stable at high temperatures, at high pH as well as high concentrations of chloride and copper ions [22–24] and the immobilized spore laccases are more compatible with almost all industrial processes.

2.2. Fungal laccases

In fungi, laccases are widely distributed in ascomycetes, duteromycetes, and basidiomycetes. These laccase producing fungi (called as wood-degrading fungi) include *Trametes* (*Coriolus*) *versicolor*, *Trametes hirsute*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Cerrena maxima*, *Phlebia radiata*, *Coriolopsis polyzona*, *Lentinus tigrinus*, *Plreurotus eryngii*, etc. Laccases are also reported in saprophytic ascomycetes of composts (*Myceliophthora thermophila*, *Aspergillus*, *Curvularia*, *Penicillium* and *Chaetomium thermophile*) and in the soil hyphomycete *Mycelia sterlia* INBI 2-26 [25–27]. Laccases have also been purified and characterized from a few fungi-forming ectomycorrhiza e.g. *Cantharellus cibarius* [28], *Lactarius piperatus* [29], *Russula delica* [30] and *Thelephora terestris* [31] or orchideoid mycorrhiza such as *Armillaria mellea* [32,33] as well as from the species of genera that contain both saprotrophic and mycorrhizal fungi e.g. *Agaricus*, *Marasmius*, *Tricholoma* and *Volvariella*.

Better penetrative ability, due to extensive hyphal organization, has been suggested to be the reason for efficient wood degradation by fungi in nature [34]. Furthermore, the high activity of laccases in wood-rotting basidiomycetes fungi suggests that the main role of fungal laccases is to depolymerize the complex cell-wall constituents such as lignin. This degradation process also involves the synergistic effects of some other enzymes and non-enzymatic components that help to establish equilibrium between enzymatic polymerization and depolymerization [35–38]. In addition to laccases, the other enzymes implicated in lignin degradation are:

1. lignin peroxidase, which catalyzes the oxidation of both phenolic and non-phenolic units,

2. manganese-dependent peroxidase,
3. glucose oxidase and glyoxal oxidase for H_2O_2 production,
4. cellobiose-quinone oxidoreductase for quinone reduction [39].

White-rot fungi, most efficient lignin degraders, are characterized by high laccase activity. It has been postulated that almost all white-rot fungi produce laccase [2,14] except for *Phanerochaete chrysosporium*, which is reported to produce a range of isoenzymes of lignin peroxidase and manganese peroxidase [40]. In white-rot fungi, manganese peroxidase in combination with either laccase or lignin peroxidase may be the minimum necessary enzymatic component for the lignin biodegradation [41]. Based on the enzyme production patterns followed, three categories of fungi may be postulated:

(i) lignin–manganese peroxidase group (e.g. *P. chrysosporium*),
(ii) manganese peroxidase–laccase group (e.g. *Dichomitus squalens*),
(iii) lignin peroxidase–laccase group (e.g. *Phlebia ochraceofulva*).

Besides degradation of biopolymers, fungal laccases are also reported to perform several other functions, such as development associated pigmentation (dihydroxynaphthalene melanins, that are produced against environmental stress), fruiting body formation, fungal morphogenesis, detoxification, sporulation, and pathogenesis [7–10]. Plant-pathogenic fungi are reported to produce laccases that are proposed to detoxify the toxic components generated by the plant defence systems [2,42]. *Botytis cinerea* that causes soft rot infections in many crop plants such as carrot, cucumbers as well as the noble rot and grey rot in grapes produces extracellular laccases, which are involved in the pathogenesis [43–45]. In *Cryptococcus neoformans*, laccases are expressed as virulence factors, thus they are considered as a major fungal pathogen in immuno-compromised individuals such as AIDS patients, organ transplant recipients and high doses corticosteroid treated patients [46]. This fungal laccase is thought to convert host catecholamines into melanin, which protects *C. neoformans*, allowing it to cause more damage to the host [47].

Laccase production in fungi is sensitive to the nitrogen concentration. Usually high nitrogen concentration is required to obtain greater amounts of laccases. For example, when *Lentinula edodes* [48], and *Rigdoporus lignonus* [49] were grown in a high nitrogen (24–26 mM) containing medium, laccase production becomes highest. In contrary to this, enhanced production of the laccase in nitrogen-limited media is also reported in *Pycnoporus cinnabarinus* [50] and *Phlebia radiate* [51].

2.3. Plant laccases

Laccases are member of multigene family in plants. The first laccase was identified in sap from *Rhus vernicifera*, the Japanese lacquer tree [52]. Subsequently, laccases were reported from variety of plants such as lacquer, mango, mung bean, peach, pine, prune, and sycamore [53]. Even multiple forms of laccases were reported from some plants. Thus, eight laccases were reported in loblolly pine (*Pinus taeda*) [54], five distinct laccases were shown to be expressed in the xylem tissues of poplar (*Populus trichocarpa*) [55]. In addition, cell suspension culture of sycamore maple (*Acer pseudoplatanus*) was reported to produce and secrete laccases like multicopper oxidases (LMCO) [56,57]. Four closely related LMCOs were identified in xylem tissues of yellow-poplar (*Liriodendron tulipifera*) [58]. LMCOs have also been reported in other species, including *Zinnia elegans* [59], tobacco (*Nicotiana tabacum*) [60], and *Zea mays* [61]. Monocot laccase from ryegrass (*Lolium perenne*) was also cloned and characterized [62]. Plant laccases perform varieties of functions, such as lignin polymerization through dehydrogenative mechanism [57],

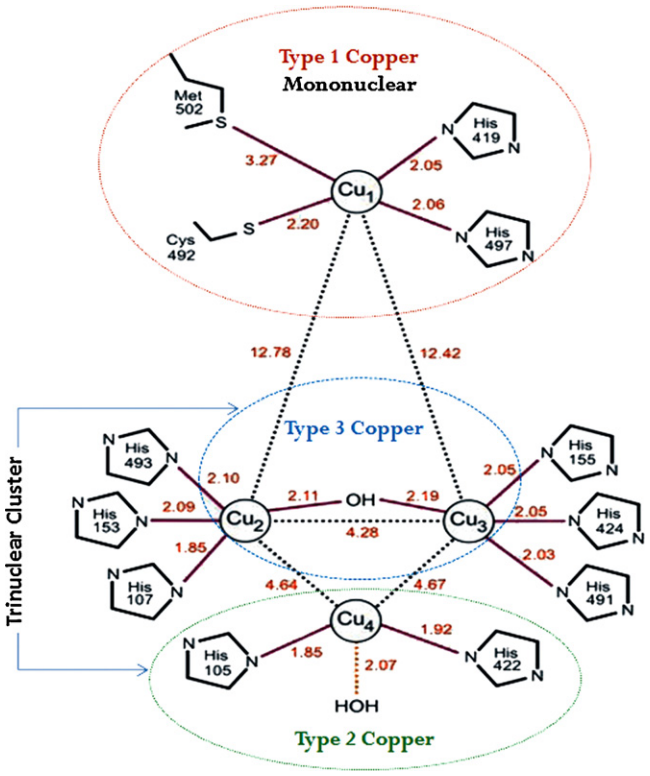

Fig. 1. Schematic representation of copper coordination centers, including interatomic distances among all relevant ligands.

wound healing [6] and iron oxidation by converting Fe(II) to Fe(III) [5,6]. Transgenic approaches, using laccase genes, for over expression as well as down regulation, have also been used in past ten years or so for utilization of plant biomass for various purposes such as energy production, phytoremediation and alteration in phenolic metabolism [63–66].

Both plant and fungal laccases are glycosylated enzymes. Plant laccases are showing a higher extent of glycosylation (22–45%) than the fungal laccases (10–25%). The carbohydrate moiety of the majority of laccases consists of mannose, N-acetyl glucosamine, and galactose. Fungal laccases often have lower molecular mass than the plant laccases [67]. On SDS-PAGE about 10–50% of molecular weight was reported to be attributed to glycosylation. The glycosylation is useful for the secretion, copper retention, thermal stability, activity of the enzyme [53].

3. Structure of copper center/active site of laccases

Laccases are dimeric or tetrameric glycoproteins, containing four copper atoms per monomer. These copper sites in laccases are categorized into three groups (Fig. 1), Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers [68,12]. The four copper atoms are differing in their characteristic electronic paramagnetic resonance (EPR) signals. Type-1 copper shows coordination with two histidines, one cysteine and one methionine as ligands. The Type-1 copper center shows an intense electronic absorption band near 600 nm ($\varepsilon = 5000\ M^{-1}\ cm^{-1}$), which is responsible for their deep blue color. The laccases which lack the typical absorption around 600 nm have also been reported. For example, a "white laccase" (containing 1 Cu, 1 Fe, 2 Zn atoms) in *Pleurotus ostreatus* [69] while "yellow laccases" (containing copper but in an altered oxidation state) in *Panus tirinus* [70]. The Type-2 copper has two histidine and water as ligands. The Type-3 copper coordinates with three histidines and a hydroxyl

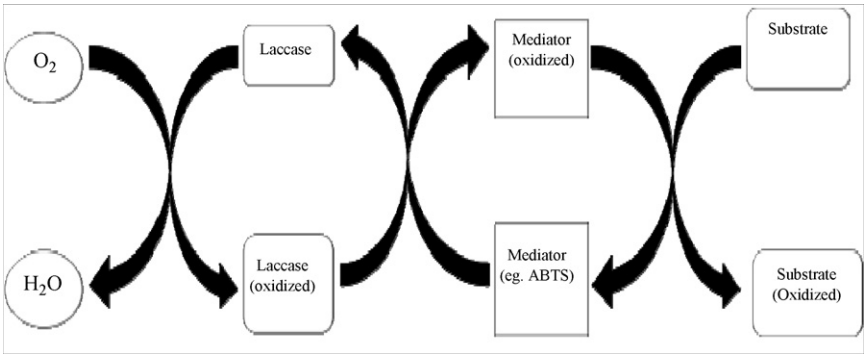

Fig. 2. Schematic representation of laccase enzyme catalysis in presence of mediator.

bridge, which maintains the strong anti-ferromagnetic coupling between the Type-3 copper atoms [71]. Type-2 copper shows no absorption in the visible spectrum and is positioned close to the Type-3 copper which shows an electron adsorption at 330 nm. Depending on the structure and properties of the copper centers laccases are also divided into low-redox potential and high-redox potential laccases. Laccases from basidiomycetes (especially white-rot fungi) are high-redox potential laccases [72], whereas, bacterial and plant laccases are the examples of low-redox potential laccases [73].

4. Mechanism of action of laccases

In contrast to peroxidases, laccases consume O_2 instead of H_2O_2 to oxidize the monolignols [57,74]. To perform catalytic function, laccase depends on Cu atoms that are distributed at the three different copper centers as mentioned above in Section 3. The laccase enzyme withdraws the electron from the substrates and converts them in free radicals, which can be polymerized. After receiving four electrons, the enzyme donates them to molecular oxygen to form water molecule (Eq. (1)) [75]. Overall, there are three major steps in laccase catalysis:

1. Type-1 Cu reduction by reducing substrate.
2. Internal electron transfer from Type-1 Cu to Type-2 and Type-3 Cu trinuclear cluster.
3. Reduction of oxygen (to water) at Type-2 and Type-3 Cu [8,12,76,77].

The overall reaction is as follows:

$$4RH + O_2 \rightarrow 4R^{\bullet} + 2H_2O \quad (1)$$

In vitro studies on small lignin model compounds [40,78], suggested that the first step of laccase mediated lignin degradation is an oxidative reaction with the loss of one electron from phenolic hydroxyl groups of lignin to produce phenoxy radicals. The radicals may spontaneously reorganize and give rise to the cleavage of alkyl side chains of the polymer. Laccase degrades both β-1 and β-O-4 dimers via C_α–C_β cleavage, C_α oxidation and alkyl–aryl cleavage.

Since laccases show activity in the absence of toxic H_2O_2, they could play a role in the early stages of lignification in living cells [79]. Thus, it has also been postulated that laccases might be the principal lignification enzymes under conditions where lignin concentration has reached a level where the middle lamella has become so hydrophobic that most of the water and H_2O_2 is excluded, whereas O_2 is still available [80].

It was also found that polymerizing activity of laccases on kraft lignin was prevented when compounds such as 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were present [75,81–83]. ABTS acts as a mediator and allows laccase to oxidize and cleave non-phenolic lignin substrates (Fig. 2) [75]. In other words, ABTS functions as a diffusible electron carrier and the action of the laccase–ABTS couple proceeds via carbon–hydrogen abstraction, with a consequent C–C bond cleavage in condensed lignins [83]. ABTS is a specific substrate for laccase in the absence of hydrogen peroxide, and a substrate for peroxidase in presence of hydrogen peroxide [84]. ABTS in solution, in reduced form, has a very faint green color, while, in oxidized form, it turns dark green so it was used to detect laccase activity. *Pycnoporus cinnabarinus* laccase efficiently degrades the lignin in the presence of 3-hydroxyanthranilate, an endogenous fungal metabolite that mediates the oxidation of non-phenolic components of lignin and thereby acts like a mediator [50]. More than 100 possible mediator compounds (e.g. 3-hydroxyanthranilic acid (HAA), N-hydroxybenzotriazole (HBT), N-hydroxyphtaimide (HPI), methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid), violuric acid (VLA)) have been investigated for their capability to oxidize lignin or lignin model compounds through the selective oxidation of their benzylic hydroxyl groups [85].

5. Physicochemical properties of laccases

Laccases are usually present as several isoenzymes having their own unique substrate specificity [62]. In addition to mono- and polyphenols, laccases have capability to oxidize various aromatic compounds, such as substituted phenols, diamines, aromatic amines and thiols, and even some inorganic compounds such as iodine, $Mo(CN)_8^{4-}$, and $Fe(CN)_6^{4-}$ [3,8,14]. The organic substrates of laccases can be divided into three groups: *ortho*- (e.g. guaiacol, *o*-phenylenediamine, pyrocatechol, dihydroxyphenylalanine, pyrogallol, caffeic acid, gallic acid, and protocatechuic acid), *meta*- (*m*-phenylenediamine, orcinol, resorcinol, and phloroglucin) and *para*- (*p*-phenylenediamine, *p*-cresol, and hydroquinone) substituted compounds with a lone electron pair. *Ortho*-substituted compounds are the best substrates for most laccases [14,86]. Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) is typically referred to as a specific substrate for laccase. Furthermore, in presence of mediators, substrate specificity of laccases can be further broadened leading to oxidation of more complex substrates [75,87].

Substrate specificity and affinity of laccase vary with changes in pH. For substrates whose oxidation does not involve proton exchange (such as ferrocyanide), the laccase activity often decreases as pH increases, whereas for substrates whose oxidation involves proton exchange (such as phenol), the pH–activity profile of laccase can exhibit an optimal pH whose value depends on source of laccase rather than substrate [81,88–90]. For phenols, the optimal pH range is between 3 and 7 for fungal laccases as well as for bacterial laccases and may increase to 9 for plant laccases [90,91]. The lower pH optima for fungal laccases may be due to the

Table 1
Km of laccases for different substrates. The pH and temperature at which Km was measured are also given.

Substrates/organisms	Km	Organisms	pH	Temperature	Brenda ID
Bacteria					
Syringaldazine	0.008	*Bacillus subtilis*	7.6	37 °C	674926
2,6-Dimethoxyphenol	0.035	*Bacillus subtilis*	7.6	37 °C	674926
K4(FeCN6)	0.027	*Bacillus subtilis*	7.6	37 °C	674926
	0.069	*Bacillus subtilis*	7.6	37 °C	674926
N,N-dimethyl-1,4-phenylenediamine	0.42	*Streptomyces griseus*	6.5	40 °C	655919
2,6-Dimethoxyphenol	0.0567	*Bacillus licheniformis*	7.0	85°C	684645
ABTS	0.049	*Bacillus subtilis*	7.6	37 °C	674926
	0.11	*Bacillus subtilis*	3.0	–	396395
Fungi					
Syringaldazine	0.0028	*Physisporinus rivulosus*	3.0	25 °C	684624
	0.0018	*Melanocarpus albomyces*	6.0	–	656736
	0.004	*Agaricus blazei*	5.5	20 °C	671510
	0.008	*Trametes versicolor*	4.5	25 °C	673096
	0.091	*Pycnoporus sanguineus*	5.0	25 °C	671552
	0.026	*Basidiomycota sp.*	4.5	25 °C	655386
2,6-Dimethoxyphenol	0.00086	*Phlebia fascicularia*	3.0	70 °C	657149
	0.026	*Trametes versicolor*	4.5	25 °C	673096
	0.203	*Pycnoporus sanguineus*	4.0	25 °C	671552
	0.43	*Pleurotus ostreatus*	7.0	25 °C	671403
	1.026	*Agaricus blazei*	5.5	20 °C	671510
	8.8	*Pleurotus ostreatus*	5.5	25 °C	655374
	56	*Cyathus bulleri*	5.2	45 °C	673816
	0.175	Lentinus edodes	4.0	50 °C	396394
Ascorbic acid	0.192	*Podospora anserine*	5.5	–	396377
Catechol	1.05	*Chaetomiaceae sp.*	7.0	30 °C	655375
	1.72	*Lentinula edodes*	3.0	30 °C	656729
Dicatechol	3.65	*Pleurotus ostreatus*	7.0	25 °C	671403
Ferulic acid	1.39	*Lentinula edodes*	5.0	30 °C	656729
	14	*Cyathus bulleri*	5.2	45 °C	673816
Ascorbic acid	0.192	*Podospora anserine*	5.5	–	396377
Catechol	1.05	*Chaetomiaceae sp.*	7.0	30 °C	655375
N,N-dimethyl-1,4-phenylenediamine	0.212	*Chaetomiaceae sp.*	7.0	30 °C	655375
Pyrogallol	0.023	*Chaetomiaceae sp*	7.0	30 °C	655375
	24.6	*Lentinula edodes*	3.0	30 °C	656729
Vanillic acid	0.28	*Trametes versicolor*	–	–	396343
	2.92	*Fomes fomentarius*	–	–	396343
O_2	0.25	*Botrytis cinerea*			396363
ABTS	0.0107	*Physisporinus rivulosus*	3.0	25 °C	684624
	0.0065	*Bacillus licheniformis*	4.0	85 °C	684645
	0.27	*Fomitella fraxinea*	3.0	70 °C	688328
	0.00134	*Trametes hirsute*	4.5	50 °C	672747
	0.00632	*Trametes hirsute*	4.5	50 °C	672747
	0.0128	*Trametes versicolor*	3.0	50 °C	675327
	0.063	*Agaricus blazei*	5.5	20 °C	671510
	0.045	*Trichophyton rubrum*	5.5	20 °C	655362
Plants					
Coniferyl alcohol	0.002	*Populus euramericana*	–	–	396398
p-Coumaryl alcohol	0.02	*Populus euramericana*	–	–	396398
o-Phenylenediamine	41	*Rhus vernicifera*	5.0	–	396383
4-Methyl catechol	1.56	*Populus euramericana*	–	–	396398
	4.5	*Acer pseudoplatanus*	6.6	–	396358
Hydroquinone	0.001	*Populus euramericana*	–	–	396398
	0.123	*Chaetomiaceae sp.*	7.0	30 °C	655375
N,N-dimethyl-1,4-phenylenediamine	3.3	*Rhus vernicifera*	7.0	25 °C	675235
O_2	0.02	*Acer pseudoplatanus*	6.6	–	396358
ABTS	0.03	*Populus euramericana*	–	–	396398

adoptability of the fungi to grow well in acidic condition, but the plant laccases exhibited their optimal pH nearer to the physiological range due to intracellular nature. The difference in pH optima suggests to be linked to their physiological functions [92].

Temperature optima of laccase activity range from 50 °C to 70 °C but few enzymes showing temperature optima below 35 °C have also been reported. For example, the *Ganoderma lucidum* laccase has highest activity at 25 °C [93]. Thermal stability of laccases varies significantly with the temperature range of the growth of the source organism. The fungal laccases usually have lower thermal stability than bacterial laccases [14]. The thermal stability of laccases has been suggested to be linked to the interaction between the copper ions of copper centers and salt bridges as well as hydrogen bonding network in the internal protein structures [94]. The more acidic isozymes of laccase were reported to have more thermostability [95].

Properties, such as, substrates used, Km, pH and temperature optima of various laccases from bacteria, fungi and plant sources are summarized in Table 1.

Laccases have been reported to be inhibited by various reagents such as small anions as halides (excluding iodide) [86], azide, cyanide, and hydroxide. These inhibitors have been suggested to bind to the Type-2 and Type-3 Cu, resulting in an interruption of the internal electron transfer and subsequent inhibition of the activity. Other inhibitors of laccases include, metal ions (e.g. Hg^{2+}, Mg^{2+}, Ca^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, and Zn^{2+}), fatty acids, sulfydryl reagents, hydroxyglycine, kojic acid, EDTA, L-cysteine, dithiothreitol, glutathione, thiourea, and cationic quaternary ammonium

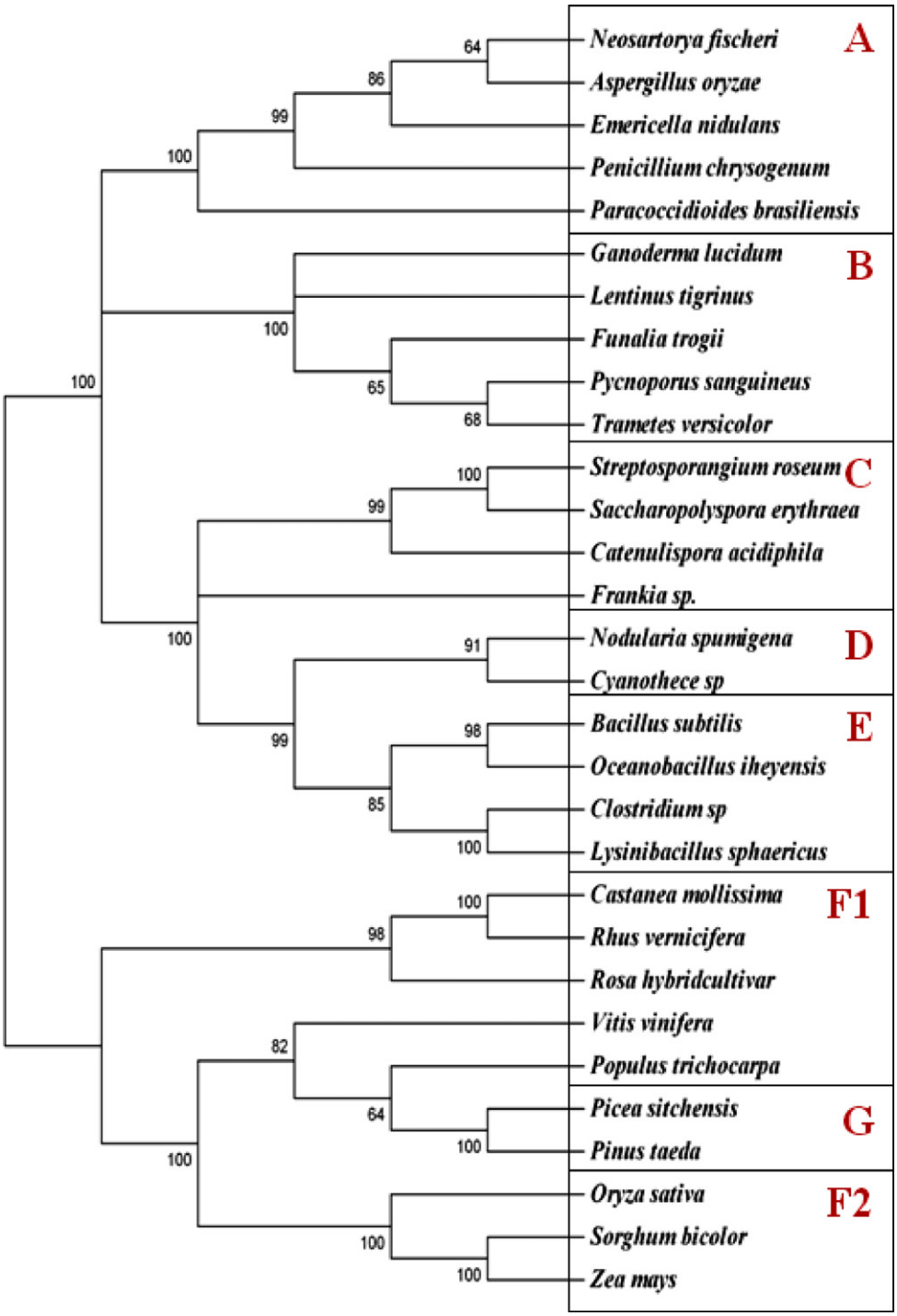

Fig. 3. Phylogenetic relationships among selected datasets from fungi, bacteria and plant laccases, derived from Neighbor-Joining method. Bootstraps values are indicated on branches. Tree is condensed for the value ≥50. In the NJ tree block 'A' represent ascomycetes fungi, 'B' – basidiomycetes fungi, 'C' – actinobacteria, 'D' – cyanobacteria, 'E' – fermicutes, 'F1' – dicots, 'G' –gymnosperms and 'F2' represents monocot angiosperms.

detergents [96,97]. These agents are suggested to affect the laccase activity by chelating the Cu(II) atoms or by modifying amino acid residues or by causing conformational change in the glycoprotein [98].

6. Phylogenetic analysis of laccases

Evolutionary relationship among the bacterial, fungal and plant laccases was investigated by constructing a phylogenetic tree based on their amino acid sequences, using Neighbor-Joining algorithm [99]. Phylogenetic analysis revealed formation of three big clusters, as shown in Fig. 3. The first cluster represents fungi while, second and third represent bacteria and plants, respectively. The fungal cluster further grouped in two sub-clusters, represent basidiomycetes and ascomycetes. Like fungi, plant cluster also subdivided into two sub-clusters, representing gymnosperms and angiosperms. The angiosperm sub-cluster further bifurcated in monocot and dicot clades. Aside from three groups, which clearly arose from a consecutive speciation and recent duplications, the rest of the sequences presented a dispersed distribution, indicating

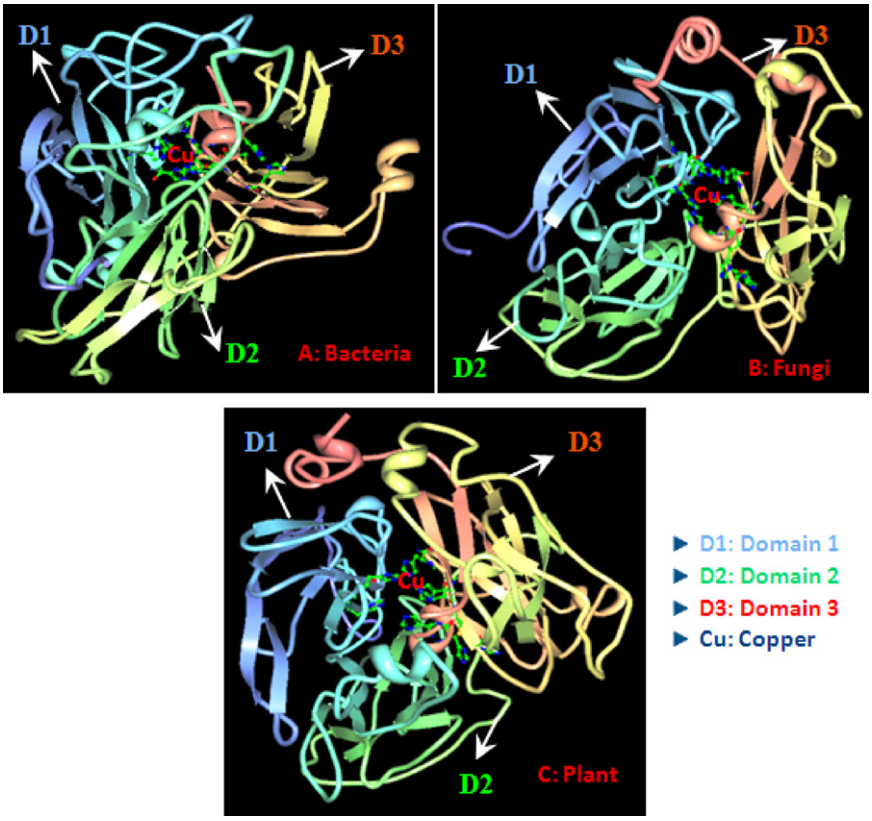

Fig. 4. Three dimensional structure of (A) bacterial laccase (*Bacillus subtilis*), (B) fungi laccase (*Tramates versicolor*), and (C) plant laccase (*Populus trichocarpa*).

more than one duplication events. In some cases, all the members of the family lay interspersed with sequences from other genera. This evidence might suggest that laccase could be useful as a robust molecular marker for organism evolutionary studies. The tree topologies presented here suggest that laccase is possibly a paralogous based enzyme. The two or more laccase genes in different organisms could be the result of duplication events, after which the enzymes evolved to perform similar biochemical processes.

7. Comparative 3-D structure analysis of bacterial, fungal and plant laccases

From previous experimental evidences [100,101] and from modeled structure, it has been shown that despite their wide taxonomic distribution and diversity of substrates, molecular architecture of laccases is common for all multicopper oxidases.

A comparative modeling study was conducted by us to provisionally explain profound differences among bacterial, fungal and plant laccases. One of the purposes in building a model was also to have a better understanding of the features that are important for catalytic activity. The three dimensional structure of bacteria (*B. subtilis*), fungi (*T. versicolor*) and plant (*P. trichocarpa*) laccases was predicted by homology modeling approach using template 1HL0, 1KYA and 1AOZ, respectively, by Modeller 9v6 [102] (Fig. 4). The qualities of each modeled structure for laccases are carried out, using PROCHECK, ProSA and PROQ. The PROCHECK analysis for modeled laccases shows that 90.1% (bacteria), 89.5% (fungi) and 86.5% (plant) residues were in favored and allowed regions in Ramachandran plot. These values match well with those for experimentally determined models. Results from ProSA gave a *z*-score of −8.28, −6.22 and −5.79 and PROQ analysis gave a LG score value of 6.006, 5.293 and 4.874 for *B. subtilis*, *T. versicolor* and *P. trichocarpa*, respectively. These results taken together suggest that the values for homology model built for bacteria, fungi and plant fall within the range of values observed for experimentally determined structures and the built models are very reliable for interpretation of structure–function relationships.

Three dimensional structure predictions, at monomeric level, for all laccases (bacteria, fungi and plant) suggest that they are composed of three sequentially arranged cupredoxin-like domains as presented in Fig. 4. These cupredoxin domains mainly formed by β-barrels (Greek key motif) consisting β-sheets and β-strands, arranged in sandwich conformation [103]. Comparative analysis of predicted models shows, first domain present at N-terminal region (blue color) in bacteria (Fig. 4A) is somewhat distorted conformation in comparison with the equivalent domain in fungi (Fig. 4B) and plant (Fig. 4C). Fig. 4A depicts, presence of a coiled section, which connects Domain 1 and Domain 2 in bacteria, is absent in fungi and plant. This coiled section also helps in packaging between Domain 1 and Domain 2 in bacteria [104]. In fungi and plant, short α-helical regions connect Domain 1 to Domain 2 and Domain 2 to Domain 3 (Fig. 4B and C). These helices also connect β-strands in structure topology. By comparing models, it is observed that, in bacteria a large loop segment link Domains 2 and 3 through external connection (Fig. 4A), whereas, in fungal and plant laccases, the corresponding link is made through internal connections (Fig. 4B and C). The Domain 2 (green color) acts as bridging element between Domain 1 and Domain 3 [90]. The structure analysis revealed, tri-nuclear copper cluster (T2/T3) embedded between Domains 1 and 3 with both domains providing residues for the coordination of the coppers. The copper interacting residue is highlighted in all modeled structures (Fig. 4). Finally, Domain 3 (red/yellow) in all modeled structures not only contains the mononuclear copper center, but also contributes to the formation of the binding site of the trinuclear copper center, which is located in the interface between Domains 1 and 3. Moreover, in all multicopper oxidases Domain 3 includes the putative substrate binding site, located at the surface of the protein, close to the Type-

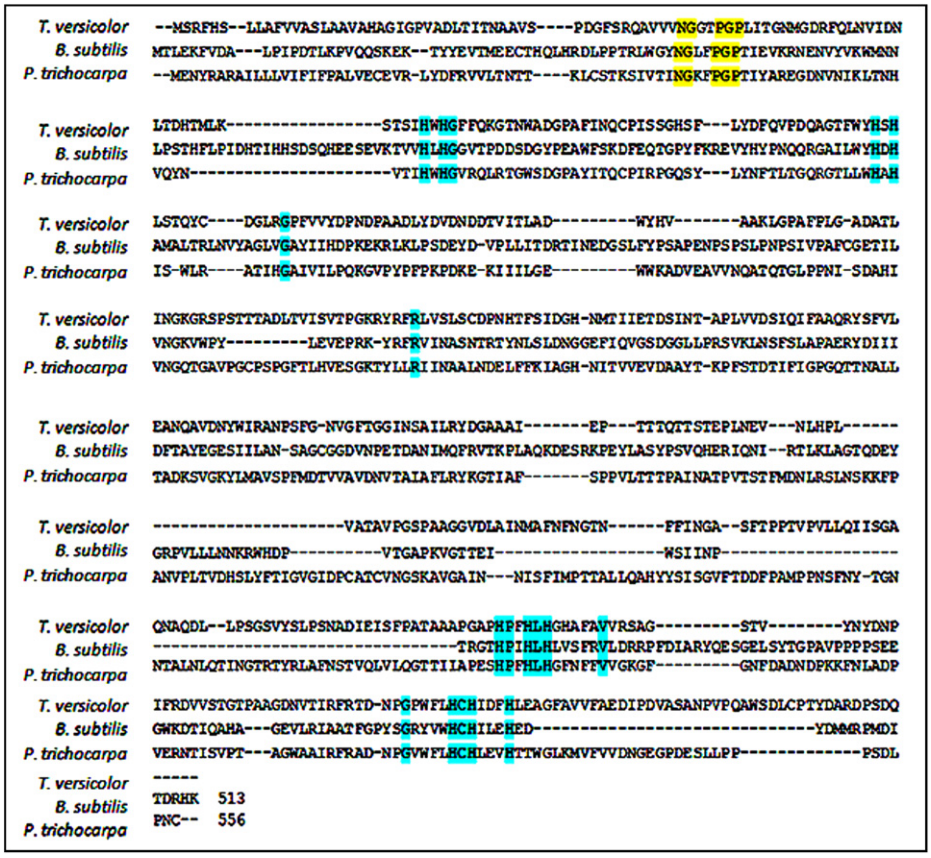

Fig. 5. Structure based sequence alignment of laccase, by COBALT multiple alignment tool. Invariant residues are highlighted in cyan and yellow color. The alignment shows highest conservation at copper interacting sites present in Domain 1 and Domain 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1 mononuclear copper center. A protruding section, formed by a loop and a short α-helix, forms a lid-like structure over the substrate binding site in bacteria. No similar element has been found in the previously analyzed 3-D structure of plant and fungal laccases. Therefore, this structural element may represent a distinctive feature of bacterial laccase. The overall structure analysis shows that, it shares a common β-barrel motif in all domains. In all laccases, the C-terminal portion is characterized by short (13 residues) α-helix stretch, stabilized by two disulfide bridges, the first bridge (e.g. in fungi -Cys-106–Cys-509) connects Domains 1–3 and second disulfide bridge (in fungi -Cys-138–Cys-226) connects Domains 1 and 2.

Multiple alignment of primary sequences of all three modeled laccases shows that, the copper binding motives are highly conserved in all sequences (Fig. 5). The similarities are more significant in the N- and C-terminal regions, corresponding to Domains 1 and 3, as the copper interacting motif is present in Domains 1 and 3 not in Domain 2. This structural conservation reflects a common reaction mechanism for the copper oxidation and the O_2 reduction in these enzymes [71,104,105]. Comparison of copper coordination distances among selected laccases from bacteria, plant and fungi, containing four copper atoms, of known three dimensional structures is given in Table 2.

Putative substrate binding pocket analysis of X-ray determined crystal structures was carried out by CASTp server. This analysis was performed in terms of molecular surface areas, molecular volumes and the cavity residues. The comparative results for surface volume analysis and the cavity residues are shown in Table 3 [42,104]. The analysis of crystal structures revealed that, bacterial (PDB ID: 1GSK) laccase has largest putative substrate binding site cavity, as compared to fungal (1KYA) and plants (1AOZ).

8. Application of laccases

A number of industrial applications of laccases such as delignification and pulp bleaching, bioremediation of contaminating environmental pollutants [106], prevention of wine decoloration, medical applications, oxidation of dye and their precursors, enzymatic conversion of chemical intermediates, and production of chemicals from lignin have been reported. Apart from the application of laccases in many agricultural, industrial, and medical areas, currently the studies on laccases are focused on laccase-based bio-oxidation, biotransformation, biosensor technology [67].

Due to high catalytic efficiency and broad substrate specificity laccases become more advantageous, as compared to other conventional chemical or microbial catalysts. However, high cost of isolation and purification, non-reusability, structure instability may be the potential practical problems for applications of laccases, but they can be overcome by immobilization of enzymes to or within solid supports [107]. Laccase immobilization has been extensively studied with a wide range of different methods and substrates [108–111]. Due to easy availability (being mostly extracellularly located) and working at broad range of pH from acidic to neutral pH and high thermostability, the fungal laccases find more industrial applications compared to those of bacteria and plants.

8.1. Delignification and pulp bleaching

About 25% of the wood pulp produced in the world is created using a mechanical pulping method, which has twice the yield of chemical pulping. Mechanical pulping has two disadvantages: it is energy intensive, and yields paper that is not as strong as paper produced from chemical processing. However, chemical

Table 2
Comparison of copper coordination distances among selected multicopper oxidases (laccases) containing four copper atoms, and of known three dimensional structure. The PDB ID for each structure is mentioned in bracket.

Copper coordination center		Laccases from different sources					
		Bacteria (1GSK)		Plant(1AOZ)		Fungi(1KYA)	
		Residue	Distance (Å)	Residue	Distance (Å)	Residue	Distance (Å)
Type 1 copper Cu(1)							
Ligand 1	Mononuclear copper center	His^{419}	2.05	His^{512}	2.05	His^{395}	2.36
Ligand 2		Cys^{492}	2.20	Cys^{507}	2.13	Cys^{453}	2.20
Ligand 3		His^{497}	2.06	His^{446}	2.09	His^{458}	2.23
Ligand 4		Met^{502}	3.27	Met^{217}	2.90	Ile^{455}	3.51
Type 2 copper Cu(4)	Trinuclear copper center						
Ligand 1		His^{105}	1.85	His^{60}	2.00	His^{64}	2.11
Ligand 2		His^{422}	1.92	His^{448}	2.09	His^{398}	1.97
Ligand 3		HOH	2.07	HOH	2.02	HOH	2.58
Type 3 copper Cu(2)							
Ligand 1		His^{107}	1.85	His^{450}	2.06	His^{66}	2.30
Ligand 2		His^{153}	2.09	His^{106}	2.16	His^{109}	2.53
Ligand 3		His^{493}	2.10	His^{506}	2.07	His^{454}	2.28
Ligand 4		OH	2.19	OH	1.99	OH	1.98
Type 3 Copper Cu(3)							
Ligand 1		His^{155}	2.05	His^{508}	2.14	His^{111}	2.28
Ligand 2		His^{424}	2.05	His^{62}	1.98	His^{400}	2.11
Ligand 3		His^{491}	2.03	His^{104}	2.19	His^{452}	2.24
Ligand 4		OH	2.11	OH	2.06	OH	1.97

pulping is expensive and produces excessive amounts of air and water pollutants. Biobleaching techniques have been considered as a potent alternative for chemical bleaching of pulp. Enzyme applications have been proposed for pulp and paper manufacture to enhance pulp bleaching, pulp refining, deinking, cellulose purification and papermaking [112,113]. Biobleaching of pulp using laccase enzyme to obtain a brighter pulp with low lignin content was also patented [114]. The pre-treatments of wood pulp with laccase (with or without a mediator) can provide milder and cleaner strategy of delignification that also respects the integrity of cellulose [115]. Treatment of laccases do not alter pulp brightness and improve auto-adhesion of fibers in medium density fiberboard [116], increase tensile strength of sheets derived of mechanical pulp and preserve tensile strength [112]. The *T. hirsute* and *T. versicolor* laccases were found to oxidize different types of lignin such as Flax soda lignin, Eucalyptus dioxane lignin, and Spruce lignin without any mediators at pH 4.5 that helps to provide an important source of raw material for pulping industry [117,118].

8.2. Bioremediation

Laccases have also shown to be useful for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures [119]. Phenolic compounds are present in wastes from several industrial processes, such as coal conversion, petroleum refining, production of organic chemicals and olive oil production [120]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [110] due to the broad substrate range of the enzyme. Laccases from white rot fungi have been also reported to perform oxidation of alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of HBT and ABTS as mediators [121]. Laccases are also able to act on polycyclic aromatic hydrocarbons (PAHs) that are toxic, carcinogenic and/or mutagens and recalcitrant environmental contaminants having tendency to bio-accumulate [122,123].

Phenols contained in the olive-mill wastewater (OMW) have a structure similar to lignin, which makes them difficult to biodegrade. Recently it was reported that the treatment of OMW with several laccase-producing fungi can remove the initial phenolic compounds (up to 78%) in 12–15 days [124]. Laccases also help in the decolorization of the OMW from black to yellow-brown [125] and to decrease its phytotoxicity, as described by the Germination Index parameter [124,126]. White-rot fungi that produce lignin-degrading enzymes are reported to be the most efficient in detoxification and decolorization of such effluents [127]. Laccase mediator system was also successfully applied in the treatment of paper mill effluent and detoxification of olive mill residue and its wastewater effluents [128,129].

8.3. Organic synthesis

Polymerizing activity of various plant and microbial laccases has been exploited for various kinds of polymer syntheses. Thus, by polymerizing various natural phenols, new cosmetic pigments, hair dyeing materials have been developed. For example, three monomer combinations, such as gallic acid and syringic acid, catechin and catechol, and ferulic acid and syringic acid result in commercially important brown, black, and red colored dyeing materials, respectively [130]. Laccases are found to be used in deodorants, toothpastes, mouthwashes, detergents [131]. Laccases are also reported in biografting of phenols and other compounds

Table 3
Analysis of surface area, volume and residues of the putative substrate binding pockets of multicopper oxidase monomers containing four copper atoms, as calculated by the CASTp server. All the structures considered for binding site analysis are X-ray crystallographic determined.

Laccase source	Molecular surface area (A^2)	Molecular volume (A^3)	Residues at binding cavity
Bacteria (1GSK)	743.1	1346.7	T^{200}, F^{208}, P^{210}, A^{212}, P^{213}, E^{214}, I^{225}, V^{226}, P^{227}, A^{228}, $F2^{29}$, C^{230}, E^{232}, T^{261}, R^{262}, T^{263}, A^{296}, P^{297}, G^{322}, C^{323}, G^{324}, K^{374}, L^{375}, A^{376}, G^{377}, T^{378}, R^{384}, P^{385}, V^{386}, L^{387}, P^{415}, T^{416}, R^{417}, G^{418}, T^{419}, H^{420}, P^{421}, Q^{443}, I^{495}, E^{497}, H^{498}, Y^{501}
Fungi (1KYA)	285.8	308.5	F^{162}, L^{164}, D^{206}, P^{207}, N^{208}, F^{239}, A^{240}, N^{264}, F^{265}, F^{332}, F^{337}, P^{391}, G^{392}, A^{393}, P^{394}, P^{396}, I^{455}, F^{457}, H^{458}
Plant (1AOZ)	255.2	214.1	L^{224}, A^{225}, A^{226}, Y^{257}, S^{258}, T^{282}, R^{283}, A^{284}, R^{285}, E^{443}, T^{444}, P^{446}, I^{509}, H^{512}

and also used to improve adhesion of fiber, particle and paperboards [113,132].

Application of laccase as a new biocatalyst in organic synthesis such as ethanol, textile dyes, flavor agents, pesticides and heterocyclic compounds [2] was also reported. For example, to improve the production of fuel ethanol from lignocellulose, a laccase from the white rot fungus *T. versicolor* was expressed in *Saccharomyces cerevisiae* [133]. The laccase-producing transformant had the ability to convert coniferyl aldehyde to ethanol at a very fast rate. It is also reported that laccase can also induce radical polymerization of acrylamide with or without mediator [134]. As, laccases can provide a remarkable number of combination possibilities of molecules for chemical synthesis, they may be applied as imperative tools for combinatorial biochemistry [73].

8.4. Application in textile industry

Laccase is also used commercially in textile industry to improve the whiteness of cotton and as well as in biostoning [135,136]. Laccase application has advantages in energy, chemicals, and water saving. Laccases were used to replace the load of pumice stones and they could bleach indigo-dyed fabrics to lighter shades [137,138]. Recently, *Stenotrophomonas maltophilia* laccase was reported to degrade synthetic dyes [139].

Laccases have potential applications in dishwashing [140] as well as in eliminating the odor on fabrics, including cloth, sofa surface, and curtain or in a detergent to eliminate the odor generated during cloth washing [141]. Laccases with mediators were also used to increase the shrink resistance of wool [142]. The polymers synthesized by laccase have been used for potential applications in development of bioactive material and textile coloration [143,144]. Laccase catalyzed grafting of functional molecules on wood and cellulose [145–147] adds new properties to fibers. In addition, use of laccase for dyeing of materials with sulfur and reduced vat dyes has been patented [148].

8.5. Treatment of beverages

Several phenolic compounds (cumaric acids, flavans, and anthocyanins) are usually present in beverages (wine, fruit juice and beer) and during their shelf life, may cause undesirable and deleterious changes such as discoloration, clouding, haze, and flavor changes. The encouraging effects of laccase action were observed on wine as well as on fruit juice [149]. Laccases can improve the flavor quality of vegetable oils, food items by removing dissolved oxygen [150]. Color of a tea-based product may be enhanced by use of fungal laccases [151]. To improve the strength of gluten structures in dough and/or baked products, laccases can be used [152,153].

8.6. Nanoparticle based biosensor application

Now-a-days, science of nanobiotechnology has grown rapidly due to its high potential impacts in almost all fields of human activity (environmental, economy, industrial, clinical, health-related, etc.). Nanoparticles, nanotubes, and nanofibers have been used extensively as carrying materials for biosensoring, and biofuel cells. A biosensor is an integrated biological probe with an electronic transducer, which converts a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding a physiological or biochemical change [154].

A number of biosensors using laccase have been developed for determination of glucose, aromatic amines and phenolic compounds [155,156]. For example, fungal laccases have been employed to estimate the phenolic content of natural juice or catechol in tea [157]. The ability of laccases to catalyze the electroreduction of oxygen via a direct mechanism, without mediator was used to develop a gas-phase oxygen biosensor in which a laccase from *R. vernicifera* and ascorbate, as reducing substrate, were both enclosed in pouches of low density polyethylene under nitrogen gas. In the presence of ascorbate, the blue chromophore prosthetic group of laccases was reduced and decolorized. When the enzyme was re-oxidized by oxygen, there was a concomitant return to the blue color that is recorded both visually and spectrophotometrically at 610 nm. Since, this oxygen biosensor is very active and stable, it was proposed as a useful tool to measure oxygen levels in products packaged under low oxygen concentrations whose quality and safety are strictly dependent on these low oxygen levels [158]. Laccase also has shown application in the design of biofuel cells [159].

8.7. Cross-linking of polysaccharides

Polymerized saccharides are useful materials for making particle boards and liner boards. Most chemicals such as, urea and formaldehyde, used in conventional polymerization are hazardous. Due to lignin oxidation properties, laccases could replace these chemicals and serve as biogluing agents [160].

8.8. Medical applications

Laccase can be used in the synthesis of complex medicinal compounds as well as heteromolecular dimmers of antibiotics via phenolic oxidation [161], phenolic oxidative coupling [162] and oxidation coupled with nuclear amination [163–165], such as, triazolo (benzo) cycloalkyl thiadiazines (a group of anti-inflammatory, analgesic agents, etc.), Vinblastine (a cytostatic, antitumor agent), mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline (for treating neoplastic diseases) [166–168]. Laccase is also reported to involve in enzyme-catalyzed production of anticancer drugs [169].

Laccase has also been reported to possess significant HIV-1 reverse transcriptase inhibitor activity. For example, laccase purified from fruiting body of *Tricholoma giganteum* was used for the assay for HIV reverse-transcriptase inhibitor activity [28].

Poison ivy dermatitis (resulted by skin contact with poison ivy, poison oak, etc.) is caused mainly by catechol-derivative toxin, urushial. Laccases have been shown to oxidize and detoxify urushial [170]. The application of laccases to oxidize iodide to iodine may be used in various industrial, medical, domestic applications such as sterilization of drinking water and swimming pool as well as disinfection of minor wounds [171]. An enzymatic method based on laccase has also been developed to distinguish morphine from codeine simultaneously in drug samples injected into a flow detection system [172].

8.9. Enzymatic and immunochemical assays

Laccase catalysis can be used to assay other enzymes including amylase, aminopeptidases (alanine, cystine or leucine-specific), alkaline phosphatases, angiotensin I converting enzymes, chymotrypsin, plasmin, thrombin, etc. [173]. Antibody or antigen conjugate laccase can be used as a marker enzyme for immunochemical assays [77].

8.10. Others applications

Laccases were also used as a versatile reporter system in filamentous fungi [174]. They could also be used to reduce harmful emission of sulfur-containing chemicals from the combustion of fossil fuels (petroleum, natural gas, liquefied coal). Laccases, the most abundant ligninolytic enzymes in soil, have also attracted

ecologists by the role in the ecosystem. It has been found that a significant decrease of laccases in forest soils resulted in elevated nitrogen doses, along with simultaneous increase in the litter layer. On the other hand, an increase of phenolic compounds in forest soil after burning resulted in an increase in laccase activity [14].

9. Concluding remarks

Laccases, widely distributed in bacteria, fungi and plants, are one of the fascinating examples of multicopper oxidase family due to their ability to carry out diverse functions of significance from the point of view of optimal utilization of plant biomass. Structurally, though all the three laccases exhibited a common 3-D architecture, at catalytic site they exhibited significant differences in conformity with their functional diversity and evolutionary relationship. Due to their diversified distributions and functions, laccases are looked upon as potential enzymes to replace the conventional chemical and mechanical processes in several industries such as the pulp and paper, textile, pharmaceutical as well as nanoparticle based biosensor.

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