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# Microbial Laccases and their Applications: A Review

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## ABSTRACT

Laccases are an interesting group of multi copper enzymes, which are widely distributed in higher plants and fungi. In fungi, laccase is present in *Ascomycetes*, *Deuteromycetes*, *Basidiomycetes* and is particularly abundant in many white-rot fungi that degrade lignin. The ability of the laccases to act on wide range of substrates has made them very important in many of the biotechnological applications. They have wide temperature and pH activity range. Their large capacities to act on both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants helped researchers to put them in various biotechnological applications like detoxification and de-colorization of industrial effluents, waste water treatment. They can also be used effectively in paper and pulp industries, textile industries for various applications and they do stand as efficient tool for bioremediation. Hence laccases have received a greater attention of researchers around the globe. This study comprehensively reviews the appreciative work done on these microbial laccases.

**Key words:** Laccase, fungi, industrial applications, mediators, fermentation, properties

## INTRODUCTION

The use of enzymes in the diverse fields of industrial application has been of greater importance in recent years. Many of such potential enzymes are widely distributed in nature; laccases are one among them, which are oldest and most studied enzymatic systems. Laccases (benzenediol: oxygen oxidoreductases, E.C 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. They are defined as oxidoreductases in Enzyme Commission (EC), which oxidizes diphenol and allied substances and use molecular oxygen as an electron acceptor (Kiiskinen *et al*., 2002; Viswanath *et al*., 2008a).

Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Thurston, 1994; Levine, 1965). In 1896, laccase was demonstrated to be present in fungi for the first time by both Bertrand and Laborde (Thurston, 1994; Levine, 1965). Since then most of the laccases which have been isolated are from fungal origin especially from white rot fungi belonging to various classes such as *Ascomycetes, Basidiomycete* and *Deuteromycetes* (Gochev and Karstanov, 2007; Kiiskinen *et al*., 2004a). Some of the bacterial laccases have also been characterized from *Azospirillum lipoferum* (Givaudan *et al*., 1993), *Bacillus subtilis* (Martins *et al*., 2002), *Streptomyces lavendulae* (Suzuki *et al*., 2003) as referred by Kiiskinen *et al*. (2004b).

Laccases have a lower redox potential (450-800 mV) than those of ligninolytic peroxidases (>1 V), so it was initially thought that laccases would only be able to oxidize phenolic substrates (Kersten *et al*., 1990). However, the range of substrates oxidized by laccases can be increased through a mediator-involved reaction mechanism. Mediators are low molecular weight compounds that are easily oxidized by laccases producing, in some cases, very unstable and reactive cationic radicals, which can oxidize more complex substrates before returning to their original state. The electrons taken by laccases are finally transferred back to oxygen to form water (McGuirl and Dooley, 1999; Wong and Yu, 1999) as referred by Osma (2009).

In contrast to most enzymes, which are generally substrate specific, laccase do act on wide range of substrates like diphenols, polyphenols, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Viswanath *et al*., 2008a). Although, most of the laccases substrates are variety of phenolic compounds, their specificity can be broadened by means of mediators such as 2, 2 '-azinobis(3-ethylbenzthiazoline-6-sulfonate) and 1-hydroxybenzotriazole (Octavio *et al*., 2006).

Discovery of novel laccases with different substrate specificities and improved stabilities is very important for industrial applications, besides developing an effective, high yielding and economic production medium to enhances its utility. Several reports regarding this aspect have been worked on both submerged and solid state fermentation. Solid state fermentation utilizing natural lignin containing substrates such as rice bran, wheat bran, coir dust, potato peel etc. (Shanmugam *et al*., 2009; Toca-Herrera *et al*., 2007) have received much attention because of its efficiency in production of microbial laccases. Where as most of the submerged fermentation research works reported thus far, have been tried to design a optimum production medium by varying concentrations of media components, introducing natural (saw dust, corn cob, bagasse particles etc.) and synthetic (veratryl alcohol, indulin, guaiacol etc.) inducers in the media (Patel *et al*., 2008; Kiiskinen *et al*., 2004a, b).

The enzymatic catalysis by laccases in different industrial applications such as textile dye bleaching, pulp bleaching and bioremediation could serve as a more environmentally benign alternative than the currently used chemical processes. Despite the availability of several reports on microbial laccases and increasing research day by day, there are hardly any reviews which encompass complete information, which could enable researchers to emphasize on works not being carried out on microbial laccases. There are few reviews which emphasize on occurrence and properties of fungal laccases (Baldrian, 2006), industrial and biotechnological applications of laccases (Couto and Toca-Herrera, 2006b), fungal laccases and their applications with reference to bioremediation (Viswanath *et al*., 2008b). Thus, there is a need to assemble information available on microbial laccases from different resources in order to bring to light their importance and direct research into those aspects which can yield much more potential laccases source and broaden their application horizon, which are eco friendly and economic too.

The present review will encompass microbial sources of laccases, optimization of production with reference to submerged and solid state fermentation, biochemical characterization, purification, molecular aspects of laccases and their potential industrial applications. It also explores the untapped potential of laccases.

**Sources of microbial laccases:** Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in higher plants and fungi. The fungi producing laccases

have been identified by the ability of laccases to oxidize different substrates such as guaiacol, Remazol Brilliant Blue R (RBBR), tannic acid, Poly R-478 etc. to specific colored products (Kiiskinen *et al*., 2004a, b).

Apart from the usual sources like soil, textile effluent, municipal waste, tree barks etc, some of the laccase producing fungi belonging to class *Ascomycete* and *Basidiomycete* are also isolated from the marine samples (Verma *et al*., 2010). *Coriolopsis byrsina*, *Cerrena unicolor*, *Diaporthe phaseolorum*, *Pestalotiopsis uvicola* are some of marine derived fungi. A few classes of bacteria also found to produce these enzymes. The most important laccase producing bacteria and fungi are listed in Table 1.

Most of the laccases reported thus far are of fungal origin, especially from white rot fungi (Kiiskinen *et al*., 2004a, b) such as *Phlebia radiata* (Niku-Paavola *et al*., 1988), *Pleurotus ostreatus* (Palmieri *et al*., 2000) and *Trametes versicolor* (Bourbonnais *et al*., 1995). Many of the *Trichoderma* species extensively studied as sources of cellulases have also been reported as sources of laccases

Table 1: List of laccase from bacterial and fungal sources

| Sources | References |
|---|---|
| Bacteria | |
| *Azospirillium lipoferum* | Givaudan *et al*. (1993), Faure *et al*. (1994) |
| *Bacillus subtilis* | Martins *et al*. (2002) |
| *S. maltophilia* AAP56 | Galai *et al*. (2009) |
| *Streptomyces coelicolor* | Dube *et al*. (2008) |
| Fungal | |
| *Stereum ostrea* | Viswanath *et al*. (2008b) |
| *Fomitella fraxinea* | Park and Park (2008) |
| *Stereum hirsutum* | Mouso *et al*. (2007) |
| *Lentinus tigrinus* | Ferraroni *et al*. (2007) |
| *Trametes versicolor* | Minussi *et al*. (2007b) |
| *Ganoderma* Sp.MK05 | Khammuang and Rakrudee (2009) |
| *Termetes* sp. | Yang *et al*. (2009) |
| *Cerrena unicolor* | D'Souza-Ticlo *et al*. (2009) |
| *Trametes* sp. | Li *et al*. (2008) |
| *Trametes versicolor*, *T. hirsuta* and *Botrytis cinerea* | Tzialla *et al*. (2009) |
| *Ganoderma lucidum* | Punnapayak *et al*. (2007) |
| *T. versicolor* | Cordi *et al*. (2007) |
| *Tramates* sp. | Li *et al*. (2008) |
| *M. parvum* | Saparrat *et al*. (2007) |
| *Pestalotigsis* sp. | Verma *et al*. (2010) |
| *Diaporthe* sp. | |
| *C. byrsiana* | |
| *C. unicolor* | |
| *T. hirsute* | Tzialla *et al*. (2009) |
| *T. versicolor* | |
| *B. cinerea* | |
| *F. troggi* | Ciullini, *et al*. (2008) |
| *H. cylindrosporum* | Ramesh *et al*. (2008) |
| *C. unicolor* | D'Souza-Ticlo *et al*. (2009) |
| *P. sanguineus* | Valeriano *et al*. (2009) |
| *T. harzianum* | Sadhasivam *et al*. (2008) |
| *T. versicolor* | Xavier *et al*. (2007) |

by Gochev and Krastanov (2007). *T. atroviride* (Holker *et al*., 2002), *T. longibrachiatum* (Velazquez-Cedeiio *et al*., 2004), *T. harzianum* (Holker *et al*., 2002) are some of those *Trichoderma* sp. studied as laccase sources, besides, some of these increased laccase production of white rot fungi in mixed cultures (Velazquez-Cedeiio *et al*., 2004), as reported by Gochev and Krastanov (2007).

*Phanerochaete chrysosporium*, *Theliophora terristrus*, *Stereum ostrea* and *Lenzitis betulina* (Viswanath *et al*., 2008b) are some of the important Basidiomycetes which have been reported as the sources of laccases.

**Mode of action of the laccase enzyme:** Laccases contain 4 copper atoms termed Cu T1 (where the reducing substrate binds) and trinuclear copper cluster T2/T3 (where oxygen binds and is reduced to water).

The four copper ions of laccase are classified into three types, referred to as Type 1 (T1), Type 2 (T2) and Type 3 (T3). Three types of copper can be distinguished using UV/visible and Electronic Paramagnetic Resonance (EPR) spectroscopy. Type 1 Cu at its oxidised resting state is responsible for the blue colour of the protein at an absorbance of approximately 610 nm and is EPR detectable, Type 2 Cu does not confer colour but is EPR detectable and Type 3 Cu atoms consists of a pair of Cu atoms in a binuclear conformation that give a weak absorbance in the near UV region but no detectable EPR signal (Thurston, 1994).

The Type 2 and Type 3 copper sites are close together and form a trinuclear centre that are involved in the catalytic mechanism of the enzyme. The T2/T3 trinuclear site is where the reduction of molecular oxygen takes place by accepting electrons from T1 site. Elucidation of the nature coordination of the copper sites in laccase by spectroscopic and DFT studies (Quintanar *et al*., 2005) reveals that the T2 copper site is coordinated to two His-N and one oxygen atom as OH- while each of the T3 coppers coordinates to three His residues. Further, both T2 and T3 copper sites have open coordination positions towards the center of trinuclear cluster with the negative protein pocket (four conserved Asp/Glu residues). Reduction of oxygen takes place via the formation bound oxygen intermediates (Zoppellaro *et al*., 2000).

There are three major steps in laccase catalysis. The Type 1 Cu is reduced by a reducing substrate, which therefore is oxidized. The electron is then transferred internally from Type 1 Cu to a trinuclear cluster made up of the Type 2 and Type 3 Cu atoms (Fig. 1). The $O_2$ molecule binds to the trinuclear cluster for asymmetric activation and it is postulated that the $O_2$ binding pocket appears to restrict the access of oxidizing agents other than $O_2$. $H_2O_2$ is not detected outside of laccase during steady state laccase catalysis indicating that a four electron reduction of $O_2$ to water is occurring (Gianfreda *et al*., 1999). As a one-electron substrate oxidation is coupled to the four-electron reduction of oxygen the reaction mechanism cannot be entirely straightforward. Laccase can be thought to operate as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen. Hence, the oxidation of four reducing substrate molecules is necessary for the complete reduction of molecular oxygen to water.

Substrate oxidation by laccase is a one-electron reaction generating a free radical. The degradation of lignin proceeds by phenoxy radical that leads to either oxidation at Cα-carbon or cleavage of bond between Cα-carbon and Cβ-carbon. This oxidation results in an oxygen-centered free radical, which can then be converted in a second enzyme-catalysed reaction to quinone. The quinone and the free radicals can then undergo polymerization (Thurston, 1994). The presence of electron withdrawing substituents at phenoxy groups and bulky groups are more difficult to be oxidised. Laccase catalysed oxidation of phenols, anilines and benzene correlates with the redox potential difference between laccase's T1 copper site and the substrate (Xu, 1996).

Laccases has been found to oxidise nonphenolic compounds and lignin in the presence of mediators -2,2'-azinobis-(3-ethylbenzthiazoline-6- sulfonate) (ABTS), 1- hydroxybenzotrizole (HBT) and 3 hydroxyanthranilic acid (Bourbonnais *et al*., 1995). As oxygen uptake by laccase in presence of ABTS is faster than in HBT, widening of the substrate range of laccase to non-phenolic subunits of lignin by the inclusion of a mediator such as ABTS is shown in Fig. 2a and b. ABTS-mediated oxidation of nonphenolic substrates proceeds via electron transfer mechanism through formation of ABTS++. Further investigation is warranted on the precise role of small molecule mediators in the catalytic mechanism of laccase.

**Laccase mediator system:** With respect to other ligninolytic enzymes, laccase can oxidize only phenolic fragments of lignin due to the random polymer nature of lignin and to the laccase lower

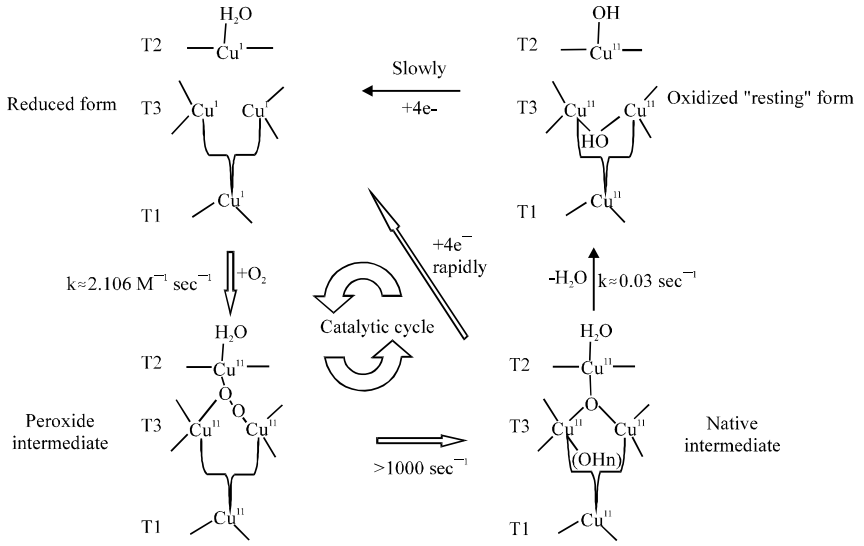


Fig. 1: Catalytic cycle of a four-copper laccase (Osma, 2009)

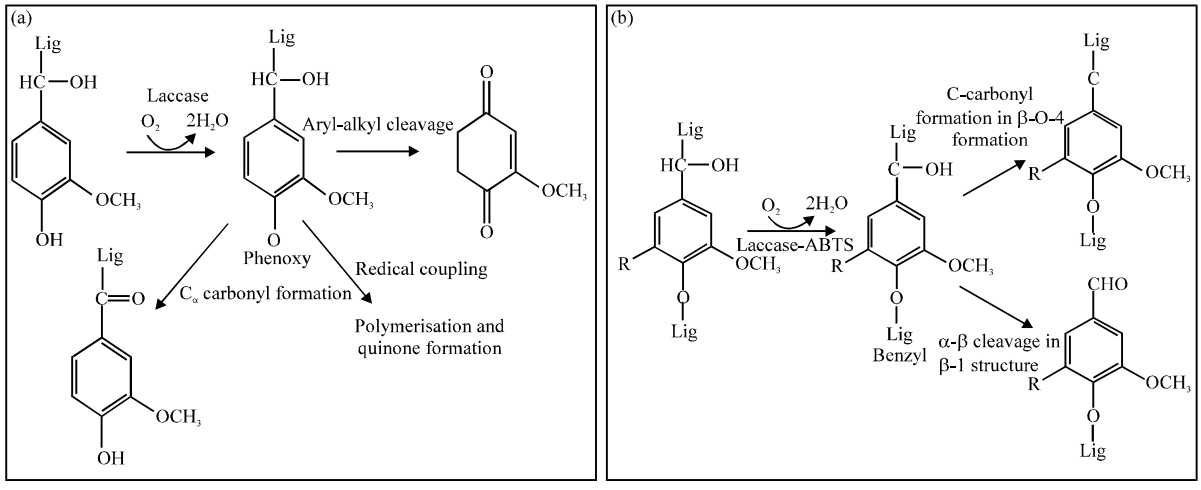


Fig. 2: Oxidation of (a) phenolic subunits of lignin by laccase and (b) non-phenolic lignin model compounds by a laccase mediator system

redox potential. Small natural low molecular weight compounds with high redox potential than laccase itself (>900 mV) called mediators may be used to oxidize the non-phenolic part of lignin. In the last years the discovery of new and efficient synthetic mediators extended the laccase catalysis towards xenobiotic substrates. A mediator is a small molecule that acts as a sort of electron shuttle: once it is oxidized by the enzyme generating a strongly oxidizing intermediate, the co-mediator (oxidized mediator), it diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size could not directly enter into the active site. Furthermore, the use mediators allows the oxidation of polymers by side-stepping the inherent steric hindrance problems (enzyme and polymer do not have to interact in a direct manner) (Fig. 3).

Alternatively, the oxidized mediator could rely on an oxidation mechanism not available to the enzyme, thereby extending the range of substrates accessible to it. It is therefore of primary importance to understand the nature of the reaction mechanism operating in the oxidation of a substrate by the oxidized mediator species derived from the corresponding mediator investigated. In the laccase-dependent oxidation of non-phenolic substrates, previous evidence suggests an Electron-Transfer (ET) mechanism with mediator ABTS, towards substrates having a low oxidation potential. Alternatively, a radical hydrogen atom transfer (HAT) route may operate with N-OH type mediators, if weak C-H bonds are present in the substrate.

More than 100 mediator compounds have been described but the most commonly used are the ABTS and the triazole 1-hydroxybenzotriazole (HBT). Various laccases readily oxidize ABTS, by free radicals, to the cation radical ABTS+ and the concentration of the intensely colored, green-blue cation radical can be correlated to the enzyme activity (ε418= 36000 $M^{-1}$ $cm^{-1}$). It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines and, upon extended oxidation and abstraction of the second electron, the corresponding dications can be obtained. The redox potentials of ABTS+• and ABTS2+ were evaluated as 0.680 and 1.09 V respectively. HBT belongs to the *N*-heterocyclic compounds bearing *N*-OH groups mediators. Consuming oxygen HBT is converted by the enzyme into the active intermediate, which is oxidized to a reactive radical (R-NO.) and HBT redox potential has been estimated as 1.1-1.2 V. Mediated laccase catalysis has been used in a wide range of applications, such as pulp delignification, textile dye bleaching, polycyclic aromatic hydrocarbon degradation, pesticide or insecticide degradation and organic synthesis. In pulp and paper industry, novel enzymatic bleaching technologies are attracting increasing attention because of concerns regarding the environmental impact of the chlorine-based oxidants currently being used in delignification or bleaching. However, synthetic mediators are toxic, expensive and generally at concentrations above 1 mM inactivate the laccase. Novel approaches to overcome this hurdles are coming up (from searching for natural mediators such as *p*-coumaric acid, 4-hidroxybenzoic acid, syringaldehyde etc.) to the directed evolution of laccases, as referred by Kunamneni *et al.* (2007).

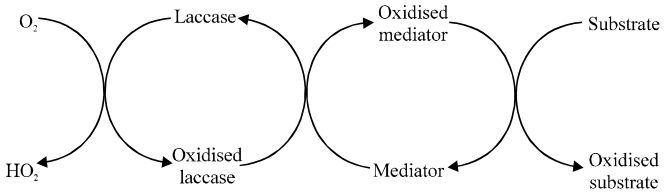


Fig. 3: Catalytic cycle of a laccase-mediator oxidation system

**General properties of laccase enzyme:** Most fungal laccases are monomeric, dimeric or tetrameric glycoproteins. Glycosylation of fungal laccase is believed to play a role in secretion, susceptibility to proteolytic degradation, copper retention and thermal stability. Upon purification, laccase enzymes demonstrate considerable heterogeneity, glycosylation content and composition of fungal glycoproteins can vary with growth medium composition.

Biochemical characteristics of purified laccases such as molecular weight, optimum pH and temperature, kinetic constants, substrate specificity and effect of inducers, inhibitors and metal ions have been reported in many research works.

**Purification of laccase:** Ammonium sulphate fractionation is being commonly employed from years to partially purify laccases from the crude filtrate of their sources. However researches reported thus far have also been experimented with much efficient methodologies such as membrane filtration techniques, various chromatographic techniques and have resulted in many commercially available purified forms of enzymes.

A single-step purification procedure for *Neurospora crassa* laccase is reported by Judewicz *et al.* (1998) using celite chromatography and obtained a specific activity of 333 U $mg^{-1}$ with 54% fold purification. Kiiskinen *et al.* (2004a, b) purified laccase from LLP13 and AH2 strains using DEAE Sepharose Fast Flow column, Phenyl Sepharose Fast Flow column. AH2 laccase was further purified with gel filtration on a Sephacryl S-100 HR column. Han *et al.* (2005) purified laccase from *Trametes versicolor*, using ethanol precipitation, DEAE-Sepharose, Phenyl-Sepharose and Sephadex G-100 chromatography. *T. versicolor* 951022 excretes a single monomeric laccase showing a high specific activity of 91,443 U $mg^{-1}$ for 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate.

Cordi *et al.* (2007), purified laccase from *Termetes vesicular* by applying the crude extract to ion exchange DEAE Sephadex A-50 followed by gel filtration on Sephacryl S- 200-HR column in a FPLC System and obtained the specific activity of 101 U $mL^{-1}$ with 34.8 fold purification. Minussi *et al.* (2007), purified laccase from *T. versicolor* (CCT 4521) using ammonium sulphate precipitation followed by Sephacryl S-200 (Sigma) column and DEAE cellulose. They obtained a purification of 41.3 folds with 43.3 units/mg specific activity. Khammuang and Rakrudee (2009) purified laccase from fruiting bodies of *Ganoderma* sp. MK05 by ammonium sulphate precipitation with 40-70% saturation and DEAE cellulose chromatography and obtained 1.34 and 3.07 fold purification, respectively. Viswanath *et al.* (2008b), purified extracellular laccase from *Stereum ostrea* and obtained up to 70-fold purification from the culture filtrate by a two-step protocol- ammonium sulphate (80% w/v) and Sephadex G-100 column chromatography. Yang *et al.* (2009) purified laccase from *Termetes* sp. using DEAE Cellulose and Superdex-75.

**Molecular weight:** The molecular weight of laccase is predicted to be in the range of 50-97 kDa from the experimental reports. An important feature is that a covalently-linked carbohydrate moiety (10-45% of total molecular mass),which may contribute to the high stability of the enzyme.

Judewicz *et al.* (1998) reported laccase of molecular weight 64.8 kDa. Lac IId (isozyme of laccase from *Cerrena unicolor*) purified by D'Souza-Ticlo *et al.* (2009) showed a molecular weight of 59 kDa and pI of 5.3 when analyzed by 2-D PAGE. Han *et al.* (2005) purified laccase enzyme from *Trametes versicolor*, with a molecular mass of approximately 97 kDa as determined through SDS-PAGE, larger than those of other laccases reported thus far. The molecular mass of

purified laccases from *Termetes* was found to be approximately 66 kDa, as reported by Cordi *et al.* (2007) through calibrated gel filtration and SDS-PAGE.

**pH and temperature:** The optimum pH value for laccases varies depending on the substrates employed, even though many reports have been reported a bell shaped profile for laccase activity. pH optima varies considerably due to reactions caused by substrate utilized, molecular oxygen or enzyme it self. The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH-_ binding to the T2/T3 centres. These two opposing effect can play an important role in determining the optimal pH of the biphasic laccase enzyme (Kunamneni *et al.*, 2007). The optimal temperature of laccase can differ greatly from one strain to another.

Cordi *et al.* (2007) examined the effect of pH on activity and found to be in the range 3.0-8.0, using syringaldazine as substrate. L1 (isozyme of laccase) showed to have pH in acidic range, with an optima of pH 4.0, whereas the optimum pH for L2 was 5.0. Laccase enzyme extracted from *Trametes versicolor* by Han *et al.* (2005) exhibited high enzyme activity over broad pH and temperature ranges with optimum activity at pH 3.0 and a temperature of 50°C. Laccase purified from *Stereum ostrea* found to be active and stable at optimal pH 6.0 and temperature 40°C (Valeriano *et al.*, 2009).

Stability experiments by Cordi *et al.* (2007) showed that L1 (isoenzyme of laccase) was stable at 60°C retaining 100% activity after 20 min incubation while the amount of residual activity at 70°C amounted to 47%. On the other hand, the L2 isoenzyme was less stable retaining only 28.1% initial activity upon 20 min incubation at 60°C. Two of the other isoenzymes exhibited an optimum temperature at 40°C and relative activities at 60 and 70°C were 65.0 and 37.0%, respectively.

Three laccases, Lac I, Lac II and Lac III enzyme extracted from mangrove (*Cerrena unicolor*) by D'Souza-Ticlo *et al.* (2009) of differing molecular masses were resolved by anion exchange chromatography. The optimum pH and temperature for Lac IId were 3 and 70°C respectively, the half-life at 70°C being 90 min. The enzyme was most stable at pH 9 and retained >60% of its activity up to 180 min at 50 and 60°C.

**Substrate specificity and kinetic constants:** Laccases can act on wide range of substrates. These enzymes catalyze one electron oxidation of a wide variety of organic and inorganic substrate, including poly phenols, methoxy-substituted phenols, aromatic amines and ascorbate with the concomitant four electron reduction of oxygen to water (Kunamneni *et al.*, 2007).

Li *et al.* (2008) compared three substrates: 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), O-dianisidine and guaiacol to assay the laccase activity from *Trametes* sp and obtained higher activity ABTS compared to others. Tzialla *et al.* (2009) used various ternary systems consisting of monoterpenes (a-pinene or D-limonene), tert-butanol and water as reaction media to enhance the catalytic performance of laccases from various fungi sources (*Trametes versicolor, T. hirsuta* and *Botrytis cinerea*).

The enzyme kinetic constants, Km and Vmax, vary from source to source, type of the substrate utilized and also other parameters used in experiment. Km value are in the range of 2-5000 μM. The Km values are different for laccases from different source organism having different substrate preference.

The Km value of the enzyme from *Trametes versicolor* by Han *et al.* (2005) for substrate ABTS is 12.8 M and its corresponding Vmax value is 8125.4 U $mg^{-1}$. Valeriano *et al.* (2009) reported that

for laccase from *Stereum ostrea*, Km and Vmax values for the substrate guaiacol were found to be 13.25 mM and 255 ηkat $mg^{-1}$ of protein, respectively. In another report by D'Souza-Ticlo *et al.* (2009), for the isozyme Lac IId (isozyme of laccase form *C. unicolor*), specificity constant (Kcat/Km) of 120 $min^{-1}$ $\mu M^{-1}$ was observed with ABTS at 70°C and pH 3. Galai *et al.* (2009), isolated laccase from bacterium *Stenotrophomonas maltophilia* AAP56. The enzyme showed Km=53 μM using syringaldazine, Km=700 μM using 2,2'-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) and Km=25 μM using pyrocatechol. The Lineweaver-Burk plot yielded a *Km* value of 28.6 μM for L1 and 5 μM for L2 using syringaldazine as substrate, by enzyme purified from *Termetes vesicular* (Minussi *et al.*, 2007).

**Effect of inhibitors on enzyme activity:** Laccase respond to inhibitors. They may inhibit enzyme activity either by binding to Type 2 or 3 copper, resulting in the interruption of internal electron transfer (metal ions such as azide halides, cyanides), amino acid modification, conformational changes of Cu chelation (metal ions, fatty acids, kojic acids), selective removal of Cu by chelating agents (EDTA,dimethyl glyoxime).

Among the various inhibitors tested by D'Souza-Ticlo *et al.* (2009) for Lac IId, isozyme (isozyme of laccase fom unicolor), activity was inhibited in the presence of sodium azide, SDS and mercaptoethanol. Approximately 56 and 48% of Lac IId activity was inhibited in the presence of Cr and W, whereas in the presence of Sn, Ag and Hg the inhibition was only 32-37%. The other metal ions did not show significant inhibition. Dube *et al.* (2008) observed that ethylene diamine tetraacetic acid (EDTA) (5 mM) totally inhibits laccase activity. EDTA, SDS and arginine have prominent inhibitory effect on laccase activity (Valeriano *et al.*, 2009).

**Isozymes of laccase:** Many laccase producing fungi secrete isoforms of the same enzyme. These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme. The number and of isoforms vary with species and also within species. The biochemical characteristics of isoenzymes vary depending upon the source and culture conditions.

Palmieri *et al.* (1997) observed two laccase isoenzymes (POXA1 and POXA2) produced by *Pleurotus ostreatus* with molecular weight of 61 and 67 kDa, pI of 6.7 and 4, respectively. Four laccase isozymes (LCC1, LCC2, LCC3 and LCC4) synthesized by *Pleurotus ostreatus* strain V-184 were purified and characterized (Mansur *et al.*, 2003). LCC1 and LCC2 have molecular masses of about 60 and 65 kDa and exhibited the same pI value (3.0). Laccases LCC3 and LCC4 were characterized by SDS-PAGE, estimating their molecular masses around 80 and 82 kDa, pI 4.7 and 4.5, respectively. When staining with ABTS and guaiacol in native polyacrylamide gels, different specificities were observed for LCC1/LCC2 and LCC3/LCC4 isozymes. Cordi *et al.* (2007) extracted two isoenzyme forms of laccase from *Trametes* sp. Three laccase isoenzymes Lac I, Lac II and Lac III from *C. unicolor* had significantly varying biochemical characteristics (D'Souza-Ticlo *et al.*, 2009).

**Immobilization:** Immobilized form enzymes are more robust and more resistant to environmental changes allowing easy recovery and multiple reuse of the enzyme. Thus researches have been directed towards a economic and efficient method of immobilization since they influence the properties of the biocatalyst. Laccase immobilization has been studied with a wide range of different immobilization methods and substrates.

Peralta-Zamora *et al.* (2003), immobilized laccase produced by *Trametes versicolor* on silica chemically modified with imidazol groups, amberlite IRA-400, glass-ceramic chemically modified with carbodiimide/ glutaraldehyde and by aminoprolyltriethoxysilane/ glutaraldehyde and montmorillonite modified by aminoprolyltriethoxysilane/glutaraldehyde. These supports were used in the decolorization of textile reactive dyes.

Prasad *et al.* (2006) observed that enhance laccase expression after immobilized PUF cubes (3109) were used compared to free mycelia (2538 U). This laccase had a molecular weight of 63 KD. Minussi *et al.* (2007), immobilized laccase on different vitroceramics supports, pyrolytic graphite and also on a carbon fiber electrode as biosensor. The maximum laccase activity was 40,774.0 U $L^{-1}$ at the 12th day. The best support for immobilization was pyrolytic graphite (glutaraldehyde treated-94% efficiency).

Alimin Abdul and Annuar (2009), fabricated an optical biosensor by using stacked films where 3-methyl-2-benzothiazolinone hydrazone (MBTH) was immobilized in a hybrid nafion/sol-gel silicate film and laccase in a chitosan film for the detection of phenolic compounds to detect catechol.

**Effect of inducers on enzyme production:** Laccase production has been found to be depended on the condition for fungal cultivation, media supporting. Lignolytic systems of white rot fungi were mainly activated during the secondary metabolic phase and were triggered by nitrogen concentration or when carbon or sulfur became limiting. Laccase are produced in low concentrations by fungi but higher concentrations can be obtained with the addition of supplements to media like veratyl alcohol, lignin, 2,3xylidine.

Galai *et al.* (2009) observed enhanced laccase activity when Triton-X-100 (0.1% v/v) was used in reaction mixture. The enzymes had improved catalytic efficiency (5 to 10 fold) in a-pinene-rich environment, while optimal reaction rates were in high-water content systems (15.5% v/v). Laccase activity was increased 2.6-fold by the addition of copper sulfate (10 mM).

Among various inducers used like gallic acid (1 mM), catechol (1 mM), ammonium tartrate (55 μM), hydroxybenzoic acid (1 mM) and vanillin (1 mM), it was observed that only ammonium tartrate increased the enzymatic activity reaching to 251 U $mL^{-1}$ of extract after 30 days in case of laccase from *Lentinula edodes* (Cavallazzi *et al.*, 2005). Valeriano *et al.* (2009) obtained high activity when inducers 2,5-xylidine or ethanol were used in specific concentration in case of laccase from *Pycnoporus sanguineus*.

**Statistical experimental designs:** Bar (2001) carried out a factorial experiment with a completely randomised design to optimize laccase production by *C. micaceus*. Three factors (carbon source, inducer and pH for assay) were replicated three times. Data were subjected to analysis of variance and means tested for significant differences with Tukey's test (Winer, 1971) as referred Marielle Bar. Molasses (4%) and malt extract (20 g $L^{-1}$) were evaluated for laccase production. Two inducers were evaluated: 500 mg $L^{-1}$ CuSO4 and 5 mg $L^{-1}$ p-hydroxybenzohydrazine. Assays for laccase activity were conducted at three different pH values (pH 4, pH 6 and pH 7) with 2, 6-dimethoxyphenol as substrate. Very low activity of laccase was observed in medium containing molasses alone. But higher activities were resulted when medium is supplemented with inducers.

Mishra *et al.* (2008) optimised the laccase production media for *C. versicolor* MTCC 138 using a Box-Benhken statistical experimental design method. Parameters such as medium pH, temperature, moisture content, inducers, ground nut shell and cyanobacterial mass were considered

for optimization. Highly influencing parameters were determined through standard Placket-Burman method and empirical model developed through Response Surface Method depicted the optimum concentrations of those factors.

Use of RSM to optimize decolorization of reactive blue 19 (RB19) dye using *Ganoderma* sp. was studied by Ohammadian-Azli *et al.* (2010). This statistical approach enabled to improve reactive blue 19 decolorization process by *Ganoderma* sp. up to 1.27 times higher than non-optimized conditions.

Gao *et al.* (2009) studied use of response surface methodology to optimize the composition of medium for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A in shaker flask cultivation.

Response Surface Methodology (RSM) was applied to optimise the decolouration of the diazo dye Reactive Black 5 (RB5) by crude laccase from the white-rot fungus *Trametes pubescens* (Roriz *et al.*, 2009).

Some of the researches have also been optimized the medium components based on statistical design methods. Placket-Burman method which allows the screening of critical components of the medium was employed in the study using *C. unicolor*. The method allows assessment of 'n' factors with n+1 + 2 experimental set up, besides effects of each component are pure in nature and are not confounded with interactions among other components for further optimization. Optimization of the concentration values of critical components being screened was further carried out with Central Composite Design (CCD). Glucose, ammonium chloride, copper sulphate, sodium chloride and Tween 80 were the five components screened and CCD was employed to optimize the concentration of carbon (glucose) and nitrogen (ammonium chloride) sources.

Laccase production by a white-rot fungus *Pycnoporus sanguineus* and its growth in a bubble column reactor were studied as a function of different inducers and superficial gas velocities. (Alimin Abdul and Annuar, 2009).

**Fermentation techniques:** Fermentation techniques can be divided into two main groups: Solid-State Fermentation (SSF) and submerged fermentation (SmF). The difference between these two techniques consists in the quantity of free flowing liquid present in the system. SSF involves the growth of microorganisms on solid materials in the absence or near-absence of free flowing water, whereas in SmF the microorganisms grow on a continuous liquid phase. There are many contributions to the field of laccase production under SmF using different microorganisms, at different scales and with the possible use of immobilization supports and the addition of inducers. Some of the most remarkable results in terms of laccase activity were obtained by the *Trametes* genus: *T. pubescens* (Galhaup *et al.*, 2002), *T. versicolor* (Font *et al.*, 2003; Tavares *et al.*, 2005) and *T. hirsuta* (Roriz *et al.*, 2009).

**Submerged fermentation:** The efficiency of the laccase producing organisms can only be exploited with the aid of media designed with optimum concentrations of every component which influences the production. Research conducted in this regard mainly aimed at picking up proper carbon sources, nitrogen sources and inducers (Table 2).

**Effect of carbon source:** The carbon source in the medium plays an important role in ligninolytic enzyme production. Jhadav *et al.* (2009) carried out optimization of laccase production medium using *P. chrysosporium* as source of laccase. Laccase production and activity was measured using

Table 2: Laccase sources, various media used for production, inducers and substrate utilized for determining the activity of laccase in submerged fermentation

| Organism | Media | Inducers | Substrates | Activity definition | References |
|---|---|---|---|---|---|
| *T. versicolor*<br>*T. pocas*<br>*T. cingulata*<br>*L. velutinus*<br>*P. sanguineus*<br>*D. concentrica*<br>*C. mollis*<br>*T.elegans* | Bonnarme (1990) | Cycloheximide | Guaiacol | - | Tekere *et al.* (2001) |
| *M. parvum* | C-limited medium | Anthracene Ethanol<br>Humic acid | 2,6 dimethoxyphenol | Amount of enzyme releasing 1picomol product per second | Saparrat *et al.* (2007) |
| *T. versicolor* | CPDA medium | | Syringaldazine | - | Udayasoorian and Prabu (2005) |
| *Pestalotigsis* sp.<br>*Diaporthe* sp.<br>*C. byrsiana*<br>*C. unicolor* | B and K Broth | | ABTS | Amount of enzyme required to oxidize 1micromol substrate per min per liter of culture broth | Verma *et al.* (2010) |
| *Terrens*<br>*T. ressei*<br>*P. chrysosporium*<br>*Polyporus* sp.<br>*Armillaria* sp. | Avicel liquid medium | | Catechol | Change in absorbance at 440 nm in 1 mL reaction mixture per min | Safari-Sinegani *et al.* (2006) |
| *Tramates* sp.<br><br><br><br>*Pleurotus* sp. | Krik's basal salt medium | | ABTS | Amount of enzyme producing 1 milimol product per min per mL | Li *et al.* (2008) |
| *Tramates* sp. | | | Guaiacol | Amount of enzyme causing 0.1 unit increase in OD of reaction mixture per minute | Unal and Kolankaya (2001) |
| *T. hirsute*<br>*T. versicolor*<br>*B. cinerea* | | 2,5-Xylidine<br>Guaiacol | ABTS<br>Syringaldizine | Amount of enzyme required to oxidize 1milimol substrate per min | Tzialla *et al.* (2009) |
| *T. versicolor* | VH Medium | 2,5-Xylidine | Syringaldizine | Amount of enzyme required to oxidize 1micromol substrate per mi per liter culture supernatant | Cordi *et al.* (2007) |
| *F. troggi* | Basidiomycete rich medium | Copper sulfate<br>Veratryl alcohol | ABTS | Amount of enzyme oxidizing 1micromol substrate per min | Ciullini *et al.* (2008) |
| *Basidiomycete* sp. | B and K Media | p-anisidine | ABTS | Units $L^{-1}$ of culture broth | Bajpai *et al.* (2006) |
| T. versicolor | TD Media | Copper Xylidine<br>Phenolic mixture | ABTS | Amount of enzyme required to oxidize 1microM substrate per min | Tavares *et al.* (2005) |
| *S. ostera*<br>*P. chrysosporium* | Olga (1998)<br>Munoz (1997)<br>Coll (1993)<br>PDB<br>Slomczyski (1995) | | Guaiacol | Amount of enzyme causing 1 mole substrate conversion per second | Viswanath *et al.* (2008b) |
| *H. cylindrosporum* | Melin's medium | Apple scraping<br>Pine nudles<br>CuSo4 | ABTS | Amount of enzyme required to oxidize 1 milimol substrate per min at 25°C | Ramesh *et al.* (2008) |

Table 2: Continued

| Organism | Media | Inducers | Substrates | Activity definition | References |
|---|---|---|---|---|---|
| *C. unicolor* | T and K medium | CuSo4 | ABTS | Amount of enzyme releasing 1 micromol product per min per liter of culture supernatant | D'Souza-Ticlo *et al.* (2009) |
| *T. versicolor* *A. niger* | PDA medium | | ABTS | Amount of enzyme oxidizing 1 micromol substrate per min at 25°C | Bajpai *et al.* (2006) |
| *P. sanguineus* | Malt extract medium | 2,5-Xylidine Ethanol | ABTS | Amount of enzyme oxidizing 1 microM substrate under standard conditions | Valeriano *et al.* (2009) |
| *T. harzianum* | | CuSo4 | ABTS | Quantity of enzyme oxidizing 1 micromol substrate per min | Sadhasivam *et al.* (2008) |
| *T. versicolor* | TDM and TaK medium | Lignin Lignosulphonates Veratryl alcohol | ABTS | Amount of enzyme causing 1 microM substrate per min | Xavier *et al.* (2007) |
| *L. edodes* | | Catechol Ammonium tartarate Gallic acid Vanillin | ABTS | Amount of enzyme oxidizing 1 milimol substrate per min | Gomez *et al.* (2005) |
| *Phanerochaete chrysosporium* | PDA media | ---- | Guaiacol | Amount of enzyme catalyzing the production of one micromole of colored product per min per ml | Jhadav *et al.* (2009) |

media containing different carbon sources. Laccase production time was standardized using composite medium containing glucose and guaiacol. Standard time for production of laccase was found to be on the 10th day. The activity was found be high in medium containing rice and maize bran than glucose as carbon source. But the activity reported in medium containing the glucose and an inducer guaiacol was very high activity. This study concluded that use of inducers in combination with alternative carbon sources like rice and maize bran would further increase the production of enzyme. D'Souza-Ticlo *et al.* (2006) obtained maximum activity of laccase when fructose was used as carbon source.

**Effect of nitrogen source:** Lignolytic systems of white-rot fungi are activated during the secondary metabolic phase of fungi and are triggered by nitrogen depletion. D'Souza-Ticlo *et al.* (2006) comprehensively analyzed the effect of type of nitrogen source, which influences the laccase production significantly. They reported the effect of $KNO_3$, glutamic acid, glycine, corn steep liquor and beef extract under stationary conditions, oxygenating the cultures every third day with pure oxygen for 1 min using tygon tubing and Pasture pipettes under sterile conditions. They obtained when glutamic acid was used as nitrogen source.

**Effect of inducers:** Laccase production with induction has been reported in the earliest days of research and yielded very good results. Tavares *et al.* (2005) established the significance of induction on *T. versicolor* culture for laccase production using $CuSO_4$ and 2, 5 xylidine as inducers.

This research was different from the previous ones in a way that it could able to develop a kinetic model depicting the precise quantity of inducers and also the sugar, which influences the higher yield of laccase in the culture. The study also emphasized upon the variation in the pH of the culture medium through out the cultivation period.

The experimental support for potential application of laccases on pulp and paper, textile industry and bioremediation was provided by the work of Xavier *et al.* (2007), which gave a detailed report on the potential of paper and pulp industrial effluents to induce production of laccase in *T. versicolor*. Different concentrations of inducers in two different media namely TDM (Tramatese Defined Medium) and TaK medium were studied along with suppression of carbon source. The study concluded that inducers alone, in combination with suppression of carbon source had different effect on laccase production.

**Solid state fermentation:** Studies on fungal enzyme production in SSF have shown that SSF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes with a higher temperature or pH stability. Also, the fermentation time is shorter and the degradation of the produced enzymes by undesirable proteases is minimized. Production of laccase from different fungal sources has been also employed through solid state fermentation (Table 3).

Safari-Sinegani *et al.* (2006) observed that fungal laccase activities in extracts of solid media were higher when compared with those in extracts of liquid media. This result may be related to (1) lower deactivation of enzymes after adsorption or immobilization on agricultural residues in solid media, and/or (2) more stimulation and production of enzymes in solid media. The addition of soil to the solid media increases laccase activity significantly. It is perhaps related to soil organic matter, especially aromatics, hydrocarbons, flavonoids and trace elements.

**Use of agro-industrial waste:** The lingocellulosic agro-industrial residues have great potential to act as substrate for the production high titres of laccase. Many of the researches have already

Table 3: Laccase production from different fungi sources grown on various natural supports under solid state conditions

| Support | Microorganism | Reference |
|---|---|---|
| Corn cob | *P. chrysosporium*<br>*P. radiate* | Cabaleiro *et al.* (2002) |
| Wheat bran, wheat straw | *P. pulmonarius* | De Souza *et al.* (2002) |
| Neem hull, wheat bran and<br>sugar cane bagasse | *P. ostreatus*<br>*P. chrysosporium* | Verma and Madamwar (2002) |
| Canola roots | *C. olla* | Shinners-Carnelle *et al.* (2002) |
| Eucalyptus grandis | *C. subvemispora* | Fenice *et al.* (2003) |
| Wheat straw | *F. sclerodermeus* | Papinutti *et al.* (2003) |
| Barley bran | *T. hirsute*<br>*T. versicolor* | Couto *et al.* (2003) |
| Corn cob | *P. pulmonaris* | Tychanowicz *et al.* (2004) |
| Chest nut, barely bran | *C. rigida* | Gomez *et al.* (2005) |
| Ground nut seeds and shells | *T. hirsuta* | Couto *et al.* (2006a, b) |
| Rubber wood saw dust Sago hampas, oil palm frond parenchyma tissue | *P. sanguineus* | Vikineswary *et al.* (2006) |
| Orange peelings | *T. hirsute* | Rosales *et al.* (2007) |
| Orange bagasse | *B. rhodiana* | Ellen *et al.* (2008) |
| Wheat straw | *L. edodes* | Kapoor *et al.* (2009) |

reported the efficiency of many such residues, work by Octavio *et al.* (2006) was one of the best among them. Sago hampas, oil palm frond parenchyma tissue (OPFPt) and rubber wood sawdust supplemented with nutrient solution and nitrogen in the form of urea were utilized as substrates with *Pycnoporus sanguineus* for laccase production. The study also emphasized the effect of inoculum age, density and nitrogen supplementation on laccase production, besides optimizing the efficiency of sodium-citrate buffer and tap water at different pH as enzyme extraction medium.

Ellen *et al.* (2008) exploited the potential of orange bagasse, major industrial food waste arising from processing orange for juice as solid support for laccase production from *B. rhodiana*. A good enzyme titre was seen in solid state fermentation without added nutrients, indicating nutrient sufficiency of orange bagasse at a solids concentration of 16% (w/v) to sustain growth and high enzyme titres.

Kapoor *et al.* (2009) reported the potential of wheat straw as a natural support for the production of laccase from *L. edodes*. The study has also reported the efficiency of several organic compounds such as rice bran, corn steep meal, peanut meal, soya meal and wheat bran as supplements to wheat straw for laccase production. Laccase source when cultivated on both supplemented wheat straw combinations and un-supplemented wheat straw (control), with 70% humidity, solid support with optimal concentration of supplement yielded a good growth and enzyme activity.

The study presented a new approach of utilising the agro-industrial waste as fermentation feed stock for the production of value-added enzymes.

**Heterologous production:** In most fungi, laccases are produced in the native hosts at levels that are too low for commercial purpose. Therefore, improving the productivity and reducing the cost of production are the major goals. Cloning of the laccase genes followed by heterologous expression may provide high enzyme yield (Table 4).

Ramesh *et al.* (2008) described cloning and sequencing of partial laccase gene from ectomycorrhizal fungus *H. cylindrosporum*. Deduced amino acid sequence of *H. cylindrosporum* showed similarity to copper binding region I and II domains at N-termini of different basidiomycetes, including *Hebeloma redicosum*, represented in the following Fig. 4.

Bulter *et al.* (2003), cloned and expressed functional fungal laccase from *M. thermophila* in *Saccharomyces cerevisiae* by directed evolution and obtained the highest yield of 18 mg $L^{-1}$, with 22 fold increase in Kcat and 170 fold enhance in total activity. A newly identified extracellular

Table 4: Laccase production in heterologous hosts

| Laccase gene | Host organism | Yield (mg $L^{-1}$) | References |
|---|---|---|---|
| *Ceriporiopsis subvermispora* lcs-1 | *Aspergillus nidulans* | 1.5 | Larrondo *et al.* (2003) |
| | *Aspergillus niger* | 1.5 | |
| *Myceliophthora thermophila* lcc1 | *Saccharomyces cerevisiae* | 18.0 | Bulter *et al.* (2003) |
| *Pleurotus sajor-caju* lac4 | *Pichia pastoris* | 4.9 | Soden *et al.* (2002) |
| *Pycnoporus cinnabarinus* lac1 | *Aspergillus niger* | 70.0 | Record *et al.* (2002) |
| *Pycnoporus cinnabarinus* lac1 | *Aspergillus oryzae* | 80.0 | Sigoillot *et al.* (2004) |
| *M. albomyces* | *Saccharomyces cerevisiae* | - | Camarero *et al.* (2004) |
| *Trametes versicolor* | *Yarrowia lipolytica* | 2.5 | Jolivalt *et al.* (2005) |
| *Trametes versicolor* lac1 and lac2 | *Pichia pastoris* | 2.8 | Bohlin *et al.* (2006) |
| *Trametes trogii* lac1 | *Pichia pastoris* | 17.0 | Colao *et al.* (2003) |
| *Cryphonectria parasitica* | *Saccharomyces cerevisiae* | - | Kim *et al.* (2010) |

Fig. 4: N-terminal amino acid sequence analysis of laccases of different sources

laccase produced by *Streptomyces ipomoea* CECT 3341 (SilA) was cloned and overexpressed and its physicochemical characteristics assessed (Molina-Guijarro *et al.*, 2009). The gene encoding *M. albomyces* laccase was expressed in *S. cerevisie* under the inducible GAL1 promoter. Pre-pro sequences of the *S. cerevisie* alpha factor gene and a stop codon in to the cDNA after the native C-terminal processing site found to enhance the production of laccase. Further the *M. albomyces* laccase was expressed as non fused and fusion protein in filamentous fungi *T. ressie*, five times higher activity was observed in non fused laccase than the fused one (Kiiskinen *et al.*, 2004a, b).

Kim *et al.* (2010) cloned a tannic acid inducible laccase3 of *Cryphonectria parasitica* in *Saccharomyces cerevisiae*. The transformants cultivation in a selective media containing tannic acid resulted in formation of brown colored precipitate around the colonies, confirming that lac3 gene responsible for laccase activity, however enzyme activity was not appreciable in non selective media as lac3 protein product found to be sensitive to be non selective media.

**Application of laccase:** Fungal laccases are of particular interest with regard to potential industrial applications, because of their capability to oxidize a wide range of toxic and environmentally problematic substrates. Oxidation reactions are comprehensively used in industrial processes, for instance in the textile, food, wood processing, pharmaceutical and chemical industries. Enzymatic oxidation is a potential substitute to chemical methods, since enzymes are very specific and efficient catalysts and are ecologically sustainable. Applications of laccase in various fields are detailed in Table 5.

**Bioremediation and biodegradation:** One of the major environmental problems, faced by the world today, is the contamination of soil, water and air by toxic chemicals. With industrialization and the extensive use of pesticides in agriculture, the pollution of the environment with organic compounds has become a serious problem. Certain hazardous compounds, such as polycyclic aromatic hydrocarbons (PAH), pentachlorophenols (PCP), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), benzene, toluene, ethylbenzene, xylene (BTEX) and trinitrotoluene (TNT) are persistent in the environment and are known to have carcinogenic and/or mutagenic effects. The ability of fungi to transform a wide variety of hazardous chemicals has arisen interest in using them in bioremediation (Alexander, 1994; Riva, 2006).

Bastos and Magan (2009) demonstrated that *T. versicolor* is a strong candidate for atrazine bioremediation in soil with low moisture and organic matter contents, such as that found in semi-arid and Mediterranean-like ecosystems. Keum and Li (2004) used laccases from *Trametes versicolor* and *Pleurotus ostreatus* to degrade hydroxy PCBs. Degradation rate constants decreased with increase of chlorination and no degradation was observed with tetra-, penta- and hexa-chloro

Table 5: Applications of laccase

| Application | Organism | Reference |
|---|---|---|
| Dye decolorization | *T. hirsuta* | Roriz *et al.* (2009) |
| | *T. hirsuta* | Couto and Toca Herrera (2006a, b) |
| | *T. versicolor* CCT 4521 | Minussi *et al.* (2007) |
| | *Stereum ostrea* | Viswanath *et al.* (2008b) |
| | *S. maltophilia* AAP56 | Dube *et al.* (2008) |
| | *Trametes sp.*strain SQ01 | Yang *et al.* (2009) |
| | *T. villosa* | Yamanaka *et al.* (2008) |
| | *T. trogii* | Ciullini *et al.* (2008) |
| | *Laetiporus sulphurous* and *Coriolus versicolor.* | Mazmanci *et al.* (2009) |
| Degradation of xenobiotics | *Stropharia rugosoannulata* | Steffen *et al.* (2007) |
| | *Stropharia coronilla* | Steffen *et al.* (2007) |
| | *Coriolopsis polyzona* | Cabana *et al.* (2007) |
| | *Rigidoporus lignosus* | Cambria *et al.* (2008) |
| Biodegradation and Bioredmediation | *T. versicolor* | Bastos and Magan (2009) |
| | *Cerrena unicolor* | D'Souza-Ticlo *et al.* (2009) |
| | *Streptomyces ipomoea* CECT 3341 | Molina-Guijarro *et al.* (2009) |
| | White-rot fungi (polyporus) | Zhao *et al.* (2010) |
| | *Ganoderma lucidum* Chaaim-001 BCU | Punnapayak *et al.* (2007) |
| Effluent treatment | *T. versicolor* | Pedroza *et al.* (2007) |
| | *Trametes versicolor* | Cordi *et al.* (2007a) |
| | *Lentinula edodes* | Cordi *et al.* (2007a) |
| | *Botrytis cinerea* | Cordi *et al.* (2007a) |
| | *Trametes trogii* | Ellouze *et al.* (2008) |
| | *Lentinus tigrinus* | Ellouze *et al.* (2008) |
| Biosensors | *Cerrena unicolor* | El Kaoutit *et al.* (2008) |
| | *Trametes hirsuta* | El Kaoutit *et al.* (2008) |
| | *Cerrena unicolor* | Karnicka *et al.* (2008) |
| | *T. versicolor* | Boussaad *et al.* (2008) |
| Biopulping | *Trametes versicolor, T. villosa* | Cordi *et al.* (2007) |
| | *Lentinula edodes* | Cordi *et al.* (2007) |
| | *Botrytis cinerea* | Cordi *et al.* (2007) |
| | *Trametes versicolor* | Oudia *et al.* (2008) |

hydroxy PCBs in non-mediated reactions. The results showed that 3-hydroxy biphenyl was more resistant to laccase degradation than 2- or 4-hydroxy analogues

A white rot fungi isolated by from paper mill, identified as *T. versicolor,* was capable of degrading phenol. $^{14}C$ synthetic lignin mineralization assay showed that it assimilated 24.3% of total label. During five days of incubation period, 71% of p-hydroxy benoic acid was utilized when glucose was used as a co-substrate and 56% degradation of protocatechoic acid using fructose. (Udayasoorian and Prabu, 2005).

Ahn *et al.* (2002) determined the potential of the laccase from *T. villosa* to remediate soil polluted with 2,4-DCP. (2,4-dichlorophenol). In Soil 1, both free and immobilized laccase removed 100% of 2,4-DCP without regard for moisture conditions. In Soil 2, immobilized laccase removed more 2,4-DCP (about 95%, regardless of moisture conditions) than free enzyme (55, 75 and 90% at 30, 55 and 100% of maximum water-holding capacity, respectively).

D'Souza-Ticlo *et al.* (2009) isolated fungi from decaying mangrove wood, identified as *Cerrena unicolor*. Partially purified laccase reduced lignin content from sugarcane bagasse pulp by 36% within 24 h at 30°C. Laccase was produced when grown in low nitrogen medium with half-strength seawater.

**Dye decolorization:** The textile industry accounts for two-thirds of the total dyestuff market and consumes large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in their composition, ranging from inorganic compounds to polymers and organic products. Due to their chemical structure dyes are resistant to fading on exposure to light, water and different chemicals and most of them are difficult to decolourize due to their synthetic origin. The development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Hou *et al.*, 2004) including synthetic dyes currently employed in the industry (Dominguez *et al.*, 2005). The use of laccase in the textile industry is growing very fast, since besides to decolorize textile effluents as commented above, laccase is used to bleach textiles and even to synthesize dyes.

Of four white rot fungi isolated by Minissui *et al.* (2001), *Lentinus edodes* displayed the greatest decolourization ability both in terms of extent and rapidity of decolourization. The dyes used were Reactive Blue 19 (0.05%), Reactive Red 195 (0.025%), Reactive Yellow 145 (0.05%) and Reactive Black 5 (0.05%).

Font *et al.* (2003), treated black liquors from a soda pulping mill with the white-rot fungus *Trametes versicolor* to detoxify and reduce colour, aromatic compounds and Chemical Oxygen Demand (COD). The fungus was used in the form of pellets in aerated reactors (fluidized, stirred and air-pulsed reactors). Reductions in colour and aromatic compounds of 70-80% and COD of 60% were achieved. During the different experiments, laccase activity was detected but neither lignin peroxidase (LiP) nor manganese peroxidase activities were detected, although *T. versicolor* is able to produce these enzymes.

Ramsay and Nugyen (2002) observed complete decolorization of Amaranth, Tropaeolin O, Reactive Blue 15, Congo Red and Reactive Black 5 with no dye sorption by *Trametes versicolor*. Cibacron Brilliant Red 3G-P, Cibacron Brilliant Yellow 3B-A and Remazol Brilliant Blue R were partially decolorized with some dye sorbed to the biomass. After decolorization, toxicity in case of few dyes remained unchanged where as for others it reduced to non-toxic levels and in others it was very toxic.

Unal and Kolankaya (2001) investigated the possibility of using crude laccase in the dechlorination of chlorine-based bleached kraft pulp. Culture supernatants of seven white-rot fungal strains and kraft-pulp samples from three stages of chlorine-based bleaching processes were used as the laccase source and substrates. The addition of a laccase inducer, xylidine, into the culture medium of *T. versicolor* led to an increase in dechlorination activity. There was also considerable reduction in the dissolved oxygen concentration due to laccase-dependent dechlorination activity was observed.

Yang *et al.* (2009) observed that the strain SQ01 was capable of decolorizing a variety of synthetic dyes, including azo, triphenylmethane and anthraquinone dyes. Of the three classes of dye, RBBR (anthraquinone ) dye was the best substrate for the enzyme. The optimal pH value for dye decolorization was determined to be pH 4.5, at which pH the decolorization yield exceeded 85% after incubation at 25, 8°C for 20 min. Of the five azo dyes tested, Orange G, FBRR and Amino

black 10B were good substrates for the laccase and were degraded to a similar extent in 12 h. Of the triphenylmethane dyes tested, Bromphenol Blue was the best substrate for the laccase, being completely degraded in 4 h. Acid Red was the worst substrate for laccase, as only 21% was degraded in 12 h. CBB G250 was also not a good substrate, with only 30% degraded in 4 h and little further decolorization thereafter for up to 20 h. Congo Red and Crystal Violet were transformed by 47% and 65%, respectively, but were never completely degraded even after extended incubation times. During incubation with the laccase in *Trametes* sp. SQ01, the maximum decolorization rates of Congo Red, Acid Red, Crystal Violet, Cresol Red and CBB G250 were observed in 12-16 h; thereafter, they were not further decolorized even if the time of incubation was prolonged.

Decolorization activity of some synthetic dyes (methylene blue, methyl green, toluidine blue, Congo red, methyl orange and pink) and the industrial effluent (SITEX Black) was achieved by the bacteria *S. maltophilia* AAP56 in the LB growth medium under shaking conditions (Dube *et al*., 2008).

D'Souza-Ticlo *et al.* (2006) also studied the effect of textile, alcohol and pulp industrial effluents on laccase production as well as their de-colorization by laccase producing fungi *in vivo*. The results of their research work concluded the effect of type of nitrogen source on laccase production and also the effect of interactions between the type of nitrogen source and different industrial effluents being used for the experiments.

**Paper and pulp industry:** In the industrial preparation of paper, the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine- or oxygen-based chemical oxidants. Although the LMS has been studied extensively, there are still unresolved problems concerning with mediator recycling, cost and toxicity. However, some environmental benefits are envisaged and the fact that LMS could be easily implemented in the existing bleaching sequences is seen as a major advantage that could possibly lead to a partial replacement of $ClO_2$ in pulp mills (Kunamneni *et al*., 2007).

**Other applications:** Laccases not only show potential for biological delignification of pulp but also for other applications. In food industry, laccase can be used in elimination of undesirable phenolics,responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine. Laccase-based hair dyes are less irritant and easier to handle than current hair dyes, since laccases replace $H_2O_2$ as an oxidizing agent in the dye formulation (Roriz *et al*., 2009).

Laccases can be used for detoxification of soils containing phenolic pollutants as well as other polluted systems due to the broad substrate range of the enzyme. Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills. Recently, increasing interest has arises on the application of laccase as a new biocatalyst in organic synthesis. Since laccases are able to catalyse electron transfer reactions without additional cofactors, their use has also been studied in biosensors to detect various phenolic compounds, oxygen or azides. The use of laccase as biosensor for the estimation of phenol or other enzymes in fruit juice, detection of morphine and codeine, catecholamines, plant flavonoids and also for electroimmunoassay have been developed. Recently, they have applications in other field such as in the design of biofuel cells (Kunamneni *et al*., 2007).

## CONCLUSION

This review summarizes the available recent and important reports about the mode of action, properties, production by fermentation, heterologous production and molecular cloning of fungal laccases and possible industrial and biotechnological use.

Laccases are blue copper proteins which catalyze oxidation reactions coupled to four electron reduction of molecular oxygen to water. Because of the versatility of potential substrates, laccases are highly interesting as novel biocatalysts in various industrial processes. One of the limitations to the large-scale application of the enzyme is the lack of capacity to produce large volumes of highly active enzyme. Thus, efforts have to be made in order to achieve cheap overproduction of laccase in heterologous hosts and also their modification by chemical means or protein engineering, to obtain more robust, active and less expensive enzymes. One should thus select a strain capable of producing high concentrations of a suitable enzyme and then optimise conditions for laccase production by the selected organism. Deeper understanding of the biochemistry of laccase will facilitate the development of novel and more economical laccase applications.

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