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Fungal Laccases and Their Biotechnological Applications with Special Reference to Bioremediation

Buddolla Viswanath[1,2*] • M. Subhosh Chandra[1,3] • K. Praveen Kumar[1] • H. Pallavi[1] • B. Rajasekhar Reddy[1]**

[1] Department of Microbiology, Sri Krishnadevaraya University, Anantapur - 515 003, Andhra Pradesh, India
[2] Present address: Department of Biotechnology, Center for Water and Waste-water Technology, Durban University of Technology. Durban, 4000, South Africa
[3] Present address: Department of Biotechnology, College of Natural Resources and Life Science, Dong-a University 840, Hadan-dong, Saha-gu, Busan-604 714, South Korea
Corresponding authors: * ViswanathB@dut.ac.za ** rajasekhar_bontha@yahoo.co.in

ABSTRACT

The exploration for efficient and green oxidation technologies has increased the interest in the use of enzymes to replace conventional non-biological methods. Among the different existing oxidant enzymes, laccases have been the subject of study due to their importance in environment protection, where enzymatic catalysis could serve as a more environmentally benign alternative than the currently used chemical processes. Fungal laccases are extracellular multi-copper oxidases mainly secreted by filamentous fungi. They have been attracting the attention of environmental scientists because of their ability to oxidise a wide variety of aromatic compounds. Though the laccases are demonstrated to have a range of promising applications, they are used in bioremediation of soils, water and the development of environmentally friendly processes in the pulp and paper industry. This paper reviews the potential applications of laccase enzymes with special reference to bioremediation.

Keywords: oxidoreductases, bioremediation, decolorization, effluent treatment, xenobiotics
Abbreviations: **ABTS**, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate); **DDT**, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; **EPR**, electron paramagnetic resonance; **NPEOs**, nonylphenol polyethoxylates; **PAH**, polycyclic aromatic hydrocarbons; **PCB**, polychlorinated biphenyls; **PCP**, pentachlorophenols; **TNT**, trinitrotoluene

CONTENTS

INTRODUCTION

Enzymatic oxidation techniques have a large potential within a great variety of industrial fields including the pulp, paper, textile and food industries. Enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones (Rodríguez Couto and Toca Herrera 2006, 2007; Mendonça *et al.* 2008). Thus, laccase is particularly a promising enzyme for the above-mentioned purposes. Laccases (EC 1.10.3.2) are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases, which oxidize diphenols and related substances and use molecular oxygen as an electron acceptor (Thurston 1994; Viswanath *et al.* 2008a). They are a group of oxidative and copper-containing enzymes whose exploitation as biocatalysts in organic synthesis has been neglected in the past, probably because they were not commercially available (Baldrian 2006; Riva 2006; Joo *et al.* 2008). Other members of this group include mammalian plasma protein – ceruplasmin and ascorbate oxidase of plants. Although laccases are known as diphenol oxidases, monophenols like 2,6-dimethoxyphenol or guiacol are used as better substrate than diphenol-catechol or hydroquinone. In spite of display of different physiological functions in different organisms, laccases basically catalyse polymerisation and depolymerisation processes. Reactions catalysed by laccases proceed by monoelectronic oxidation of a suitable substrate molecule to the corresponding reactive radical (Riva 2006). The redox process takes place with assistance of a cluster of copper ions that form catalytic core of the enzyme (Wong 2008). Laccases are of particular interest with regard to potential applications, because of their capabilities to oxidize a wide range of environmentally dangerous substrates. Greater attention on laccase, an ecofriendly enzyme and a green catalyst in recent past is generating information that appeared in a number of reviews (Baldrian 2006; Riva 2006; Singh and Chen 2008; Wong 2008) in the last couple

Received: 25 May, 2008. Accepted: 29 November, 2008.

Invited Review

of years. These reviews dealt laccase-producing organisms, structure of enzyme, reaction mechanisms and general applications. In the present review, emphasis is focussed on molecular biology of laccase genes, heterologous expression of laccase genes and bioremediation.

DISTRIBUTION AND PHYSIOLOGICAL FUNCTIONS

Laccases are common enzymes in nature, being widely found in plants and fungi as well as in some bacteria and insects (Thurston 1994; Gianfreda *et al.* 1999; Mayer and Staples 2002; Claus 2004; Kiiskinen *et al.* 2004; Dong *et al.* 2005; Minussi *et al.* 2007a). The physiological functions of these biocatalysts, which are mostly secreted and sometimes intracellular, are different in various organisms but they all catalyse polymerization or depolymerization processes (Riva 2006). The first laccase was reported in 1883 from *Rhus vernicifera*, the Japanese lacquer tree (Reinhammar 1984), from which the designation laccase was derived and the enzyme was characterized as a metal-containing oxidase by Bertrand (1985). Laccases have subsequently been detected in all species of family Anacardiaceae, and other plants – *Acer pseudoplatanus*, *Pinus taeda*, *Aesculus parviflora* and *Populus eruamericana* (Bligny and Douce 1983; De Marco and Roubelakis-Angelakis 1997; Ranocha *et al.* 1999, Hüttermann *et al*. 2001; Mayer and Staples 2002). Plant laccases are found in the xylem, where they presumably oxidize monolignols in the early stages of lignification (Bao *et al.* 1993; O'Malley *et al.* 1993; Gavnholt and Larsen 2002; Mayer and Staples 2002) and also participate in the radical-based mechanisms of lignin polymer formation (Hoopes and Dean 2004). In addition, laccases have been shown to be involved in the first steps of healing in wounded leaves (De Marco and Roubelakis-Angelakis 1997). However, the occurrence of laccases in higher plants appears to be far more limited than in fungi (Mayer and Staples 2002; Hoegger *et al.* 2006; Baldrian 2006).

Only a few bacterial laccases have been described hitherto. The first bacterial laccase was detected in the plant root-associated bacterium '*Azospirillium lipoferum*', where it was shown to be involved in melanin formation (Givaudan *et al.* 1993; Faure *et al.* 1994). An atypical laccase containing six putative copper-binding sites was discovered in *Marinomonas mediterranea*, but no functional role has been assigned to this enzyme (Sanchez-Amat *et al.* 2001). *Bacillus subtilis* produces a thermostable CotA laccase which participates in pigment production in the endospore coat (Martins *et al.* 2002). Although there are also some other reports about laccase activity in bacteria (Givaudan *et al.* 1993; Martins *et al.* 2002; Claus 2003; Dubé *et al.* 2008), it does not seem probable that laccases are common enzymes in certain prokaryotic groups. Bacterial laccase-like proteins are intracellular or periplasmic proteins (Claus 2003; Baldrian 2006). Currently, all marketed laccases are of fungal origin, but the recent identification and structure determination of a bacterial laccase may eventually broaden the horizon for this enzyme class (Enguita *et al*. 2003). It remains to be seen whether bacterial enzymes can be expressed at levels sufficient for their commercialization.

Laccases are widespread in many fungal species belonging to ascomycetes and basidiomycetes and the enzyme has already been purified from many species. Laccase production in Phytopathogenic ascomyycetes like *Gaeumannomyces graminis* (Edens *et al.* 1999), *Magnaporthe grisea* (Iyer and Chattoo 2003), *Melanocarpus albomyces* (Kiiskinen *et al.* 2002), *Monocillium indicum* (Thakker *et al.* 1992), some soil ascomycete species from the genera *Aspergillus*, *Curvularia* and *Penicillium* (Banerjee and Vohra 1991; Rodríguez *et al.* 1996; Scherer and Fischer 1998), as well as some fresh water ascomycetes (Abdel-Raheem and Shearer 2002; Junghanns *et al.* 2005), was reported. Among yeasts, to date, laccase was only purified from the human pathogen *Cryptococcus* (*Filobasidiella*) *neoformans* (Williamson 1994). This laccase is involved in the synthesis of melanin which was responsible for protection of yeast from animal host oxidative immune response (Liu *et al*. 1999) and fungicides (Ikeda *et al.* 2003). Among physiological groups of fungi, laccases are typical for the wood-rotting basidiomycetes and a related group of litter-decomposing saprotrophic fungi i.e., the species causing lignin degradation. *Agaricus bisporus* (Wood 1980), *Botrytis cinerea* (Marbach *et al.* 1984), *Coprinus cinereus* (Schneider *et al.* 1999), *Phlebia radiata* (Niku-Paavola *et al.* 1988), *Pleurotus ostreatus* (Sannia *et al.* 1986), *Cerrena unicolor* (Kim *et al*. 2002), *Stereum ostrea* (Viswanath *et al.* 2008b), *Phlebia radiata* (Campoy *et al.* 2008), *Fomitella fraxinea* (Park and Park 2008), *Stereum hirsutum* (Mouso *et al.* 2007), *Lentinus tigrinus* (Ferraroni *et al.* 2007) and *Trametes versicolor* (Rogalski *et al.* 1991; Minussi *et al.* 2007b) are some examples of basidiomycetes that produce laccases. Almost all species of white-rot fungi were reported to produce laccases with different production levels (Hatakka 2001). The majority of laccases characterized so far have been derived especially from white-rot fungi because of their abundance (Gianfreda *et al.* 1999; Kiiskinen *et al.* 2004). Fungal laccase plays a role in pigment formation in spores, detoxification of phenol compounds produced during lignin degradation and acts synergesticlly with other enzymes in the breakdown of lignin. In addition to plants, bacteria and fungi, laccases or laccase-like activities have been found in some insects, where they have been suggested to be active in cuticle sclerotization (Sidjanski *et al.* 1997; Dittmer *et al.* 2004).

LACCASE REACTION MECHANISM

Laccases occur often as isozymes with monomeric or dimeric protein structures (Thurston 1994). Most monomeric laccase molecules contain four copper ions in their structure that can be classified in three groups using UV/visible and electron paramagnetic resonance (EPR) spectroscopy (Leontievsky *et al.* 1997). The type I copper (T1) is responsible for the intense blue colour of the enzymes at 600 nm and is EPR-detectable, the type II copper (T2) is colourless, but EPR-detectable, and the type 3 copper (T3) consists of a pair of copper ions that give a weak absorbance near the UV spectrum but no EPR signal (Palmieri *et al.* 1998). The T2 and T3 copper sites are close together and form a trinuclear centre that are involved in the catalytic mechanism of the enzyme (Palmieri *et al.* 1998; Solomon *et al.* 2001; Palmer *et al.* 2003; Quintanar *et al.* 2005; Ferraroni *et al.* 2007; Augustine *et al.* 2008). The three-dimensional structures of a few fungal laccases (Hakulinen *et al.* 2002; Piontek *et al.* 2002) and the CotA laccase from *Bacillus subtilis* (Enguita *et al*. 2003) have been characterised. All fungal laccases show a similar architecture consisting of three sequentially arranged domains of a β-barrel type structure (**Fig. 1**). The active site is well conserved with four copper sites T1 is located in domain 3 with copper lying in a shallow depression and trinuclear copper cluster is at the interface between domains 1 and 3 with each domain providing ligand residues at the coordination of copper ions. The T1 copper is coordinated with His-N and Cys-S as conserved equatorial ligands (Palmer *et al*. 1999). The axial position has Leu or Phe that does not participate in the coordination. The copper-thioether bond and noncoordination residue strongly influence the redox potential of the enzyme. Laccases from different sources displays a wide range of redox potentials. The T1 site of laccase of *T. versicolor* shows a high redox potential of 780-800mV (Pointek *et al*. 2002) where as the plant *R. vernifera* enzyme has a value of 420 mV (Reinhammar 1984). The T1 site is the primary electron acceptor site where the enzyme catalyses four $1e^-$ oxidations of a reducing substrate (Huang *et al.* 1999a, 1999b). 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

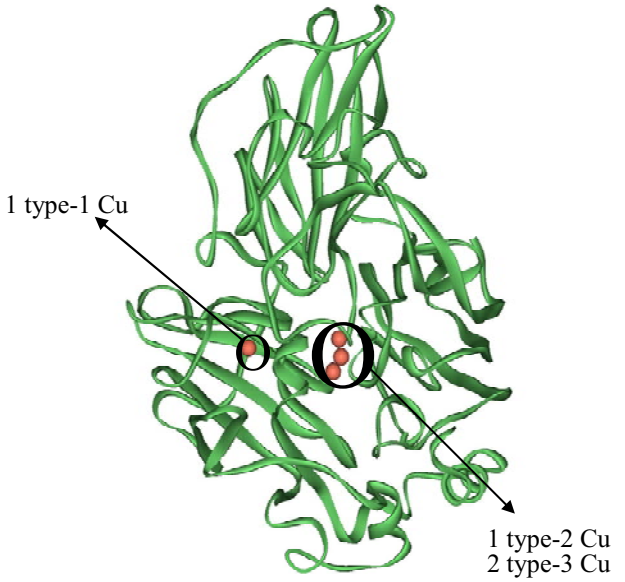

Fig. 1 Ribbon representation of the X-Ray crystallographic structure of *Trametes versicolor* laccase. Modified from figure at www.chem.ox.ac.uk/icl/faagroup/fuelcell.html.

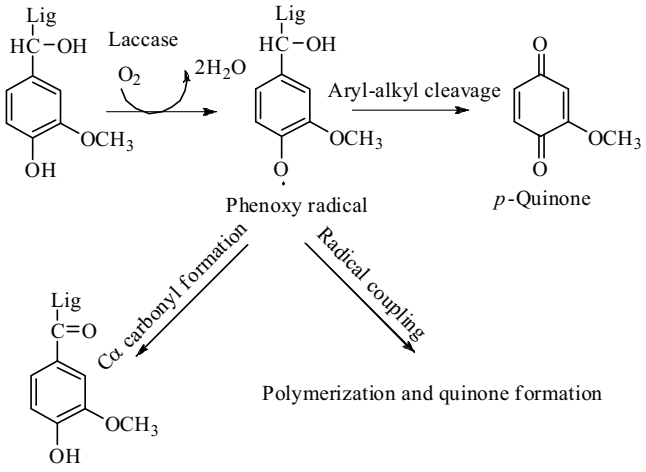

Fig. 2 Oxidation of phenolic sub-units of lignin by laccase.

three His residues. Further, both T2 and T3 copper sites have open coodrination 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).

Laccase promotes substraction of one electron from phenolic hydroxyl groups of lignin to form phenoxy radical (**Fig. 2**). The degradation of lignin proceeds by phenoxy radical that leads to either oxidation at C_α-carbon or cleavage of bond between C_α-carbon and C_β-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), I-hydroxybenzotrizole (HBT) and 3 hydroxyanthranilic acid (Bourbonnais *et al.* 1995, 1998). As oxygen uptake by laccase in presence of ABTS is faster than in HBT, widening of the substrate range of laccase to

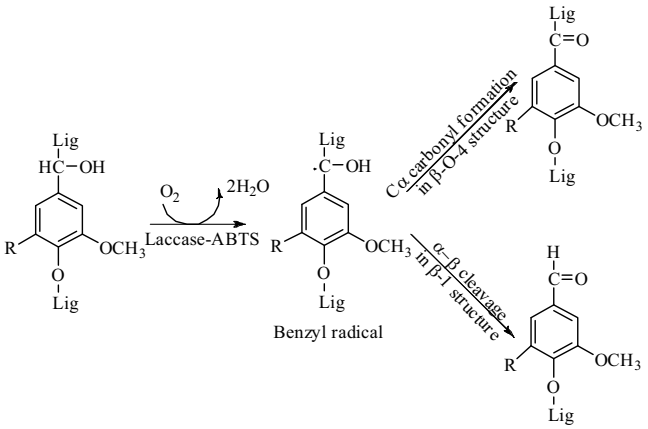

Fig. 3 Oxidation of non-phenolic lignin subunits by laccase and ABTS.

non-phenolic subunits of lignin by the inclusion of a mediator such as ABTS is shown in **Fig. 3**. 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.

MOLECULAR BIOLOGY OF LACCASES

Isozymes of laccases in lignolytic organisms are encoded by multiplicity of laccase gene sequences (Mander *et al*. 2006). For example, the number of *laccase* genes in *Rhizoctonia solani* is four (Wahleithner *et al*. 1996), five in *Trametes* species (Yaver and Golightly 1996; Yaver *et al.* 1996; Hoshida *et al*. 2001), three in the Basidiomycete I–62 (Mansur *et al.* 1997), at least two in *Agaricus bisporus* (Smith *et al.* 1998), at least three in *Pleurotus species* (Giardina *et al.* 1999; Soden and Dobson 2001; Palmieri *et al.* 2003; Rodríguez *et al*. 2008), four in *Podospora anserine* (Fernandez-Larrea and Stahl 1996), two in *Lentinula edoeles* (Zhao and Kwan 1999) or three in *Gaumannomyces graminis* (Litvintseva and Henson 2002). For the first time, laccase genes were isolated and sequenced about 20 years ago from the fungi *Neurospora crassa* (Germann and Lerch 1986), *Aspergillus nidulans* (Aramayo and Timberlake 1990), *Coriolus hirsutus* (Kojima *et al.* 1990), and *Phlebia radiata* (Saloheimo *et al.* 1991). Since then, sequencing of laccase genes has increased considerably. Laccase genes in lignolytic fungi have been cloned and characterized (Kojima *et al* 1990; Saloheimo *et al.* 1991; Coll *et al* 1993; Giardina *et al.* 1995; Yaver *et al*. 1996; Jonsson *et al.* 1997). The overall intron–exon structure of all three laccase genes in *G. graminis* (Litvintseva and Henson 2002) and in *P. ostreaus* (Giardina *et al*. 1999) is distinct. Analysis of *laccase* genes in different organisms points out conserved sequences in genes that encode copper binding regions of N-terminal domain of laccase enzyme. These sequences are *laccase* gene-specific and have become basis for PCR–based screening for the presence of the laccase genes in organisms (Pointing *et al.* 2005). However, the number of laccase genes of which the corresponding protein products have been experimentally characterized is significantly lower. To date, there are about 50 such enzymes, most of which are fungal laccases (Morozova *et al.* 2007). A typical laccase gene codes for a protein of 500-600 amino acids with a molecular weight usually ranging from 60 to 90 kDa, when determined by SDS-PAGE (Kiiskinen 2004; Jolivalt *et al.* 2005; Viswanath *et al.* 2008b). The difference between the molecular weight predicted from the peptide sequence and the experimentally obtained molecular weight is caused by glycosylation, which typically accounts for about 10-20% of the total MW (Froehner and Eriksson 1974; Coll *et al.* 1993; Giardina *et al.* 1996; Wahleithner *et al.* 1996; Dedeyan *et al.* 2000; Galhaup *et al.* 2002). Although laccase production in organisms is known to be influenced by a number of physiological and

environmental factors, little work has been done to examine the regulation of expression of *laccase* genes at molecular level (Fernandez–Larrea and Stahl 1996; Mansur *et al*. 1997). Probing by PCR-based and Northern blot methods indicates that expression of *laccase* genes in organisms was differentially regulated. For instance, *lcc*1 gene in a pathogen *G. graminis* was transcribed constitutively under all conditions whereas *lcc*2 was copper–inducible (Litvintseva and Henson 2002). Transcription of *lcc*3 was observed only when the fungus was grown in association with the host plant. Similar observation on induction of transcription of *laccase* gene in another pathogen – *Botrytis cinera* occurred with only addition of pectin product of the host plant to the culture (Pezet 1998). The animal pathogen – *C. neoformis* expresses laccase that is involved in biosynthesis of melanin-like pigment in mouse tissue. Transcript levels of *lcc1* and *2* in Basidiomycete I-62 were inducible by veratryl alcohol during different stages of life cycle whereas *lcc3* was not inducible (Mansur *et al.* 1998). Addition of 2,5 xylidine to the culture increased *lcc1* mRNA levels in *Trametes villosa* (Yaver and Golightly 1996) but did not affect *lcc*2 mRNA levels.

Laccase production is subject to complex regulation by nutrients in the culture medium during the fungal growth (Soden and Dobson 2001; Cavallazzi *et al.* 2005; Dekker *et al*. 2007). Expression of laccase in some fungi is regulated by nitrogen-limiting conditions (Kirk and Farrell 1987; Perie and Gold 1991; Eggert *et al*. 1996; Pointing *et al.* 2000) while in others nitrogen sufficiency results in enhanced enzyme production. The effect of various nutrient nitrogen concentrations on expression of *lcc* genes at molecular level in *T. Versicolor* was examined (Collins and Dobson 1997). There was a direct correlation between concentration of nitrogen nutrient in growth medium and the level of *lcc* expression by *T. versicolor*. In Basidiomycetes 1-62 under nonlimiting nitrogen conditions, *lcc1* and *lcc2* transcript levels increased 100-fold over limting conditions (Mansur *et al.* 1998). The use of 1% cellulose as a carbon source increased both *lcc1* mRNA and *lcc2* mRNA of *Lentinula edodes* (Zhao and Kwan 1999). A medium with high nitrogen has been shown to induce transcription of laccase genes in the Basidiomycete I-62 (CECT 20197) and in *Pleurotus sajor-caju* (Soden and Dobson 2001).

Copper is also often a strong inducer of laccase gene transcription, and this has been suggested to be related to a defense mechanism against oxidative stress caused by free copper ions (Fernandez-Larrea and Stahl 1996; Collins and Dobson 1997; Palmieri *et al.* 2000; Soden and Dobson 2001; Galhaup *et al.* 2002; Litvintseva and Henson 2002; Dekker *et al.* 2007). In addition to copper, other metal ions such as Mg^{2+}, Cd^{2+} or Hg^{2+} can stimulate laccase expression (Scheel *et al.* 2000; Soden and Dobson 2001; Galhaup *et al.* 2002). Metal response elements (MRE) with consensus sequences are found in the promoter regions of two fungal *laccase* genes of *P. anserine* (Fernandez–Larrea and Stahl 1996) and the Basidiomycete PM 1 (Coll *et al.* 1993). Involvement of these elements on copper induction of expression of *lcc* genes needs to be further assessed.

Certain aromatic compounds that are structurally related to lignin precursors, such as 2,5-xylidine or ferulic acid, have also been shown to increase laccase gene transcription in *Trametes villosa*, *T. versicolor* and *Pleurotus sajor-caju* (Yaver *et al.* 1996; Collins and Dobson 1997; Soden and Dobson 2001). Current understanting of differential expression of *laccase* genes in lignolytic organisms is fragmentary and needs to be further improved.

HETEROLOGOUS PRODUCTION OF LACCASES

The most important obstacles to commercial application of laccases are the lack of sufficient enzyme stocks and the cost of redox mediators. In order for laccases to be effective in environmental detoxification, large amounts of active enzyme are needed. Since an inexpensive source of laccase must be obtained for the some potential applications to become a reality. There have been several reports of heterologous expression of recombinant laccases (**Table 1**). *Laccase* genes from *Myceliophthora thermophila* (Bulter *et al.* 2003), *Trametes versicolor* (Cassland and Jonsson 1999; Klonowska *et al.* 2005), *Coriolus hirsutus* (Kojima *et al.* 1990) and *Melanocarpus albomyces* (Kiiskinen and Saloheimo 2004) in *Saccharomyces cerevesiae* were expressed. Similarly, expression of *laccase* genes from *Pleurotus sajor – caju* (Soden *et al.* 2002), *Trametes trogii* (Colao *et al.* 2006) and *Pycnoporus cinnabarinus* (Otterbein *et al.* 2000) was in *Pichia pastoris* (Hong *et al.* 2002), those from *Pleurotus ostreus* (Piscitelli *et al*. 2005; Faraco *et al.* 2008) in *Kluveromyces lactis*, *Trametes versicolor laccase gene*s in *Yarronia lipolytica* (Jolivolt *et al.* 2005) and the *laccase* gene of *Coriolus hirsitus* (Sonoki *et al.* 2005) and *Lentinula edodes* (Sakamoto *et al.* 2008) was in tobacco, *Myceliophthora laccase* gene in *Aspergillus oryzae* (Berka *et al.* 1997), and the *Pycnoporus laccase* gene (Record *et al.* 2002) and *Trametes versicolor* in *Aspergillus niger* (Bohlin *et al.* 2006). Laccase production levels have often been improved significantly by expression in heterologous hosts, but the reported levels are still rather low for industrial applications (**Table 1**). The common problems associated with heterologous expression of fungal enzymes are incorrect folding of the polypeptide and inefficient codon usage of expression organisms, resulting in non-functional or low yields of enzyme. The incorrect substitution of carbohydrate residues during glycosylation of proteins, which is due to preferential utilization of specific carbohydrates by the expression organism, may pose an additional problem to heterologous expression. These problems are being overcome by using more advanced organisms as expression hosts whose codon usage and molecular folding apparatus are suitable for correct expression of these proteins.

The importance of adequate copper concentration for proper laccase folding was further corroborated by studies in which two genes related to copper-trafficking in *T. versicolor* were over-expressed in *S. cerevisiae* expressing *T. versicolor lcc*3 gene; the heterologous laccase production by *S. cerevisiae* was improved up to 20-fold (Uldschmid *et al.* 2003). The effect was suggested to result from more efficient transport of copper to the Golgi compartment (Uldschmid *et al.* 2003).

Directed evolution has also been used for improving heterologous laccase production. A site directed mutation/ random mutation in the *Myceliophthora thermophila* laccase gene accompanied by evolution resulted in the highest

Table 1 Laccase production in heterologous hosts

Laccase gene	Production host	Laccase production ($mg1^{-1}$)*
Ceriporiopsis subvermispora lcs-1 (Larrondo *et al.* 2003)	*Aspergillus nidulans*	1.5
	Aspergillus niger	1.5
Myceliophthora thermophila lcc1 (Bulter *et al.* 2003)	*Saccharomyces cerevisiae*	18
Pleurotus sajor-caju lac4 (Soden *et al.* 2002)	*Pichia pastoris*	4.9
Pycnoporus cinnabarinus lac1 (Record *et al.* 2002)	*Aspergillus niger*	70
Pycnoporus cinnabarinus lac1 (Sigoillot *et al.* 2004)	*Aspergillus oryzae*	80
Trametes versicolor (Jolivalt *et al.* 2005)	*Yarrowia lipolytica*	2.5
Trametes versicolor lac1 & lac2 (Bohlin *et al.* 2006)	*Pichia pastoris*	2.8
Trametes trogii lac1 (Colao *et al.* 2006)	*Pichia pastoris*	17

* The reported production levels have been obtained in shake flask cultivations, except in the case of *Phlebia radiata* laccase which was produced in a laboratory fermenter.

reported laccase production level in *S. cerevisiae* but also enhanced K_{cat} of the enzyme (Bulter *et al.* 2003). A similar strategy with *Fome lignosus* yielded mutant laccase enzyme with four substitutions (Hu *et al.* 2007). This mutant enzyme exhibited improved kinetic properties due to rapid movement of water along water channel in the enzyme molecule. A themostable laccase that tolerates high concentrations of solvents is the genetic product of five rounds of directed evolution in *Saccchromyces cerevisiae* (Zumarraga *et al*. 2007). Thus, efforts have to be made in order to achieve cheap overproduction of this biocatalyst in heterologous hosts and also their modification by chemical means of protein engineering to obtain more robust, active and tailormade enzymes.

INDUSTRIAL APPLICATIONS

Laccases of fungi 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. Laccases are currently studied intensively for many applications and they are already used in large scale in the textile industry (Tzanov *et al.* 2003; Rodríguez Couto and Toca Herrera 2006; Casieri *et al*. 2008). Related to textile bleaching, in 1996 Novozyme (Novo Nordisk, Denmark) launched laccase enzyme in denim finishing: DeniLite. 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. Together with low molecular weight redox-mediator compounds, laccases can generate a desired worn appearance on denim by bleaching indigo dye (Campos *et al.* 2001). The potential use of laccase for bleaching has been investigated and this has even led to the esoteric suggestion of using laccase in the presence of hydroxyl stilbenes as hair dyes (Onuki *et al.* 2000). Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases are able to oxidize toxic organic pollutants, such as polycyclic aromatic hydrocarbons (Manzanares *et al.* 1995; Canas *et al.* 2007; Martin *et al.* 2007; Camarero *et al.* 2008) and chlorophenols (Gianfreda *et al.* 1999; Michizoe *et al.* 2005; Ford *et al.* 2007). The most useful method for this application would probably be inoculating the soil with fungi that are efficient laccase-producers, because the use of isolated enzymes is not economically feasible for soil remediation in large scale. The current practical applications of the use of laccase, has led to a search for source of the enzyme from white-rot fungi, and the use of mediators, which promote or facilitate enzyme action.

Laccases, when acting on lignin, can display both ligninolytic and polymerizing (cross-linking) abilities. The substrate range of laccase can be extended to cover both phenolic and non-phenolic compounds by means of laccase-mediator systems (LMS), which makes laccase suitable for, e.g., biobleaching of lignocellulosic pulps. As laccase/LMS can also help remove pitch, phenolic contaminants, and dyes from wood-based materials and water, laccase technology is virtually applicable to the entire production chain of paper products from pulping to recovery of secondary fibers and effluent treatment. Indeed, most of the published research and applications of laccase/LMS in the forest products industry relate to the pulp and paper sector, where particular emphasis has been placed on studying laccase/LMS for use in biobleaching and mill water treatment (Petri and Andreas 2008). Emerging research areas include the tailoring of lignocellulosic materials by laccase-assisted biografting of phenols and other compounds, and the use of laccase for adhesion enhancement in binderless wood boards (Petri and Andreas 2008). Recently, the utility of laccases has also been applied to Nanobiotechnology (Rodríguez Couto and Toca Herrera 2006). This is an increasing research field mainly due to the fact that laccases are able to catalyse

Table 2 Recent industrial applications of fungal laccases.

Application	Laccase source	Reference
Decolorization of dyes	*Trametes medusta*	Rehorek *et al.* 2004
	Trametes versicolor	Blanquez *et al*. 2004
	Pycnoporus cinnabarinus	Camarero *et al*. 2004
	T. versicolor	Camarero *et al*. 2004
	Pleurotus ostreatus	Hou *et al.* 2004
	Pleurotus eryngii	Camarero *et al*. 2004
	T. villosa	Knutson and Ragauskas 2004
	T. versicolor	Tavares *et al.* 2004
	T. versicolor	Moreira *et al.* 2004
	Trametes hirsuta	Rodriguez Couto *et al.* 2004a
	T. hirsuta	Rodriguez Couto *et al.* 2004b
	T. hirsuta, *T. versicolor*	Rodriguez Couto *et al.* 2004c
	P. ostreatus	Palmieri *et al*. 2005
	Coriolopsis rigida	Gomez *et al.* 2005
	Funaria trogii	Unyayar *et al*. 2005
	T. hirsuta	Dominguez *et al.* 2005
	T. medusta	Michael *et al.*2005
	Trametes trogii	Levin *et al.* 2005
	T. hirsuta	Rodriguez Couto *et al.* 2005
	Collybia sp.	Mc Erlean *et al*. 2006
	Collybia dryophila	Baldrian and Snajdr 2006
	Mycena inclinata	Baldrian and Snajdr 2006
	T. versicolor	McErlean *et al*. 2006
	P. ostreatus	McErlean *et al*. 2006
	P. ostreatus CCBAS477	Baldrian and Snajdr 2006
	P. sajor-caju	Murugesan *et al.* 2006
	Pycnoporus coccineus	Chairattanamanokorn *et al.* 2006
	Rhizoctonia solani	Mc Erlean *et al*. 2006
	Stropharia rugosoannulata	Baldrian and Snajdr 2006
	T. versicolor CCBAS 614	Baldrian and Snajdr 2006
	T. hirsuta	Rodriguez Couto *et al.* 2006
	T. hirsuta	Rodriguez Couto and Toca Herrera 2006
	T. versicolor CCT 4521	Minussi *et al*. 2007b
	Stereum ostrea	Viswanath *et al.* 2008b
Degradation of xenobiotics	*Trametes pubescens*	Nicotra *et al.* 2004
	Panus tigrinus	Zavarzina *et al.* 2004
	Cladosporium sphaerospermum	Potin *et al.* 2004
	Myceliophtora thermophila	Nicotra *et al.* 2004
	Rhus vernicifera	Moeder *et al*. 2004
	T. versicolor	Dodor *et al.* 2004
	T. versicolor	Mun-Jung *et al.* 2004
	P. ostreatus, *T. versicolor*	Keum and Li 2004
	Clavariopsis aquatica	Junghanns *et al.* 2005
	Stachybotrys chartarum	Mander *et al.* 2006
	Stropharia rugosoannulata	Steffen *et al.* 2007
	Stropharia coronilla	Steffen *et al.* 2007
	Coriolopsis polyzona	Cabana *et al.* 2007
	Rigidoporus lignosus	Cambria *et al.* 2008
Effluent treatment	*Rhus vernicifera*	Durante *et al.* 2004
	Panus tigrinus	D' Annibale *et al*. 2004
	Pycnoporus coccineus	Jaouani *et al*. 2005
	Phanerochaete chrysosporium	Lee *et al.* 2006
	T. versicolor CCT 4521	Minussi *et al*. 2007b
	T. versicolor	Pedroza *et al.* 2007
	Trametes versicolor	Minussi *et al.* 2007a
	Trametes villosa	Minussi *et al.* 2007a
	Lentinula edodes	Minussi *et al.* 2007a
	Botrytis cinerea	Minussi *et al.* 2007a
	Trametes trogii	Ellouze *et al.* 2008
	Lentinus tigrinus	Ellouze *et al.* 2008

Table 2 (Cont.)

Application	Laccase source	Reference
Biosensors	*Agaricus bisporus*, *T. versicolor*	Timur *et al*. 2004
	Aspergillus niger	Timur *et al*. 2004
	Agaricus bisporus, *T. versicolor*	Vianello *et al*. 2004
	C. unicolor	Jarosz-Wilkolazka *et al.* 2004
	T. versicolor	Gomes *et al.* 2004
	T. versicolor	Ferry and Leech 2005
	T. versicolor	Roy *et al.* 2005
	C. unicolor	Jarosz-Wilkolazka *et al.* 2005
	Coriolos versicolor	Liu *et al.* 2006
	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	*Phanerochaete chrysosporium, Pleurotus eryngii*	Sigoillot *et al*. 2005
	Trametes versicolor, *T. villosa*	Minussi *et al.* 2007a
	Lentinula edodes	Minussi *et al*. 2007a
	Botrytis cinerea	Minussi *et al.* 2007a
	Trametes versicolor	Oudia *et al.* 2008
Organic synthesis	*Rigidoporus lignosus*	Garavaglia *et al.* 2004
	Lentinus tigrinus	Ferraroni *et al.* 2007
	Myceliophthora thermophila	Zumárraga *et al.* 2007
	Pleurotus ostreatus	Festa *et al.* 2008
Food industry	*Phellinus robustus*	Songulashvili *et al.* 2006
	Ganoderma adspersum	Songulashvili *et al.* 2006
	Trametes hirsuta	Selinheimo *et al*. 2006
	Trametes hirsuta	Schroeder *et al.* 2008
	Trametes hirsuta	Flander *et al*. 2008

electron transfer reactions without additional cofactors and to the development of several techniques for the immobilisation of biomolecules such as micropatterning, self-assembled monolayers and layer-by-layer technique. These techniques can be used to immobilize laccases preserving their enzymatic activity. **Table 2** shows several recent applications of laccases with reference to bioremediation.

LACCASES - ROLE IN BIOREMEDIATION

Laccases have received much attention from researchers during the past decades due to their broad substrate range and to the fact that they use molecular oxygen as the final electron acceptor instead of hydrogen peroxide as used by peroxidases. This makes laccases highly interesting for a wide variety of processes, such as textile dye decolourization, pulp bleaching, effluent detoxification, biosensors and bioremediation. 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. Eighty billion pounds of hazardous organopollutants are produced annually in the United States and only 10% of these are disposed of safely (Reddy and Mathew 2001). 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). Enzymatic treatment is currently considered an alternative method for the removal of toxic xenobiotics from the environment (Gianfreda *et al.* 1999).

The laccase of the ectomycorrhizal fungus *Xerocomus chrysenteron* responds to DDT stress in various ways, suggesting a large potential of biodegradation or mineralization of DDT (Chao *et al.* 2008). Biodegradation of 2,4-dichlorophenol using response surface methodology by the laccase of *Pleurotus* sp. is one of the recent applications demonstrated by Bhattacharya and Banerjee (2008). The mechanism(s) of bisphenol A (BPA) to induce cell proliferation and the occurrence of its bioremediation by treatment with laccase are reported by Bolli *et al.* (2008). BPA, a naturally-occurring pollutant that can be used as a model of environmental estrogen action complexity, promotes human cancer cell proliferation via ER-alpha-dependent signal transduction pathways. BPA oxidation by laccase impairs the binding of this environmental estrogen to ER-alpha loosing all its ER-alpha-dependent effect on cancer cell proliferation. Moreover, the laccase-catalyzed oxidation of BPA reduces the BPA cytotoxic effect (Bolli *et al.* 2008). Recently, aqueous solutions polluted by BPA have been bioremediated by using laccase (*Trametes versicolor*) immobilized on hydrophobic membranes in non-isothermal bioreactors (Ricupito *et al.* 2008). Mohamad *et al.* (2008) described the design of a laccase of *Trametes versicolor* with broader substrate spectrum in bioremediation. They described the application of evolutionary trace (ET) analysis of laccase at the ligand binding site for optimal design of the enzyme. In this attempt, class specific sites from ET analysis were mapped onto known crystal structure of laccase. These findings provide a foundation to the design of laccase with a broader substrate spectrum for further expansion of laccase application in industry and bioremediation.

Degradation of xenobiotics

One of the most efficient processes to remove pollutants from environment is through biodegradation of xenobiotics. It is the process by which living organisms degrade or transform hazardous organic contaminants into less toxic compounds. Screening of indigenous microbes from the pollutant contaminated site for their degradation potential is one way to approach the problem. Thus, microorganisms that can degrade various pollutants have been isolated with the eventual goal of exploiting their metabolic potential for the degradation of polluted sites (Spain *et al.* 2000; Dua *et al.* 2002; Furukawa 2003).

Laccases are able to oxidize a broad range of xenobiotic compounds including chlorinated phenolics (Bollag *et al.* 2003; Colao *et al.* 2006), pesticides (Torres *et al.* 2003; Gorbatova *et al.* 2006; Ford *et al.* 2007) and polycyclic aromatic hydrocarbons (Pozdnyakova *et al.* 2004; Tekere *et al.* 2007; Koschorreck *et al.* 2008). Moreover, polycyclic aromatic hydrocarbons, which arise from natural oil deposits and utilization of fossil fuels, were also found to be degraded by laccases (Pointing 2001; Hu *et al.* 2007; Galli *et al.* 2008). Laccase purified from a strain of *Coriolopsis gallica* oxidized carbozole, *N*-ethylcarbozole, fluorine and dibenzothiophene in presence of 1-hydroxybenzotriazole and 2.2'-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid as free radical mediators (Bressler *et al.* 2000). Patel *et al.* (2008) established the effective role of laccase of *Pleurotus ostreatus* HP-1 in bioremediation of PAHs contaminated sites. Laboratory experiments have demonstrated that phenols and aromatic amines may be removed from water by the application of laccase (Dec and Bollag 2000). The underlying mechanism of the removal involves enzymatic oxidation of the pollutants to free radicals or quinones that undergo polymerization and partial precipitation (Dec and Bollag 2000). Laccase from white-rot fungus, *Trametes hirsuta*, has been used to oxidize alkenes (Niku-Paavola and Viikari 2000). The oxidation is the effect of a two-step process in which the enzyme first catalyses the oxidation of primary substrate, a mediator added to the reaction, and then the oxidized mediator oxidizes the secondary substrate, the alkene, to the corresponding ketone or aldehyde. In addition to substrate

oxidation, laccase can also immobilize soil pollutants by coupling to soil humic substances – a process analogous to humic acid synthesis in soils (Bollag and Myers 1992). The xenobiotics that can be immobilized in this way include phenolic compounds and anilines such as 3,4-dichloroaniline, 2,4,6-trinitrotoluene or chlorinated phenols (Ahn *et al.* 2002). The immobilization lowers the biological availability of the xenobiotics and thus their toxicity. A laccase produced in the yeast, *Pichia pastoris*, was engineered by site-directed mutagenesis to improve the rate of electron transfer between the copper-containing active site of laccase and an electrode (Gelo *et al.* 1999). Thus, laccase may be usefully engineered to improve the efficiency of particular bioremediation processes.

Decolourization of dyes

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 (Riu *et al.* 1998). The chemical reagents used are very diverse in their composition, ranging from inorganic compounds to polymers and organic products (Banat *et al.* 1996). There are about 1,000,000 commercially available dyes with over 7×10^5 tonnes of dyestuff produced annually (Zollinger 2002). Representatives of different classes of synthetic dyes, classified in the colour index (C.I.) according to chemical structure (Wesenberg *et al.* 2003). 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. Government legislation is becoming more and more stringent, especially in the more developed countries, regarding the removal of dyes from industrial effluents (Poots and McKay 1976). Concern arises, as several dyes are made from known carcinogens such as benzidine and other aromatic compounds. Most currently existing processes to treat dye wastewater are ineffective and not economical. Therefore, 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 (Rodríguez Couto *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 (Setti *et al.* 1999; Kandelbauer *et al.* 2004). *Flavodon flavus* decolourized several synthetic dyes like Azure B, Brilliant Blue R in low nitrogen medium (Raghukumar 2000). Alternatively, laccase, along with stabilizers, may be suitable for treatment of wastewater (Soares *et al.* 2001a, 2001b). Partial decolorization of two azo dyes and complete decolorization of two triphenylmethane dyes (bromophenol blue and malachite green) was achieved by cultures of *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase (Pointing and Vrijmoed 2000). *Trametes hirsuta*, and a laccase purified from the fungus, was able to degrade triarylmethane, indigoid, azo and anthraquinonic dyes used in dyeing textiles (Abadulla *et al.* 2000b) as well as 23 industrial dyes (Rodríguez *et al.* 1999). The purified laccase of white rot fungi *Pleurotus ostrea and Stereum ostrea*, decolorized textile dyes i.e., Remazol black-5, Remazol blue-19 and Remazol orange-16 (Palmieri *et al.* 2005; Viswanath *et al.* 2008b). More than 90% of decolorization activity was observed after 16 h incubation with laccase at 20 *n*kat/ml for all the textile dyes used in their study. The degradation of azo dyes, which are the most widely used colorants, has been studied most (Heinfling *et al.* 1997; Chagas and Durrant 2001; Martins *et al.* 2003; Ciullini *et al.* 2008; Tauber *et al.* 2008). Other classes, such as anthraquinone (Knapp *et al.* 1995; Swamy and Ramsay 1999; Lu *et al.* 2007; Guo *et al.* 2008), phthalocyanin (Knapp *et al.* 1995; Heinfling *et al.* 1997; Huang *et al.* 2007) and polymeric dyes (Glenn and Gold 1983; Camarero *et al.* 2005) were also shown to be susceptible to biodegradation by laccases of white rot fungi. The demand for removal of synthetic dyes released from textile industries using laccase is being increased tremendously (Abadulla *et al.* 2000a; Zille *et al*. 2003; Baldrian and Snajdr 2006; Viswanath *et al.* 2008b; Casieri *et al.* 2008). Therefore, searching for potential laccases to cope with this demand is an important task in the area of dye degradation.

Effluent treatment

Laccases from fungi offer several advantages of great interest for biotechnological applications of industrial effluent treatment. As they exhibit broad substrate specificity, they can bleach kraft pulp or detoxify agricultural byproducts including olive mill wastes or coffee pulp (D'Annibale *et al.* 2000). Laccase of an isolate of the fungus, *Flavodon flavus,* was shown to decolourize the effluent from a kraft paper mill bleach plant (Raghukumar 2000). Laccase purified from white-rot basidiomycete, *T. villosa* degrades bisphenol A, an endocrine-disrupting chemical (Setti *et al.* 1999). Nonylphenols have increasingly gained attention because of their potential to mimic the action of natural hormones in vertebrates (Ying *et al.* 2002). They result from incomplete biodegradation of nonylphenol polyethoxylates (NPEOs), which have been widely used as non-ionic surfactants in industrial processes. Both nonylphenols and NPEOs are discharged into the environment, mainly due to incomplete removal of wastewater treatment facilities (Ying *et al.* 2002). Nonylphenols are more resistant to biodegradation than their parent compound and hence are found worldwide in wastewater treatment plant effluents and rivers (Heemken *et al.* 2001). Due to their hydrophobicity, they tend to absorb onto surface water particles and sediments and accumulate in aquatic organisms. Consequently, nonylphenols represent a serious environmental and human health risk. Laccases from aquatic hyphomycete *Clavariopsis aquatica*, has proved to degrade xenoestrogen nonylphenol (Junghanns *et al.* 2005). In addition to the potential role of such degradation processes for natural attenuation processes in freshwater environments, laccase also offers new perspectives for biotechnological applications such as wastewater treatment.

Simple bioinformatics searches that incorporate multiple sources of data offer a quicker and more rapid means of identifying new potential targets for bioremediation as compared to conventional method. Protein-ligand docking tool can be used to screen pollutants for their susceptibility to degradation by already characterized enzyme. As the substrate specificity differs from one laccase to other, laccase from different sources can be utilized for degrading different pollutants. Although docking has been successfully used for drug screening (Drews 2000), its utility in predicting the pollutants which can be potential targets for bioremediation has not been acknowledged so far (Suresh *et al.* 2008).

The most important obstacles for reaping benefits from non-specific reaction mechanism of laccase for bioremediation purpose in maintenance of clean environment are lack of inexpensive enzyme stocks and redox mediators. Rapid progress has been made over the past few years and current level of understanding of regulation of expression of *laccase* genes has to be improved further. Efforts have to be put forth for expression of *laccase* genes in heterologous hosts with larger capacity for secretion of protein. It is expected that laccases will be able to compete with other processes such as elemental chlorine free totally chlorine bleaching and effluent treartment. Tailor-made laccase for specific purposes will become a possibility.

CONCLUSIONS

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. This review provided infor-

mation on distribution and physiological functions of laccases with their biotechnological applications with special reference to bioremediation with recent account. In addition, deeper understanding of the biochemistry of laccase will facilitate the development of novel and more economical laccase applications.

ACKNOWLEDGEMENTS

Buddolla Viswanath was supported by a grant from University Grants Commission. We are very grateful to the University Grants Commission, New Delhi, India for financial support and encouragement.

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