



## Laccase - The Wonder Enzyme for a Variety of Industries

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### Abstract

Laccases constitute a family of copper-containing oxidase enzymes which catalyze the oxidation of various aromatic compounds (particularly of phenolic and aromatic amines) and some inorganic ions, and simultaneously reduce oxygen to water. The laccase molecule is either a dimeric or tetrameric glycoprotein containing four copper atoms per monomer, which are distributed across three redox sites. It was discovered by Yoshida in 1883 in *Rhus vernicifera* (Japanese lacquer tree). Laccase molecules are of common occurrence in higher plants, some fungi, insects and bacteria. These are now considered as the industrial enzymes because of their wide substrate specificity. Besides discussing the production and activity of laccases in various organisms, this article examines their wider potential for diverse biotechnological applications (e.g. in biosensor technology, cosmetics, food improvement, wine and beer stabilization, medical diagnosis, pharmaceutical industry, agriculture, petrochemicals, paper and pulp industry) as well as their use in detoxification and bioremediation of synthetic dyes. Further, it elucidates the process of enzyme immobilization, as immobilized enzymes also have a variety of applications.

**Keywords:** Laccase Enzyme; Immobilized Laccase; Laccase Production; Laccase-based Oxidation; Oxidation Mechanisms; Laccase Applications

### Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multinuclear copper oxidase (MCO) widespread in the eukaryotes

and the prokaryotes including bacteria, fungi, insects and plants [37]. This enzyme oxidizes an array of aromatic and non-aromatic compounds via a catalyzed radical reaction involving the molecular

oxygen ( $O_2$ ). The effectiveness of laccase as an enzyme was first demonstrated in exudates from *Rhus vernicifera*, the Japanese lacquer tree, in 1883 [18] and subsequently in certain fungi [19]. Laccases belong to a diverse group of proteins called multi-copper oxidases and are capable of oxidizing a variety of substrates and simultaneously reducing oxygen to water [1,18]. The proteins consisting of more than 100 amino-acid residues may have 1 to 9 Cu atoms per molecule. Laccases supposed to be monomeric, dimeric or tetrameric glycoproteins have four copper atoms per monomer located in catalytic sites. The Cu atoms are of three different classes (Type 1, Type 2 and Type 3). Type 1 copper (Cu1, ligated by at least one Cys and two His) is paramagnetic and responsible for oxidation of substrate. Type 2 copper (Cu2, ligated by two His) and two copper atoms of type 3 (Cu3, each ligated by three His) conforming to a trinuclear cluster have a key role in converting the molecular oxygen to two water molecules [1,36]. Typically, laccase is a monomeric glycoprotein consisting of nearly 500 amino acids that are arranged in three  $\beta$ -barrel cupredoxin domains, having a mononuclear Cu centre located in the third domain, and a trinuclear Cu centre that occurs between the first and the third domains (Figure 1a). In the Type 1 mononuclear Cu centre (Figure 1b), an intense absorption band appears at 600 nm due to a ligand-to-metal charge transfer between the Cu atom and cysteine sulphur, which gives rise to the blue color of the protein. A characteristic EPR (electron paramagnetic response) signal appears due to high covalency at the Cu site. An EPR signal also arises from the Type 2 Cu site located in the trinuclear centre (Figure 1b and 1c), but it is not accompanied by visible bands in the absorption spectra. The two Cu ions of Type 3, also located in the trinuclear centre, do not produce an EPR, possibly due to antiferromagnetic coupling in the presence of a bridging ligand, presumably hydroxyl. This site also exhibits an absorption band at 330 nm due to charge transfer between a hydroxyl-bridging group and the Cu atoms ( $OH^- Cu^{2+}$ ) [18].

In general, laccases have strong abilities to oxidize both organic (ascorbic acid, organic amines, phenolate siderophores) and inorganic (metal ions such as Cu (I), Fe(II) and Mn(II)) substrates [18,25]. Furthermore, laccase can transfer four electrons from a reducing substrate to one oxygen molecule, reducing it to water and is the only enzyme known to catalyze this four-electron-transfer reaction [50]. It is a versatile moiety for use in biotechnology because of its wide substrate scope, ability to use oxygen as an electron acceptor, and the absence of dependence on cofactors

and peroxide. It has also attracted interest in the environmental remediation sector for its ability to transform or degrade various toxic compounds ubiquitous in contaminated wastewaters. Laccase-based oxidation is a good substitute for the more common chemical oxidation brought about via reactions with hydroxyl radicals ( $\cdot OH$ ) for removing the organic (and sometimes inorganic) contaminants present in water and wastewater [37]. This is because inorganic chemical oxidation is often too expensive to implement practically when most advanced oxidation process systems require a continuous input of chemical reagents to maintain the operation. In comparison, laccase-catalysed reactions, which can be carried out under mild reaction conditions, are characterized by enhanced specificity, greater oxidation capacity and a lower yield of by-products. It also does not require hazardous organic solvents, extremely high or low pH or an increased temperature or pressure [47]. Also, these reactions are cost-effective because of the abundance of molecular oxygen in nature [37].

Laccase is effective as a biocatalyst in established and emerging biotechnological applications due to its nonspecific and high oxidation ability without the aid of any cofactors [33]. It is also useful in the agriculture sector, where it can remediate the soil contaminated with herbicides, pesticides and other chemicals/explosives [32]. It is used in the preparation of some anticancer drugs and in detoxification of cosmetic products [33]. Moreover, it can form industrially important polymers [36,37].

As a potential oxidative enzyme with broad substrate specificity towards aromatic compounds, laccase is capable of degrading the synthetic chemicals (xenobiotics) having hydroxyl and amine groups [32]. However, the comparatively low stability of the free enzyme hampers its large-scale application [18]. Protein stability can be enhanced through immobilization by preventing autolysis or proteolysis [36]. Immobilized enzymes are being used since 1916, when Nelson and Griffin discovered that invertase when absorbed to charcoal, can hydrolyse the sucrose [12]. The present review is an effort to encompass the laccase production by different organisms, understand its immobilization and mode of action, examine its biotechnological and industrial applications, and evaluate its bioremediation potential.

### Mechanism of action within the laccase molecules

In laccase-catalysed oxidation, Type 1 copper (Cu1) acts as the primary electron acceptor (Figure 1a). Electrons then shift through

a His-Cys-His tripeptide to a trinuclear cluster (TNC) containing Type 2 and Type 3 copper (Cu2 and Cu3) atoms (Figure 1b) which reduce  $O_2$  to  $H_2O$  [18]. The cavity on the surface of the enzyme, which contains Cu1 is quite large and can accommodate very many substrates. Cu1 facilitates the entrance of an electron into the catalytic site and regulates the catalytic action, which is dependent on the reduction of Cu1 [50]. However, Cu1 carries a relatively low redox potential (420-790 mV) in comparison to the normal hydrogen

electrode; this limits laccase action to phenolic moieties, have considerably low redox potential that causes electron abstraction by Cu1 [27]. A triangular arrangement of three Cu2/Cu3 ions allows for oxygen binding and reduction with the help of the four electrons transferred from Cu1 [50]. A substantial acidic group in the triangular cluster forms a hydrogen bond with a water molecule, which interacts with the oxygen species between the T3 coppers (Figure 1c).

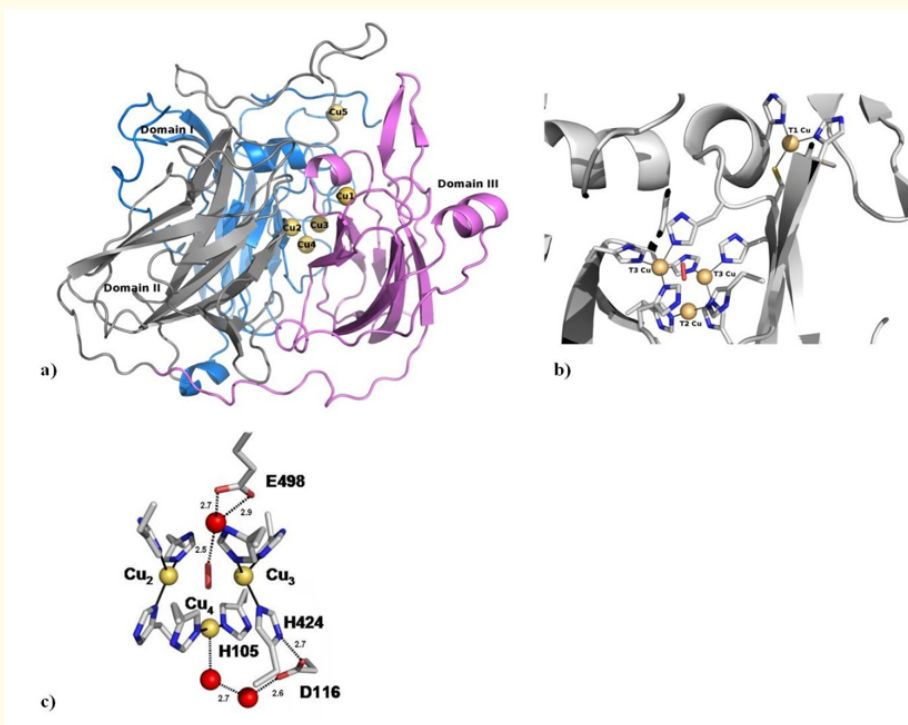


Figure 1 (a-c)

An example of the three-dimensional structure of one laccase protein *CotA*, which is a component of the endospore coat of *Bacillus subtilis* and displays all activities of laccase. It is also called *CotA* laccase. (a) Three-dimensional structure of *CotA* with each cupredoxin area pitted in different colors; area I (blue), area II (grey) and area III (violet). Copper atoms are shown as yellow spheres, corresponding to the mononuclear coppers 1 and 5, and the trinuclear centre, containing coppers 2, 3 and 4. (b) Structural pattern of the catalytic copper centres, the mononuclear Type 1 copper centre (T1) where copper atom is interconnected by a cysteine and two histidines, and the tri-

nuclear centre consisting of a Type 2 copper atom (T2) and two Type 3 (T3) copper atoms. The cysteine residue (C492), which aligns the T1 copper atom, is adhered to two of the histidine residues (H491 and H493), which organize the two T3 coppers in the trinuclear centre. This indicates the path for transfer of electrons from the T1 copper centre to the trinuclear centre. (c) Close view of the *CotA* trinuclear centre - substantial acidic groups are marked (E498 and D116) and also the histidine ligands to the copper that form hydrogen bonds with D116, From [18].

### Mode of action

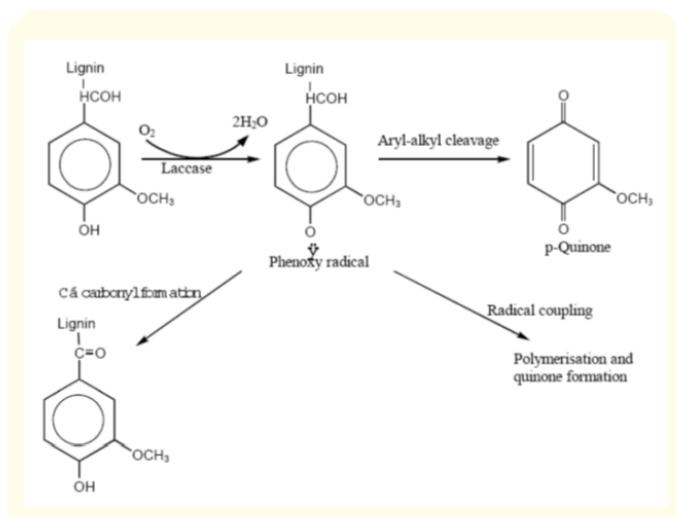
Polyphenolase, which contains copper proteins, oxidises aromatic compounds. Copper proteins help in oxidizing the benzene-ring-containing compounds, in which oxygen acts as the last electron receptor. Polyphenols are oxidized by a group of enzymes that have oxidase activities, e.g. catechol oxidase (EC 1.10.3.1); laccases (EC 1.10.3.2); cresolase (EC 1.18.14.1) [10]. The basic reaction mechanism of laccase involves the formation of two water molecules with a concomitant electron loss of a single oxygen molecule. This abstracted electron causes oxidation of a variety of benzene-ring-containing compounds [30]. Laccase has a significant role in the degradation of aromatic compounds leading to the generation of cation, which is normally less stable, and gets converted into some stable product in the presence of laccase (e.g., quinone phenol) or non-enzymatic reactions (e.g., hydration, degradation or polymerization) [14]. The redox mechanism occurs due to the presence of four copper atoms that constitute the central part of this reaction.

Substrate molecules are bound near the T1-copper center, which is shallower than the oxygen-binding center, and converted through abstraction of one electron into free radicals that may continue experiencing oxidation or radical-coupling reactions, giving rise to oligomers or polymers. The abstracted electron moves from the T1 center to the trinuclear cluster via a cysteine-histidine pathway (super exchange pathway), which is built by overlapping the redox active molecule orbitals of T1 coordinating cysteine, backbone atoms and T3 copper coordinating histidine residues [15]. The trinuclear center, a key player in the catalytic mechanism, is composed of Type 2 and 3 copper. Attachment of oxygen molecules to the trinuclear cluster and inhibition of further entry of other molecules precede the commencement of catalytic process. The T2Cu site reacts with two molecules of histidine and one molecule of water, while T3Cu site reacts with three histidines and hydroxide molecules. Finally, laccase converts the oxygen molecule to water via a two-step reaction, i.e. the first electron is reduced by T2Cu and T3Cu, whereas the second electron is reduced with the help of the peroxide mediator, which is directly entertained by the T2Cu site and T1Cu linked to T3Cu by covalent Cys-His bonds [30].

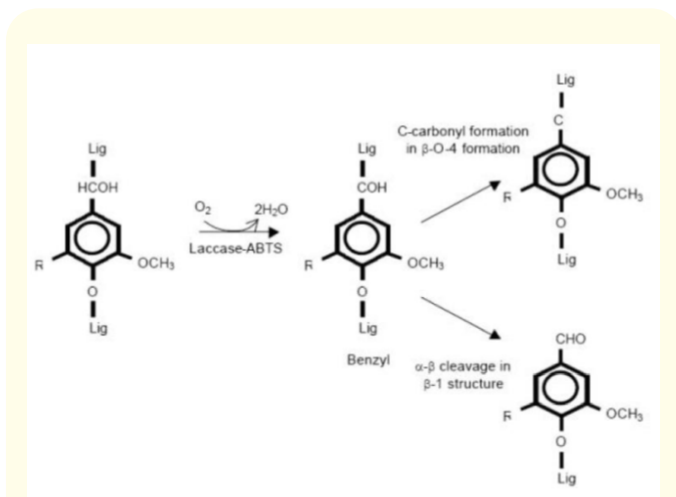
### Substrates and mediators for laccase

Laccase is a highly substrate-specific enzyme, which oxidizes a wide range of substrates including phenolic compounds, phenylpropanoids, azo dyes and indigo dyes [35]. It improves the synthesis as well as breakdown reactions of so many organic and aromatic compounds. The laccase-catalyzed reaction normally slows down in the presence of a bulky substrate characterized with a high redox potential. As a result of all these reactions, environmentally harmful substances, such as pollutants, may be broken down leading to an eco-friendly environment, or the synthesis of complex compounds may give rise to non-toxic products thus causing bioremediation [27]. Phenolic compounds such as hydroquinone and catechol are the most appropriate substrates for most of the laccases, but methoxy-substituted phenolic compounds, syringaldazine, guaiacol and DMP (2,4-di-methoxy phenol), are also used in various reactions [27]. Laccase may not oxidize some substrates directly because of their large size, low diffusion into the active pocket or high redox potential. Therefore certain reactions are carried out in the presence of different mediators. These redox mediator are first oxidized by laccase and the oxidized radical produced then reacts with bulky or high redox potential substrates. ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was used as the first artificial mediator [10]. N-heterocycles bearing N-OH, such as violuric acid, N-hydroxy-N-phenyl acetamide, or N-hydroxybenzotriazole, are among the most effective mediators [30,31].

In the phenolic substrates, oxidation by laccase results in the production of a free radical, which may undergo a second enzyme-catalyzed oxidation or a non-enzymatic reaction such as hydration, disproportionation or polymerization (Figure 1d). The bonds of the natural substrate lignin, which is split by laccase, include,  $\alpha$ - oxidation,  $\alpha$ -C $\beta$  cleavage and aryl-alkyl cleavage [18]. Laccases are known for their role in biodegradation of lignin, but their low oxidation potential keeps their action limited to phenolic compounds [12]. However, in the presence of mediator compounds, laccases exhibit a great oxidation potential and can oxidize nonphenolic lignin compounds (Figure 1e) [10].



**Figure 1d:** Oxidation of phenolic subunits of lignin by laccase. [Adopted from work [10] with authors' permission].



**Figure 1e:** Oxidation of a non-phenolic lignin compound by laccase [Adopted from [10] with authors' permission].

### Sources of laccase

The enzyme laccase is found in a wide variety of organisms including plants, fungi, bacteria, and insects. Each of these different sources of laccase is discussed in detail below.

### Plants

Laccase has been reported from several plant species including bean, lacquer, mango, mung, peach, pine, prune and sycamore

[9]. Other less common plant sources include *Rhus vernicifera* [17], *Pinus taeda* and *Acer pseudoplatanus* [12], and *Populus euramericana* [36]. In plants, laccase is involved in lignin synthesis via polymerization of lignin units [15] and takes part in early lignification stages by catalyzing the monolignols oxidation [15]. Besides the natural production of laccase in plants, laccase gene has been expressed in crop plants for large-scale commercial production, for instance, in the maize embryo [9,12]. Laccases participate in the wound-healing processes and defense reactions against external stresses [48]. However, they do not act on highly complex structures such as the phenolic compounds containing several aromatic rings [15].

### Fungi

Fungal laccase have great utility in facilitating lignin degradation and it is produced by several fungi belonging to Ascomycetes, Deuteromycetes and Basidiomycetes, wherein the white rot fungi are particularly effective in laccase production [2]. However, there is still no report of laccase production in the lower fungi (Zygomycetes and Chytridiomycetes), which are yet to be studied in great detail [9]. Laccase from *Monocillium indicum* was the first of the Ascomycetes to show peroxidative activity [16]. The well-recognized laccase producers such as *Chaetomium thermophilum*, *Pleurotus eryngii* and *Trametes versicolor*, and a few *Trichoderma* species, can produce polyphenol oxidases [18]. Several white-rot fungi and geophilous saprophytic fungi produce laccase, as do some edible mushrooms such as *Pleurotus ostreatus* (oyster mushroom), *Agaricus bisporus* (champignon) and *Lentinula edodes* (rice mushroom) [19]. A number of wood-rotting fungi, for instance, *Coriopsis polyzona*, *Cerrena maxima*, *Lentinus tigrinus*, *Tamarix gallica*, *Trametes hirsuta* (*Coriolus hirsutus*), *Thapsia villosa* and *Tabebuia ochracea* also produce laccase [13].

Laccase is also involved in fungal morphogenesis [13], formation of spores and fruiting body, fungal protection from toxic compounds, delignification of lignocellulosic material [48], and the synthesis of melanin [14]. Laccase from the wood-colonizing Basidiomycetes has been extensively examined, purified and characterized at both the protein and gene levels [10]. A limited number of researchers have also used cyanobacterial bloom biomass, dye effluent or groundnut shell (GNS) as culture media for producing laccase from *Coriolus versicolor*. When using a GNS-derived medium and a cyanobacterial bloom at a ratio of 9:1 (dry weight basis), laccase production of  $10.2 \pm 2.2 \text{ U ml}^{-1}$  was obtained at 28°C and pH 5 [14].

The half-life of laccase can be as little as 74 min at 60°C with  $K_m$  and  $V_{max}$  of 0.29 mM and 9.49 mol min<sup>-1</sup>, respectively, as determined by kinetic analysis with the mediator 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [17]. Production of thermostable laccase was suppressed substantially by azide and hydroxylamine [13]. [17] showed that *Phanerochaete chrysosporium* NCIM 1197 secreted extracellular laccase and also examined the impact of various inducers on laccase production, finding that copper sulphate increased the production maximally (by 3.5 times) in comparison to the control [13]. Laccase production under (a) solid state fermentation, (b) batch fermentation and (c) static liquid culture was the maximum (i.e. 48.9 ± 1.8, 30.2 ± 1.6 and 22.6 ± 1.2 U L<sup>-1</sup> respectively) after five days [17].

*Trametes pubescens* MB 89 is expected to become the main laccase source for industrial production; where the formation of extracellular laccase is substantially increased by adding small quantities of Cu (II) to a simple glucose medium [14]. When glucose, usually an inhibitory substrate, was used as the primary carbon source, significant laccase production by *T. pubescens* began only after the complete consumption of glucose in the culture medium [50]. Synthesis of laccase was markedly affected by the nitrogen source used [14] and the maximum laccase activity was 330 U mL<sup>-1</sup> in an optimized medium containing glucose (40g L<sup>-1</sup>), meat-derived peptone (10g L<sup>-1</sup>) and MgSO<sub>4</sub>·7H<sub>2</sub>O (1g L<sup>-1</sup>) with 2.0 mM Cu as an inducer [50].

### Bacteria

In bacteria, laccase is present mainly inside the cell in the form of a periplasmic protoplast [9,28]. Bacterial laccase was first reported in *Azospirillum lipoferum*, a bacterium associated with plant roots, where it was involved in melanin formation [50]. A number of studies have since found laccase in various bacteria including *Bordetella compestrus*, *Bacillus subtilis*, *Caulobacter crescentus*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Yersinia* sp., *Pseudomonas aeruginosa* and *P. syringae* [4,9,31,51]. A laccase-producing strain *Stenotrophomonas maltophilia* has been used for synthetic dye degradation [31]. Although the bacterial laccase is characterized by a low redox potential (0.45-0.54 V), it is active and remains stable when exposed to high temperatures (46 hrs at 60°C, pH 7-9) and salt concentrations [46,48]. These features are useful for industrial applications, because many industrial processes conducted under harsh conditions could inactivate other types of laccase. Application of bacterial laccase for treating the

textile wastewater (i.e. dye decolorization) affirms that it has the industrial potential [31,39].

Laccase with six copper-binding sites were detected in the marine bacterium *Marinomonas mediterranea* [5]. Previous studies have shown that laccases have the ability to perform well under varying environmental conditions, for instance, at neutral pH or in the presence of high concentrations of Cu<sup>2+</sup> and Cl<sup>-</sup>. For example, the laccase enzyme produced by *Sinorhizobium meliloti*, comprised two subunits each with a molecular weight of 45 kDa and functioned well at pH 6.2 [39], whereas another enzyme synthesized by *Pseudomonas putida* had a single subunit of 59 kDa and worked effectively at pH 7.0 [31]. Both of these enzymes oxidized laccase substrates such as syringaldazine. [12] optimized the laccase production of the filamentous bacterium *Streptomyces psammoticus* MTCC 7334 by varying the physical and nutritional attributes of the submerged fermentation using the Response Surface Methodology (RSM). Increase in temperature, incubation period, agitation, and yeast extract or trace elements concentrations enhanced the laccase production significantly.

A new laccase gene called *CotA* was isolated from the bacterium *Bacillus licheniformis* and was subsequently cloned and expressed in *Escherichia coli* [31]. The purified recombinant protein *CotA* was shown to be a typical blue multi-copper oxidase (MOC) with a molecular weight of ~65kDa which exhibited activity to several laccase mediators such as 2, 6-dimethoxyphenol (2, 6-DMP), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and syringaldazine (SGZ) [39].

### Insects

Laccase enzyme is also found in various insects including *Bombyx*, *Calliphora*, *Drosophila*, *Diptera*, *Lucilia*, *Manduca*, *Musca*, *Oryctes*, *Phormia*, *Papilio*, *Rhodnius*, *Sarcophaga*, *Schistocerca*, *Tenebrio* and *Coptotermes formosanus* [49]. In fact, 78% of the insect origin proteins classified by [16] are insect laccases, which are active in cuticle sclerotization [7]. At some stage during sclerotization, insect cuticular proteins are oxidatively coupled with catechols, a post-translational process termed as catecholization, and then become cross-linked, forming oligomers and subsequently polymers [41]. The salivary glands of *Nephotettix cincticeps* secrete a laccase (diphenoloxidase) in watery saliva [7]. This laccase possibly enhances the oxidative gelling that occurs in the stylet sheath, and rapidly oxidizes the highly toxic monolignols to non-toxic polymers [7].

Analysis of the insect species sequenced genomes has shown that there are two laccase genes in *Tribolium castaneum* and *Bombyx mori*, four genes in *Drosophila melanogaster* and five in *Aedes aegypti* and *Anopheles gambiae* [15]. The insect laccase gene has also been cloned [7] and two main forms (viz. *laccase-1*, *laccase-2*) have been identified [42]. *Laccase-1* expressed in malpighian tubules, midgut, fat body and epidermis of *Manduca sexta* (tobacco hornworm), oxidized the toxic compounds ingested by insects [42]. *Laccase-2* has two isoforms (*Lac2A*, *Lac2B*) arising from alternative splicing of the exon [41]. These isoforms are able to catalyze larval, pupal and adult cuticle tanning in *Tribolium castaneum* (red flour beetle) and are thus critical for insect life [7].

### Immobilized enzyme

The limitations of laccase such as low stability, and high sensitivity to the process conditions can be overcome by immobilization technique [47], which improves the activity and stability of the biocatalyst in both aqueous and organic phases, provided that the support permits the diffusion of the substrate to the active site of the enzyme [43,47]. It also facilitates simple recovery of enzymes by centrifugation, sedimentation or other physical separation methods. Immobilized enzymes can also encounter several drawbacks, such as mass transfer limitations or interaction between the enzyme and the support that may reduce its catalytic potential [43]. Immobilization of laccases can improve their efficiency by increasing their thermo-stability, resistance to harsh chemical reagents and other extreme conditions [10]. Another advantage is that immobilized laccases can be more easily separated from reaction products, which makes continuous bioreactor operation more practical [8,10]. However, immobilization can also potentially reduce the enzyme activity due to changes in conformation or heterogeneity when placed on a support [10].

The process of enzyme immobilization involves fastening of the enzyme to an insoluble substrate support [8]. There are two basic methods for enzyme immobilization, as the enzyme-support link can take place by physical or chemical interactions. Physical coupling methods comprise the enzyme entrapment within a tridimensional matrix, its encapsulation in an organic or inorganic polymer, and its adsorption to the support surface by ionic exchange; covalent bonding enables the irreversible binding of enzyme to the support matrix [10]. Of the various alternatives, the large specific surface

area characteristic of nanomaterials makes this type of support an ideal candidate for enzyme immobilization [13]. The efficiency of ionic exchange depends on the pH and ionic strength of the medium and the hydrophobic nature of the nanoparticle (NP) surface [13]. NPs are supposed to offer a homogeneous core-shell structure that may be functionalized to react with the nucleophilic groups on the enzyme [43]. The majority of enzymes are covalently attached to lysine amino groups typically present on the protein surface [22]. A number of factors, like pH, ionic strength, protein concentration, additives, and the porous or non-porous nature of NPs may influence the biocatalyst and the strength of covalent bonding between the enzyme and the support.

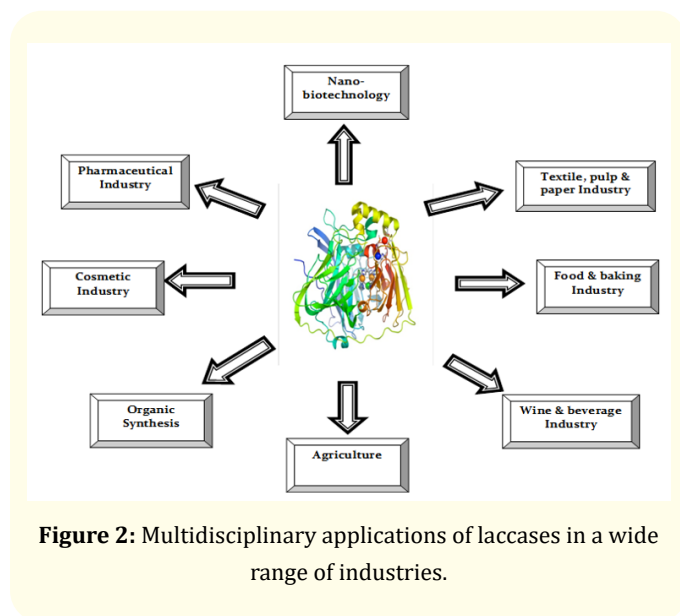
In the recent past, efforts have been made to immobilize the laccase enzyme on different types of NPs including the silver and gold NPs [12], chitosan-coated magnetic NPs [14], and carbon nanotubes. Two different immobilization procedures are normally followed, i.e. ionic exchange between the enzyme and the NP, and covalent bonding of the enzyme protein with NP surface, using glutaraldehyde or carbodiimide as cross-linkers [43]. Glutaraldehyde, a bifunctional and versatile agent, may react with various enzyme moieties, mainly with primary amino groups of proteins, and then also with other groups such as thiols, phenols and imidazoles [15].

### Laccase applications

Laccases have a role in several biotechnological applications (Figure 2). These are used for cleaning industrial effluents due to their ability to oxidize a vast range of phenolic and non-phenolic compounds [43]. These are also used in cosmetics, drug development, and medical diagnostics and for removing toxins from contaminated soils [43]. In general, laccases have enormous potential for removing xenobiotic substances and for making polymeric products [43]. Increasing the laccase-based synthesis of organic compounds, biooxidation, biotransformation and development of biosensors are also attracting the attention of active researchers [43].

### Biotechnological applications

The laccase-based biocatalysts, which are energy-saving and biodegradable, are becoming popular, as they support the development of efficient, environment-friendly and sustainable industries [9]. Some of the current industrial applications of laccase are discussed below.

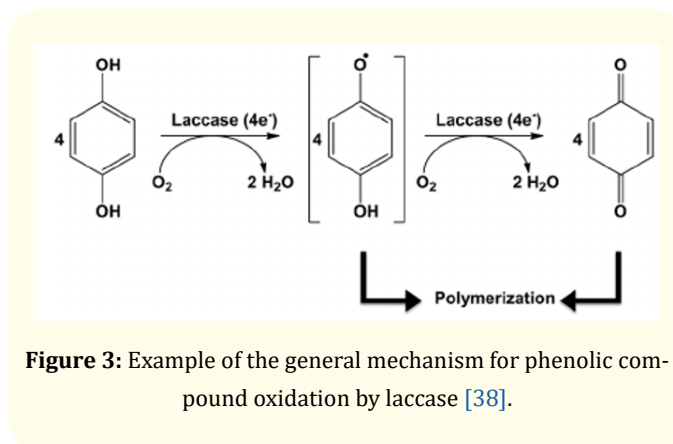


**Figure 2:** Multidisciplinary applications of laccases in a wide range of industries.

### Pulp and paper industries

The pulp and paper industry is well known for generating significant amounts of environmental pollutants because of the ubiquitous use of high concentrations of chemicals such as bisulfites, calcium oxide, chlorine or chlorine dioxide, hydrochloric acid, sodium sulfide, sodium carbonate and sodium hydroxide [16]. The wastewater from the paper industry is also typically characterized by dark brown discoloration, a high organic matter content (20-110 kg COD per air-dried ton of paper), high levels of absorbable organic halides (AOX) and a variety of other toxic pollutants. Pulping, the initial stage of the process of paper preparation, is the major source of contamination because wood chips are treated to remove lignin at this stage. Lignins are the most abundant group of biopolymers in the terrestrial environment [23] and constitute the main non-carbohydrate component of wood. In paper production industry, lignin in the wood pulp is separated from the pulp and degraded via chemical oxidation typically using either chlorine or oxygen. Lignin-degrading enzymes such as laccase provide a greener alternative for biodegradation than these traditional chemical oxidants [23]. Laccases also find application in other industries where chlorolignins or phenolic compounds are produced [23]. They can also reduce the overall toxicity of the residual phenolic solutions via initial promotion of decomposition of phenols and promotion of the subsequent polymerization of pollutant phenols with naturally occurring ones (Figure 3) [38]. Phenolic effluents as well as polycyclic aromatic hydrocarbons (PAHs) are treated by several techniques using either laccases as such or immobilized laccases [38].

Laccases can also remove lipophilic substances responsible for pitch deposition in paper pulps, thus improving the chemical, mechanical, and physical properties of the pulp via (a) formation of radicals that react with lignin or (b) functionalization of lignocellulosic fibers [23].



**Figure 3:** Example of the general mechanism for phenolic compound oxidation by laccase [38].

### Textile industry

Large amounts of water, energy and harsh chemicals are consumed during textile processing. In the conventional washing process, uncoloured fabric is passed through a series of chemical treatments such as desizing, scouring, mercerization, bleaching and washing, to make the finished fabric [17]. The conventional chemical processes are generally severe and cause fibre damage. Laccase is mainly used in the bleaching process to increase the fabric whiteness and in the process of bio-stoning to save energy and water [18].

More than 10,000 different dyes are used in the textile and printing industries worldwide, with about 10% of the used dyestuffs effluxed to the environment as waste [16]. Traditionally, wastewater from the textile dyeing processes is treated by chemical and physical processes, including electrokinetic coagulation, electrochemical destruction, irradiation, ozonation and precipitation [44], which involves high costs and operational problems. Since the laccase readily biodegrades most of dyes, many laccase-based methods have been developed to treat the excessive synthetic dye concentrations [44]. Laccases can also degrade indigos [26], prevent fabric shrinkage [17], decolorize wool from azo-dyes [48] and improve yarn regularity, all under mild reaction conditions, thus keeping the process environment-friendly [26]. They also catalyze textile dye bleaching and functionalize the specific textile fiber molecules, where modification of protein fibers, such as cotton and wool, improves fiber properties. For example, fibers treated with laccase enzyme can enhance the



fixation of dye to wool [18], resulting in economic advantage due to reductions in the amount of the dye used for obtaining deeper colors [18].

### Food industry

Since many laccase substrates (carbohydrates, phenols, thiol-containing proteins and unsaturated fatty acids) are important components of our food and beverages, laccase can also be applied to alter the color and appearance of foods or beverages [29]. During the food storage, elevated levels of O<sub>2</sub> lead to a detrimental oxidation and decreased food quality. Thus laccase is used as an oxygen scavenger for better food packaging and for the elimination of unwanted phenolic compounds in baking, beer and wine stabilization and in juice processing [34,40]. Laccase is also employed in ascorbic acid determination, sugar beet-pectin gelation, and for treating the olive mill wastewater [18].

### Wine and beverage stabilization

Laccase is used as a stabilizer in the manufacture of beer, fruit juice, musts and wine to increase their shelf life [9]. Musts and wines contain a variety of chemical compounds including ethanol, organic acids, phenolic compounds and salts. The aroma of wine is determined by alcohol and organic acids, while the color and taste depends largely on the phenolic compounds present. However, elevated polyphenols concentration have undesirable effects on the production and characteristics of wine [45], whereas polyphenol oxidation in wines (and musts) causes undesirable changes in color and flavor, a process called maderization. Laccases have been proposed as an alternative to physical and chemical adsorbents for the selective removal of specific polyphenols during maderization, so that polyphenolic substances can be oxidized, polymerized and removed [9]. Thus, the high quality and stable wines may be prepared 'naturally' by laccase treatment with little or no addition of SO<sub>2</sub>.

Haze formation in beer, commonly referred to as "chill haze", is an aesthetic problem that occurs due to the presence of naturally occurring pro-anthocyanidin and polyphenols. Chill haze normally develops at low temperatures due to the formation of insoluble complexes. Beer is sufficiently warm to re-dissolve these complexes above room temperature. However, on standing at room temperature, phenolic rings are slowly replaced by sulphhydryl groups, ultimately leading to the formation of a permanent haze, which cannot be re-dissolved. Thus to reduce the polyphenol oxidation, laccase is used as an O<sub>2</sub> scavenger to increase the shelf life of beer.

Application of laccase as a juice stabilizer is very common. The phenolic compounds and their oxidative products naturally present in the fruit juice regulate its distinctive color and taste. However, both aroma and color change determinately due to increased phenolic/polyphenol polymerization and oxidation. These changes, referred to as 'enzymatic darkening', are normally attributed to high polyphenol concentrations. With the aid of membrane filtration, laccase treatment is able to remove both phenols and substrate-enzyme complexes, resulting in color stability with some turbidity [29]. Laccase treatments are distinctly more effective than the conventional methods [29].

### Baking industry

Laccase can also be used in the bakery industry to increase the gluten structure strength in doughs and baked products [10]. In the bread-making process, the action of laccase, when added to dough, improves the freshness, flavor, stability, strength, softness and texture, and reduces its stickiness, thus improving the overall machinability [13]. Addition of laccase also improves many baked-product characteristics, including the crumb structure, softness and volume. One significant advantage of using laccase is that a small portion of poor quality flour can be used during the backing to obtain the same result as could be obtained from using 100% of a higher quality (more expensive) flour, thus resulting in cost savings.

### Petrochemical industry

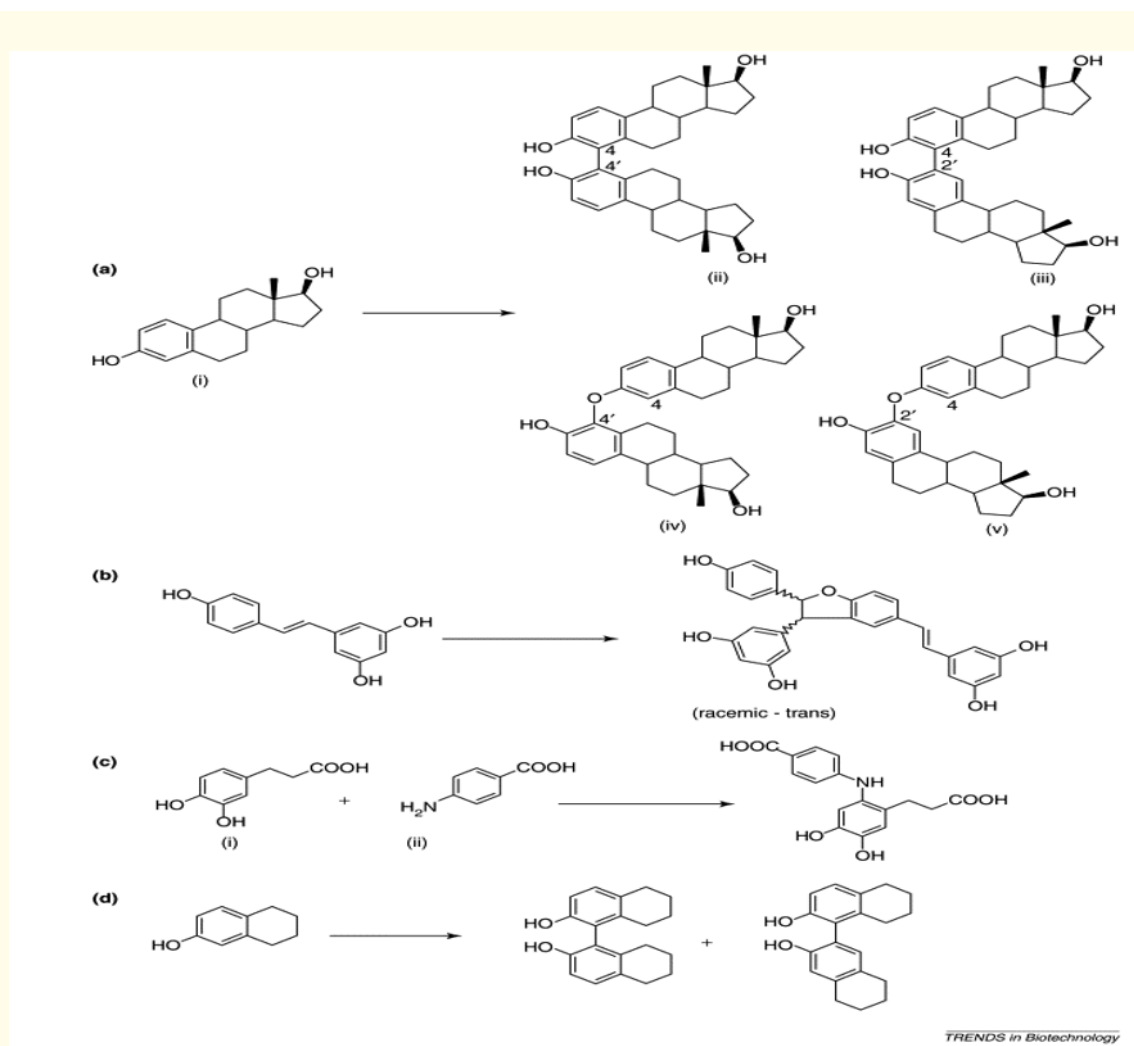
The petrochemical industry is a major source of hazardous wastes and petroleum hydrocarbon contamination [14]. Bioremediation of petrochemical contaminants by natural populations of microorganisms has been one of the basic strategies for removing hydrocarbon pollutants [14] and is also much cheaper than many other conventional techniques [14]. Laccase can easily degrade polycyclic aromatic hydrocarbons (PAHs) occurring naturally in fossil fuels and oil deposits [6]. For example, a laccase-mediator system (LMS) could successfully oxidize a range of compounds including alkenes, carbazole, dibenzothiophene, ethylcarbazole and fluorine [24]. The LMS also proved to be useful for the oxidation of PAHs and many other pollutants [3]. For instance, oxidation of naphthalene could be carried out by immobilized laccase from *Trametes versicolor*, using various immobilization strategies [16].

### Organic synthesis

Over the last two decades, laccases have become popular as a new biocatalyst for organic synthesis that can be used with a variety

of substrates and is a promising green alternative to classical oxidative chemicals [10]. Enzymatic polymerization by laccase has drawn considerable attention because laccases or laccase-mediator systems are capable of generating polymers that cannot be produced via conventional chemical synthesis. Laccases promote oxidative coupling of small organic compounds, paving new routes for the synthesis of biological compounds and chemicals such as amino acids and anti-oxidants (Figure 4) [14]. Laccases have a wide applicability and are employed for functional group oxidation, coupling of phenols and steroids, formation of carbon-nitrogen bonds and synthesis of complex natural products [15]. Moreover, the *Tinea versicolor* laccase ex-

pressed in *Saccharomyces cerevisiae* markedly facilitated fuel ethanol production from renewable raw materials like lignocellulose. Lignocellulosic materials have the ability to hydrolyze to sugars, which could be fermented to ethanol by microorganisms, such as the yeast *Saccharomyces cerevisiae* [18]. Ethanol derived from lignocellulosic materials can be used as an environment-friendly liquid fuel [18]. Laccase is also employed for producing ‘artificial urushi’, which is a synthetic substitute for ‘urushi’ (oriental lacquer), a natural resinous sap obtained from the Japanese Laquer tree via enzymatic polymerization followed by curing of catechol derivatives [17].



**Figure 4:** Illustrations of synthetic applications of the so-called laccase-mediator system (LMS): (a) Oxidation of indigo to isatin, (b) oxidation of adlerol to adlerone, (c) oxidation of benzyl alcohols to the representing aldehydes, and (d) oxidation of alkyl β-glucosides to their equating glucuronosides.

## Pharmaceuticals

Laccases find broad applications in the pharmaceutical industry due to their ability to synthesize the complex medically important compounds including anesthetics, antibiotics, anti-inflammatory agents and sedatives such as cephalosporins, dimerized vindoline, mitomycin, penicillin X dimer, triazolo (benzo) cycloalkyl thiadiazines, and vinblastine [9].

The first chemical of high pharmaceutical value obtained using a laccase enzyme was actinocin, which was derived from 4-methyl-3-hydroxyanthranilic acid. Actinocin has anticancer properties and blocks DNA transcription in tumor cells [24]. Another anticancer drug prepared using laccase was vinblastine, which is used for treating leukemia. While the plant *Catharanthus roseus* naturally produces small amounts of vinblastine, laccase allows for the production of commercial quantities. The two precursors of vinblastine, namely katarantine and vindoline, are produced in large quantities and purified easily, and laccase is used for their conversion into vinblastine with a 40% conversion rate [24]. Enhancement of such conversion reactions by laccase has greatly augmented the production of various pharmaceutically important compounds including the antibiotics [13].

Catechins, consisting of small tannin units, are the natural antioxidants present in a variety of herbs, tea and vegetables. Owing to their free-radical-scavenging properties, they have a preventive role against different diseases including cancer and the inflammatory and cardiovascular disorders [24]. Recently, laccase was used to oxidize a number of catechins, resulting in numerous products with improved antioxidant properties [24].

For their ability to isolate new dimeric derivatives of the  $\beta$ -estradiol hormone, laccases are also used in the synthesis of hormone derivatives. Moreover, laccase oxidation of isoeugenol or coniferyl alcohol and totarol gave rise to new dimeric derivatives including a mix of dimeric and tetrameric derivatives, whereas oxidation of substituted imidazole by laccase resulted in more complex substances [24]. Aliphatic and aromatic amines may be converted to 3-(3, 4-dihydroxyphenyl)-propionic acid with the help of laccase-based oxidation [24]. All these products have immense therapeutic significance.

## Cosmetics

The ability of laccase enzymes to polymerize the natural phenols has been exploited for the development of novel cosmetic pigments, deodorants, hair-dyeing materials, and personal-hygiene products

(e.g. diapers, detergents, mouthwashes, soaps and toothpastes) [33]. Laccase-based hair dyes in which laccases replace  $H_2O_2$  are less irritant and safer to handle, compared to the conventional hair dyes [33]. Recently, laccase use has been extended to the production of dermatological and cosmetic preparations, such as the skin-lightening proteins [33], wherein the protein-engineered laccase reduces allergenicity [33].

## Agriculture

Recently, laccase has found agricultural applications and has been used to remediate soil contaminated by herbicides, pesticides and certain explosives [9, 21, 32]. Remediation of phenyl-urea-based herbicides, which are widely used for weed mitigation on cereal crops, can cause oxidation reactions in the agricultural ecosystems leading to pollution. Laccase is effective here because it can transform the N, N'-dimethyl-N-(hydroxyphenyl) urea herbicide to an insoluble phenolic compound (p-benzoquinone, at pH=3), which is readily metabolized by fungi. Laccase also enhances the pesticide and herbicide degradation in conjunction with the redox mediators such as 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS),  $MnSO_4$  and Tween-80 [32]. For example, 40.9 % of glyphosate was remediated by the action of laccase and ABTS, whereas addition of  $Mn^{2+}$  and Tween 80 degraded 62.8% glyphosate after 24 h. Moreover, ABTS,  $Mn^{2+}$  and Tween 80 showed a synergistic effect, removing 90.1% of glyphosate in 24 h [32]. Moreover, some recent findings indicate that the bacterial laccase has a potential against a wide range of plant pathogens due to realizing the antimicrobial activity [32]. These authors have reported that the extracellular laccase of *Pseudomonas putida* LUA15.1 successfully controlled a wide range of both fungal (*Phytophthora capsici*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Alternaria zinniae*, *Dematophora necatrix*, *Sclerotium rolfsii*) and bacterial (*Ralstonia solanacearum*, *Agrobacterium tumefaciens*, and *Xanthomonas axonopodis*) plant pathogens [32].

## Nano-biotechnology

Laccases have been applied to nano-biotechnology, an emerging research field, wherein they catalyze (without additional cofactors) the electron-transfer reactions. Greater attention is now being paid to laccases, and a number of techniques such as micro-patterning, self-assembled monolayers, and layer-by-layer methods have been devised to immobilize laccases and preserve their enzymatic activity [50]. Further, the role of laccase as a biosensor or bioreporter for detecting various azides, oxygen or phenolic compounds has also been investigated [50]. A number of laccase-based electro-

immunoassay techniques have been used for determination of adrenaline, aromatic amines, codeine, catecholamines, glucose, morphine, plant flavonoids and various phenolic compounds. Recently, laccases have found application in biofuel cell design [50], synthesis of gold nanoparticles [50] and in the production of biofuels [50] and nanostructured materials (nanoparticles, nanotubes and nanofibers) [50].

### Biosensor technology

Because of acting on a wide range of substrates and reacting with phenolic compounds, laccase can be useful in biosensor technology. Oxygen and various reducing substrates (especially phenols and anilines), which are catalyzed by laccase, can be detected easily when combined with a physical instrument, which acts as a biosensor. The laccase-based biosensors are of two types: (a) those which monitor spectrum variation of enzyme at an absorbance of 600 nm, and (b) those which monitor voltage changes from a modified oxygen electrode [16]. [51] immobilized the alkali-tolerant laccase on a nitrocellulose membrane, which could react linearly to different substrates, such as syringaldazine, catechol, catechin and L- DOPA, even at low concentrations.

### Bioremediation

The process of bioremediation aims at removing the fractious compounds of an environment. It uses microorganisms to remove the polluting organic compounds by metabolizing them to carbon source. Laccase from fungus is considered as a potential tool for removing from wastewater the pollutants that are normally difficult to remove [11]. Microorganisms that produce laccases are used in bioremediation of contaminated soil and biodegradation of toxic waste [11]. Many laccase-producing plants and saprophytic fungi transform in the natural environment various phenolic and other aromatic and cyclic compounds into non-toxic derivatives by oxidative transformation (as, for instance, dehalogenation of chlorophenols or their transformation into benzopyrene) and/or bind such compounds to form chemically neutral polymeric compounds, e.g. immobilisation of toxins during humus formation [27], often with the assistance of laccase-mediator systems. [27] demonstrated that the laccase isolated from *Trametes hirsuta* can oxidize the aliphatic and cyclic alkenes, a large group of compounds polluting the environment. Oxidation reactions were catalysed only in the presence of mediators, like HBT and violuric acid, to produce the corresponding aldehydes and ketones. In the presence of ABTS, the purified laccase from *Cariollopsis gallica*

catalysed the decomposition of carbazole, N-ethylcarbazole, dibenzothiophene and fluorene with a 60 - 100% efficiency, depending on the compound being degraded. These polycyclic hydrocarbons, generated mainly through petroleum and coal combustion, are among the major atmospheric pollutants [27]. Likewise, in the presence of HBT, laccase from *Trametes versicolor* could cause efficient degradation of the herbicide isoproturon [27]. Laccases catalyse the formation of oligomeric TCS products of little environmental concern [27].

### Environmental protection

The rate of the biodegradation of organic compounds, an efficient and eco-friendly mechanisms for the removal of toxic pollutants, is often determined and explained by a kinetic constant [20]. It has been reported that caffeine, acetaminophen, estradiol and ibuprofen like contaminants can be easily degraded due to their high biodegradation constant, while tetracycline, carbamazepine, and iopamidol, by contrast, have a low biodegradation constant and therefore are not easily biodegradable [20]. Efforts have been made to enhance the biodegradation of persistent pharmaceutical ECs through oxidoreductase enzyme, nitrifier and fungal cultures [20]. Numerous biotic and abiotic factors such as the redox potential, molecular features, microbial diversity, temperature, availability of ECs, pH, toxicity of ECs and primary substrates have a role in the overall degradation of harmful pollutants apart from their physicochemical properties [20].

### Conclusion

Laccases constitute a group of enzymes that are common in nature and can be used in many industrial processes because of their versatility and potential to catalyze the oxidation of many different phenolic compounds. Laccases are produced by various natural sources including bacteria, fungi, higher plants and insects. In addition to industrial applications, laccases also have numerous biotechnological applications because of their natural capacity to oxidize a broad range of both phenolic and non-phenolic compounds. They are also utilized in the beverage industry to improve stability and quality, in the pharmaceutical industry, where their high oxidation potential helps in the preparation of various useful drugs and in the remediation sector for oxidation of toxic industrial pollutants. On the whole, their versatile potential to act on a variety of substrates and remediate a number of pollutants makes them useful across many industries including the cosmetics, pulp, paper, petrochemicals textile, wine, food, pharmacy, biosensors

and bioremediation. A deeper understanding of the mechanism of laccase action and laccase biochemistry would not only facilitate more efficient and economical applications of this enzyme to the modern industrial processes, but also pave the way for exploring the novel and hitherto unidentified prospective applications.

### Future Perspectives

Given that laccases have immense application in areas of nanobiotechnology and in the industrial sector; efforts need to be made to exploit the nutritive potential of industrial wastes for the more economical production of laccase. Solid wastes as well as the wastewater from textile-processing industries can be of immense utility in this context. However, a limiting factor in the application of laccase on a larger scale is the lack of technologies to produce such high activity enzymes in large volumes at a low cost. The use of laccase enzymes in the biosensors and bioremediation opens up new hopes and possibilities. Future research must therefore focus on these aspects.

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