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Impacts Statements:

Bacterial laccase is multi-copper oxidase extracellular isozyme with broad range substrate range specificity. It is mainly induced in industrial waste degradation for environmental safety. Its molecular and biochemical properties in bacterial system is fragmentary and very less known in literature. Since the bacterial cell is more versatile for its nutritional and environmental adaptation. Hence the knowledge for laccase application of bioremediation for various industrial wastes will open ample opportunities for large scale application due to slight variation in the structural and functional properties of bacterial and fungal laccases. There is limitation at the large scale application. Thus, this manuscript will support to researcher for the understanding thermotolerant mechanism and application of this enzyme at alkaline conditions.

Properties of Bacterial Laccases and Their Application for Bioremediation of Industrial Wastes

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Abstract:

The bioremediation process of industrial waste can be made more efficient by using ligninolytic enzymes laccase which is obtained from fungus, bacteria, higher plant, insect and also in lichen etc. Laccase are catalyzed in the mono-electronic oxidation of substrate expenditure of molecular oxygen, this enzyme belong to the multicopper oxidases and participate in the cross linking of monomers, involved in degradation of wide range industrial pollutants. In recent years these enzyme have gained application in industries of pulp and paper, textile and food industry. There are numerous reviews on laccase, however still a lot of information is unknown due to their broad range of function and application. In this review the bacterial laccase has been focused on the bioremediation of various industrial pollutants. A brief description on structural molecular and physicochemical properties has been done. Besides, the mechanism by which reaction is catalyzed, physical basis of thermostability and, enantioselectivity which requires more attention for researcher, and laccase application in various fields of biotechnology has been pointed out.

Keywords: Bacterial laccases; Biotechnological application; Industrial waste; Pulp paper industry; Textile industry

1. Introduction:

There are many potential ligninolytic enzymes (LiP, MnP and Laccases) that are widely distributed in nature in different category of taxa.[1,2] The demand for these enzymes has increased in the recent year due to their potential applications in the diverse biotechnological areas. Among these laccase is most potential with broad range of specificity, and well studied ligninolytic enzyme, which is highly versatile in nature with wide variety of industrial applications. Laccase is an isozyme predominantly present in microbial community which are encoded by different genes and expressed in different organelle and it can be readily detected by gel electrophoresis. Consequently, laccase have been reported in different molecular form i.e. *LCC 1, LCC 2, LCC 3* and *LCC 4* which one is obtained from *Pleurotus ostreatus.*[3] Laccases (EC 1.10.3.2) are polyphenol oxidases that catalyze the oxidation of various aromatic compounds, particularly those with electron-donating groups such as phenols (−OH) and anilines ($-NH_2$), by using molecular oxygen as an electron acceptor.[4] Further biochemically laccases are monomeric, dimeric or tetrameric glycoproteins and have ubiquitous in nature with four copper atoms and have three types of copper where Type 1 copper (T1Cu) is responsible for the oxidation of substrate and also responsible for the blue color of enzyme, have strong electronic absorbance around 610nm and electro-paramagnetic resonance (EPR) detectable, type 2 copper (T2Cu) is colorless it is also EPR detectable and type 3 copper (T3Cu) gives a weak absorbance near the UV spectrum (330nm) but not detectable by EPR.[5] Laccases use molecular oxygen to oxidize a variety of aromatic and non-aromatic hydrogen donors via a mechanism involving radicals. These radicals can undergo further laccases catalyzed reaction and/or non- enzymatic reaction such as polymerization, and hydrogen abstraction. Therefore, laccase has also the ability to oxidize phenolic and nonphenolic substrate. The phenolic substrate oxidation by laccases result in formation of an aryloxyradicals an active species that is converted to a quinone in the second stage of the oxidation. Though, typical substrate of laccases known to be diphenol oxidase, monophenol e.g. Sinapic acid or Guaiacol can also oxidize polyamines, aminophenols, lignin, aryl diamine, inorganic ions and it may mitigate the toxicity of some polycyclic hydrocarbon.[6] However 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS is substrates which are most commonly used, does not form quinone and is not pH dependent. For this reason it is used to calculate international unit of laccase activity. The structurally, laccases contain 15-30% carbohydrate and have molecular mass 60-90 kDa with acidic isoelectric point around pH 4.0 due to this enzyme shows high stability.[6,7] Laccase can

oxidize wide range of molecules and nearly 100 different types of compound have been identified as substrate which varies from one laccase to another.

Most of study has been focused on white-rot fungus in which *Phlebia floridensis* showed higher thermostability at pH 4.5[8], *Trametes versicolor*, for dye decolorization[9], *Phanerochaete chrysosporium* has been reported for high laccase activity on solid medium [10]. The biostimulatory effect of 2,5 xylidine has been reported in *Pholiota mutabilis, leurotus ostreatus,* and *Tarmetes versicolor* for laccase production.[11] In another study Shingo et al., reported for degradation of phenolic lignin model compound, 4,6-di(tert-butyl)guaiacol, by *Coriolus versicolor* and production of laccase.[12] Recently, in bacterial laccase polyphenol oxidase activity has been reported in an *Azospirillum lipoferum* [13], Phenol oxidases, laccase were isolated from cell extracts of the soil bacterium *Pseudomonas putida* F6.[14] Four strains of the bacterial genus Streptomyces (*S. cyaneus, S. ipomoea, S. griseus and S. psammoticus*) and the white-rot fungus *Trametes versicolor* were studied for their ability to produce active extracellular laccase in biologically treated wastewater with different carbon sources.[15] Similarly, *Proteus mirabilis, Bacillus sp., Raoultella planticola* and *Enterobacter sakazakii* are used for degradation of persistent organic pollutant from biomethanated distillery spent wash but, it is still lacking for industrial application. [16]

The low substrate specificity makes this enzyme interesting for biotechnology in various purposes, in many industries of pulp-paper and textile industries and bioremediation of various industrial pollutants. Laccases have been predominantly reported in fungi for reduction of colour from complex distillery waste water.[17] But at large scale of laccase production from fungus and applications have limitation due to its slow growth rate, unfavorable submerged aquatic environment and desired low pH range. In contrast bacteria have wide range of bioremediation activity by providing optimum nutrient and environmental condition through biostimulation process. Recently laccases have been proficiently applied in nano biotechnology due to their ability to catalyze electron transfer reaction without any mediator/cofactor.[18] Therefore, the bacteria is used for the different category of waste water treatment containing complex pollutants. However, the information on bacterial laccase in the available literature is scattered. Moreover, the detail properties of bacterial laccase and their biotechnological application is very less known. Furthermore the mechanism of enzyme enantioselectivity and stability at high pH and temperature is also reported in few literatures only. Therefore the present review has updated on bacterial laccase molecular properties, mechanism, its substrate and there thermostability has been highlighted which needs attention of researcher for further study for industrial application.

2. Laccase producing bacterial species:

Laccases have been mostly isolated and characterized from plants and fungi, and only fungal laccases are used currently in biotechnological applications for the detoxification of complex industrial wastewater. [17] Unfortunately, these enzymes usually work only efficiently under mild acidic conditions (pH 4-6) whereas the temperature range (30-55 ^{0}C) for catalytic activity is suboptimal. In contrast, little is known about bacterial laccases, which broad range substrate specificity for industrial application. Although recent rapid progress in molecular study the whole genome analysis revealed that these enzymes are widespread in bacteria. Since bacterial genetic tools and biotechnological processes are well established, so developing bacterial laccases would be much more significantly important.

Insert table 1 here

Bacterial laccases applications are still limited for industrial use but predominantly used in bioremediation of various industrial wastes. The first bacterial laccases were detected in the plant root associated bacterium *Azospirillum lipoferum.*[47] Thereafter, laccase has been discovered in a number of bacteria including *Bacillus subtilis, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas syringae,* γ- proteobacterium *Pseudomonas aeruginosa,* and *Yersinia pestis.* [48,49] Recently, *Stenotrophomonas maltophilia* strain has been found to be laccase producing, which has been used to degrade synthetic dyes. Bacterial laccases are also reported for decolourization and detoxification of post methanated distillery effluent and chlorolignin containing pulp paper mill waste. [50,51] In the table 1 the detail of bacterial species and their functions are mentioned. The spore coat protein name as Cot A of *Bacillus subtilis* (Gram positive soil bacterium) is copper dependent laccases, which one is best-studied bacterial laccases. This is the first laccase reported from the endospore coat protein designated as endospore coat component of *Bacillus subtilis* and also first bacterial laccase structurally elucidate in detail structure of bacterial laccase. Cot A gene codes for a 65 kDa protein which belong to the outer spore coat. Cot A gene participates in the biosynthesis of the brown spore pigment which is melanin like product. Cot A protects bacterial spore from UV light and H_2O_2.

Further the laccase- like genes CueO has been reported from *E. coli* and CopA from the *Pseudomonas syringae* and *Xanthomonas campestris* also has been considered to encode Pseudo-laccase due to their dependence on 2, 4- dimethoxyphenol oxidation.[52] A protein

encoded by ORF bh2082 of *Bacillus halodurans* C-125 has been identified as a potential bacterium laccase by genome mining, which showed Cu^{2+} resistance properties.[53]

3. Molecular and physicochemical properties of Laccases:

Laccases are monomeric, dimeric and tetrameric glycoprotein, generally having fewer saccharide compounds (10-25%) in fungi and bacteria than in the plant enzymes. The carbohydrate are covalently linked which are 10-45% of total molecular mass, due to this property enzyme showed higher stability. Mannose is one of the major components of the carbohydrate attached to laccases. The molecular weight of laccases is predicted to be in the range of 50-97 kDa from various experimental reports recently in *Bacillus pumilus,* laccase mol. wt. has been estimated to be 58 kDa, and also laccases molecular weight is 64.8 kDa. [54,55] Fig. 4 showing the three dimensional structure of bacterial and fungal laccase (*Bacillus subtilis & Trametes versicolor*).

Insert figure 1 & 2 here

Since laccase constitute three molecular from of isozyme where reported that *Lac I, Lac II* and *Lac III.* D'souza Ticlo et al. reported that Lac II^{nd} mol. wt 56 which is isozyme of laccase from *Cerrena unicolor*. Further, through SDS-PAGE analysis the molecular mass of *Trametes versicolor*, ≃97 KDa has been reported.[57] The above report showed that laccase mol. wt. is varying from one organism to other. The biochemical properties of spore coat protein Cot A of *Bacillus subtilis* has been reported similar with multicopper oxidase including laccase.[58] Further, studies has revealed that Cot A contain all the structural feature of laccase including the reactive surface-exposed copper center (T1) and two buried copper centers (T2 and T3). The most thermostable laccases have been isolated from *Streptomyces lavendulae* with half-life 100 minutes at 70 ^{0}C and in *Bacillus subtilis* for Cot A is reported 112 minutes at 80°C. [59,60] Amino acid sequence alignment of cot A with *Coprinopsis cinereus* laccase and zucchini ascorbate oxidase used to generate structural model as shown in fig 3 which has been proposed by Lıgia O. Martins et al. [61] Since the redox potential of any substrate also play very important role for the laccase activity. The redox potential (is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced) of bacterial laccases ranges from 0.4-0.5 V in bacteria [62] but they are active and stable at high temperature (66 h at 60 ^{0}C), pH 7-9 and salt concentrations. [63,64]

Insert figure 3 here

As it is known that the first gene and c-DNA sequence were recorded for laccases from the fungi *Neurospora crassa, Aspergillus nidulans, Coriolus hirsutus* and *Phlebia radiate*. Since then the number of laccases gene sequenced and has increased considerably. The sequences mostly encode polypeptides of approximately 500 to 600 amino acid in *Bacillus subtilis* (Cot A gene) which is 513 amino acid long and mol. wt. is 65 KDa.[60]

Insert figure 4 here

Typical eukaryotic signal peptide sequences of about 21 amino acids are found at the N-terminal of the protein sequences. In addition to the secretion signal sequence, laccase genes from *Neurospora crassa*, *Podospora anserina*, *Myceliopthora thermophila* and *Coprinopsis cinereus* contain regions that code for N-terminal cleavable propeptides. These laccases also have C-terminal extensions of controversial function, i.e. the last amino acids from the predicted amino acid sequence are not present in the mature protein. Fig. 5 shows that active site of copper oxidase. Active site of CueO (T1Cu, T2Cu, and T3Cu) in ribbon form shown in different colour (blue, green and orange respectively) and also shows that Asp 112 is located behind the tri-nuclear copper center.

Insert figure 5 here

There are also numerous laccase isozyme are able to degraded industrial pollutants. Here we have discused about the transcript levels of laccase genes lcc 1, lcc 2, and lcc 3 (*Basidomycete sp*.) in the presence of veratryl alcohol. The *lcc1* gene is inducible in the early stage of growth and *lcc 2* gene is also inducible but only when organism reaches the stationary phase, where as the transcript level of *lcc 3* were un-induced and repressed by glucose[56] shown in fig. 1. And also fig. 2, shows that *Basidomycete sp.* I-62 genomic DNA digested with three restriction enzymes (*Pst I, Sca I, and Sam I*) and specific probe are used. In fig. 2 Southern blot containing total DNA from *Basidomycetes* completely digested by three restriction enzymes has been hybridized under low stringency condition, similarly in highly stringent condition gave a different pattern of bands for each gene. Thus, it has been confirmed that the chosen probe were specific for each laccase gene and adequate to perform transcription analysis.

4. Substrate of Laccases and its bioassay:

Laccases can oxidize a wide range of molecules more than hundred different types of compound have been identified as substrate for laccase. There are various natural and synthetic substrates which are mentioned in table 2, used for laccase assay. All substrates cannot be directly oxidized by laccases, either because of their large size which inhibit 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 are oxidized by the laccase and their oxidized forms are then able to interact with high redox potential substrate targets.[65] Most commonly used substrate are the ABTS, by the free radicals. Although polyphenol oxidases are copper proteins are able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor. Polyphenol oxidases are associated with three types of activities.[66] Catechol oxidase or o-diphenol: oxygen oxidoreductase (EC 1.10.3.1)

a) Laccases or p-diphenol: oxygen oxidoreductase (EC 1.10.3.2)
b) Cresolase or monophenols monooxygenase (EC 1.18.14.1)

D. Faure et al., compared commercial fungal laccase and catechol oxidase, purified from *Pyricularia oryzae* and *Agaricus bisporus*, respectively, with bacterial laccase from *A. lipoferum* by using several substrates and phenol oxidase inhibitors.[67] Five classes of chemical compounds were investigated as substrates for laccase:

(1) L-tyrosine and several substituted monophenols such as *p*-coumaric and *o*-hydroxyphenylacetic or salicylic acids;
(2) *o*-diphenols (catechol, pyrogallol, guaiacol, and protocatechic, gallic, and caffeic acids), L-3, 4- dihydroxyphenylalanine and *o*-aminophenol, which could be oxidized by both laccase and catechol oxidase;
(3) *p*-diphenol and *p*-substituted aromatic compounds as typical *p*-phenol oxidase (*p*-PO) substrates such as hydroquinone, *p*-cresol, *p*-aminophenol and *p*-phenylenediamine;
(4) *m*-diphenols such as resorcinol, orcinol, 4-hexylresorcinol, and 5- pentadecylresorcinol;
(5) Other laccase substrates such as syringaldazine, 1-naphthol, ABTS, and 4- and 5-hydroxyindoles. The range of substrates used by *A. lipoferum* laccase was similar to that used by *P. oryzae* laccase.

Insert table 2 here

In fig. 6 (a) the simplest case is the one in which the substrate molecules are oxidised to the corresponding radicals by direct interaction with the copper cluster. However, the substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of "redox mediators", which act as intermediate substrates for laccases, whose oxidized radical forms are able to interact with the bulky or high redox potential substrate targets Fig. 6 (b)

Insert figure 6 here

It is very difficult to define laccases according to substrate because substrate range varies from one organism to another.[81] Laccase have ortho and p-diphenol activity. Immobilized commercial laccase is able to degrade meta, ortho and para-substituted methoxyphenol, chlorophenols and cresols, but the substituted from these three types of phenols are oxidized in different orders and to different extents. [82] Laccases are remarkably nonspecific as to the including substrate from one laccases to another.[83] Laccases substrate specificity and its affinity can also vary inhibited by various reagents such as halides (excluding iodide), azide, cyanide and hydroxide.[84]

Bioassay of laccase activity is measured based on the oxidation of various substrate in presence of suitable buffer (i.e. sodium acetate) in acidic pH (5.0). The oxidation product is measured by spectrophotometer taking the absorbance at 420 nm or 450 nm depending upon substrate specificity. ABTS has been most commonly used substrate for laccase bioassay because it acts as cooxidant that can interact with laccase to accomplish electron transfer and it is chemically oxidized in two steps via $ABTS^{+}$ and $ABTS^{2+}$. Anisyl alcohol, and benzyl alcohol can be better oxidized by $ABTS^{2+}$ than by $ABTS^{+}$. Assay through ABTS method has been given by Bourbonnais et al. and further used by M. M. Atalla et al. In this method reaction mixture contained 600 µL sodium acetate buffer (0.1 M, pH 5.0 at 27 ºC), 300 µL ABTS (5 mM), 300 µL culture filtrate and 1400 µL distilled water. The mixture is then incubated for 2 min at 30 ^{0}C and the absorbance was measured immediately in one-minute intervals. One unit of laccase activity has been defined as activity of an enzyme that catalyzes the conversion of 1 mole of ABTS per minute[85,86,] Laccase activity has been measured by another substrate guaiacol described by Arora and Gill and by Arora and Sharma . In this method reaction mixture contained 10 mM sodium acetate buffer (pH 5), 2 mM guaiacol and 0.2 ml of culture supernatant was incubated at 25ºC for 2 h and the absorbance was read at

450 nm. The relative enzyme activity has been expressed as colorimetric units/ml (CU/ml). .[87,88]

5. Mechanisms of bacterial laccases

5.1. Laccases Catalytic Mechanism: The use of molecular oxygen as the oxidant and water is generated byproduct are very common catalytic features. Laccases catalysis mechanism occurs due to the reduction of the oxygen molecule including the oxidation of one electron with the wide range of aromatic compounds which include polyphenol and also methoxyl substituted monophenol and aromatic amines.[89,90] Since, laccases have 4 copper atom known as CuT1, at this site reducing substrate are bind and the trinuclear Cu cluster T2/T3, this one responsible for electron transfer from T1Cu to the T2Cu and the T3Cu trinuclear cluster reduction of oxygen to water at trinuclear center, T1Cu, T2Cu, T3Cu are detected by UV/visible and electro-paramagnetic resonance (EPR) spectroscopy. [91]

It is believed that laccase catalysis involve following mechanism:

a) Reduction of type 1 copper by reducing substrate.
b) Internal electron transfer from the type 1 (T1Cu) to the type 2 (T2Cu) and type 3 copper (T3Cu).
c) Reduction of oxygen to water at the type 2 (T2Cu) and type 3 (T3Cu) site.

The type 1 copper (T1Cu) gives blue color of protein (absorbance about 600 nm depends on the intensity of the Scys- Cu^{II} bond and the ligand field strength [92,93] (type 2 copper does not give any colour but it is EPR detectable and type 3 copper (T3Cu) contains a pair of atom in binuclear conformation that gives a weak absorbance (330nm) but not detected by EPR.[5] Spectroscopy combined with crystallography has provided a detail description of the active site in laccase. Type 2 copper and Type 3 copper form a trinuclear center which one is involve in the catalytic mechanism. In the catalysis mechanism oxygen molecule bind to the trinuclear cluster of asymmetric activation and it is postulated to restrict the access of oxidizing agent. During steady state, laccase catalysis indicated that O_2 reduction takes place.[91] The bonds of the natural substrate, lignin, that are cleaved by laccase include Cα-Cβ cleavage and aryl cleavage. Subsequently, the lignin degradation is proceeded by phenoxy radicals lead to the oxidation at α- carbon or cleavage of bond between α-carbon -β carbon. This oxidation result in an oxygen-centered free radicals, which can be converted into a second enzyme catalyzed reaction to quinone. Then the quione and the free radicals can then undergo polymerization.[5]

Insert figure 7 here

The Type 2 center coordinate with two histidine ligands and water as ligands. The Type 3 coppers are each 4-coordinate, having three histidines (His) ligands and bridging hydroxide (Fig. 7). Reduction of oxygen by laccase appears to occur in two $2e^-$ steps. The first is rate determining step. In this Type 2/3 bridging mode for the first $2e^-$ reduced, the peroxide-level intermediate would facilitate the second $2e^-$ reduction (from the Type 2 and 1 center) in that the peroxide is directly coordinated to reduce Type 2 copper, and the reduced Type 1 is coupled to the Type 3 by the covalent Cys–His linkages.

The above information is described about the blue copper oxidase i.e. blue laccase, but there are some authors reported the yellow laccase. Both laccases (yellow and blue) have similar molecular mass (70 kDa)[94] and specific activity and also it is observed that the yellow laccase are obtained from culture grown on solid state medium while blue forms were isolated from culture grown on liquid medium without lignin.[95] The yellow laccases are formed by modification of the blue form with low molecular weight lignin decomposition product, and some non- blue laccase (Yellow), have high redox potential allowing them to oxidize non- phenolic compounds without any mediator. [96] Therefore, it is assumed that yellow form of laccase is as a result of binding of aromatic product of lignin degradation with the blue laccase. It has been postulated that yellow laccase might contain endogenous mediators derived from lignin, which carry out role of exogenous mediator in the reaction of non- phenolic compounds.[97,95] Due to the insufficient information of yellow laccase it requires more attention of researcher who works on this field.

5.2. Thermostability and Enantioselectivity of bacterial Laccase: Laccases are highly stable, industrially important enzyme capable of oxidizing a large range of substrates. Thermostable enzymes allow high process temperatures with associated higher reaction rates and less risk of microbial contamination. [98,99] The molecular determination of thermostability is not well understood and cannot be generalized across protein families.[100] Thermostability is playing an important role in the enzyme catalysis, several sequence and structural factor are involved in this phenomenon. Some of the mechanism/indicator of increased thermostability include: a more highly hydrophobic core, tighter packing (compactness), deleted loop, greater rigidity (e.g. through increased Proline content in loop), higher secondary structure content, greater polar surface area, fewer thermolabile residue, increased H-bonding, higher isoelectric point, disulfide bridge, more salt bridge and buried polar interaction.[101] Amino acid like Glycine, Lysine, Tyrosine and Isoleucine are preferred in mesophiles.[102] Isoleucine is preferred to

leucine in hydrophobic region of the structure because the side chain carbon can exist which can result in tighter chain packing.[103] And also enhance thermostability of laccase from *Bacillus* HR03 using site directed mutagenesis of surface loop. In which glutamic acid 188 was substituted with 2 positive (Lysine and Arginine) and 1 hydrophobic (Alanine) residues.[104] There are some bacteria which show the thermostability at different temperatures.

a. Cot A from *B. substilis* at 75 °C showed maximum activity [60] and at 80 ^{0}C wild type Cot A have a life of 4 hr. whereas recombinant Cot A from E. coli have a half life of 2 hr.

b. And also *T. thermophilum* laccase have optimum activity at 92 ^{0}C with a half life of 4 hr. at 80 ^{0}C attains 60% activity after incubation for 10 min. at 100 ^{0}C.

Enantioselectivity is the degree to which one enantiomer of a chiral product is preferentially produced in a chemical reaction. In other words enantioselectivity is the result of difference between the enantiomers, not only in activation enthalpy but also in activation entropy.[105] Traditionally, an enzyme has been regarded as a lock to which only one of the enantiomeric keys would fit. Later, this model has been modified to incorporate enzyme flexibility and became a glove that only fits one hand. It would be important to note that accrual with smaller particle sizes increases reaction rates and enantiomeric ratios during biocatalyzation.[106] Production of enantioselective compounds for biomedical application has become possible by immobilized enzymes. [107,108] It also plays a central role within life and is manifold exploit by using biocatalysis as a key step I the development of fine chemicals and drug synthesis. But how it helps, mechanism is not completely known therefore it needs to focus more to elucidate thermostability and enantioselectivity of laccase.

6. Laccases Immobilization:

Immobilized commercial laccase has been reported to degrade meta, ortho and para-substituted methoxyphenol, chlorophenols and cresols, and also some recalcitrant compound because when enzyme is immobilized, it becomes more durable, vigorous and resistant towards the alteration in environment and allowing for easy recovery and multiple reuse of these enzyme.

Laccase immobilization by alginate gel has been carried out according to the method of Lu et al. [109] In this method 2% sodium alginate and 2% calcium chloride was used to prepare the alginate immobilized laccase. *Trametes versicolor* on silica chemically modified with imidazol groups, are used in the decolorization of textile dyes.[110] Laccase immobilization by gelatin has been carried out according to the method of Crecchio et al.[111] Hence 2.5% gelatin was used to immobilize the laccase. Immobilization of laccase (LCC2) by

polyacrylamide gel has been reported by Trevan and Grover. [112] Buffered Monomer Solution (2.0 g of acrylamide and 0.1 g of bisacrylamide in 10 ml of 100 mM sodium phosphate buffer [pH 6.0]) was used to immobilize the laccase.

Immobilization of laccasses can protect them from denaturation by organic co-solvent, increase their stability. [113]And also it facilitates the separation of reaction products and have a good catalytic capacity over many reactions cycles.[114-116] Laccases are well known biocatalysts in transforming phenolic or aromatic amines and are considered some of the most promising enzymes for future industrial applications. [117] The immobilized spore laccases are more compatible with almost all industrial processes. Laccases from the bacteria are highly active and much more stable at high temperatures and high pH value.[118,119]

The enzyme based biosensor, the stabilization of enzymatic activity on the biological recognition element is of great importance. The fabricated an optical biosensor by using staked films where 3- methyl-2 benzothiazotinone hydrazone (MBTH) has been immobilized in a hybrid nafion/sol-set silicate film and laccase in a chitosan film for the detection of phenolic compounds to detect catechol.[120] A disposable biosensor based on immobilization of laccase with silica spheres on the multi-walled carbon nanotubes (MWCNTs)- doped screen-printed electrode (SPE) has been determine DA based on a non-oxidative mechanism in a rapid, selective and sensitive way.[121] The magnetically separable particles have also attracted much attention as a means to facilitate reuse and reduce mechanical stress during separation.

There are several techniques may be applied to immobilize enzymes on solid supports. They are mainly based on chemical and physical mechanism.[122] Enzyme may be immobilized by a variety of method (adsorption, entrapment, cross linking and covalent bonding also self-immobilization) mainly based on chemical and/or physical mechanism. Since the methods for the immobilization procedures greatly influence the properties of the resulting biocatalyst, immobilization strategy determines the process specifications for the catalyst. Therefore the general goal of the immobilization is to obtain stable catalysts with long life times and low cost, we think that the combination of these techniques will enhance: (i) the adsorption of laccase on appropriate substrate, (ii) the life time of the laccase activity and (iii) reutilization of the substrate/ Laccase product.

7. Application of Laccases for industrial waste detoxification:

Laccase have broad range substrate specificity due to this, it oxidized wide range of pollutants present in industrial wastes. The bacterial laccases are playing important role in bioremediation of industrial waste because it oxidizes both the toxic and nontoxic substrate

and also laccase include the cleaning of industrial effluents, mostly from industries of paper and pulp, textile and distillery industries. Among the biological agents, laccases represent an interesting group of ubiquitous, oxidoreductase enzymes that show promise of offering great potential for biotechnological application.[91] In particular, laccases from fungi have found wide applications ranging from the pharmaceutical sector to the pulp and paper industry to reduce the kappa number (an indication of the residual lignin content or bleach ability of wood pulp) and enhance the bleaching of kraft pulp when they are used in the presence of chemical mediators, such as ABTS[123] The role of fungal laccases for detoxification of molasses distillery wastewater has been reported by Pant and Adholeya.[17,124]

As well as, decontamination of phenolic pollutants in soil could be directed towards their immobilization (humification) by oxidative coupling catalyzed by laccases, reducing their biodisponibility so far.[125-129] On the adverse, pollutants might undergo oxidative transformation (degradation) by laccases to easily up-taken products by soil microflora[130] (Fig. 8). In the paper industries laccase are play important role, in which one of the most studied application are laccase-mediator bleaching of kraft pulp and efficiency of which has proven in mill-scale trials.[131]

Insert figure 8 here

7.1. *Oxidaion of phenolic and non- phenolic substrate:* Laccases catalyze by subtraction of one electron from phenolic hydroxyl groups of phenolic lignin model compounds, such as vanillyl glycol, 4,6-di(t-butyl)guaiacol, and syringaldehyde, to form phenoxy radicals, which generally undergo polymerization via radical coupling. The reaction is also accompanied by demethylation, formation of quinone, resulting in ring cleavage.[132] The degradation of phenolic β-1 lignin substructure models occurs via the formation of phenoxy radicals that leads to Cα- Cβ cleavage, Cα oxidation, alkyl-aryl cleavage, and aromatic ring cleavage.[133] Laccase catalyzes oxidation of phenols, anilines, and benzenethiols correlates with the redox potential difference between laccases T1 copper site and the substrate. The presence of electron-withdrawing o- and p-substituents reduces the electron density at the phenoxy group and, hence, more difficult to be oxidized the phenolic substrate further (less susceptible to electron transfer to the T1 copper site). Bulky substituents, which impose steric interference with substrate binding, cause a decrease in reactivity.

Laccase has been found to oxidize non-phenolic model compounds and β-1 lignin dimers in the presence of a mediator, indicating that the enzyme plays a significant role in

depolymerization of lignin and pulp delignification.[134] The most studied mediator for laccase, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate (ABTS), 1-hydroxybenzotriazole (HBT), and 3-hyroxyanthranilic acid (HAA). The oxidation is different for ABTS and HBT involving a di-cation and a benzotriazolyl-1-oxide radical, respectively[135] Oxygen uptake by laccase is faster with ABTS than HBT, but the oxidation of non-phenolic substrate is comparable for both mediators. ABTS-mediated oxidation of non-phenolic substrate proceeds via an electron transfer mechanism.[136] In the oxidation process the ABTS is first oxidized to the radical cation ($ABTS.^{+}$) and then to the di-cation ($ABTS^{2+}$) with redox potentials of 472 mV (ABTS/$ABTS.^{+}$) and 885 mV ($ABTS.^{+}$/$ABTS^{2+}$), respectively [137] .The di-cation is the active intermediate responsible for the oxidation of the non-phenolic substrate. HBT-mediated oxidation of non-phenolic substrate involves initial oxidation of HBT to $HBT.^{+}$ by laccase, followed by immediate deprotonation to form the N-oxy radical. The latter abstracts the benzylic H-atom from the substrate, converting it to a radical. The oxidation of veratryl alcohol by laccase- ABTS and by laccase-HBT (radical H- abstraction mechanism) showed in fig 9. HBT/$HBT.^{+}$, with an E_o value of 1.08 V, has a mediator efficiency with laccase that is higher than that of the ABTS. [136] The use of laccase/HBT for bleaching of paper pulps and for the removal of lipophilic extractives has been described. Recently, two lignin-derived phenols, syringaldehyde and acetosyringone, have been shown to act as effective laccase mediators for the removal of lipophilic compounds from paper pulp.

Insert figure 9 here

The degradation of non-phenolic β-O-4 model compounds, which represent the major substructure in lignin, has been studied using laccase-mediator systems. Four types of reactions, β-ether cleavage, Cα- Cβ cleavage, and Cα-oxidation and aromatic ring cleavage, are catalyzed by laccase– (Butylated hydroxytoluene) BHT coupled system[132]. In the oxidation of a non-phenolic β-O-4 lignin model dimer, 1-(4-ethoxy-3-mthoxyphenyl)-1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)propane, the coupled enzyme/HBT system catalyzes 1e− oxidation of the substrate to form β-aryl radical cation or benzylic (Cα) radical intermediates. Electron density of the aromatic ring affects the 1e− oxidation by the laccase/1-HBT couple. Substrates containing electron-donating groups favor aromatic ring cleavage products. The β-aryl radical cation is converted to the product via an aromatic ring cleavage, and the benzylic radical is cleaved at the Cα- Cβ bond similar to a Baeyer-Villiger reaction. The β-ether cleavage of the β-O-4 lignin substructure is caused by reaction with the

Cα-peroxy radical intermediate produced from the benzylic radical.[138] The rate of oxidation depends on the k.cat of the laccase for the mediator and the stability of the enzyme to inactivation by the free radical of the mediator. Because the nonphenolic lignin model dimer is chemically oxidized by the HBT radical, it may infer that oxidation of the Cα in the substrate occurs more easily than that of the π electron in the aromatic ring[139]. The work on oxidation of veratryl alcohol may also infer that laccase/ ABTS favors the formation of aryl radical intermediate, while laccase/HBP proceeds with the benzylic radical formation.

7.2. *Pulp paper industry:* Laccase has been reported for the degradation and decolourization of chlorophenol, chlorolignin containing black liquor and pulp paper mill waste water. [140] Pulp paper mill effluent and black liquor containing primary mixture of all pigments, extract of plant along with cellulose and some heavy metals. Lignin is an insoluble complex polymer of phenolic compounds and up to 90% of the lignin is solubilized and removed during pulping process. Filamentous fungi are able to efficiently degrade wastewater by the action of several extract enzyme classes. The laccases have attracted considerable interest for application in pulp bio-bleaching also for industrial application. During lignin degradation, laccase are thought to act on small phenolic lignin fragments which substrate reacts with the lignin polymer, resulting in its degradation. Conventional methods of delignifying or decolourizing paper pulp involve either chlorine or oxygen based chemical oxidant (e.g. ClO_2 and O_2).[141] In the pulp paper industry the chemical bleaching are very much effective but these methods have serious drawbacks due to generation of hazardous chemical and chlorinated by product. Hence, laccases delignification systems that overcome these drawbacks and can be easily adapted to current pulp production line are attractive alternatives. The direct decolourization and degradation of pulp paper mill effluent include direct dechlorination, cleavage of aromatic rings, and mineralization of polycyclic aromatic hydrocarbons, decolorization of pulp mill effluent. Consequently, metabolic product has been detected through GC/MS-MS analysis.

7.3. *Distillery industry:* Recently the bacterial laccase also has been reported for the degradation and decolourization of amino carbonyl complex containing distillery effluent. Though, the distillery effluent contains the complex mixture of phenolics metals. Sulfur compounds and melanoidins (amino carbonyl compounds). The detection of metabolic products by GC/ MS-MS analysis from bacterial decolourization of post methanated distillery effluent given strong evidence for metabolization of complex colourant by laccase. [50] Further

a laccase-producing white rot fungus Trametes sp. has been tested for bioremediation. Maximum effluent decolourization of 73.3% and chemical oxygen demand (COD) reduction of 61.7% was achived after 7 days of fungal treatment to 20% v/v of distillery wastes in culture medium. Under these condition, a35-fold increase in laccase production by this fungus was observed. [142]

7.4. *Textile industry & Azo dye:* The textile industry containing chemical reagents used are very diverse in chemical composition, ranging from inorganic compound to polymers and organic products. [143] Laccase has been reported to prevent back- staining of dyed of printed textiles. As part of the washing solution laccase could quickly bleach released dyestuff, 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 finishing dyed cotton fabric. Another interesting laccase application in this field is oxidative transformation and consequent coupling of dye precursor to the collegen matrix in hides. Under laccase catalysis, soluble dye precursor could be absorbed, oxidized, and polymerized to give the desired tanning effect. The process could improve dyeing efficiency, reduced cost, or provide improved hide characteristics.[144] Laccases have been broadly studied for the degradation of azo dyes. [145,146] (In the fig. 10 showing the proposed mechanism of laccase degradation of 3-(2-hydroxy-1-naphthylazo) benzenesulfonic acid.[147] Laccase, manganese peroxidase, lignin peroxidase, and aryl alcohol oxidase activities were found in crude extracts from solid-state cultures. However, only the laccase activity has been found to correlate with color removal, and the subsequently purified preparations were able to perform this reaction in vitro.

7.5. *PAH degradation:* Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants. These are produced during the incomplete combustion of fossil fuels and organic wastes and also as a result of various industrial processes. [148,149] There have been many studies on the biodegradation of various aromatic hydrocarbon, but their low water solubility and subsequent low degradation rates interrupt the bioremediation of PHA polluted environment.[150] Therefore, knowledge of microorganism having a high PAH degrading capability is essential for efficient remediation of PAH contamination. White rot fungi produce extracellular enzyme laccase which oxidize PAHs to corresponding PAH quinones and subsequently degrade the material further to CO_2.

7.6. *Detection of Phenol, aniline, Oxygen:* Laccase has been used for detection of various pollutants by developing of biosensor and diagnostic kits. For detecting phenol, anilines, or other reducing substrates, three types of laccase based sensor have been reported. The first type detects the photometric change resulted 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 replaces O_2 as the acceptor for the electrons flown from the substrate (through laccase).[144]

Insert figure 10 here

Insert table 3 here

Laccase has been studied intensively for myriad applications other than bioremediation. These applications are discussed briefly below and summarized in Table 3.

Insert figure 11 here

Laccases have been employed for several biotechnological application showing in fig. 11 and also in organic synthesis as the oxidation of functional groups, the coupling of phenol and steroids, medical agents i.e. anesthetics, anti- inflammatory, antibiotics and sedatives etc.

8. Difference of bacterial laccases from fungus

The largest group of enzyme in the latter study has been indeed fungal laccase, as reported in earlier section [124], which clearly separated in two clusters of sequences from *Ascomyceteses* and sequences from *Basidomyceteses*. The sequences were selected for the study by the presence of four conserved Cu- oxidase consensus patterns and the study also included a number of bacterial protein sequences. A couple of bacterial sequences were excluded, as they could not be aligned without loss of resolution in phylogenetic analysis.

Comparison of amino acid sequences of bacterial laccases reveals a large degree of diversity. This variation is reflected in several molecular parameter of the laccase to approximetly 65, 70, and 75 kDa for *Bacillus subtilis, Streptomyces lavendulae*, or *Streptomyces cyaneus* laccase. [159,60]Three dimensional structure of bacteria (*B. subtilis*), and fungi (*T. versicolor*) laccases was predicted by homology modeling approach using template1HL0, 1KYA and 1AOZ, respectively, by Modeller 9v6.[160] (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), and 89.5% (fungi) residues were in favored and allowed regions in Ramachandran plot. These values match well with those for experimentally determined model. Result from ProSA gave a z-score (standardizing data on one scale) of −8.28 and −6.28 and protein quality predictor (ProQ) analysis gave a LG score (log of a P-value and MaxSub ranges from 0-1, were 0 is insignificant and 1 very significant) value of 6.006 and 5.293 respectively. These results taken together suggested that the values for homology model built for bacteria and fungi fall within the range of value observed for experimentally determined structure and the built models are very reliable for interpretation. Three dimensional structure predictions, at monomeric level, for all laccases (bacteria, and fungi) suggested 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 of β -sheets and β -strands, arranged in sandwich conformation. 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) Fig. 4A depicts, presence of a coiled section, which connects Domain 1 and Domain 2 in bacteria, is absent in fungi. This coiled section also helps in packaging between Domain 1 and Domain 2 in bacteria.[161]

In fungi and plant, short α-helical regions connect Domain 1 to Domain2 and Domain 2 to Domain 3 (Fig. 4B). 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 laccases, the corresponding link is made through internal connections (Fig. 4B). The Domain 2 (green color) acts as bridging element between Domain 1 and Domain 3.[162]The structure analysis revealed, tri-nuclear copper cluster (T2/T3) embedded between Domains 1 and 3 with both domains provide in residues for the coordination of the coppers. The copper interact in 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 Type1 mononuclear copper center. A protruding section, formed by a loop and a short alpha-helix, forms a lid-like structure over

the substrate binding site in bacteria. There is no similar element has been found in the previously analyzed 3-D structure of fungal laccases. Therefore, this structural element may represent a distinctive feature of bacterial laccase. The overall structure analysis shows that, it shares a common beta-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 1and 2.[64] There are various significant functions carried out by bacterial and fungal laccase shown in Table 4.

Insert table 4 here

9. Conclusions:

Ligninolytic enzymes are playing important role in biotransformation of recalcitrant compounds, in which laccase holds large potential for the economical treatment of waste water containing phenolic compound, PHA, chemical pesticides, synthetic dyes and various emerging pollutants. In some cases enzymatic oxidation of phenolic compounds can generate by-product that convert the blue laccase into yellow laccase (YL), which have no requirement to any mediator to degraded pollutants like blue laccase according to some author. Thus there is need to more attention of researcher in this area. Laccases are highly versatile in nature and they have various applications such as waste water detoxification, and textile dye transformation, in food technology, personal and medical care application and biosensor and analytical application. The biotechnological significance of bacterial laccase has led to increase its demand in recent days**.**

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1014 Table.1: There are some known bacterial species/ laccase- like protein and their function

S.No.	Species	Their possible function	Ref.
1.	*Leptothrix discophora* SS1	Detoxification of Mn^{2+}, destruction of toxic oxygen species	19
2.	*Pseudomonas maltophila*	Shows nucleoside oxidase activity	20
3.	*Pseudomonas syringae pv. Tomato* (cop A)	Cu^{2+} resistance activity	21
4.	*Streptomyces antibioticus*	Shows phenoxazinone synthesis activity	22
5.	*Azosprillum lipoferum*	Pigmentation, oxidation of phenolic compounds, and also electron transport	23,24
6.	*Xanthomonas campesteris* (copA)	Cu^{2+} resistance	25
7.	*Bacillus sp.* (mnxG)	Sporulation, Mn^{2+} oxidation	26
8.	*Bacillus sphaericus*	Sporulation and pigmentation	27
9.	*Phormidium valderianum*	Resistant to higher nitrogen concentration	28
10.	*Pseudomonas fluorescens* GB-1	Mn^{2+} oxidation, destruction of toxic oxygen species	29
11.	*Marinomonas mediterranea* (ppoA)	Pigmentation	30,31
12.	*Aquifex aeolicus* (suf 1)	Work as cell division protein	32
13.	*Pseudomonas putida* GB 1	Mn^{2+} oxidation	33-35
14.	*Bacillus subtilis* (cotA)	Pigmentation of spores, UV and H_2O_2 resistance	36
15.	*E. coli* (yacK)	Cu^{2+}, oxidation of phenolate- siderophores ferrooxidase activity	37,38
16.	*Pseudomonas sp.* (CumA)	Mn^{2+} oxidation	39
17.	*α- proteobacterium* SD21	Mn^{2+} oxidation	39
18.	*Pseudomonas aerophilum* (pae1888)	Unknown	40
19.	*γ- proteobacterium* JB	Oxidation of toxic compounds	41,42
20.	*Bacillus halodurans* C-125 (lbh 2082)	Cu^{2+} resistance	43
21.	*Shinorhizobium meliloti*	Thermostable	44
22.	*Stenotrophomonas maltophilia*	Degrade synthetic dyes	45
23.	*Rhodococcus sp.*	Reduction of synthetic dye	46

Table.2: Natural and synthetic mediator of laccases

S. No.	Natural mediator	Ref.
1.	Acetosyringone	68
2.	Syringaldehyde	68
3.	Vanilin	68
4.	Acetovanillone	68
5.	Sinapic acid	69
6.	Ferulic acid	69
7.	p- coumaric acid	68
8.	Reduced glutathione	70
9.	Cystine	70
10.	Aniline	70
11.	4 hydroxybenzyl alcohol	70
	Synthetic mediator	
12.	1- hydroxylbenzotrizole (HBT)	71
13.	N- hydroxyphthalimide (HPI)	72
14.	Violuric acid (VLA)	73
15.	N- hydroxylacetanlide (NHA)	74
16.	2,2,6,6-Tetramethylpiperidine- N-oxyl (TEMPO)	75
17.	Acetohydroxamic acid	76
18.	2,2,5,5- Tetramethylpyrrolidine-N-oxyl (PROXYL)	73
20.	2,2'-Azinobis-(3-ethylbenzothia- zoline 6-sulfonic acid) (ABTS)	77,78
21.	Guaicol	79
22.	Methyl syringate	80

1026 Table.3: Sectors where the laccases are used in various applications

S. No.	Industries	Main Application	Ref.
1.	Food industry	Brewing; Colour enhancement in tea etc. Cork modification; Wine stabilization; Oxidizing Phenolic compound in apple juice.	151
2.	Textile industry	Denim bleaching; Denim finishing; Denim bleaching and shading.	152
3.	Wood industry	Modification and polymerization of lignin; Increase the mechanical strength and thickness.	
4.	Organic synthesis, Medical, Pharmaceutical, Cosmetics and Nanotechnology	Polymers production; Coupling of phenols and Steroids; Medical agents; Carbon-nitrogen bonds construction; Complex natural products synthesis; Personal hygienic products; Biosensors and bioreporters	153
5.	Bioremediation	Polycyclic aromatic hydrocarbons (PAHs) degradation; Biodegradation of xenobiotics	154
6.	Paper industry	Pulp bleaching; Paper pulp delignification.	123,155
7.	Distillery industry	Removal of dark brown color.	156-158

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1029 TABLE: 4. Functional difference of bacterial laccase from fungus

S. No.	Bacteria	Fungus	Reference
1.	Melanin formation, endospore coat protein synthesis	Delignification , pigmentation, fruiting body formation as well as pathogenesis	163,164
2.	Localization in bacteria most of the laccases are intracellular	Localization of fungal laccase is extracellular	165
3.	Bacterial laccase have low- redox potential	Fungal laccase (especially white rot fungi) have high redox potential	166
4.	The bacterial thermal stability is higher	The fungal laccase usually have lower thermal stability than bacterial laccase	64

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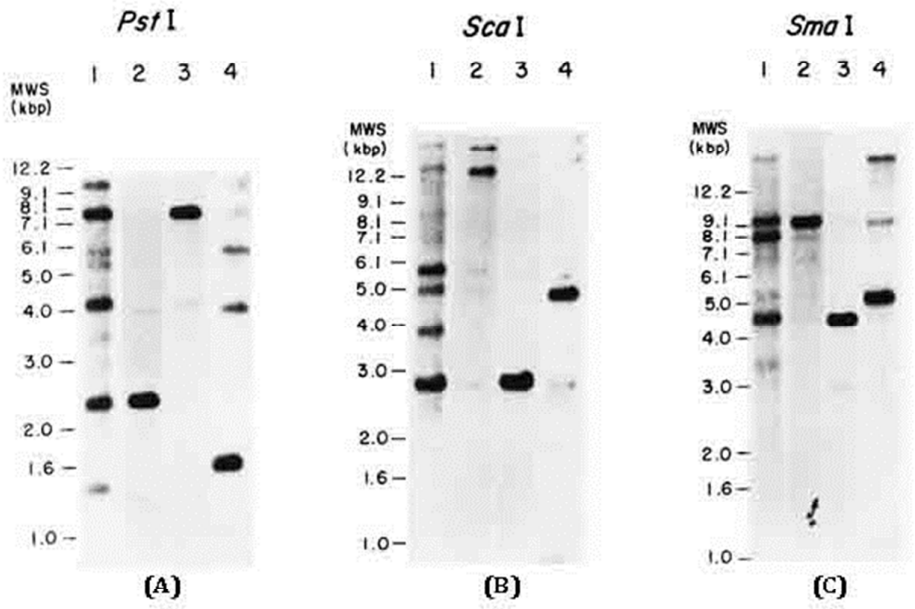

FIGURE 1: Northern analyses of *lcc1, lcc2,* and *lcc3*transcription patterns. (A) Total RNA samples from mycelium growing on inducing medium; (B) total RNA samples from mycelium growing on fructose medium. The actin hybridization shown in panel B corresponds to a longer exposure time. EtBr, (ethidium bromide).[72]

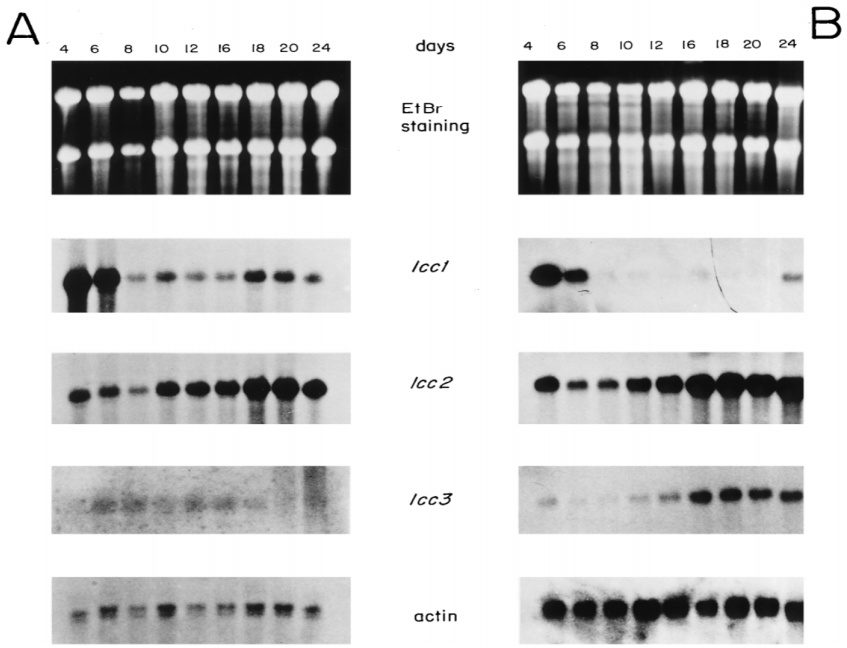

FIGURE 2:Southern analyses of *Basidiomycete* I-62 genomic DNA digested with three restriction enzymes (*PstI, ScaI, and SmaI*) and hybridized with a heterologous probe from the *Basidiomycete* PM1 *lac1*laccase gene under low-stringency hybridization conditions (lanes 1), an *lcc1*-specific probe (lanes 2), an lcc2-specific probe (lanes 3), and an lcc3-specific probe (lanes 4). MWS, molecular sizes.[72]

```
1A0Z    SQI----RHYKWEVEYMFWAPNCNE--NIVMGINGQFPGP---TIRANA-GDSVVVELTNKLHT--------------------EGVVIH-WH-GILQR
1A65    ---QIVNSVDTMTLTNANVSPDGFT--RAGILVNGVH----GPLIRGGK-NDNFELNVVNDLD-NPTM---------------LRPTSI-HW-HGLFQR
CotA    -------YEVIMEECTHQLHRDL--PPTRLWGYNGLFPGP---TIEV-KRNENVYVKWMNNLPSTHFLPIDHTIHHSDSQHEEPEVKTVVH-LH-GGVTP
                        :          **          *      :   ::  * *                                  :     *

1A0Z    GTPWADGTASISQCAINPG---ETFFYNFTV-DNPGTF---FYH-GH-LG-----MQRSAGLYGSLIVDPPQ-GKKE-PF-HYDGE------INLLLSDW
1A65    GTNWADGADGVNQCPISPG---HAFLYKFTPAGHAGTF---WY-HS-HFG-----TQYCDGLRGPMVIYDD---N-DPHAALYDEDDEN---TIITLADW
CotA    DDSDGYPEAWFSKDFEQTGPYFKREVYHY-PNQQRG--AILWYH-DH-A-MALTRLNVYAGLVGAYIIHDPKEKRLKLPSDEYDVPLLITDRTINEDGSL
                  :     *       *::    : *     :*             :   ** *  ::              **

1A0Z    WHQSIH--KQEVGLSSKPI-RWI--GEPQTILLNGRGQF-DCSIAAKYDSNLEP---CKLKGSESC-A-PYIFHVSPKKTYRIRIASTTALAALNFAIGN
1A65    YHIP--APSI---------QG--AA-QPDATLINGKGRYVG--------------GPA--------AE-LSIVNVEQGKKYRMRLISLSCDPNWQFSIDG
CotA    FYPS--APENPSPSLPNPSIV--PAFCGETILVNGKV-------------------------------W-PYLEVEP-RKYRFRVINASNTRTYNLSLDN
        ::                        :: *:**:                                  *   : **:*:   :     ::::

1A0Z    --HQLLVVEADG-NYVQPFYTSDIDIYSGESYSVLITTDQNPSENYWVSV---GTRARHP---NTPP--------GLTLLNYLPN---SVSK-LPTSPPP
1A65    --HELTIIEVDG-ELTEPHTVDRLQIFTGQRYSFVLDANQPV-DNYWIRA---QPN---KGRNGL--AGTFANGVNSAILRYAGA---ANA-DPT-TS-A
CotA    GG-DFIQIGSDGGLLPRSVKLNSFSLAPAERYDIIIDFTAYEGESIILANSA--------------G--------CGGDVNP---ETDANIMQFR-VT--
           ::  :  **          : :   : *  ::       :   :                                  :        :

1A0Z    QTPAWDDFDRSKN-------FTYRIT-AAMGSP-KPPVK--------FNRRIFLLNTQNVINGYVKWAINDVSLALPPTPYLGAMKYNLLHA-------F
1A65    -------------NPNPAQLNEADLH-AL--IDPAA---PGIPTPGAADVNLRFQLGFSG----GRFTINGTAYESPSVPTLLQIMSG----AQSANDLL
CotA    -------------K--PL-AQK-D-ES-------RK---PKYLASYPSVQHERIQNIRTL-----KLAGTQDEYGRPVLLLNNK----------------
                                                                :          : :       *

1A0Z    DQNPPPEVFPEDYDIDTPPTNEK-------TRIGNG--VYQFKIGEVVDVILQN-AN-MMKENLS-ETH-PWH-LH-GHDFWVLGY---G-DGKF--S-A
1A65    ------------------------------------PAGSVYELPRNQVVELVVPA-G-VL------GGP-HPF-HL-HGHAFSVVRSAGSS----------
CotA    ----------------------RWHDPVT-------ETPKVGTTEIWSIINPTR----------GTH-PIH-LH-LVSFRVLDRRPF-DIARYQE-SG
                                        :    ::  :   :                    *   *     * *:

1A0Z    EEESSLNLKNPP-----LRNTVVIFPY-GWTAIRFVADNPGVWAFHCH---IEPH-LHMGM-GVVFAEGVEK-V--GRIPTKALA-CGGTAKSLINNPKN
1A65    ----TYNFVNPV-----KRDVVSLGVTGDEVTIRFVTDNPGPWFF---HCHIEF-HLMNG-LAIVFAEDMANTVDANNPPVEWAQLC-------------
CotA    ELSYTGPAVPPPPSEKGWKDTIQAHAGEVLRIAATFGPYSGRYVWHCH---ILEH-EDYDM-MRPMDITDPHK--------------------------
           :    *       :: :                 * : :     *                :     :

1A0Z    P-----------------
1A65    -EIYDDLPPEATSIQTVV
CotA    ------------------
```

FIGURE 3: Amino acid sequence alignment of CotA with *C. cinereus* laccase and zucchini ascobate oxidase used to generate the structural model. The CotA sequence was then aligned against the primary alignment and used to generate structural models that were checked using Modeler and PROCHECK (Laskowski et al.; see "Materials and Methods"). The alignment was changed to correct for problems detected using the above-mentioned software. Several of these cycles were performed to optimize the alignment, and the structural models were obtained. Two dots indicate similarity, whereas an asterisk indicates identity. Gaps were introduced to maximize the quality of the final structural model for CotA.[61]

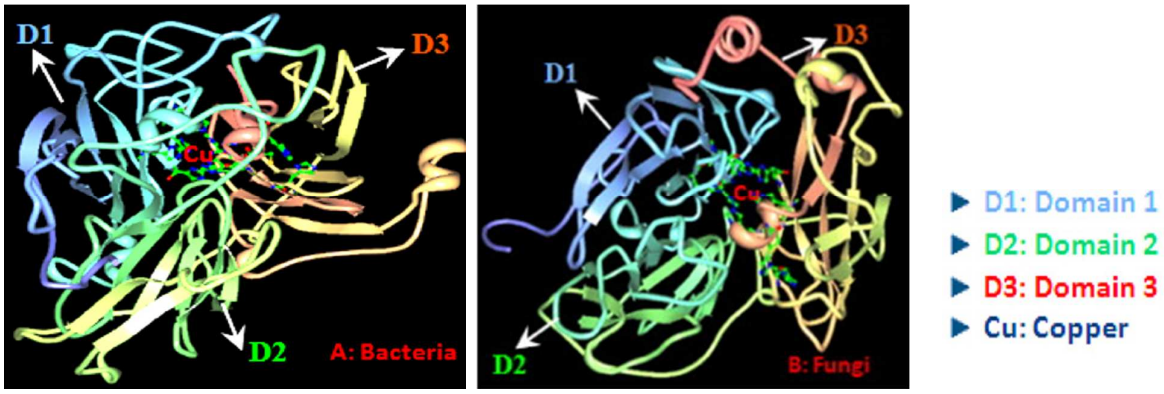

FIGURE 4: Three dimensional structure of bacterial and fungal laccase (*Bacillus subtilis& T. versicolor*)

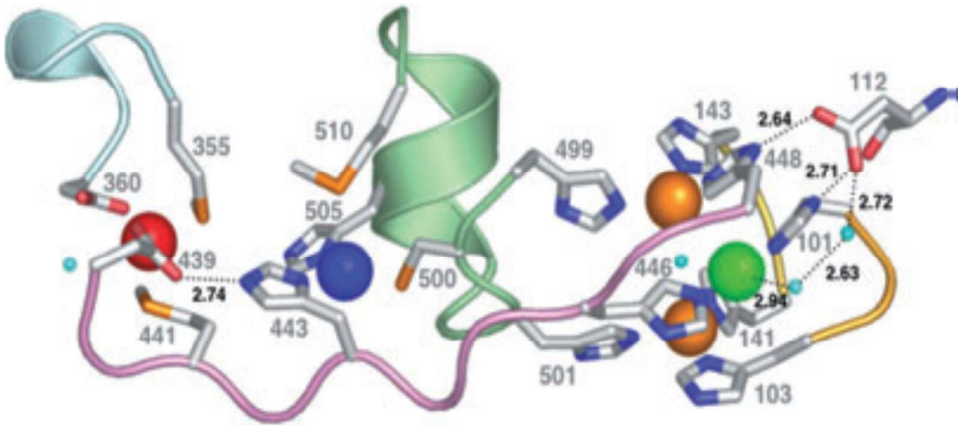

FIGURE 5: Active site of CueO. Type I Cu, type II Cu, and type III Cu's are indicated by blue,green, and orange balls, respectively. The Cu(II) bound at the substrate-binding site is indicated by the red ball. The backbone peptides colored water blue, green, magenta, orange, and yellow show that each Cu center is closely connected with each other. Asp112 is located behind the trinuclear Cu center, forming hydrogen bonds with the imidazoles coordinating toa type II and a type III Cu directly and indirectly through a water molecule. Drawn with PyMol for PDB data, 1N68.

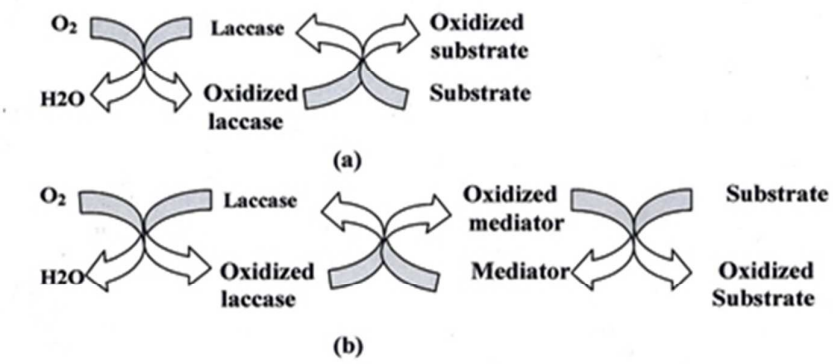

FIGURE 6: Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence **(a)** or in the presence **(b)** of redox mediators.

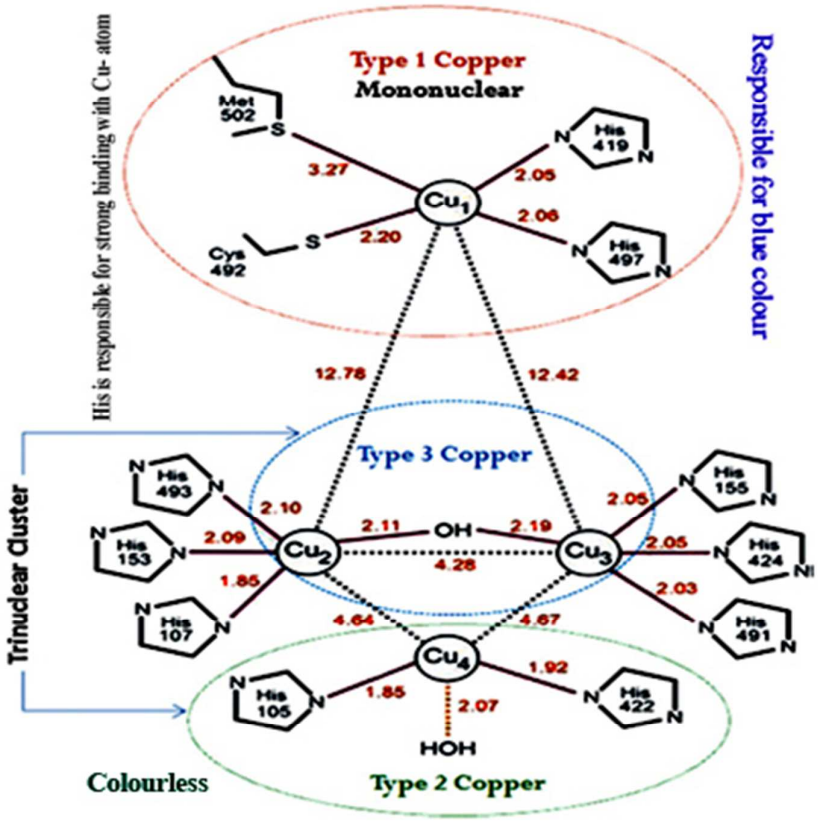

FIGURE 7: The laccase active site showing the relative orientation of the copper atoms including interatomic distances among all relevant ligands.

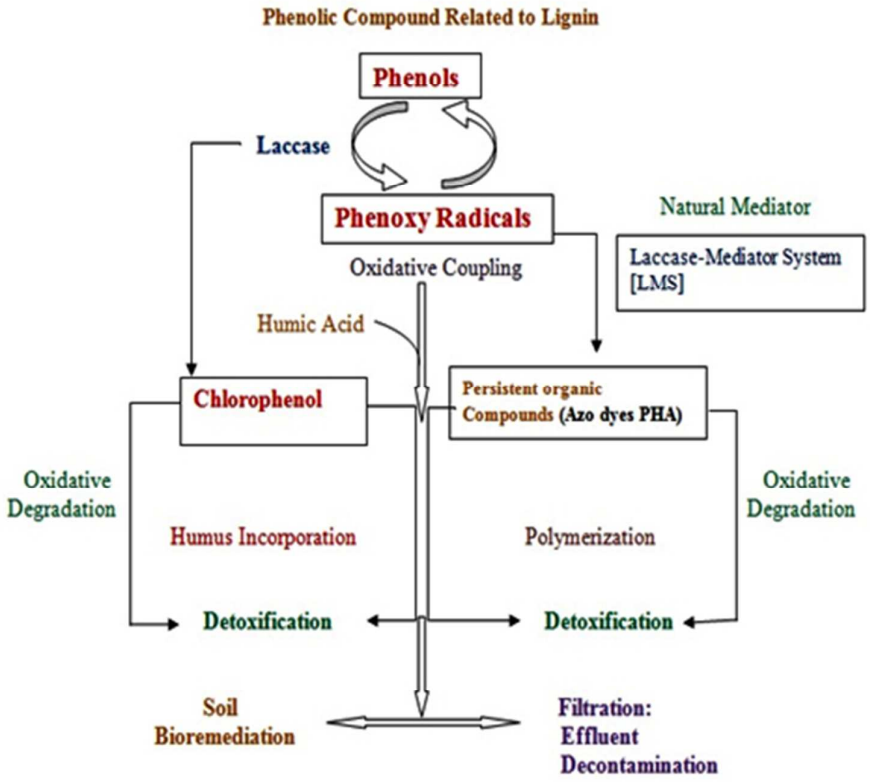

FIGURE 8: Possible role of laccase and their natural phenolic mediators in soil bioremediation and detoxification of industrial waste.[154]

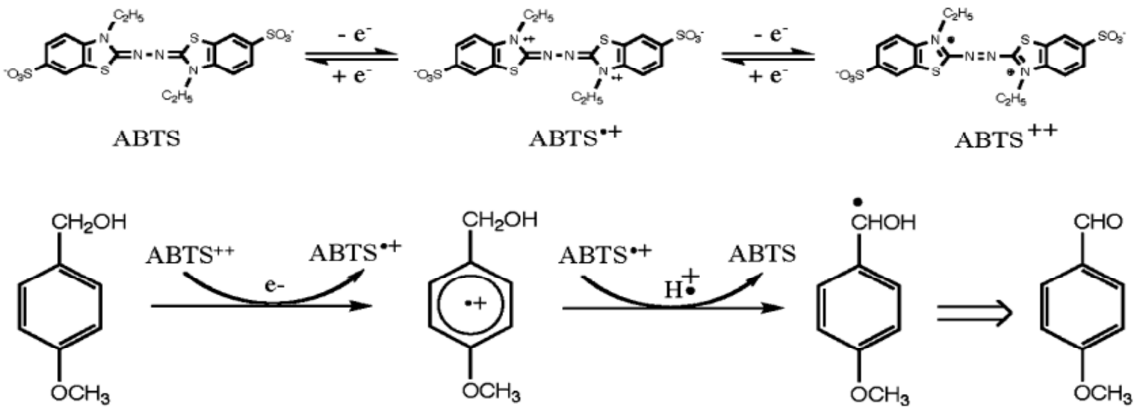

Figure 9: Radical H-atom abstraction and electron transfer mechanism

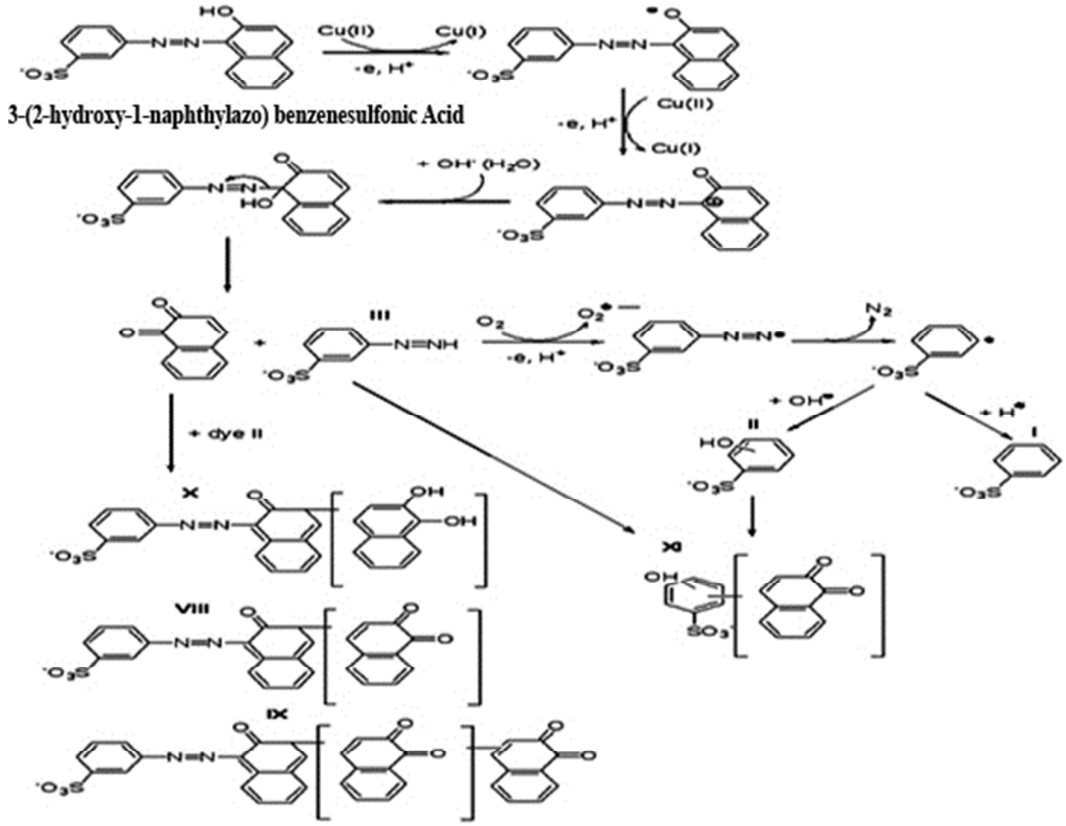

FIGURE 10: Mechanism of laccase degradation of 3-(2-hydroxy-1-naphthylazo) benzenesulfonic Acid.[147]

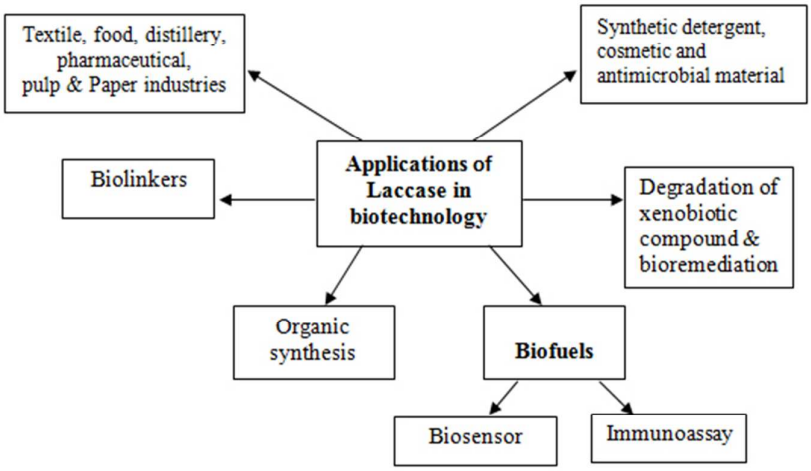

FIGURE 11: Various applications of laccases in biotechnology.