GENERAL INTRODUCTION

The search for efficient and green oxidation technologies has increased the interest in the use of enzymes to replace the conventional non-biological methods. Among the different existing oxidant enzymes, laccases (EC 1.10.3.2; para-benzenediol: oxygen: oxidoreductase) has been the subject of intensive research in the last decades due to their wide range of substrate specificity. The trouble-free purification processes, considerable level of stability in the extracellular environment, lack of inhibition and the inducible expression of laccases in most white rot fungal species makes this enzyme both suitable and attractive for biotechnological applications [Arora and Sharma, 2010; Murugesan et al., 2010; 2009b; Majeau et al., 2009; Couto et al., 2009; Sette et al., 2008; Couto and Toca-Herrera, 2006].

1. Laccase

Laccases are monomeric, dimeric or tetrameric glycoproteins with four copper atoms (belonging to three types: 1, 2 or 3) per monomer located at the catalytic site. Type 1 (T1) copper is responsible for the oxidation of the substrate and imparts the blue color to the enzyme. Laccases use molecular oxygen to oxidize a variety of aromatic and non-aromatic hydrogen donors via a mechanism involving radicals. These radicals can undergo further laccase-catalyzed reactions or nonenzymatic reactions such as polymerization, hydration or hydrogen abstraction. For phenolic substrates, oxidation by laccase results in formation of an aryloxy radical, an active species that is converted to a quinone in the second stage of the oxidation. Quinone intermediates can spontaneously react with each other to form soluble or insoluble colored oligomers, depending on substrate and environmental parameters [Walker, 1988].
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Laccase can decarboxylate phenolic and methoxyphenolic acids [Agematu et al., 1993], and also attacks methoxyl groups through demethylation [Chhabra et al., 2009; Gomare et al., 2009; Leonowicz et al., 2001]. Dehalogenation of substituents located in the ortho or para position may also occur in the case of substituted compounds [Schultz et al., 2001].

2. Nature of distribution

Laccase was first discovered by Yoshida [1883] in the sap of lacquer tree Rhus vernicifera and the enzyme has been characterized in great detail later by Huttermann et al., [2001]. However, the report of laccase in other plant species is more limited and partially characterized. These laccases are from Typhonium flagelliforme [Kagalkar et al., 2010], Zea mays [Caparrós-Ruiz et al., 2006], Lolium perenne [Gavnholt et al., 2002], Pinus taeda [Sato et al., 2001], Populus euramericana [Ranocha et al., 1999], Liriodendron tulipifera [LaFayette et al., 1999], Rhus succedanea [Solomon et al., 1996], Nicotiana tobacco [Kiefer-Meyer et al., 1996] and Acer pseudoplatanus [Sterjiades et al., 1992]. In plant, laccase participates in the formation of polymer lignin via radical-based mechanisms [Hoopes and Dean 2004; Ranocha et al., 2002].

A few years later after the discovery of the plant laccase by Yoshida [1883], fungal laccases were discovered by Bertrand [1986]. The majority of laccases characterized so far were isolated from white rot fungi, and the reports of their presence in more and more fungal species have been published [Baldrian, 2006; Couto and Toca-Herrera, 2006; Claus, 2004; Thurston, 1994]. So far, more than 100 white rot fungal laccases have been purified and characterized. The laccase producing fungal strains are Fusarium incarnatum [Chhaya and Gupte,
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2010], Streptomyces cyaneus [Moya et al., 2010], Ganoderma lucidum [Murugesan et al., 2009b], Streptomyces coelicolor [Skalova et al., 2009], Lentinus edodes [Nagai et al., 2009], Trametes pubescens [Enayatzamir et al., 2009], Pleurotus florida [Pant and Adholeya, 2009], Pleurotus eryngii, Pleurotus ferulae [Punelli et al., 2009], Fome sclerodermeus [Papinutti et al., 2008], Cerrena unicolor [Michniewicz et al., 2008], Pleurotus sapidus [Linke et al., 2007], Fome lignosus [Hu et al., 2007], Coprinopsis cinerea [Kilaru et al., 2006], Pleurotus sajor-caju [Murugesan et al., 2006], Pleurotus ostreatus [Palmieri et al., 2005], Trametes hirsuta [Pereira et al., 2005], Funalia trogii [Unyayar et al., 2005], Pycnoporus cinnabarinus [Camarero et al., 2004], Cantharellus cibarius [Ng and Wang, 2004], Pleurotus pulmonarius [De Souza and Peralta, 2003], Irpex lacteus [Kasinath et al., 2003], Trametes versicolor [Claus et al., 2002], Marasmius quercophilus [Tagger et al., 1998], Trametes villosa [Yaver and Golightly, 1996], Botrytis cinerea [Slomczynski et al., 1995] and Neurospora crassa [Huber and Lerch, 1987]. For a detailed review of fungal laccases kindly refer Arora and Sharma [2010].

The first report on bacterial laccase was from the rhizospheric bacterium Azospirillum lipoferum [Givaudan et al., 1993]. Laccase and laccase-like activity has been reported from some of the bacteria, these are Sphingobacterium sp. [Tamboli et al., 2010], Pseudomonas sp. [Telke et al., 2010], Brevibacillus laterosporus [Gomare et al., 2009], Agromyces salentinus and Sinorhizobium morelense [Kellner et al., 2008], Comamonas sp. [Jadhav et al., 2008], Ralstonia solanacearum [Hernandez-Romero et al., 2005], Bacillus subtilis [Driks, 2004], Streptomyces lavendulae [Suzuki et al., 2003], Streptomyces griseus [Endo et al., 2003], Sinorhizobium meliloti [Castro-Sowinski et al., 2002], Bacillus subtilis
[Hullo et al., 2001], *Marinomonas mediterranea* [Solano et al., 1997], *Pseudomonas syringae* [Mellano and Cooksey, 1988], *Bacillus sphaericus* [Claus and Filip, 1997] and *Escherichia coli* [Brown et al., 1995]. The laccase enzyme has also been detected in different insects, e.g., *Bombyx, Calliphora, Diploptera, Drosophila, Lucilia, Manduca, Musca, Oryctes, Papilio, Phormia, Rhodnius, Sarcophaga, Schistocerca* and *Tenebrio* [Arora and Sharma, 2010].

3. Physiological function

The physiological function of these biocatalysts, which can be secreted or intracellular, is different in various organisms but they all catalyze polymerization or depolymerization processes. It has been proposed that laccases are involved in cuticle sclerotization in insects and in the assembly of UV-resistant spores in *Bacillus* species.

In plants they are involved in cell wall formation and, together with peroxidases, in lignification. Laccases are among the main enzymes involved in delignification processes by white rot fungi [Mayer and Staples, 2002]. It plays a role in the morphogenesis and differentiation of sporulating and resting structures in basidiomycetes as well as lignin biodegradation of wood in white rot fungi [Soustrade and Escarmant, 1997].

Laccase is responsible for pigment formation in mycelia and fruiting bodies, improves cell-to-cell adhesion, assists in the formation of rhizomorphs and is also responsible for the formation of a polyphenolic glue that binds hyphae together [Thurston, 1994]. Additionally, these enzymes can protect fungal pathogens from toxic phytoalexins and tannins, thus they are an important virulence factor in many fungal diseases [Mayer and Staples, 2002].
4. Laccase structure

Laccases are glycoproteins which often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular weight of the monomer ranges from 50 to 130 kD. The carbohydrate moiety of laccases consists of mannose, N-acetylglucosamine and galactose ranging from 10 to 45% of the protein mass. This carbohydrate moiety is believed to be responsible for the stability of the enzyme [Morozova et al., 2007a; Claus, 2004]. For the catalytic activity, the active site of laccases contains four copper atoms which are one Type-1 (T1) copper and a three-nuclear cluster consisting of one Type-2 (T2) and two Type-3 (T3) coppers. T1 copper atom is located at the distance of about 12 Å from the T2/T3 site, and T2 copper atom is located at the distance of about 4 Å from T3 copper atoms [Garavaglia et al., 2004; Enguita et al., 2003; Palmer et al., 2001]. The T1 copper has a trigonal coordination with two histidine and one cysteine, and the axial ligand of T1 is methionine in the bacterial (CotA) [Enguita et al., 2003] and leucine or phenylalanine in fungal laccases. The T1 copper confers the typical blue color to multicopper proteins due to the strong absorption around 600 nm. This intense absorption caused by the covalent copper-cysteine bond. Moreover, T1 copper is the site where substrate oxidation takes place because of its high redox potential.

T2 copper is coordinated by two histidines and T3 coppers are coordinated by six histidines. T2 copper shows only weak absorption in the visible region and reveals paramagnetic properties in electron paramagnetic resonance (EPR) studies. While in T3 copper, a binuclear copper site with copper paired antiferromagnetically through a hydroxyl bridge, exhibit the absence of an EPR
signal. The T3 site can be characterized by electron absorption at 330 nm (oxidized form) [Quintanar et al., 2005; Solomon et al., 1996]. In addition, the trinuclear cluster (T2/T3 site) is where the reduction of molecular oxygen and release of water takes place. Scheme of laccase active site is illustrated in figure 1.

![Diagram of laccase active site](image)

**Figure 1. Active site of laccase [Enguita et al., 2003]**

The three-dimensional structure has been determined for fungal laccases from *Coprinus cinereus* [Zhukhlistova et al., 2008; Ducros et al., 1998], *Cerrena maxima* [Lyashenko et al., 2006], *Trametes versicolor* [Bertrand et al., 2002; Piontek et al., 2002], *Pycnoporus cinnabarinus* [Antorini et al., 2002], *Melanocarpus albomyces* [Hakulinen et al., 2002], *Rigidoporus lignosus* [Garavaglia et al., 2004] and endospores of *Bacillus subtilis* [Enguita et al., 2004]. A cartoon representation of the three-dimensional structure of the *Cerrena maxima* laccase is shown in figure 2.
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Figure 2. The overall structure of the laccase from *Cerrena maxima* at 1.9 Å resolution showing the three domains (coloured blue, orange and green for domains I, II and III, respectively) and the position of the copper centres (yellow spheres) [Lyashenko et al., 2006].

5. Catalytic mechanism and properties

Laccase catalysis is proposed to comprise three major steps [Bento et al., 2005; Solomon et al., 2001]:

1. Type-1 copper is reduced by accepting electrons from the reducing substrate.
2. Electrons are transferred ~13 Å from T1 copper to the trinuclear T2/T3 cluster.
3. Molecular oxygen is activated/reduced to water at the trinuclear T2/T3 cluster.

Figure 3 shows the catalytic cycle of laccase showing the mechanism of four electron reduction of a dioxygen molecule to water at the enzyme copper sites [Shleev et al., 2006].
Figure 3. Catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites [Shleev et al., 2006].

Dioxygen molecule interacts with the completely reduced trinuclear cluster (T2/T3) via a 2e\(^-\) process (k \(\approx 2 \times 106 \text{ M}^{-1}\text{s}^{-1}\)) to produce the peroxide intermediate which contains the dioxygen anion [Cole et al., 1991]. One oxygen atom of the dioxygen anion bound with the T2 and T3 copper ions and the other oxygen atom coordinated with another copper ion of T3. The peroxide intermediate undergoes a second 2e\(^-\) process (k > 305 s\(^{-1}\)) [Palmer et al., 2001], and the peroxide O-O bond
is split to produce a native intermediate which is a fully oxidized form with the three copper centers in the trinuclear site mutually bridged by the product of full O₂ reduction with at least one Cu-Cu distance of 3.3 Å. This native intermediate form of laccase was confirmed by the combination of Cu K-edge X-ray spectroscopy (XAS) and magnetic circular dichroism (MCD) studied by Solomon et al. [2001]. Moreover, a combination of model studies and calculations has further demonstrated that the three copper centers in the trinuclear cluster are all bridged by a μ₃-oxo ligand [Yoon et al., 2005]. This structure has a single μ₃-oxo ligand bridging all three coppers at the center of the cluster, with the second oxygen atom from O₂ either remaining bound or dissociated from the trinuclear site as shown in the native intermediate structure in figure 3.

Figure 4. Decay mechanism of the native intermediate to the resting laccase [Yoon et al., 2007]

The μ₃-oxo bridged structure of the native intermediate provides a relatively stable structure that serves as the thermodynamic driving force for the 4e⁻ process of O₂ reduction, and also provides efficient ET pathways from T1 site to all of the copper centers in the trinuclear cluster [Yoon et al., 2005]. This efficient ET pathways lead to the fast reduction of the fully oxidized trinuclear
cluster in the native intermediate to generate the fully reduced site in the reduce form for further turnover with O₂. The native intermediate can slowly convert to a completely oxidized form called “resting” laccase which has the T2 copper isolated from the couple-binuclear T3 centers. The decay of the native intermediate to the resting enzyme proceeds via successive proton-assisted steps as illustrated in figure 4 [Yoon et al., 2007].

The first proton binds at μ3-oxo center and then the second proton binds at T3 OH-bridge. Finally, the three copper centers in the trinuclear cluster are uncoupled to form the resting form of laccase. The slow decay of the native intermediate is due to the rearrangement of the μ3-oxo-bridge, the rate limiting step, from inside to outside of the cluster. The T1 site of this resting laccase can be reduced by a substrate. However, the electron-transfer rate onto the trinuclear cluster (T2/T3) is too low to be significant for catalysis [Lee et al., 2002].

6. Oxidation mechanism

The redox potential of the T1 copper site is directly responsible for the catalytic capacity of the enzyme. The mechanism of interaction between a laccase T1 site and its substrate seems to be identical among fungal laccases [Smirnov et al., 2001]. However, important differences between laccase active sites have been described by Hakulinen et al. [2002].

In its native state, the enzyme holds copper atoms in the monovalent state as Cu⁺. When dioxygen binds at the trinuclear cluster formed by T2 and T3 copper, the four copper atoms are oxidized (Cu²⁺) in two steps, while passing through a peroxide-level intermediate [Solomon et al., 2001]. Oxygen is then reduced to divalent oxygen, and subsequently becomes water.
Oxidation of substrate is always carried out by T1 copper. Electrons are transferred from the T1 site to the trinuclear cluster by a His-Cys-His tripeptide motif. Each substrate is oxidized by a successive one electron oxidation step. The capture of four electrons by the substrate returns the enzyme to its native state.

The stoichiometric ratio corresponding to the molar ratio of substrate/dioxygen transformation is generally 4/1, i.e., four electrons withdraw from four substrate molecules per one dioxygen reduced. If substrate molecules donate more than one electron, a lower ratio may be observed. Kurniawati and Nicell [2008] developed an equation to relate this ratio to phenol concentration.

![Diagram](image)

**Figure 5.** (a) Scheme of laccase-catalyzed redox cycles for substrate oxidation; (b) The example of the oxidation of hydroquinone by laccase [Mayer and Staples, 2002].

Laccase can catalyze the oxidation of a variety of compounds including ortho and para-diphenols, polyphenols, aminophenols, polyamines, lignins, aryldiamines, and a number of inorganic ions [Morozova et al., 2007a; Riva, 2006; Mayer and Staples, 2002]. Laccase can abstract an electron from substrates which
produces a free radical, and reduce oxygen to water. The simplified scheme of laccase-catalyzed redox cycles for substrate oxidation and the example of the oxidation of hydroquinone by laccase are illustrated in figure 5.

7. Characteristics of laccases

Laccases characterized so far belong mostly to the group of wood-rotting white-rot basidiomycetes [Baldrian, 2006]. Catalytic activity is measured with relatively susceptible laccase substrates, such as azinobis (3-ethylbenzathiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), (N-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)) syringaldazine or guaiacol. Laccases have a specific substrate, called syringaldazine [Thurston, 1994]. The oxidation of syringaldazine, in the absence of hydrogen peroxide, combined with an inability to oxidize tyrosine is an indicator of laccase activity. The reaction product can differ with pH, which affects the chain reaction initiated by the primary radical. ABTS is by far the most commonly used substrate, as it does not form quinines and is not pH dependent. For this reason, it is used to calculate International Units of laccase activity. The turnover number ($k_{\text{cat}}$) varies with each specific protein and substrate and can be diverse: laccase from Agaricus blazei oxidizes ABTS at only $k_{\text{cat}} = 21 \text{s}^{-1}$, whereas $k_{\text{cat}}$ for Pleurotus ostreatus laccase was reported as 350,000 s$^{-1}$ [Baldrian, 2006]. However, Terron et al. [2004] highlighted the fact that tannic acid or structurally related molecules in biological samples could interfere with laccase activity measurement with ABTS.

Laccase can also oxidize polyamines, aminophenol, lignins, aryl diamine, some inorganic ions, and may also diminish the toxicity of Triclosan [Murugesan et al., 2010], Polycyclic aromatic hydrocarbons (PAHs) [Pozdnyakova et al.,
2006a], organophosphorus pesticides and azo dyes [Torres et al., 2003]. Some xenobiotic compounds, such as PAHs and pentachlorophenol can be oxidized by some laccases, but with very low catalytic constants and high Michaelis–Menten constant ($K_m$).

Fungal laccases typically exhibit pH optima in the range from 3.5 to 5.0 when the substrates are hydrogen atom donor compounds, and the pH-dependence curve is bell shaped [Kurniawati and Nicell, 2008; Shleev et al., 2006; 2005; Baldrian, 2004]. The optimum pH for phenolic compounds can actually increase at higher pH to a limit. The limit for increasing the pH during substrate oxidation results from the balance between the redox potential difference between the substrate and the inhibition of the T2/T3 copper site by the binding of OH⁻ ion [Xu, 1997; 1996].

The optimal temperatures of laccases usually do not differ from other extracellular ligninolytic enzymes within the range of 50 to 70 °C [Baldrian, 2006]. Many laccases are relatively stable over time in extracellular fluid, but further improvement in stability of the enzyme may still be required for industrial applications. The half life at 50 °C of purified enzyme can range from minutes for the enzyme produced by Bacillus cinerea [Slomczynski et al., 1995] to 50–70 h for the enzyme from Trametes sp. [Smirnov et al., 2001]. Separation of fungal laccase by native PAGE, mono-Q chromatography and isoelectrofocusing has resolved several different isoenzymes per species. Sathishkumar et al. [2010] assessed the thermostability of Pleurotus florida laccase isoenzyme by zymogram analysis and reported that L1 isoenzyme was more stable than L2 isoenzyme. Besides the temperature and pH of the medium, other factors may also lower laccase activity.
The laccase reaction is usually performed in organic solvents as many toxic compounds of interest are hydrophobic. Although many laccases remain stable in organic media, denaturation also occurs, in addition to changes in the enzyme-substrate interaction [Torres et al., 2003]. Furthermore, the solvent may slow down the reaction and have an influence on intermediate formation [Couto and Toca-Herrera, 2006]. Inactivation of laccases by organic solvents is strain specific but, in general, higher concentrations of organic solvent (>30%) inhibit laccase activity [Al-Adhami et al., 2002]. Organic solvents can also restrict environmental use of laccase. Kurniawati and Nicell [2006] developed a kinetic model for phenol oxidation by laccase. They observed an inactivation mechanism that was directly proportional to substrate conversion due to the attack on the enzyme by free radicals.

A wide spectrum of compounds has been described to inhibit laccase. These inhibitors include small inorganic anions such as azide, cyanide, fluoride and hydroxide. These ions bind with the T2/T3 site and this prevents the electron transfer from T1 site onto T2/T3 site and inhibits the enzymatic activity [Gianfreda et al., 1999; Xu, 1996]. Other inhibitors such as metal ion (Hg^+), fatty acids, quaternary ammonium detergents, have been shown to either replace or chelate the copper centers, or denature the protein [Yaropolov et al., 1994]. Polymerization of phenol can be controlled by adjusting the appropriate hydrophobicity level of the medium [Mita et al., 2003]. In many cases, the reaction products of a simple organic substrate are numerous (Figure 6). One electron donor substrate gives dimeric products if steric overcrowding is low enough to permit that non-enzymatic reaction [Smirnov et al., 2001].
Some pollutants can be mineralized completely by fungi through an unknown mechanism. Results obtained by Ryan et al. [2007] suggest that phenol (guaiaicol) can be used as a carbon source by *Trametes versicolor* if cultures were at least 8 d old. This suggests that some by-products of laccase activity can serve as substrates for other enzymes, depending on the microbial species, reaction conditions, culture age, type of substrate used and substrate concentration. These findings underline the complexity of laccase activity in combination with other extracellular enzymes.

The most widespread methods used to measure activity in waste material involve testing laccase breakdown of common dyes, based on the assumption that decolorization of dyes and biodegradation of aromatic pollutants is highly correlated [Field et al., 1993]. Nevertheless, this correlation is based on studies carried out with Reactive dye 478 and anthracene, both of which can be oxidized by peroxidases and laccases. Laccase activity was not monitored in these experiments. Disappearance of color is a simple and rapid way to measure laccase activity, but the catalytic properties and the substrate range remain hypothetical. Decolorization tests on two or three structurally different dyes at appropriate pH values may give more informative results about the catalytic activity of laccase in an extracellular liquid [Couto and Sanroman, 2006]. Demonstration of a clear proportional relationship between a specific dye and laccase substrate oxidation by laccase still remains to be established. Even activity measurement, which can be taken as a good standard indicator of laccase catalytic capacity for substrate oxidation, is not yet standardized, leaving many questions unanswered.
**Figure 6. Example of laccase-catalysed oxidation.** (A) Catechol, a typical substrate; (B) Two possible major oxidation products from the herbicide Dymron; (C) Major by-products of bisphenol A (BPA) oxidation; (D) Example of oxidative decarboxylation; (E) Oxidative polymerization of phenol derivatives in aqueous organic solvents forms polymers of phenylene and oxyphenylene units [Couto and Sanroman, 2006].
8. Laccase mediators

The standard redox potential range for laccase activity is usually between 0.5–0.8V, i.e., not high enough for oxidation of several xenobiotic compounds. The discovery of “mediators” – small molecules that can extend the enzymatic reactivity of laccase towards several “uncommon” substrates – stimulated interest in laccases for detoxification and industrial purposes [Khlifi et al., 2010; Moya et al., 2010; Morozova et al., 2007b; Call and Mucke, 1997; Bourbonnais and Paice, 1990]. Mediators are easily oxidizable substrates that can act as redox intermediates between the active site of the enzyme and a non-phenolic substrate (Figure 7).

Figure 7. Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence (a) or presence (b) of chemical mediator [Bourbonnais and Paice, 1990].

The most commonly used mediators are ABTS and 1-hydroxybenzotriazole (HBT). The use of HBT is often reported in the literature as a reference mediator, but HBT has been shown to inactivate laccase over time, possesses high toxicity even at low concentrations, and is not as effective as other artificial mediators, for example, 1-nitroso-2-naphthol-2,6-disulfonic acid (NNDS) [Moldes and Sanromán, 2006]. An adequate mediator should have a high number of redox cycles, a stable oxidized form, a high oxidation power, and should not undergo
secondary polymerization. Mediators are progressively removed from the reaction medium undergoing polymerization, except in the case of 2,2',6,6'-tetramethylpiperidinyl-1-oxy (TEMPO) and its cognates, which are recycled by laccases [Astolfi et al., 2005].

Three different mechanisms of mediated oxidation can occur, depending on the chemical structure [Fabbrini et al., 2002]. They are:

(1) Electron transfer (ABTS),
(2) Transfer of radical hydrogen (N—OH group), and
(3) Ionic oxidation (TEMPO).

The electron transfer route is possible with low oxidation potential substrate, while N—OH group mediators attack weak C—H bonds. N-heterocycles containing a N—OH group are the most effective mediators for lignin degradation. One example of laccase mediator system (LMS) application is the Lignozym®-process consisting of Trametes versicolor laccase with HBT as mediator, which has been tested in pilot plant trials [Call and Mucke, 1997]. The LMS process was also introduced into the textile industry for indigo oxidation [Galante and Formantici, 2003]. Sustainable processes using such artificial mediators for detoxification are not feasible in practice due to the high cost of the common mediators described so far, the toxicity and potential toxicity of their by-products, and the fact that mediator recycling is impossible. Furthermore, polymerized mediators can form a dark precipitate leading to residual coloration of the wastewater [Ryan et al., 2007].

In addition, common mediators were shown to inactivate laccase even under mild conditions (pH 6 and 25 °C) [Kurniawati and Nicell, 2008]. Thus,
substitution with a natural, harmless and low cost mediator should be investigated. It is generally accepted that some compounds generated during natural degradation of lignin by white-rot fungi may act as mediators. Oxidized lignin units and molecules found in fungal metabolism were investigated. Camarero et al. [2005] screened 44 different natural laccase mediator compounds with a recalcitrant dye as substrate and demonstrated that acetylaminone and syringaldehyde were more efficient than synthetic mediators such as NNDS or HBT. Natural mediators could be obtained from syringyl-rich lignins, such as eucalyptus and non-woody plants by biological and enzymatic degradation [Kirk and Farrell, 1987].

Mediators from natural origins should not be considered automatically as risk-free. A cytotoxicity study with the mediator 4-hydroxybenzoic acid, a natural metabolite excreted by fungi, revealed high toxicity towards mouse fibroblasts [Maruyama et al., 2007]. Oxidized mediators possess specific affinity towards different functional groups of the targeted substrate, but do not act as substrates for different laccases.

Successful use of natural mediators was demonstrated using a high molar ratio of mediator/substrate, but excess mediator could generate unwanted by-products [Cantarella et al., 2003]. There is still no perfect mediator that can be applied to all reactions. Therefore, further screening experiments must be carried out to optimize oxidation efficiency of other recalcitrant pollutants.

LMS rely on the redox reversibility of the reaction of the radical with the substrate as well as on the balance between the stability and reactivity of the mediator radical, which, in addition, should not inhibit the enzyme activity [Camarero et al., 2005]. The optimal pH for oxidation by LMS is influenced
strongly by the stability of the oxidized mediator rather than by the stability of laccase itself [Fabbrini et al., 2002]. In the case of mediator N−OH, the oxidation rate is dependent on the redox potential difference between the mediator (should be low) and the laccase (should be high) [Fabbrini et al., 2002; Xu et al., 2000].

Addition of mediator usually enhances the oxidation efficiency of the pollutant, but is not required if the culture broth contains some lignocellulosic substrates. Phenolic molecules generated during the degradation of complex substrates may act as natural mediators as they can be oxidized easily [Evans et al., 1994]. Some fungi also produce metabolites that can mediate oxidation of nonphenolic molecules [Eggert et al., 1996].

9. Laccase production by fungus

Almost all fungi studied to date possess intracellular laccase; nevertheless, extracellular laccases are produced in higher amounts, which is a prerequisite for industrial purposes. The culture growth medium can be synthetic, natural or semi-synthetic, for example, solid lignocellulosic waste in artificial liquid medium.

9.1. Influence of parameters on laccase production

Various parameters like nutrients (carbon and nitrogen source), inducers and environmental parameters (pH, temperature, influence, oxygen and incubation period) highly influence the laccase production.

9.1.1. Carbon source

Carbon sources, such as glucose, mannitol and cellobiose, usually give higher laccase activity than substrates that are assimilated more slowly (cellulose and lactose) [Mikiashvili et al., 2006]. In some cases, an increase in laccase
activity is concomitant with an increase in growth, but certain studies have shown that the specific activity of laccase can be increased by the choice of carbon source. For example, Mansur et al. [1997] observed that the use of fructose instead of glucose resulted in a 100-fold increase in specific laccase activity. However, the total laccase activity profile was similar for fructose and glucose. Also, Couto et al. [2007] demonstrated that sequential addition of different carbon sources, such as fructose followed by glycerol, caused an increase in the laccase production rate in Trametes hirsuta relative to glucose alone or cellulose supplementation.

For many species, glucose is a typical repressor of laccase production. Inducers and optimal carbon sources are species- and strain specific. Two different strains of Pleurotus ostreatus [Mikiashvili et al., 2006] and Trametes versicolor [Mikiashvili et al., 2005] showed different laccase activity in the medium depending on the type of carbon source supplemented. Carbon concentration is particularly important. Excess concentration usually represses laccase expression, but certain minimal carbon source levels appear to be essential to maintain culture viability and to sustain laccase production over time. A considerable increase in laccase production is observed when glucose becomes limited [Thiruchelvam and Ramsay, 2007; Galhaup et al., 2002b].

Optimal laccase production over an extended period of time could be obtained with a fed-batch strategy with a low feed of carbon source, or by using a natural substrate that can slowly liberate carbon sources during fermentation, for example, cereal bran or fruit peelings. For Trametes pubescens, the fed-batch mode doubled laccase activity as compared to batch fermentation [Galhaup et al., 2002b].
9.1.2. Nitrogen source

The concentration of nitrogen optimal for laccase production seems to be more ambiguous than that of carbon. In nature, nitrogen limitation has been a major factor in enhancing ligninolytic enzyme production. N-limited medium has been shown to favour laccase production in some species, for example *Pycnoporus sanguineus* [Pointing et al., 2000] and *Pleurotus ostreatus* [Hou et al., 2004]. For several different species, ligninolytic activity often started after depletion of an initially high nitrogen concentration in the medium, which suggested activation by N limitation.

Many reports have demonstrated that high nitrogen concentration in the medium was favorable for laccase production with *Trametes gallica* [Dong et al., 2005; Dong and Zhang, 2004], *Lentinus edodes* [Hatvani and Mecs, 2002], *Trametes versicolor* [Collins and Dobson, 1997] and *Trametes pubescens* [Galhaup et al., 2002a]. However, conflicting results regarding optimal nitrogen concentration abound in the literature. For example, Kaal et al. [1995] and Hou et al. [2004] obtained contradictory results with two different strains of *Pleurotus ostreatus*. Indeed, laccase expression varies depending on the growth conditions, composition of the medium, source of nitrogen (organic, inorganic), and strain. Tryptone and peptone are often preferred N-sources over asparagine, yeast extract and ammonium tartrate [Dong et al., 2005; Galhaup et al., 2002b]. It was established that the use of a complex substrate, such as lignocellulosic waste, needed higher energy input for fungal growth. Mycelium must grow on waste particles to produce an enzyme arsenal to liberate nutrients and inducers. A positive effect of additional nitrogen sources may be a result of the production of more biomass.
In general, studies confirm the idea that nitrogen usually represses laccase expression, but expression of some enzymes is less sensitive to this repression [Kachlishvili et al., 2006]. Fungal laccases that are not inhibited by nitrogen may be more attractive for scale-up, as biomass growth may be speeded up, improving laccase activity.

9.1.3. Laccase inducers

The use of an appropriate inducer can greatly enhance laccase production and can be a prerequisite for effective production at large/industrial scale. An inventory of molecules used as inducers has been compiled by Ikehata et al. [2004]. The most effective and commonly used inducers of laccase in fungi are 2,5-xylydine and copper (CuSO₄). Copper atoms serve as cofactors in the catalytic core of laccase; thus, a minimum concentration (μM range) of copper ions is necessary for production of the active enzyme. Copper ions are also an inducer of laccase in several fungi: Pleurotus florida [Palvannan and Sathishkumar, 2010], Trametes trogii [Levin et al., 2005], Panus tigrinus [Chernykh et al., 2005], Trametes pubescens [Galhaup et al., 2002a], Pleurotus ostreatus [Palmieri et al., 2000], Marasmius quercophilus [Tagger et al., 1998], Phanerochaete chrysosporium [Dittmer et al., 1997], Neurospora crassa [Huber and Lerch, 1987] and many others. Copper regulates laccase at the level of gene transcription Ceriporiopsis subvermispora [Álvarez et al., 2009], Trametes pubescens [Galhaup et al., 2002a], Pleurotus sajor-caju [Soden and Dobson, 2001] and Trametes versicolor [Collins and Dobson, 1997]. Optimal copper concentration varies among species and within strains of a single species. For example, high copper concentration (400 μM CuSO₄) gave high laccase activity with Trametes
versicolor [Minussi et al., 2007] and Pleurotus ostreatus produced maximum laccase enzyme at 150 μM copper concentration [Palmieri et al., 2000], but it was shown that copper concentrations as low as 6 μM can inhibit laccase from Marasmius quercophilus [Tagger et al., 1998]. Excess copper may have a toxic effect on fungal biomass and thus decrease laccase production. Addition of copper during the exponential phase of fungal growth gives optimal laccase activity while minimizing the inhibitory effect of copper on fungal growth [Revankar and Lele, 2006; Galhaup and Haltrich, 2001]. Chernykh et al. [2005] reported that Pycnoporus tigrinus laccase production was 10-fold stimulated by the combined addition of 2mM CuSO₄ and 1mM 2,4-dimethylphenol to a glucose/peptone liquid medium.

Among other interesting molecules are the natural aromatic compound guaiacol [Ryan et al., 2007]; veratryl alcohol, a secondary metabolite [Dekker and Barbosa, 2001]; and ethanol, an inexpensive agricultural by-product [Lomascolo et al., 2003]. Heat shock and a few lignin monomers, amino acids and vitamins can also act as inducers of laccase [Fink-Boots et al., 1999]. Degradation of lignocellulosic agricultural residues can liberate efficient natural inducers and may surpass the effect of artificial inducers [Pickard et al., 1999]. There are only a few reports describing the effects of addition of a combination of two inducers. Jang et al. [2006] showed that 2,5-xylidine together with ABTS increased laccase activity to 359%, relative to 284% for 2,5-xylidine or 130% for ABTS when used alone.

In the future, it will be of interest to evaluate the potential of some common lignocellulosic waste materials in the presence of another inducer and a sufficient concentration of copper. The concentration of inducer has an important impact on
laccase production and is specific to species or strains, as shown by Minussi et al. [2007]. The time at which the inducer is supplemented in a particular culture is important for optimal induction with *Trametes pubescens* [Galhaup et al., 2002b]. If wastewater is used as an inducer, culture age is of significant importance: older cultures contain more degradative enzymes and mature cells, and are more tolerant to toxic effluent.

For example, phenolic effluent added to a 12 d old *Trametes versicolor* culture, laccase production was induced; however laccase activity inhibited in 8 d or 4 d old cultures [Ryan et al., 2007]. Different inducers – artificial, lignocellulosic materials or metals – can result in varied isoenzyme profiles, which might give different enzymatic activities [Lorenzo et al., 2006; Moldes et al., 2004]. In summary, in terms of nutrients, appropriate carbon and nitrogen concentrations as well as the co-factor copper and other inducers are absolutely necessary to obtain optimal production of laccase.

### 9.2. Environmental parameters

The mechanisms of metabolism in microorganisms are used and controlled by its environmental conditions [Monteiro and De Carvalho, 1998]. Generally, a good laccase producer can grow under room temperature (25 °C to 30 °C) and slightly acidic (4.5 and 5.5) conditions. When pH is not controlled during fermentation, a decrease in the culture pH occurs in the “growth phase” or “enzyme production phase”, and is associated with the release of lignolytic enzymes and other excreted acidic factors [Galhaup et al., 2002b; Gao et al., 2006]. It is important to note that laccase production cannot be generally correlated to fungal growth [Galhaup et al., 2002b; Arora and Sandhu, 1987]. However,
Papinutti *et al.* [2008] found evidence to support the idea that laccase is produced by *Fomes sclerodermeus* in a growth-dependent manner.

Laccase production may be improved if studies on optimal aeration rate or oxygen concentration during fermentation are undertaken. In the case of *Botryosphaeria* sp., laccase titre was greatly improved in liquid shake flask culture if aeration was provided by the use of baffled flasks in comparison to flasks without baffles [Dekker and Barbosa, 2001].

10. **Laccase production optimization**

Optimization of laccase production has been carried out using various mathematical and statistical models, such as Plackett–Burman design experimental design [Chhaya and Gupte, 2010; Niladevi *et al.*, 2009; Levin *et al.*, 2005], Response Surface Methodology [Chhaya and Gupte, 2010; Niladevi *et al.*, 2009; Quaratino *et al.*, 2008; Murugesan *et al.*, 2007; Trupkin *et al.*, 2003; Vasconcelos *et al.*, 2000], Taguchi orthogonal array [Krishna Prasad *et al.*, 2005], Box–Behnken design [Teerapatsakul *et al.*, 2007] and multivariate regression analysis using unconstrained optimization programming [Jing *et al.*, 2007].

10.1. **Plackett-Burman design**

Plackett-Burman (PB) designs are experimental designs presented by Plackett and Burman [1946]. Their goal was to find experimental designs for investigating the dependence of some measured quantity on a number of independent variables (factors), each taking *L* levels, in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments. Interactions between the factors were considered negligible. The solution to this problem is to find an experimental design in which each
combination of levels for any pair of factors appears the same number of times. A complete factorial design would satisfy this criterion, but the idea was to find smaller designs.

Experimental designs of this type exhibit an extremely high degree of confounding. This is not surprising when one considers that a full eleven-factor, two-level design would require 2,048 (2^11) individual experiments, involving 11 main effects, 55 second-order interactions, and no fewer than 1,981 further interactions of orders ranging from 3 to 11. Because they are so highly confounded, Plackett–Burman designs cannot be used to evaluate individual main effects and interactions between them, although they are of great value in screening experiments, as mentioned previously. In these experiments, a comparatively large number of factors may have an influence on the response. Thus, it is of value to distinguish those that have an effect from those that do not. Projections of two-level designs can be used to investigate main effects and interactions of the factors retained, although these cannot support a fully quadratic model, which require at least three levels. However, there are also economical three-level designs that can be used for screening when a second-order fit is desirable. They can be used as initial building blocks for some small, second-order designs. For the case of more than two levels, Plackett and Burman rediscovered designs that had previously been given by Bose and Kishen [1940] at the Indian Statistical Institute.

10.2. Response surface methodology

Response Surface Methodology (RSM) which is an efficient statistical method for the optimization of multiple variables was employed to predict the best
performance condition. The main advantages of RSM are the reducing number and cost of experiments [Shieh et al., 1995]. It is widely used to optimize process parameters, especially in determining optimum conditions for chemical investigations and maximizing yields in biocatalyzed synthesis [Gunawan et al., 2005]. Statistical experimental design techniques are useful tools for screening for nutrients with significant impact on growth rate as they can provide statistical models, which aid in understanding the interactions among the process parameters at varying levels. Furthermore, calculations of the optimal level of each parameter for a given target can be performed. It is widely used in order to improve product yield, reduce development time and overall process costs [Ren et al., 2008; Kammoun et al., 2008; Pan et al., 2008]. The optimum values of the selected variables were obtained by solving the regression equation and by analyzing the response surface contour plots [Myers and Montgomery, 2002]. RSM has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolites production [Deepak et al., 2008; Liu and Wang, 2007].

11. Laccase fermentation

Laccases are generally produced during the secondary metabolism of white-rot fungi growing on submerged fermentation (SF) or solid-state fermentation (SSF) or Semi-solid-state fermentation (SSSF).

Liquid batch or SF is frequently used for laccase production with fungi, despite the fact that this mode is quite different from the natural living conditions of these organisms. Fermentation parameters can be controlled easily in liquid batch culture, and existing bioreactor configurations have provided satisfactory laccase production [Thiruchelvam and Ramsay, 2007]. Filamentous fungi growing
in liquid medium in shake flasks usually form small compact pellets that vary in size depending on the type of agitation, or the configuration of the stir bar. Thiruchelvam and Ramsay [2007] demonstrated that pellet size could have important impact on laccase productivity and obtained uniform pellets of 1 mm of \textit{Trametes versicolor} with the aid of an "O-ring"-type stir bar.

12. Laccase purification

Laccase purification was achieved from different fungal strains by many researchers (Table 1). Purification procedures for fungal laccases depend on species, laccase properties and the purity level required for subsequent applications. Successive column chromatography procedures are commonly used as purification techniques, for example, a combination of high pressure liquid chromatography (HPLC) and gel filtration [Rebrikov \textit{et al.}, 2006], or a combination of anion-exchange chromatography and gel filtration [Pozdnyakova \textit{et al.}, 2006b]. In the beginning, or in between steps, samples are sometime concentrated by ammonium sulfate precipitation or ultrafiltration, followed by dialysis [Iyer and Chattoo, 2003].

These methods yield a quality product but they are time consuming and usually not very productive. An old process for enzyme purification — foam fractionation — may offer an excellent alternative. This method was used successfully for efficient isolation of laccase from \textit{Pleurotus sapidus} [Linke \textit{et al.}, 2007]. Using a modified culture medium (without yeast extract and MgSO\textsubscript{4}), adjusted parameters and cetyl trimethylammonium bromide (CTAB) as surfactant, more than 70\% of enzyme activity was recovered with no denaturing effect; however, the different isoenzymes were not extracted proportionally. Cheap,
simple and efficient laccase purification procedures are proving essential to make wastewater bioremediation economically feasible, and foam fractionation is a highly promising method in this regard. Its use in continuous mode for laccase enrichment suggests its potential for high volume fermentation [Gerken et al., 2006].

Methods for the purification of all the different isoenzymes from a given strain, or effective isoenzyme ratios, must also be developed. Temperature and pH have significant impacts on laccase extraction. Acidic conditions are more favorable for laccase recovery compared to neutral or alkaline media. Substantial yield improvements have been obtained at 25 °C as opposed to 4 °C in the recovery of laccase from *Pycnoporus sanguineus* [Vikineswary et al., 2006]. Higher temperature augments laccase solubility but samples should not be allowed to exceed 4 °C for more than a few hours [Hublick and Schinner, 2000]. These latter authors tested the stability of laccase from *Pleurotus ostreatus* at different temperatures. At 4 °C, enzyme activity decreased slightly after 80 d, while no activity remained after 20 d at 25 °C. Maximum stability over time was achieved in high ionic buffer at pH 10 with 10 mM benzoic acid and 35% ethylene glycol. Thus, the shelf life of purified enzymes remains an important exploratory field, requiring the development of not only stable, but also economical and eco-friendly formulations.
Table 1. Purification of laccase from different fungal strains

<table>
<thead>
<tr>
<th>Organism Name</th>
<th>Purification Steps</th>
<th>Laccase Activity (U/ml)</th>
<th>Specific Activity (Crude) U/mg</th>
<th>Specific Activity (Purified) U/mg</th>
<th>Purification Fold</th>
<th>Yield (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trichoderma atroviride</strong></td>
<td>1. Ultrafiltration 2. Q cartridge 3. Superdex 75 4. Mono-Q</td>
<td>0.296</td>
<td>1.6</td>
<td>69.3</td>
<td>42.1</td>
<td>47.8</td>
<td>Chakroun et al., 2009</td>
</tr>
<tr>
<td><strong>Lentinula edodes</strong></td>
<td>1. Ultrafiltration 2. Toyopearl DEAE-650M 3. Toyopearl Butyl-650M 4. Superdex 75 HR 10/30 5. Mono Q HR 5/5</td>
<td>18,100</td>
<td>0.0510</td>
<td>155</td>
<td>3040</td>
<td>2.49</td>
<td>Nagai et al., 2009</td>
</tr>
<tr>
<td><strong>Funaalia trogii</strong></td>
<td>1. (NH4)2SO4 2. AE chromatography 3. Ultrafiltration 4. GF Chromatography</td>
<td>19.9</td>
<td>33</td>
<td>92</td>
<td>2.8</td>
<td>44</td>
<td>Patrick et al., 2009</td>
</tr>
<tr>
<td><strong>Xylaria polymorpha</strong></td>
<td>1.Ultrafiltration 2.DEAE-sepharose FF 3.Superdex 200</td>
<td>14,380 (Total activity)</td>
<td>127.7 (Total activity)</td>
<td>823.2</td>
<td>6.4</td>
<td>31</td>
<td>Liers et al., 2007</td>
</tr>
<tr>
<td><strong>Pycnoporus sanguineus</strong></td>
<td>1.(NH4)2SO4 precipitation 2. DE 52 3. DEAE Toyopearl 4. Mimetic Yellow II</td>
<td>165 (Total activity)</td>
<td>0.034</td>
<td>32.9</td>
<td>967</td>
<td>38</td>
<td>Litthauer et al., 2007</td>
</tr>
<tr>
<td><strong>Cerrena maxima</strong></td>
<td>1. Ultrafiltration 2. (NH4)2SO4 precipitation 3. Ion-exchange on DEAE-cellulose 4.DEAE-Toyopearl 650M 5. HPLC</td>
<td>4.5</td>
<td>10</td>
<td>209</td>
<td>30</td>
<td>32</td>
<td>Lyashenko et al., 2006</td>
</tr>
<tr>
<td><strong>Cyathus bulleri</strong></td>
<td>1.Ultrafiltration 2.DEAE-Sepharose 3.Sephaeryl S-200</td>
<td>21</td>
<td>49.1</td>
<td>4,022.4</td>
<td>81.9</td>
<td>44.9</td>
<td>Salony and Mishra, 2006</td>
</tr>
</tbody>
</table>

Continued....
<table>
<thead>
<tr>
<th>Organism</th>
<th>Purification Steps</th>
<th>Yield (nkat/ml)</th>
<th>Activity (nkat/mg)</th>
<th>Specific Activity (nkat/mg)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pleurotus sajor-caju</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. DEAE-cellulose 3. Sephadex G-100</td>
<td>14</td>
<td>120.0</td>
<td>1,244.4</td>
<td>Murugesan et al., 2006</td>
</tr>
<tr>
<td><em>Pleurotus pulmonarius</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. Ion-exchange 3. Gel filtration</td>
<td>85,000</td>
<td>3,346</td>
<td>19,750</td>
<td>De Souza and Peralta, 2003</td>
</tr>
<tr>
<td><em>Maugiinia sp.</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. Anion exchange 3. HIC Concentrate</td>
<td>26</td>
<td>1449</td>
<td>100</td>
<td>Palonen et al., 2003</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. DEAE Sepharose 3. Mono Q 4. Superdex 7</td>
<td>3</td>
<td>130</td>
<td>292</td>
<td>Palmieri et al., 2003</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em></td>
<td>1. Q-Sepharose 2. Superdex-75</td>
<td>2.5</td>
<td>10</td>
<td>300</td>
<td>Jung et al., 2002</td>
</tr>
<tr>
<td><em>Pleurotus florida</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. DEAE Sephadex A -50 3. Biogel P-200</td>
<td>5.0</td>
<td>8.3</td>
<td>2.86</td>
<td>Das et al., 2001</td>
</tr>
<tr>
<td><em>Rigidoporus lignosus</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. DEAE-Sephacel 3. Mono-Q pooled peak</td>
<td>0.36</td>
<td>0.65</td>
<td>15.4</td>
<td>Cambria et al., 2000</td>
</tr>
<tr>
<td><em>Pleurotus florida</em></td>
<td>1. (NH$_4$)$_2$SO$_4$ precipitation 2. DEAE Sephadex A-50 3. Biogel P-200</td>
<td>5000 nkat</td>
<td>83.3</td>
<td>1720</td>
<td>Das et al., 2000</td>
</tr>
<tr>
<td><em>Coniothyrium minitans</em></td>
<td>1. Acetone 10% 2. Ultrafiltration 3. HIC</td>
<td>0.92</td>
<td>0.52</td>
<td>9.89</td>
<td>Dahiya et al., 1998</td>
</tr>
</tbody>
</table>
13. Molecular features of laccases

Laccases are glycoproteins with molecular weight of 50-130 kDa. The carbohydrate moiety of the majority of laccases consists of mannose, \( N \)-acetylglucosamine, and galactose and constitutes about 45% of the protein mass in laccases of plant origin. Fungal laccases have lower carbohydrate contents (10-20%). In laccases from the basidiomycete *Pleurotus eryngii* the carbohydrate moieties are 1 and 7% [Munoz *et al.*, 1997], whereas in laccases from *Coriolopsis fulvocinerea* [Shleev *et al.*, 2004] and *Bacillus cinerea* [Slomczynski *et al.*, 1995] carbohydrate contents are 32 and 49%, respectively, i.e. are high for fungal laccases. Many researchers think that the carbohydrate moiety of the molecule is responsible for the stability of the protein globule. Deglycosylation of laccase from *Ganoderma lucidum* completely inhibited the enzyme activity [Ko *et al.*, 2001]. The carbohydrate moiety has also been supposed to protect the enzyme molecule against proteolysis and inactivation by free radicals [Thurston, 1994; Yoshitake *et al.*, 1993].

14. Laccase isoenzymes

Genes encoding isoenzymes are differentially regulated and may be inducible or constitutively expressed during the life of the cell. The proportion of different laccase isoenzymes produced may depend on culture age and substrate [Moldes *et al.*, 2004]. Genetic analysis has revealed that isoenzymes often originate from different laccase genes in the genome. Kilaru *et al.* [2006] identified seventeen different laccase genes, nine of which were active, in analyzing the complete sequenced genome of *Coprinopsis cinerea*. Thirty-one putative laccase genes from *Trametes versicolor* genomic sequences are known (NCBI GenBank).
The characterization of these isoenzymes has revealed diverse molecular weight, pH, inducibility and substrate specificity. Different combinations of isoenzymes are expressed in media of different composition, in the presence or absence of inducer, and depending on the mode of incubation. Dong et al. [2005] tested 12 different media with *Trametes gallica* under static or shaking conditions and found twenty different isoenzyme patterns. The isoenzymes from a particular microorganism usually possess different kinetic properties, which results in broadened substrate specificity and may have an adaptive value for rotting fungi that grow on complex substrates such as hard wood, and in changing environments [Shleev et al., 2007]. To determine the most appropriate species or isoenzyme for a specific substrate, systematic testing of different laccases under standardized conditions would be essential prior to practical applications.

Laccases are the major extracellular components of the lignin-degrading system of the white rot fungi belonging to the *Pleurotus* genus [Baldrian et al., 2005]. Thus, the fungus *Pleurotus ostreatus* secretes eight different laccase isoenzymes, six of which have been isolated and characterized [Palmieri et al., 2003; Giardiana et al., 1999; Palmieri et al., 1997]. The predominant isoenzymes (POXC) is a 59 kD protein with pI 2.9; three isoenzymes (POXA2, POXB1, and POXB2) have the same molecular weight of about 67 kD, with pI 4.1 for two of them and pI 2.9 for the third. Two other isoenzymes of this protein (POXA1b and POXA1w) have molecular weight of 61 kD and pI 6.7-6.9, and two more isoenzymes (POXA3a and POXA3b) are heterodimers (pI 4.1-4.3) consisting of two subunits: of 61 kD and 16 or 18 kD. Formation of *Pleurotus ostreatus* isoenzymes is induced by copper ions in the culture medium and regulated on the level of gene transcription [Palmieri et al., 2003, Palmieri et al., 2000]. Although
laccase POXC is the predominant isoenzyme, the highest level of mRNA has been found for isoenzyme POXA1b [Palmieri et al., 2003].

The fungus *Trametes pubescens* produces two laccase isoenzymes with the same molecular weight of 67 kDa and pI values of 5.1 and 5.3 [Nikitina et al., 2005], the fungus *Ceriporiopsis subvermispora* produces four isoenzymes (68-71 kD, pI values from 3.4 to 4.7) [Fukushima and Kirk, 1995], and the flat polypore *Ganoderma lucidum* produces five laccase isoenzymes with pI values of 3.0, 4.25, 4.5, 4.8, and 5.1 and molecular weights ranging from 40 to 66 kDa [Ko et al., 2001].

The number of isoenzymes found depends on conditions of cultivation, in particular on the presence of an inducer in the medium [Dong et al., 2005; De Souza et al., 2004; Palmieri et al., 2003; Galhaup and Haltrich, 2001]. The fungus *Pleurotus pulmonarius* produces three laccase isoenzymes, two of which (L1 and L2) are constitutive and the isoenzymes L3 can be detected only when the fungus is cultured in the presence of inducers [De Souza et al., 2004], whereas the fungus *Marasmius quercophilus* strain 17 produces three constitutive and four inducible forms of the enzyme [Farnet et al., 2000]. Although most of fungal laccases are monomeric proteins, enzymes consisting of several subunits are also known. Thus, laccases from the wood rot fungi *Phellinus ribis* [Min et al., 2001], *Pleurotus pulmonarius* [De Souza and Peralta, 2003], and *Trametes villosa* [Yaver et al., 1996], and the mycorrhizal fungus *Cantharellus cibarius* [Ng and Wang, 2004] consist of two similar subunits with molecular weights typical for monomeric laccases. Oligomeric laccases have been isolated from some ascomycetes. Thus, gel filtration shows the molecular weight of laccase from *Monocillium indicum* to
be 100 kDa, and SDS electrophoresis reveals in it three subunits: 24, 56, and 72 kDa [Thakker et al., 1992]. The fungus Gaeumannomyces graminis produces an enzyme consisting of three 60 kD subunits [Edens et al., 1999], and a laccase from the ascomycete Podospora anserina is a tetramer consisting of 80 kDa subunits [Molitoris and Esser, 1970].

Furthermore, these different isoenzymes can have different roles in the physiology of different species or in the same species under different conditions [Assavanig et al., 1992]. Various laccase encoding gene sequences have been reported from a range of ligninolytic fungi. These sequences encode for proteins between 515 and 619 amino acid residues and close phylogenetic proximity between them is indicated by sequence comparisons [Bourbonnais et al., 1995].

15. Laccase gene family

Laccase gene family can be used as a potential tool to define its moonlighting functions. The copy numbers of laccase genes vary among fungi. A laccase gene family in which the genes encoding two of five laccases were located on the same chromosome of Trametes villosa [Yaver et al., 1996], and three laccase genes were found to be clustered within approximately 11 kb of each another in the plant pathogenic fungus, Rhizoctonia solani [Sharma and Kuhad, 2008; Wahleithner et al., 1996]. Giardiana et al., [1996] isolated two-phenol oxidase genes (POX1 and POX2) that showed 84% homology with each other and thus demonstrated the existence of a multigene family that encoded for isoforms of laccase in Pleurotus ostreatus. Coprinus cinereus also contains a laccase gene family consisting of at least three genes [Yaver et al., 1999]. The presence of
multiple gene families for the secreted laccases requires systematic genetic analysis to elucidate their functions. Gene families probably produce closely related proteins that are subtly different in their activities, allowing transformation of a wider range of substrates or showing differential regulation [Mansur et al., 1997]. Moreover, until transcripts for all the laccase genes are not detected, the possibility that some of the non-expressed laccase genes are pseudogenes or are expressed under different physiological conditions cannot be ruled out. Phylogenetic data of the moonlighting protein i.e., Fet3, CotA and polyphenol oxidase from various sources proves that diverse paralogus laccase genes may have descended from progenitor gene or master gene, which has duplicated and diverged prior to speciation.

16. Heterologous expression and genetic amelioration

Heterologous expression is a common and efficient industrial process for producing appreciable amounts of a protein of interest. Several fungal laccase genes have been cloned and heterologously expressed in the filamentous fungus *Aspergillus niger* [Rodríguez et al., 2008; Larrondo et al., 2003; Record et al., 2002], *Aspergillus oryzae* [Hoshida et al., 2005; Ducros et al., 1997; Yaver et al., 1996] and *Trichoderma reesei* [Bailey et al., 2007; Kiiskinen et al., 2004]. The latter gave high laccase production levels.

Yeasts are suitable as hosts for heterologous protein production because they combine a high capacity for growth, the easy manipulation of unicellular organisms and a eukaryotic organisation enabling post-translational modifications. Laccase genes have been heterologously expressed in the yeasts *Saccharomyces cerevisiae* [Necochea et al., 2005; Piscitelli et al., 2005; Kiiskinen and Saloheimo,
### General Introduction

**Table 2. Decolorization of synthetic dyes using fungal laccases**

<table>
<thead>
<tr>
<th>Laccase source</th>
<th>Dye</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trametes versicolor</em></td>
<td>Reactive blue 19</td>
<td>Champagne <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>Pleurotus florida</em></td>
<td>Reactive blue 198</td>
<td>Sathishkumar <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>Lentinus crinitus</em></td>
<td>Reactive blue 220</td>
<td>Niebisch <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>Trametes versicolor</em></td>
<td>Reactive Green 19</td>
<td>Bayramoğlu <em>et al.</em>, 2010</td>
</tr>
<tr>
<td></td>
<td>Reactive Red 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reactive Brown 10</td>
<td></td>
</tr>
<tr>
<td><em>Trametes trogii</em></td>
<td>Gentian Violet</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xylidine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Congo Red</td>
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<tr>
<td></td>
<td>Malachite Green</td>
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<tr>
<td></td>
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<td>Remazol Brilliant Blue R</td>
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<td>Indigo Carmine</td>
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<td></td>
<td>Anthraquinone Blue</td>
<td>Levin <em>et al.</em>, 2010</td>
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<td>Commercial laccase</td>
<td>Black Novacron R</td>
<td>Khouni <em>et al.</em>, 2010</td>
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<td>Blue Bezaktiv S-GLD 150</td>
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<td><em>Streptomyces cyaneus</em></td>
<td>Acid Yellow 17</td>
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<td>Chromotrope 2R</td>
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<td>Crocin Orange G</td>
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<td>Methyl Orange</td>
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<td>New Coccine</td>
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<td>Orange II</td>
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<td>Tropacolin O</td>
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<td></td>
<td>Tartrazine</td>
<td>Moya <em>et al.</em>, 2010</td>
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<td>Malachite green</td>
<td>Yan <em>et al.</em>, 2009</td>
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<td>Crystal violet</td>
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<td><em>Cyathus bulleri</em></td>
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<td>Chhabra <em>et al.</em>, 2009</td>
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<td>Jadhav <em>et al.</em>, 2009</td>
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<td>Orange-16</td>
<td>Srinivasan and Murthy,</td>
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<td>Reactive Red-35 dyes</td>
<td>2009</td>
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<td><em>Trametes pubescens</em></td>
<td>Reactive Black 5</td>
<td>Enayatzamir <em>et al.</em>, 2009</td>
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<td><em>Trametes pubescens</em></td>
<td>Reactive Black 5</td>
<td>Roriz <em>et al.</em>, 2009</td>
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*Continued...*
| **Schizophyllum commune** | Cibacron Red FN-2BL | Bhatti et al., 2008 |
| **Streptomyces psammoticus** | Acid orange, Methyl orange, Bismarck brown | Niladevi et al., 2008 |
| **Coriolus versicolor f. antarcticus** | Malachite green | Diorio et al., 2008 |
| **Pichia methanolica** (recombinant strain) | Remazol Brilliant Blue R | Guo et al., 2008 |
| **Funalia trogii** | Azo textile dyes | Ciullina et al., 2008 |
| **Streptomyces coelicolor** | indigo dye | Dubé et al., 2008 |
| **Cerrena unicolor** | Azo dyes, Anthraquinone dyes | Michniewicz et al., 2008 |
| **Pleurotus ostreatus** | Acid black 10 BX | Katuri et al., 2008 |
| **Irpex lacteus** | Reactive Orange 16, Remazol Brilliant Blue R | Svobodov et al., 2007 |
| **Pleurotus ostreatus, Coriolus versicolor and Funalia trogii** | Remazol Brilliant Blue Royal, Drimaren Blue CL-BR | Erkurt et al., 2007 |
| **Pycnoporus sanguineus** | Anthraquinone dye | Lu et al., 2007 |
| **Pycnoporus sanguineus** | Acid Blue 62, Acid Blue 281, Reactive Blue 19 | Vanhulle et al., 2007 |
| **Ischnoderma resinosum** | Reactive Black 5, Reactive Red 22 | Kokol et al., 2007 |
| **Ganoderma lucidum** | RBBR and RB 5 | Murugesan et al., 2007 |
| **Trametes versicolor** | Grey Lanaset G | Romero et al., 2006 |
| **Pleurotus calyptratus** | Orange G, Remazol Brilliant Blue R | Eichlerova et al., 2006 |
| **Trametes trogii** | Malachite green | Levin et al., 2005 |
| **Pleurotus ostreatus** | Remazol Brilliant Blue R | Palmieri et al., 2005 |

*For a detailed review kindly refer Couto and Toca Herrera [2006]*
Therefore, the development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure [Chhabra et al., 2009; Murugesan et al., 2009b; Salony and Bisaria, 2006; Hou et al., 2004; Abadulla et al., 2000], including synthetic dyes currently employed in the industry [Srinivasan and Murthy, 2009; Murugesan et al., 2007; Couto, 2007] [Table 2]. Recently, several publications describing the textile effluent treatment [Khlifi et al., 2010; Khouni et al., 2010] and degradation of synthetic dyes used in textile industries [Moya et al., 2010; Srinivasan and Murthy, 2009; Murugesan et al., 2009b; Erkurt et al., 2007] have been reported with special emphasis in the use of fungal laccases.

17.1.2. Denim finishing

In the textile finishing industry, enzymatic degradation of indigo could have a potential both in stone-wash process and for the treatment of dyeing effluents. Several steps are involved in the manufacture of denim garments between dyeing and the final stone-washing where excessive amounts of indigo are removed from the fabrics and discharged with the wastewater. The traditional technology of producing a stone-washed look in denim fabric involves the wash of the fabrics in the presence of pumice to generate the desired erosion of the fabrics. Subsequently, the fabrics are partially bleached by a treatment with sodium hypochlorite, followed by neutralisation and a rinsing step all causing substantial environmental pollution [Pedersen and Schneider, 1998]. In 1996, DeniLite™, the first industrial laccase and the first bleaching enzyme was launched by Novo Nordisk, Denmark. In 1999, DeniLiteII™ was launched based on a new type of laccase with higher activity than that of Denilite™. Also, in 2001, the company
Zytex (Zytex Pvt. Ltd., Mumbai, India) developed a formulation based on LMS capable of degrading indigo in a very specific way. The trade name of the product is Zylite.

Campos et al. [2001] reported the degradation of indigo both in effluents and on fabrics using purified laccases from Trametes hirsuta and Sclerotium rolfsii in combination with redox-mediators and reported that bleaching of fabrics by the laccases correlated with the release of indigo degradation products. Pazarlioglu et al., [2005] demonstrated that a phenol-induced laccase from Trametes versicolor was an effective agent for stone-washing effects of denim fabric without using a mediator. Moreover, they found that Trametes versicolor laccase without a mediator was more effective than commercial laccase (obtained from recombinant Aspergillus niger, Novo Nordisk, Denmark) with a mediator.

17.1.3. Cotton bleaching

The purpose of cotton bleaching is to decolorize natural pigments and to confer a pure white appearance to the fibres. Mainly flavonoids are responsible for the color of cotton [Ardon et al., 1996; Hedin et al., 1992]. The most common industrial bleaching agent is hydrogen peroxide, which is usually applied at alkaline pH and temperatures close to boiling. However, radical reactions of bleaching agents with the fiber can lead to a decrease in the polymerization. Furthermore, a huge amount of water is needed to remove hydrogen peroxide from fabrics, which can cause problems in dyeing. Therefore, replacement of hydrogen peroxide by an enzymatic bleaching system would not only lead to better product quality due to less fiber damage but also to substantial savings on washing water needed for the removal of hydrogen peroxide.
Tzanov et al. [2003a] reported for the first time the enhancement of the bleaching effect achieved on cotton fabrics using laccases in low concentrations. In addition, the short time of the enzymatic pre-treatment sufficient to enhance fabric whiteness makes this bio-process suitable for continuous operations. Also, Pereira et al. [2005] showed that a laccase from a newly isolated strain of *Trametes hirsuta* was responsible for whiteness improvement of cotton most likely due to oxidation of flavonoids. Basto et al. [2007] proposed a combined ultrasound-laccase treatment for cotton bleaching. They found that the supply of low ultrasound energy enhanced the bleaching efficiency of laccase on cotton fabrics.

**17.1.4. Wool dyeing**

Shin et al. [2001] showed that laccase was able to color wool fabric that was previously padded with hydroquinone. Montazer et al. [2008] and Tzanov et al. [2003b] also proved the ability of laccases for wool dyeing. They used a dye bath prepared with a dye precursor (2,5-diaminobenzenesulfonic acid), dye modifiers (catechol and resorcinol) and laccase, without any dyeing auxiliaries. Additionally, the enzymatic reaction was carried out at pH and temperature values safe to the wool material. Furthermore, they showed that by prolonging the contact time between wool, enzyme, precursor and modifier, deeper colors were obtained in contrast to the conventional process in which deeper colors are attained by increasing the amount of dye. This makes wool dyeing with laccase an economically attractive alternative to the conventional process, which uses high amounts of water, auxiliaries, acid and energy. This laccase application is a promising technology especially for the coating of natural and synthetic materials.
17.1.5. Anti-shrink treatment for wool

A process conventionally used for wool shrink-proofing is chlorination. This process degrades the exo-cuticle of the wool, forming cysteic acid residues and protein losses. This process has been replaced by proteinases treatment due to their high specificity and much lower environmental impact. However, proteinase treatment leads to protein degradation, resulting in deterioration of fiber strength and limited shrink resistance [Breier, 2002]. A patent application about the use of laccase from *Trametes versicolor* plus a mediator to increase the shrink resistance of wool was published [Yoon, 1998]. Also, Lantto *et al.* [2004] found that wool fibers can be activated with laccase if a suitable mediator is present. Therefore, the use of laccase for anti-shrink treatment of wool seems very attractive.

17.2. Dye synthesis

Setti *et al.* [1999] described the ability and efficacy of laccases from *Pyricularia oryzae* to form red azo dyes by the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and phenols. Mustafa *et al.* [2005] showed that the oxidation of ferulic acid by laccase in a biphasic hydroorganic medium led to the production of stable yellow colored products, which were only soluble in the organic phase being, thus, easily recovered. The synthesis of colorants by laccase in this medium is currently extended to other phenolic and polyphenolic compounds. Enaud *et al.* [2010] reported the synthesis of an environmental friendly azoanthraquinone dye using *Perenniporia ochroleuca* laccase through enzymatic coupling of aromatic amine monomers.
17.3. Paper industry

The industrial preparation of paper requires separation and degradation of lignin in wood pulp. Environmental concerns urge to replace conventional and polluting chlorine-based delignification/bleaching procedures. Oxygen delignification processes have been industrially introduced [Carter et al., 1997], but pre-treatments of wood pulp with ligninolytic enzymes might provide milder and cleaner strategies of delignification that are also respectful of the integrity of cellulose [Kuhad et al., 1997]. Although extensive studies have been performed to develop alternative bio-bleaching systems, few enzymatic treatments exhibit the delignification/brightening capabilities of modern chemical bleaching technologies. Lignin-oxidizing enzymes directly attack the lignin structure of pulp; as a result, these oxidative enzymes improve the efficiency of kraft pulp bleaching [Martinez et al., 2005; Bajpai, 2004; Call and Mücke, 1997].

Laccases can be used in the enzymatic adhesion of fibers in the manufacturing of lignocelluloses based composite materials such as fiberboards. Laccases have been proposed to activate the fiber bound lignin during manufacturing of the composites [Suurnakki et al., 2010], thus, resulting in boards with good mechanical properties without toxic synthetic adhesives [Huttermann et al., 2001].

Sigoillot et al. [2005] compared various fungal enzymes in the bleaching of high-quality paper pulps and found the LMS to posses the highest pulp delignification potential. Also, the joint use of laccase with mediators has proved a highly efficient choice for delignifying flax pulp [Fillat et al., 2010; Fillat and Roncero, 2009; Camarero et al., 2004; García et al., 2003].
Another possibility is to functionalise lignocellulosic fibers by laccases in order to improve the chemical or physical properties of the fiber products. Preliminary results have shown that laccases are able to graft various phenolics acid derivatives onto kraft pulp fibers [Chandra and Ragauskas, 2002; Lund and Ragauskas, 2001]. This ability could be used in the future to attach chemically versatile compounds to the fiber surfaces, possibly resulting in fiber materials with completely novel properties such as hydrophobicity or charge. Recently, several publications describing the pulp and paper industry effluent treatment and biobleaching process have been reported with special emphasis in the use of LMS [Eugenio et al., 2010; Sadhasivam et al., 2010; Moldes and Vidal, 2008; Moldes et al., 2008; Malaviya and Rathore, 2007].

17.4. Food industry

Laccases can be applied to certain processes that enhance or modify the color appearance of food or beverage. In this way, an interesting application of laccases involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine. Alberts et al. [2009] was investigated the enzymatic degradation of aflatoxin by white rot fungi through laccase production in different liquid media. A significant correlation was observed between laccase activity and aflatoxin degradation exhibited by representatives of Peniophora and Pleurotus ostreatus cultivated in minimal salts media. The degradation of aflatoxin by white rot fungi could be an important bio-control measure to reduce the level of this mycotoxin in food commodities.

A laccase from Trametes hirsuta was applied for the elimination of off-flavor substances in apple juice caused by microbial contamination. The evaluation
using GC-MS showed that enzymatic treatment could reduce the off-flavor in apple juice significantly [Schroeder et al., 2008]. Laccases are interest in baking due to its ability to cross-link biopolymers. Thus, Selinheimo et al. [2006] showed that a laccase from the white-rot fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough. Xu [2005] and Minussi et al. [2002] have described the potential applications of laccase in different aspects of the food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor.

17.5. Nanobiotechnology

During the past two decades, bioelectrochemistry has received increased attention. Progress on bioelectrochemistry has been integrated into analytical applications, e.g. in biosensors working as detectors in clinical and environmental analysis [Park et al., 2010; Haghighi et al., 2003]. 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. Moreover, biosensors for detection of morphine and codeine [Bauer et al., 1999], catecholamines [Ferry and Leech, 2005], plant flavonoids [Jarosz-Wilkolazka et al., 2004] and also for electroimmunoassay [Kuznetsov et al., 2001] have been developed.

Nanotechnology contributes to the development of smaller and more efficient biosensors through controlled deposition and specific adsorption of biomolecules on different types of surfaces, achieving micro and nanometer order. Hammond and Whitesides [1995] have introduced a method to pattern ultrathin
ionic multilayer films with micron-sized features onto surfaces building a patterned alkanethiol monolayer with ionic functionality onto a gold surface.

Chen et al. [2001; 1998] showed a biotechnological application of such micro patterned surfaces: the production of islands of micrometer size of extracellular matrix, where the pattern of these islands could determine the position and distribution of bovine and endothelial cells. The control of the nature and the density of the groups (e.g. alkys, amides, alcohols) of a surface built with assembled monolayers has been used successfully to investigate the non-specific adsorption of proteins [Sigal et al., 1998]. Regarding laccases, the immobilisation has an important influence on the biosensor sensitivity [Park et al., 2010; Freire et al., 2001]. Martele et al. [2003] have shown that micro patterning is an efficient method for the immobilization of laccases on a solid surface in order to develop a multi-functional biosensor. Also, Roy et al. [2005] found that cross-linked enzyme crystals (CLEC) of laccase from Trametes versicolor could be used in biosensor applications with great advantage over the soluble enzyme. Cabrita et al. [2005] have immobilised laccase from Coriolus versicolor on N-Hydroxysuccinimide-terminated self-assembled monolayers on gold. This procedure could be useful for the further development of biosensors.

In addition, an enzyme electrode based on the co-immobilisation of an osmium redox polymer and a laccase from Trametes versicolor on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, attaining nanomolar detection limits [Ferry and Leech, 2005].
Laccase electrodes have aroused a considerable attention as biocathode for the development of biofuel cells [Ding et al., 2010; Ressine et al., 2010; Willner et al., 2009; Tan et al., 2009; Habrioux et al., 2008], for example, small transmitter systems [Calabrese et al., 2002; Chen et al., 2001]. Biofuel cells are extremely attractive from an environmental point of view because electrical energy is generated without combusting fuel, thus, providing a cleaner source of energy.

The bioelectrochemical studies provide unequivocal evidence of efficient O$_2$-bioelectrocatalysis by laccase in the three dimensional chip structure. The designed 3D biochips might be useful in the future development of efficient biofuel cells and highly sensitive biosensor systems [Ressine et al., 2010].

17.6. Soil bioremediation

Laccases and LMS are able to oxidize toxic organic pollutants, such as various xenobiotics, PAHs, chlorophenols, and other contaminants [Hamada and Saito, 2006; Kajiuchi et al., 2006; Shirai et al., 2005; Suzuki et al., 2004; Suzuki et al., 2003; Pointing, 2001; Durán and Esposito, 2000]. Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others [Aggelis et al., 2003; Schlosser et al., 1999]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [Guebitz et al., 2004; Sun et al., 2006; Kozaki et al., 2003]. Laccase was found to be responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone [Leontievsky et al., 2000]. LMSs have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene [Niku et al., 2000; Bressler et al., 2000]. Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form
of the herbicide: laccases are able to convert the diketonitrile into the acid [Mougin et al., 2000]. Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil [Duncan, 2004]. LMS has been extensively studied in the oxidation of recalcitrant PAHs, main components of several ship spills. In this sense, LMS is being included in several enzymatic bioremediation programs [Alcalde et al., 2006].

17.7. Antimicrobial agent detoxification

Triclosan, an antimicrobial agent, is an emerging and persistent environmental pollutant that is often found as a contaminant in surface waters and sediments. Murugesan et al. [2010] reported the detoxification of triclosan through enzymatic transformation. This was achieved by ether bond cleavage of diphenyl ether in the triclosan by *Ganoderma lucidum* laccase.

17.8. Synthetic chemistry

In the future laccases may also be of great interest in synthetic chemistry, where they have been proposed to be applicable for oxidative deprotection [Semenov et al., 1993] and production of complex polymers and medical agents [Kunamneni et al., 2008; Nicotra et al., 2004; Kurisawa et al., 2003; Uyama and Kobayashi, 2002; Mai et al., 2000]. Mustafa et al. [2005] synthetised phenolic colourants by using an industrial laccase named Suberase® (Novo Nordisk A/S, Bagsvaerd, Denmark).

17.9. Pharmaceutical sector

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are attracting active research efforts. Laccase
can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc. [Nicotra et al., 2004; Juelich et al., 2001], including triazolo (benzo) cycloalkyl thia-diazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline [Molino et al., 2004; Stahl et al., 2002].

One potential application is laccase-based *in situ* generation of iodine, a reagent widely used as disinfectant [Danielsen et al., 2003]. Wang and Ng [2004] carried out the assay for HIV reverse-transcriptase inhibitory activity using *Tricholoma giganteum* laccase purified from its fruiting body. The capability of laccase to synthesize new compounds might also be used to generate new therapeutic compounds for treatment of microbiol infection or cancer. Another laccase has been shown capable of fighting aceruloplasminemia (a medical condition of lacking ceruloplasmin, a multi-Cu serum oxidase whose ferroxidase activity regulates iron homeostasis) [Harris et al., 2004].

17.10. Cosmetics

A novel application field for laccases is in cosmetics. For example, laccase based hair dyes could be less irritant and easier to handle than current hair dyes [Pereira and Burgaud, 2005; Plos, 2004; Shichiri et al., 2003; Koike, 2002]. Cosmetic and dermatological preparations containing proteins (including laccase) for skin lightening have also been developed [Yokoyama et al., 2006; Tachihara et al., 2006; Golz-Berner et al., 2004]. Laccases may find use as deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers [Markussen and Jensen, 2006; Miyamoto et al., 2005; Kozato et al., 2005; Enomoto, 2005; Hiramoto and Abe, 2004]. Protein engineered laccase may be used to reduce allergenicity [Roggen, 2001].
AIM AND OBJECTIVES

Considering the advantages of laccase enzyme in biotechnological application, the present work was undertaken to explore the potential utilization of laccase from *Pleurotus florida* NCIM 1243 with the following objectives.

Objectives

1. To screen and optimize the laccase production from *Pleurotus florida* NCIM 1243 by Plackett–Burman design and Response Surface Methodology.

2. To purify the dye decolorizing laccase isoenzyme by ultrafiltration, ammonium sulfate precipitation, anion exchange and size-exclusion chromatography.

3. Application of *Pleurotus florida* NCIM 1243 laccase in bioremediation processes
   
   (i) To decolorize the synthetic dyes by laccase and to identify the decolorization mechanism.

   (ii) To perform *in vitro* treatment of textile and paper industrial effluents by laccase.

4. To optimize the reactive dye RBBR decolorization by laccase using Response Surface Methodology.

5. To clone and express the laccase gene from *Pleurotus florida* NCIM 1243 into *Saccharomyces cerevisiae* CEN.PK2-1C.