LACCASE: PROPERTIES AND APPLICATIONS

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Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases that are widely distributed among plants, insects, and fungi. They have been described in different genera of ascomycetes, some deuteromycetes, and mainly in basidiomycetes. These enzymes catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, amino-phenols, methoxyphenols, aromatic amines, and ascorbate, with the concomitant four electron reduction of oxygen to water. Laccase is currently the focus of much attention because of its diverse applications, such as delignification of lignocellulosics, crosslinking of polysaccha-rides, bioremediation applications, such as waste detoxification, and textile dye transformation, food technologic uses, personal and medical care applications, and biosensor and analytical applications. This review helps to understand the properties of this important enzyme for efficient utilization for its biotechnological and environmental applications.

Keywords: Laccase; Lignin; Bioremediation; Dye decolorization; White rot fungi

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INTRODUCTION

Laccase (EC 1.10.3.2, p-diphenol oxidase) is one of a few enzymes that have been studied since the nineteenth century. Yoshida first described laccase in 1883 from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Thurston 1994; Levine 1965). However in 1896, for the first time, both Bertrand and Laborde demonstrated laccase to be a fungal enzyme (Thurston 1994; Levine 1965). Laccases are copper-containing enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines and ascorbate with the concomitant four electron reduction of oxygen to water (Galhaup et al. 2002). Laccase is a member of the large blue copper proteins or blue copper oxidases; other enzymes in this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin (Thurston 1994). The ability of laccases to oxidize phenolic compounds as well as their ability to reduce molecular oxygen to water has led to intensive studies of these enzymes (Thurston 1994).

Occurrence

Laccase is most widely distributed in a wide range of higher plants and fungi (Benfield et al. 1964) as well as in bacteria (Diamantidis et al. 2000). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears,

and various other vegetables (Levine 1965). It has been detected in various plant species, including lacquer, mango, mung bean, peach, pine, prune, and sycamore, etc. Laccase is also found to be present in a dozen insects of genera that include *Bombyx*, *Calliphora*, *Diploptera, Drosophilia, Lucilia, Manduca, Musca, Oryctes, Papilio, Phormia, Rhodnius, Sarcophaga, Schistocerca*, and *Tenebrio* (Xu 1999). Most of the laccase described in literature was isolated from higher fungi. Laccases have been isolated from ascomycetes, deuteromycetes, and basidiomycetes fungi (Assavanig et al. 1992). Laccase from *Monocillium indicum* was the first laccase to be characterized from an ascomycete showing peroxidative activity (Thakker et al. 1992). In fungi, ascomycetes and deuteromycetes have not been a focus for lignin degradation studies as much as the white-rot basidiomycetes. Most common laccase producers are the wood rotting fungi *Trametes versicolor*, *Trametes hirsuta*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Cerena maxima*, *Coriolposis polyzona*, *Lentinus tigrinus*, *Pleurotus eryngii*, etc. (Morozova et al. 2007). Although laccase enzymes are widely distributed in plants and fungi, laccase activity has been reported only in few bacteria, including *Azospirillum lipoferum, Marinomonas mediterranea, Streptomyces griseus, and Bacillus subtilis* (Octavio et al. 2006).

Molecular Properties of Laccase

Laccase is a glycosylated monomer or homodimer protein generally having fewer saccharide compounds (10-25 %) in fungi and bacteria than in the plant enzymes. The carbohydrate compound contains monosaccharides such as hexoamines, glucose, mannose, galactose, fucose, and arabinose (Rogalski and Leonowicz 2004). On SDS-PAGE, most laccases show mobilities corresponding to molecular weight of 60-100 kDa, of which 10-50% may be attributed to glycosylation. Mannose is one of the major components of the carbohydrate attached to laccase. Glycosylation in laccase is responsible for secretion, proteolytic susceptibility, activity, copper retention, and thermal stability (Xu 1999).

Most laccases studied are extracellular proteins, although intracellular laccases have been detected in several fungi and insects. Fungal laccases have isoelectric points (pI) ranging from 3 to 7, whereas plant laccase pI values range to 9. The main difference between the two enzymes is that fungal enzymes have their pH optima between pH 3.6 and 5.2, while laccase from *Rhus vernicifera* have pH optima between 6.8 and 7.4. The low pH optima of the fungal enzyme may be because they are well adapted to grow under acidic conditions, while the plant laccase being intracellular have their pH optima nearer to the physiological range. Thus the differences in pH optima may be because of the dissimilarity in physiological functions. In addition to their variation in pH, these enzymes also differ in their function. Fungal enzyme is responsible in mechanism for removing toxic phenols from the medium in which these fungi grow under natural conditions, while the plant enzymes are involved in synthetic process such as lignin formation (Benfield et al. 1964).

Purified laccases exhibit a characteristic blue appearance from their electronic absorption around 600nm. Typical UV- Visible spectra of laccase (at resting state) show two maxima around 280 and 600 nm and one shoulder near 330nm. The ratios of the absorbance at 280 nm to that at 600 nm are generally 14 to 30, and the ratio of the

absorbance at 330nm to that at 600 nm is 0.5 to 2 (Xu 1999). In the holoenzyme form, most laccases have four copper atoms per monomer (Coll et al. 1993), although the laccase from *Phlebia* was reported to have two copper and one pyrroloquinoline quinone as the prosthetic group. These copper atoms are 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 and has a strong electronic absorption around 600nm 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 atoms that give a weak absorbance near the UV spectrum but no EPR signal. The T2 and T3 copper sites are close together and form a trinuclear centre (Leontievsky et al. 1997) in which binding dioxygen and four-electron reduction to water occur.

Copper of the first type can be acted upon by solvents including water, and removed from the enzyme molecule by various complexes. Copper of the second type is easy to eliminate, which can frequently be done during purification procedures. On the other hand, type 2 copper can be reconstructed both in aerobic and anaerobic conditions. The study of laccase derivatives showed that type 2 copper is bound to three nitrogen atoms. A water molecule is the fourth copper ligand. Type 2 copper has proven to play an important role in structural nonspecific stabilization of anionic bonding in the copper 3 active site. It has also been found that a type I copper center, which lacks a liganding methionone, is relatively unstable. This is true for all fungal laccases (Rogalski and Leonowicz 2004).

Although most laccases adhere to these descriptions, there are certain highly purified laccases that do not show these typical characteristics. It was demonstrated that the laccases isolated from solid state fungal cultures were yellow-brown and did not have typical blue oxidase spectra, showing simultaneously atypical EPR spectra. The comparison of N- terminal amino sequences of the *Phlebia radiata*, *Panus tigrinus*, *Coriolus versicolor*, and *Phlebia tremellosus* laccases showed high homology between blue and yellow-brown laccase forms. The yellow enzyme seems to have an altered oxidation state of copper in the active center, which is probably caused by the integration of aromatic lignin-degradation products. Interestingly, evidence has been provided that yellow laccases are capable of oxidizing nonphenolic lignin models and veratryl alcohol directly in presence of O_2 but in the absence of any diffusible mediator (Rogalski and Leonowicz 2004). Not all laccases are reported to possess four copper atoms (Thurston et al. 1994) per monomeric molecule. One of the laccases from *Pleurotus ostreatus* is said to confer no blue colour and was described by the author to be a white laccase (Palmieri et al. 1997). It was determined by atomic absorption that the laccase consisted of 1 copper atom, 1 zinc atom, and 2 iron atoms instead of the typical four coppers.

Classification According to Substrate Specificity

Laccase (EC 1.10.3.2) is a blue copper protein, but it also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor (Mayer 1987).

Polyphenol oxidases are associated with three types of activities (Mayer 1987):

- Catechol oxidase or *o*-diphenol: oxygen oxidoreductase (EC 1.10.3.1)
- Laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)
- Cresolase or monophenol monooxygenase (EC 1.18.14.1)

These different enzymes can therefore be differentiated on the basis of substrate specificity (Walker and McCallion 1980). There is, however, difficulty in defining laccase according to its substrate specificity, because laccase has an overlapping range of substrates with tyrosinase. Catechol oxidases or tyrosinases have *o*-diphenol as well as cresolase activity (oxidation of L-tyrosine). Laccases have ortho and paradiphenol activity, usually with more affinity towards the second group. Only tyrosinases possess cresolase activity, and only laccases have the ability to oxidize syringaldazine (Thurston 1994; Eggert et al. 1996). There has been only one report of an enzyme exhibiting both tyrosinase and laccase activity (Sanchez-Amat and Solano 1997). Secondly, laccases are remarkably nonspecific as to the inducing substrate, and the range of substrates oxidized varies from one laccase to another (Wood 1980).

Thurston (1994) stated in a review that simple diphenols like hydroquinone and catechol are good substrates (for most laccases, but not for all), but that guaiacol and 2,6-dimethoxyphenol (DMP) are often better. Para-phenylenediamine is a widely used substrate, and syringaldazine (N,N'-bis 3,5 dimethoxy-4 hydroxybenzylidene hydrazine) is a unique substrate for laccase only. Thus, laccase oxidizes polyphenols, methoxy–substituted phenols, diamines, and a vast range of other compounds (Thurston 1994).

Another difficulty in defining laccase according to substrate is that the substrate range varies from one organism to another. *Neurospora crassa* laccase (Germann et al. 1988) only effectively oxidizes para and ortho-diphenols – with the exception of phloroglucinol, while laccase from *Pyricularia oryzae* preferred phloroglucinol as a substrate above other substituted monophenols. Laccases from *Cerrena unicolor* and *Trametes versicolor* oxidize meta-substituted phenols, but to varying degrees. Laccase from *Cerrena unicolor* oxidizes para-substituted phenols to the greatest extent (Filazzola et al. 1999), while *Trametes versicolor* laccase oxidises ortho-substituted phenols to the greatest extent (Jolivalt et al. 1999). An immobilized commercial laccase was able to degrade meta, ortho, and para-substituted methoxyphenols, chlorophenols, and cresols, but the substituted phenols from these three types of phenols are oxidized in different orders and to different extents (Lante et al. 2000). Reports suggest that many different reactions have been catalyzed by laccases from different fungi. A comparative study concerning properties of fungal laccases indicated that all the laccases in the study had the ability to oxidize methoxyphenolic acids, but to different degrees, and the oxidation efficiencies were also dependant on pH (Bollag and Leonowicz 1984). Phenolic compounds that were oxidized very slowly by laccase have recently been used to increase the storage stability of laccase activity for *Trametes versicolor* (Mai et al. 2000). The increased stability of laccase could have technological importance, as there are so many potential applications for laccase.

Mode of Action of Laccase

Laccase only attacks the phenolic subunits of lignin, leading to Cα oxidation, Cα-Cβ cleavage, and aryl-alkyl cleavage (Figure 2.2.1.3a). It is believed that laccase catalysis involves

1. reduction of the type 1 copper by reducing substrate
2. internal electron transfer from the type1 to the type 2 and type 3 copper
3. reduction of oxygen to water at the type 2 and type 3 copper site

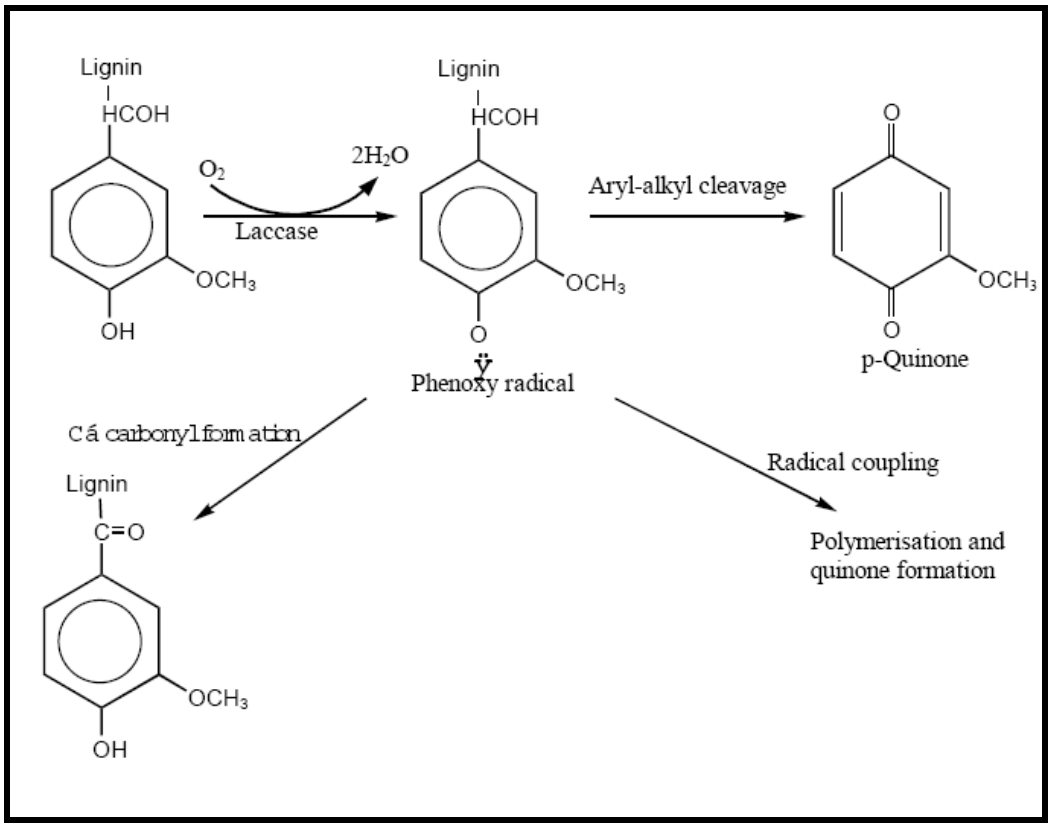

Figure 1. Oxidation of phenolic subunits of lignin by laccase (Archibald et al. 1997)

The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the formation of a free (cation) radical. The radical is in general unstable and may undergo further laccase-catalyzed oxidation (e.g., to form quinone from phenol) or nonenzymatic reactions (e.g., hydration, disproportion or polymerization) (Xu 1999). The electron transfer from substrate to type 1 copper is probably controlled by redox potential difference. A lower oxidation potential of substrate or a higher redox potential of laccase (type 1 site) often results in a higher rate for substrate oxidation.

It seems that the binding pocket of reducing substrate (or the type 1 copper site) is quite shallow and has limited stearic effect on simple phenol substrate. In contrast, the O_2 binding pocket (or the type 2 and type 3 copper sites) appears to restrict the access of oxidizing agents other than O_2. Activation of O_2 likely involves chemical bond formation on the trinuclear copper cluster. Solomon et al. (1996) proposed that under turnover conditions, the electron transfer from the substrate to the type 1 site (the initial electron acceptor from substrate) is the rate-determining step.

Laccases are similar to other phenol-oxidizing enzymes, which preferably polymerize lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups (Bourbonnais et al. 1995). However, the substrate range of laccase can

be extended to non-phenolic subunits of lignin by the inclusion of a mediator such as 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

Laccase Mediator System

Laccases were thought to play a role in the biodegradation of lignin, but it was restricted to phenolic compounds because of the low oxidation potentials of these enzymes (Reid and Paice 1994). Application of these enzymes in the presence of mediator compounds resulted in a high oxidation capability, leading to the oxidation of nonphenolic lignin model compounds [Fig. 2].

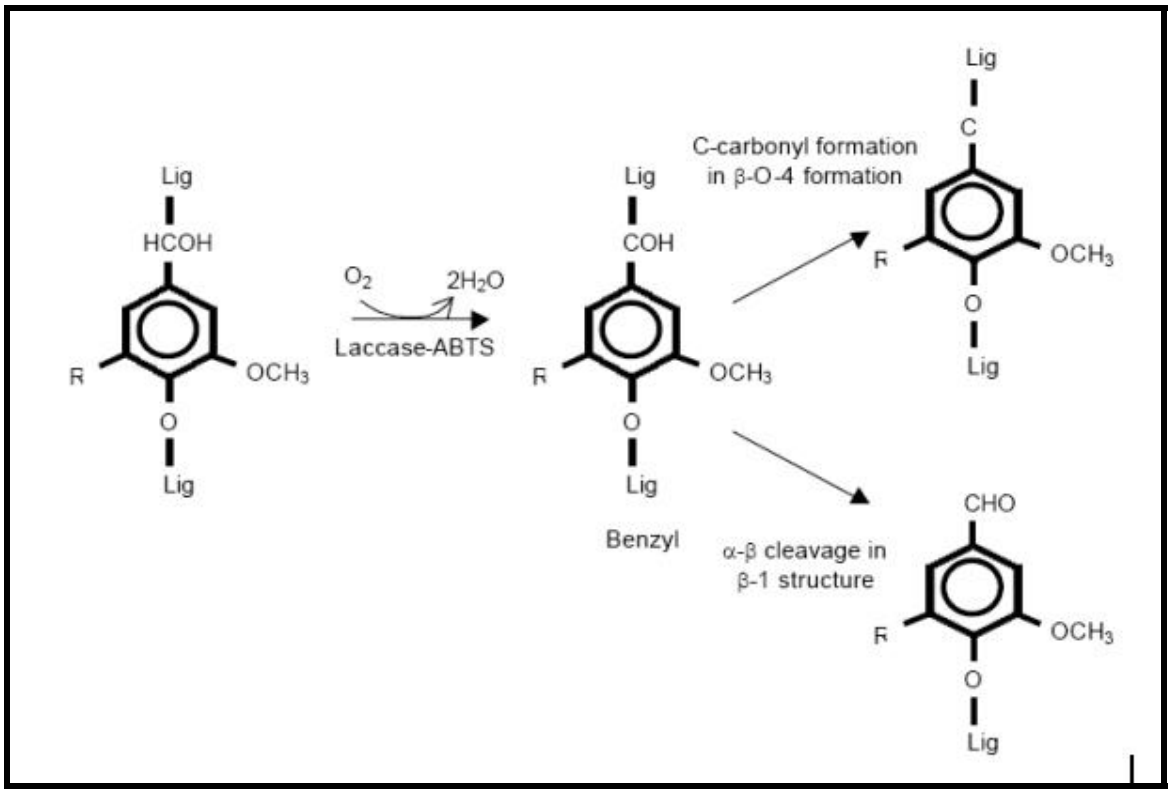

Figure 2. Oxidation of nonphenolic lignin model compounds by a Laccase Mediator System (Archibald et al. 1997)

The laccase mediator system (LMS) was originally developed to solve problems in bio-bleaching of wood pulps and was first described by Bourbonnais and Paice (1990) with the use of ABTS as the first mediator. The first attempt at using laccase mediator couples for delignification in the pulp industry was the development of Lignozym process, which was described by Call and Mucke (1997). According to Bourbonnais et al. (1995), the delignification of kraft pulp by laccase can be supported by a number of external synthetic, low molecular mass dyes, or other aromatic hydrogen donors. ABTS [2,2' azinobis—(3-ethylbenzenthiazoline-6-sulphonic acid)] was the first mediator shown to be effective in the delignification of kraft pulp and lignin transformation by laccase.

The reaction mechanism mediated by ABTS appears to proceed as follows: Oxygen activates laccase, and the mediator is oxidized by the enzyme. The oxidized

mediator diffuses into pulp and oxidized lignin, disrupting it into smaller fragments, which are easily removed from the pulp by means of alkaline extraction. The application of the laccase mediator system on hardwood kraft pulp resulted in a reduction of kappa number, demethylation, and depolymerization of kraft lignin (Archibald et al. 1997; Reid and Paice 1994).

The LMS was successfully applied to the oxidation of aromatic methyl groups, benzyl alcohols (Johannes et al. 1998), polycyclic aromatic hydrocarbons (Johannes et al. 1998; Majcherczyk et al. 1998; Johannes and Majcherczyk 2000) and bleaching of textile dyes (Rodriguez et al. 2004a).

Isozymes

Many laccase-producing fungi secrete isoforms of the same enzyme (Leontievsky et al. 1997). These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme (Archibald et al. 1997). The number of isozymes present differs between species and also within species depending on whether they are induced or non-induced (Assavanig et al. 1992). Table 1. gives a list of different fungal laccase and its isozymes.

Isozymes can differ markedly in their stability, optimal pH and temperature, and affinity for different substrates (Assavanig et al. 1992; Heinzkill et al. 1998). Furthermore, these different isozymes 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).

Functions of Laccase

In some fungi, the reactions of laccase are unrelated to ligninolysis. Laccase 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 (Thurston 1994). 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 polyphenolic glue that binds hyphae together. Various plant pathogens also produce extracellular laccases that enable the fungus to overcome the immune response of the host (Thurston 1994). Laccase also facilitates the detoxification of the plant tissue via the oxidation of antifungal phenols or deactivation of phytoalexins (Assavanig et al. 1992). It has been postulated that laccase is involved in various cellular and microbial activities. Recent studies on the physiological function of laccase include those on plant cell wall biosynthesis, phytopathogenesis, wood material degradation and humification, insect sclerotization, bacterial melanization, and melanin related virulence for humans (Xu 1999).

Table 1. Properties of Purified Fungal Laccase

Organism	Number of isozymes	Molecular mass (kDa)	References
Trametes multicolor	5	63	Leitner et al. 2002
Trametes trogii	2	70	Garzillo et al. 1998
Ganoderma lucidum	3	65-68	Ko et al. 2001
Pycnoporus cinnabarinus	1	81	Eggert et al. 1996
Coriolus hirsutus	1	73	Shin and Lee 2000
Ceriporiopsis subvermispora	2	71 68	Fukushima and Kirk 1995
Trichoderma	1	71	Assavanig et al. 1992
Trametes sanguinea M85-2	1	62	Nishizawa et al. 1995
Ganoderma lucidum	2	40 66	D'Souza et al. 1999
Chaetomium thermophilium	1	77	Chefetz et al. 1998
Chalara paradoxa CH 32	1	67	Robles et al. 2002
Pleurotus ostreatus	1	59	Sannia et al. 1986
Coriolus hirsutus	1	55	Koroljova-Skorobogat'ko et al. 1998
Polyporus versicolor	2	60 ~ 65	Mosbach 1963
Botrytis cinerea 61-34	1	74	Slomczynski et al. 1995
Monocillium indicum	1	72	Thakker et al. 1992
Neurospora crassa	1	65	Froehner and Eriksson 1976
Podospora anserine	3	70 80 390	Thurston 1994

PURIFICATION OF LACCASE

In general, plant laccases are purified from sap or tissue extracts, whereas extracellular fungal laccases are purified from culture medium (fermentation broth) of the selected organism. Various protein purification techniques are frequently employed in purifying laccase. Typical purification protocols involve ultrafiltration, ion exchange, gel filtration, hydrophobic interaction, or other electrophoretic and chromatographic techniques.

Table 2. enlists different methods for laccase purification. Affinity chromatography using a phenolic group as ligand can increase purification efficiency. The purity of a laccase preparation is often measured by SDS-PAGE and by the ratio of the absorbance at 280nm to that at 600 nm.

Table 2. Different Methods for Laccase Purification

Organism	Purification step	Yield and Purification fold		Reference
Trametes multicolor	Ultrafiltration	100 %	-	Leitner et al. 2002
	Q-Sepharose	39.8 %		
	Superdex 75	31.8 %		
Chaetomium thermophilium	Ultrafiltration	84 %	3	Chefetz et al. 1998
	DEAE Sepharose	79 %	5	
	Concanavalin A-Sepharose 4B	40 %	10	
Ganoderma lucidum	DEAE Sepharose	74.9 %	2.6	Ko et al. 2001
	Preparative electrophoresis	28.6 %	32.4	
Pycnoporus cinnabarinus	Ultrafiltration	87 %	7	Eggert et al. 1996
	DEAE M	75 %	20	
	Butyl toyopearl	56 %	32	
	S-400	47 %	36	
Trametes sanguinea	DEAE Toyopearl	78 %	-	Nishizawa et al. 1995
	Phenyl Toyopearl	73 %		
Polyporus versicolor	Hydroxyl apatitie	58 %	-	Mosbach 1963
	Zone electrphoresis	65 %		
Coriolus hirsutus	DEAE cellulose	-	17.5	Koroljova-Skorobogat'ko et al. 1998
	DEAE Toyopearl		167.5	
Pleurotus ostreatus	Ultrafiltration			Sannia et al. 1986
	DEAE Sepharose	18 %		
	ConA- Sepharose	12 %	27	
Coriolus hirsutus	DEAE Sepharose	62.1 %	3.3	Shin and Lee 2000
	Sephacryl S-200 HR	57.2 %	3.7	
	Hitrap SP	45.4 %	8.2	
	Mono S	32.3 %	14.5	
Trametes versicolor	Sephadex G-25	95 %	0.91	Rogalski et al. 1991
	Syringyl- CPG	35.9 %	13.11	
	Vanillyl- CPG	9.2 %	109.19	
Fomes fomentarius	Sephadex G-25	92.4 %	0.94	Rogalski et al. 1991
	Syringyl- CPG	41 %	8.12	
	Vanillyl- CPG	15.6 %	41.4	
Ceriporiopsis subvermispora	Acel anion exchange	54 %	-	Fukushima and Kirk 1995
	Mono Q (L1)	34 %		
	Phenyl Superose	15 %		

CHARACTERIZATION OF LACCASE

Influence of pH on laccase activity

The pH optima of laccases are highly dependent on the substrate. For phenols, the optimal pH can range from 3 to 7 for fungal laccases and up to 9 for plant laccases. When using ABTS as substrate, the pH optima are more acidic and are found in the range between pH 3 and pH 5 (Heinzkill et al. 1998). In general, laccase activity has a bell-shaped profile with an optimal pH that varies considerably. This variation may be due to changes to the reaction caused by the substrate, oxygen, or the enzyme itself (Xu 1997). The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH^-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to the disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the bi-phasic laccase enzymes (Xu 1997). The role of the T1 copper in the pH optima of the enzyme was confirmed by Palmieri et al. (1997), who found that the T1 copper was absent in laccase enzymes exhibiting more neutral pH optima. Table 3 gives a list of optimum pH and kinetic parameters for different laccases.

Influence of temperature on laccase activity

The optimal temperature of laccase can differ greatly from one strain to another. Laccase isolated from *Ganoderma lucidum* showed optimum temperature 20-25°C and was found to be stable between 10- 50°C for 4 hours (Ko et al. 2001). Laccases isolated from *Marasmius quercophilus* (Farnet *et al*. 2000) were found to be stable for 1 h at 60°C. Farnet et al. (2000) further found that pre-incubation of enzymes at 40°C and 50°C greatly increased laccase activity.

Influence of inhibitors on laccase activity

In general, laccases respond similarly to several inhibitors of enzyme activity (Bollag and Leonowicz 1984). In a study conducted by Bollag and Leonowicz (1984) it was found that azide, thioglycolic acid, and diethyldithiocarbamic acid all inhibited laccase activity, whereas EDTA affected laccase activity to a lesser extent. Small anions such as halides (excluding iodide), azide, cyanide, and hydroxide bind to the type 2 and type 3 copper, resulting in an interruption of the internal electron transfer and activity inhibition. Other inhibitors include metal ions (e.g. Hg^{+2}), fatty acids, sulfhydryl reagents, hydroxylglycine, kojic acid, and cationic quaternary ammonium detergents.

APPLICATIONS OF LACCASE

Laccase in the Paper Industry

Biological pulping

Making paper from wood requires separation of the wood fibres from each other and then reforming them into a sheet. In wood, lignin glues the fibres together. These fibres can be separated either by degradation and removal of lignin (chemical pulping), or

by physically tearing the fibres apart (mechanical pulping). Chemical and mechanical pulps have different market niches. Many paper products contain both pulp types, in variable proportions depending on the required properties. Mechanical pulp is cheaper than chemical pulp because of its high yield (up to 95 % by weight of the starting material, in contrast to the yield from chemical pulping of wood is usually less than 50%), and capital cost. However the high lignin content of the mechanical pulp fibres detracts from the quality of the paper; because the fibres have little flexibility, they do not bond together, the paper has lower strength, and there is a tendency of the pulp to yellow on exposure to sunlight. In addition, mechanical pulping requires a lot of electrical energy, which in turn increases the cost.

Table 3. Kinetic Parameters of some laccases

Species	Substrate	pH	K_m	Reference
Armillaria mellea	p-phenylenediamine	3.5	1.7 mM	Rehman and Thurston 1992
Botrytis cinerea	2,6- Dimethoxyphenol	3.5	0.1 mM	Slomczynski et al. 1995
Ceriporiopsis subvermispora	Guaiacol	3.5	1.6 mM	Fukushima and Kirk 1995
Cerrena unicolor	Syringaldazine	5.5	-	Xu 1999
Coprinus cinereus	ABTS	4	-	Schneider et al. 1999
Chalara paradoxa	Syringaldazine	6.5	3.40 mM	Robles et al. 2002
Coriolus hirsutus	Catechol	4.5	145.6 μM	Koroljova Skorobogat'ko et al. 1998
Fomes annosus	Syringaldazine	4.6	-	Bollag and Leonowicz 1984
Ganoderma Lucidum	ABTS	3.5	0.0037 mM	Ko et al. 2001
Lentinus edodes	Syringaldazine	5.0	-	Bollag and Leonowicz 1984
Monocillium indicum	0-dianisidine	3.0	0.025mM	Thakker et al. 1992
Pholiota mutabilis	Syringaldazine	5.2	-	Bollag and Leonowicz 1984
Pleurotus osteatus	Syringaldazine	6.5	-	Sannia et al. 1986
Pycnoporus cinnabarinus	Guaiacol	4	-	Eggert et al. 1996
Rhizoctonia practicola	2,6 dimethoxyphenol	6.8	0.26 mM	Bollag and Leonowicz 1984
Sporotrichum pulverulentum	Syringaldazine	5.0	-	Bollag and Leonowicz 1984
Trametes hirsutus	Guaiacol	4	10.9 μM	Shin and Lee 2000
Trametes sanguinea	Dimethyl phenelyenediamnine	5	-	Nishizawa et al. 1995
Trametes versicolor	Catechol	4.6	0.02 mM	Mosbach 1963
Trametes multicolor	ABTS	3.5	14.1μM	Leitner et al. 2002

Since wood and the other raw materials for pulping are biological in origin, and readily degraded in natural environment, biological lignin degradation, for use in the pulping process, provides new alternatives. Treating aspen chips with *Phanerochaete chrysosporium* before kraft pulping (the most widely used chemical pulping method) improved the tensile strength, brightness, and yield. The effects of biological treatment of coarse mechanical pulp after primary defibration reduced the energy required for secondary refining of the pulp. Pretreatment of wood chips with ligninolytic fungi to decrease the energy requirement for consequent mechanical pulping and to increase the strength of the pulp produced has been the most successful approach. For example, incubating aspen chips for four weeks with *Phlebia brevispora* decreased the refining energy requirement by 47%. Pretreatment of hard wood with *Phlebia tremellosa* produced an 80% increase in the tensile strength. The first sustained effort towards this goal took place in the laboratory of Karl-Eriksson at the Swedish Forest Products Laboratory. It was observed that the tensile strength, yield, and light scattering coefficiant of biomechanical pulps are similar to those of chemithermomechanical pulp (CTMP). Thus biomechanical pulps are likely to compete with CTMP for a share in the market (Reid 1991).

Biobleaching by laccase

Wood fibre has a multilayered structure consisting primarily of cellulose, hemicellulose, and lignin. Lignin is an insoluble complex polymer of phenolic compounds. Up to 90% of the lignin is solubilized and removed during the pulping process. The remaining lignin is a major cause of residual color in the pulp and must be removed by oxidative degradation or bleaching. The bleaching process requires application of harsh chemicals and conditions that are energy intensive. Conventional methods of delignifying or decolorizing paper pulp involve either chlorine or oxygen-based chemical oxidants (e.g. ClO_2 and O_2) (Bajpai and Bajpai 1992). Although very effective, these methods have serious drawbacks, such as disposal of chlorinated byproducts or loss of cellulose fibre strength. Microbial or enzymatic delignification systems that overcome these drawbacks and can be easily adapted to current pulp production line are attractive alternatives. Filamentous fungi are able to efficiently degrade lignin by the action of several secreted enzyme classes. Of these, laccases have attracted considerable interest for application in pulp bio-bleaching. During lignin degradation, laccases are thought to act on small phenolic lignin fragments that then react with the lignin polymer, resulting in its degradation. Alternatively, artificial mediator compounds can be provided to accelerate the delignification process (Kerovuo et al. 2008). Laccase could be applied as a biobleaching agent (as an alternative to conventional chemical oxidants) to pulp. The residual lignin in pulp could be degraded by laccase, resulting in pulp decolorization. For example, *Trametes versicolor* laccase, under the mediation of *N*-hydroxy compounds, delignified Kraft pulp effectively in pilot plant scale.

Laccase in Waste Detoxification and Decontamination

Laccase has been used to oxidatively detoxify or remove various aromatic xenobiotics and pollutants found in industrial waste and contaminated soil or water. Laccase catalysis could result in direct degradation or polymerization/ immobilization.

Reported examples of direct degradation by laccase include direct dechlorination, cleavage of aromatic rings, mineralization of polycyclic aromatic hydrocarbons, decolorization of pulp or cotton mill effluent, and bleaching of textile dyes. The processes include polymerization among pollutants themselves or copolymerization with other nontoxic substances (such as humic materials). Polymerized pollutants often become insoluble or immobilized, thus facilitating easy removal by such means as adsorption, sedimentation, or filtration (Xu 1999).

Laccase in Decolorization of Dyes

Approximately 10,000 different dyes and pigments are produced annually world wide and used extensively in the dyeing and printing industries. The total world colorant production is estimated to be 800,000 tons per year, and at least 10 % of the used dyestuff enters the environment through wastes (Palmieri et al. 2005). Most of the dyes are very stable to light, temperature, and microbial attack, making them recalcitrant. These industrial effluents are toxic and are characterized by high chemical oxygen demands (COD)/ biological oxygen demand (BOD), suspended solids, and intense colour.

Physical and chemical methods such as adsorption, coagulation-flocculation, oxidation, filtration, and electrochemical methods may be used for colour removal from wastewater. These methods are quite expensive and have operational problems. Hence there is a need to develop a practical biological method of dye waste treatment that can be used for a wide range of wastes (Kapdan et al. 2000).

Bacterial anerobic reduction of azo dyes (which comprise a large percentage of synthetic dyes) generates colorless, deadend aromatic amines, which are generally more toxic than the parent compounds. Bacterial aerobic dye degradation has been confined to chemostat-enriched cultures adapted to a single dye (Swamy and Ramsay 1999a). Since effluent contains a range of dyes, successful decolorization of single dye does not indicate the suitability of an organism for a decolorization system. A biodecolorization system should sustain high activity upon repeated exposure to various dyes. Ligninolytic fungi have been shown to possess a remarkable potential for degrading various types of dyes.

White rot fungi are the most efficient ligninolytic organisms capable of degrading various types of dyes such as azo, heterocyclic, reactive, and polymeric dyes (Novotny et al. 2004a). The decolorization of dyes by white rot fungi was first reported by Glenn and Gold (1983), who developed a method to measure the ligninolytic activity of *P. chrysosporium* based upon the decolorization of sulphonated polymeric dyes. White rot fungi offers significant advantages for decomposition of recalcitrant compounds. Ligninolytic enzymes produced by white rot fungi are substrate-nonspecific, and therefore they can degrade wide variety of recalcitrant compounds. Because the enzymes are extracellular, the substrate diffusion limitation into the cell, generally observed in bacteria, is not encountered. These organisms do not require preconditioning to particular pollutants, because enzyme secretion depends on nutrient limitation, nitrogen, or carbon rather than presence of pollutant. In addition, extracellular enzymes enable white rot fungi to tolerate high concentration of pollutants (Kapdan et al. 2000). This paved the way for a wealth of studies on the decolorization of dyes under conditions in which white rot fungi produce lignin-modifying enzymes. The capability of white rot fungi to degrade

various dyes is due to extracellular nonspecific and non-stereoselective enzyme systems composed of laccases (EC 1.10.3.2), lignin peroxidases (EC 1.11.10.14), and manganese peroxidases (EC 1.11.1.13) (Heinzkill et al., 1998). Laccases catalyze the oxidation of both phenolic and nonphenolic compounds (Bourbonnais and Paice 1997) and thus can mineralize a wide range of synthetic dyes (Swamy and Ramsay 1999a). This nonspecific mechanism of laccase makes it a versatile biocatalyst suitable for several applications such as biopulping, biobleaching, and industrial wastewater treatment. Due to the stringent environmental legislation, the textile industry is seeking to develop effective wastewater remediation technologies. The development of processes based on laccase enzyme seems an attractive solution due to the potential of these enzymes in degrading dyes of diverse chemical structure. Therefore considering the importance of laccases in removal of dyes from industrial effluents studies to find the optimal production conditions are currently required.

Laccase in Degradation of Polyaromatic Hydrocarbon

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds comprising benzene rings arranged linearly, angularly, or in clusters. These are ubiquitous environmental contaminants found in air, soil, and aquatic environments (Han et al. 2004). Most aromatic hydrocarbons are toxic to living organisms, and some of them and their metabolites are mutagenic and carcinogenic to humans. PAHs are resistant to biodegradation and therefore tend to accumulate to substantial levels in the environment and have been detected in a wide range of soils and sediments. There have been many studies on the biodegradation of various aromatic hydrocarbons, but their low water solubility and subsequent low degradation rates hamper the bioremediation of PAH-polluted environments (Levin et al. 2003). Therefore, knowledge of microorganisms having a high PAHs degrading capability is essential for efficient remediation of PAHs contamination. To date, most biodegradation studies have been focused on degrading bacteria. However, white rot fungi that can degrade lignin and various recalcitrant aromatic compounds have several potential advantages over other degrading microorganisms. Compared to most degrading enzymes of bacteria which have narrow substrate specificity, the ligninolytic enzymes of these fungi are very nonspecific and extracellular. Therefore, white rot fungi can degrade various insoluble organic pollutants. The lignin degrading system is induced more in response to nutrient exhaustion than by the presence of pollutants. This allows the fungi to degrade pollutants to essentially nondetectable levels. Under such conditions, white rot fungi produce certain extracellular enzymes lignin peroxidases, manganese peroxidases, and laccases. These ligninolytic enzymes of white rot fungi oxidize PAHs to corresponding PAH quinones and subsequently degrade the material further to CO_2. Laccase of *T. versicolor* oxidizes most of the 16 PAHs listed by the US EPA as priority pollutant chemicals. Benzo[α]pyrene and perylene are partially converted to polymeric products. Small amounts of quinones and ketones are the main oxidation products from anthracene, benzo[α]pyrene, and fluorene. Laccase in combination with 1-hydroxybenzotriazole oxidizes acenaphthene and acenaphthylene to 1,2-acenaphthenedione and 1,8-naphthalic acid. Purified Lac from *Trametes versicolor* has been shown to oxidize a range of 3-5 ring PAH in the presence

of the chemical mediators HBT and ABTS (Collins et al. 1996; Johannes et al. 1996; Majcherczyk et al. 1998).

Laccase in the Textile Industry

Textile dye transformation by laccase

Laccase has been reported to prevent back-staining of dyed or printed textiles. As part of the washing solution, laccase could quickly bleach released dyestuff, thus resulting in the reduction of processing time, energy, and water needed to achieve satisfactory quality of the textile. Laccase-catalyzed textile dye bleaching may also be useful in finishing dyed cotton fabric. Another interesting laccase application in this field is oxidative transformation and consequent coupling of dye precursors to the collagen matrix in hides. Under laccase catalysis, soluble dye precursors could be adsorbed, oxidized, and polymerized to give the desired tanning effect. The process could improve dyeing efficiency, reduce cost (by using inexpensive precursors), or provide improved hide characteristics (Xu 1999).

Laccase for denim processing

Replacing conventional chemical oxidants (e.g., hypochlorite), a laccase-based system has been shown capable of bleaching indigo dye in denim and achieving various bleached appearances on the fabric. By themselves, laccases cannot decolorize indigo on denim. However, when combined with an appropriate mediator compound, rapid decolorization takes place. The oxidation reaction catalysed by this oxidoreductase enzyme involves transfer of electrons from a donor to an acceptor substrate. In this case, electrons are transferred from indigo to oxygen, via the enzyme and mediator compound. The mediator is a low molecular weight organic compound to which the insoluble indigo molecule is accessible. The conjugated double bond between the two carbonyl groups in indigo is cleaved, and the dye chromophore is destroyed. The end result is oxidation of indigo to isatin, and further to anthranilic acid, along with the concomitant reduction of oxygen to water. The combined laccase mediator system specifically decolorizes indigo in denim processing applications. Sulfur-black dye (sulfur-bottom denims) and natural off white colors of the fill yarns are not oxidized. The resulting finish is an authentic washdown of the denim with a characteristic gray cast. The laccase mediator reaction stops by itself, when the active mediator is depleted by the reaction. Thus, the extent of decolorization can easily be targeted and obtained repeatedly by choosing the correct dosage and time. In the case of high wash down finishes, the risk of overtreatment with enzymatic decolorization is low compared to hypochlorite bleaching. The benefits provided by laccase processing include minimal strength loss, reduced time, reproducible performance (i.e. easy process control), reduced water consumption and a safe alternative to hypochlorite and bleaching technologies (Mueller and Shi 2001).

The first commercial product was introduced in 1996 as a liquid slurry. The product exhibited good performance, but handling characteristics were not ideal. A fully formulated solid laccase has been commercially available for the denim market since 1999. The newest commercial product contains a laccase enzyme, mediator, buffer, and granulate components. Today, customized formulations of laccase are routinely used to target specific garment wet process conditions (Mueller and Shi 2001).

Medical and Personal Care Applications of Laccase

Poison ivy dermatitis (resulting from skin contact with poison ivy, poison oak, or poison sumac) is caused mainly by urushiol, which is a catechol-derivative toxin. Oxidized urushiol (an o-quinone derivative), however, is nontoxic. Laccase has been shown to oxidize, polymerize, and detoxify urushiol, thus reducing the effect of poison ivy dermatitis.

Another potential application of laccase in the field is laccase-based generation of iodine insitu. Laccase can oxidize iodide to produce iodine, a reagent widely used as a disinfectant. The application of laccase-iodide salt binary iodine generating system (for sterilization) may have several advantages over the direct iodine application. First, the iodide salt is more stable and much safer than I_2 in terms of storage, transport, and handling. Second, the release of iodine from a laccase-iodide system could be easily controlled (by means such as adjusting laccase concentration). The system may be used in various industrial, medical, domestic, and personal care applications, such as sterilization of drinking water and swimming pools, as well as disinfections of minor wounds.

Current hair dyeing or waving processes often involve oxidative or additive chemicals that have unpleasant odours, are irritant (or even harmful) to tissues, or are difficult to handle. A laccase-based system may overcome these drawbacks by replacing harsh chemicals and operating at milder conditions (in terms of pH and solvents). Laccase catalyzed oxidation, transformation, and cross-linking of various precursors have been reported to result in satisfactory hair dyeing or waving. In addition to providing an easier to handle hair care procedure, a laccase-based system may also improve or complement the cosmetic effect achieved by conventional chemical methods (Xu. 1999; Kainz et al. 2003).

Many body, domestic, and industrial odours are caused by sulfides, thiols, ammonia, amines, short chain fatty acids, or other volatile organic compounds. Being able to oxidize various thiols and other sulphur containing compounds, laccases have been studied for deodourant application. Rather than just masking the malodor with fragrances as conventional deodorants do, a laccase system would degrade the offensive molecules or even kill the microbes that generate them (Schmid and Urlacher 2007).

Laccases are also used as catalysts for the manufacture of anticancer drugs and even as ingredients in cosmetics (Kunamneni et al. 2008)

Laccase in the Food Industry

Bioremediation of food industry waste water

Laccase is a well known enzyme studied in bioremediation because of its ability to degrade phenolic compounds (Gianfreda et al. 1999). Aromatic compounds, including phenols, aromatic amines, constitute one of the major classes of pollutants and are heavily regulated in many countries (Karam and Nicell 1997). The presence of these compounds in drinking and irrigation water or in cultivated land represents a significant health hazard. Laccase immobilized on organogel supports remove naturally occurring and xenobiotic aromatic compounds from aqueous suspensions (Crecchio et al. 1995). Laccase immobilized by adsorption on polyethersulphone showed chemical and physical

properties potentially useful for decreasing phenol concentration in a model wastewater solution (Lante et al. 2000).

Beer factory waste water: Some fraction of beer factory waste water represents an important environmental concern due to their high content of polyphenols and dark brown colour. *Coriolopsis gallica*, a white rot fungus producer of laccase, was able to degrade this high tannin containing wastewater. Pyrolysis /gas chromatography/mass spectrometry results showed a decrease in polyphenols pyrolysis products, mainly phenol and guaiacol, with the incubation time (Yague et al. 2000).

Distillery waste water: Distillery waste water is generated during ethanol production from fermentation of sugarcane molasses called vinasses. It produces an ecological impact due to high content of soluble organic matter and its intense dark brown colour. A laccase-producing white rot fungus *Trametes sp*., was tested for bioremediation. Maximum effluent decolorization of 73.3% and chemical oxygen demand reduction of 61.7% was achieved after 7 days of fungal treatment to 20% ^{v}/v of distillery wastes in culture medium. Under these conditions, a 35-fold increase in laccase production by this fungus was observed (Gonzalez et al. 2000).

Olive mill wastewaters (OMW): OMW are a characteristic by product of olive oil production and a major environmental problem in the Mediterranean area. Greco et al. (1999), compared the utilization of a polyphenoloxidase naturally immobilized in olive husk and purified laccase from *Trametes versicolor* in the bioremediation of OMW. Both enzymatic systems showed relevant activity towards phenol polymerization. Treatment of OMW effluent with purified laccase from *Pleurotus ostreatus* significantly decreased the phenolic content (up to 90%) but no reduction of its toxicity was observed (Maritirani et al. 1996).

Beverages

Wine stabilization: Wine stabilization is one of the main applications of laccase in the food industry. Musts and wines are complex mixtures of different chemical compounds such as ethanol, organic acids, salts, and phenolic compounds. Alcohol and organic acids are responsible for the wine aroma, while the color and the taste depend on the phenolic compounds present. The sensorial properties of fresh wines should remain constant until consumption; that is, they should be sufficiently stable at least during the first year of storage. Due to a complex sequence of events, where the polyphenols play an important role, oxidative reactions in musts and wines catalyzed by iron, copper, and enzymes which involve aldehydes, amino acids, and proteins, cause turbidity, color intensification, and aroma and flavor alterations. This oxidation phenomenon is called madeirization.

Different methods have been used in order to prevent the decolorization and flavor alteration in wines, such as the removal of phenolic groups with polyvinylpyrrolidone (PVP), and the use of sulfur dioxide to block oxidizers, among others. An alternative for the physical and chemical adsorbents could be the use of enzymes that selectively target specific polyphenols during the madeirization process. These polyphenolic substances would be oxidized by the enzymes, polymerized, and then removed by clarification. One enzyme studied for this purpose is laccase (Minussi et al. 2002). Laccase presents some important requirements when used for the treatment of

polyphenol elimination in wines, such as optimum pH around 2.5-4.0, stability in acid medium, and reversible inhibition with sulphite. Polyphenol content, color, haze stability, and sensorial quality of Riesling wines prepared with and without oxidation of the must, or with must oxidation and laccase treatment was compared. The results showed that wines made by laccase treatment were the best, suggesting that a stable and high quality wine can be made with little or no added SO_2.

Fruit juice processing

It is well known that browning, both enzymatic and chemical, is one of the major faults in beverages. Various pre and post treatments are available to avoid post turbidity and discoloration of fruit juices. Stabilization of beverages by gelatin, bentonite, silica gel, etc., is a wide-spread conventional treatment (Gokmen et al. 1998). Various enzymatic treatments have been proposed for fruit juice stabilization, and laccase is one among them (Piacquadio et al. 1997). The results reported on laccase apple juice treatments are contradictory. Gokmen et al. (1998) reported that laccase treatment increased the susceptibility of browning during storage. Golden Delicious apple juice was treated with conventional method (SO_2 added as meta bisulfite, PVPP, bentonite) and laccase free or immobilized on metal chelate regenerable carrier. The enzymatically treated apple juice was less stable than the conventionally treated juice. However application of laccase in conjunction with cross flow filtration (ultrafiltration) in continuous process without the addition of fining agents led to a stable and clear apple juice. Stutz (1993) proved that it is possible to produce clear and stable juices/ concentrates with a light colour by means of ultrafiltration and laccase with out any large additional investment. Treatment with laccase acting at a compatible pH followed by "active" filtration or ultrafiltration improved color and flavour stability as compared to conventional treatments by addition of ascorbic acid and sulfites.

Baking

In the bread making process it is a practice to add bread and/or dough improvement additives to the bread dough, the action of which results in improved texture, volume, flavour, and freshness of the bread, as well as improved machinability of the dough. Enzymes (amylases, proteases, cellulases, etc.) have been used as dough and/ or bread improving agents. Si (1994) suggested that when laccase is added to dough used for producing baked products, it may exert an oxidizing effect on the dough constituents and thereby serve to improve the strength of gluten structures in dough and/or baked products. The use of laccase results in an increased volume, an improved crumb structure, and softness of the baked product, as well as increased strength, stability and reduced stickiness, thereby improving the machinability of the dough. The effect on the dough has been found to be particularly good when poor quality flour has been used.

Biosensor and Diagnostic Applications of Laccase

Detection of phenol, aniline, oxygen, and other substances

Laccase catalysis (coupled with various physical instruments) could be useful as biosensors for detecting O_2 and a wide variety of reducing substrates (especially phenols and anilines). Two types of laccase based O_2 sensor are widely used. One type monitors

visible spectral changes (at 600 nm) of laccase, resulting from the reoxidation of the type 1 copper (I) in laccase by O_2. Another type monitors current or voltage changes from a modified oxygen electrode on which O_2 reduction is enhanced under the electrocatalysis of immobilized laccase.

For detecting phenols, anilines, or other reducing substrates, three types of laccase based sensors have been reported. The first type detects the photometric change resulted from the oxidation of a chromogenic susbtrate, the second type monitors the O_2 concentration change that is coupled to the substrate oxidation, and the third type uses an electrode that replaces O_2 as the acceptor for the electrons flown from the substrate (through laccase). For these applications laccase is either immobilized or free in solution, and the coupled physical converter is either optical, amperometric, or piezo effect in nature (Xu, 1999).

Laccase assisted enzymatic and immunochemical assays

Studies suggest that laccase can be used as a new enzymatic label for enzyme immunoassay (Slorobogat'ko et al. 1994). Laccase catalysis can be used to assay other enzymes. In these assays, either the enzyme of interest catalyzes the production of a compound whose subsequent oxidation by laccase generates detectable physical change, or a product from a laccase catalysis (whose production is accompanied by a detectable physical change) is quenched by the activity of the enzyme of interest. The strategy has been applied to assay various hydrolases, transferases, and oxidoreductase (Schmid and Urlacher 2007).

Laccase covalently conjugated to biobinding molecules (antibody, antigen, DNA, RNA, biotin, and streptavidin) can be used as a marker enzyme for immunochemical histochemical, cytochemical, or nucleic acid detection assay. In these assays, the binding moiety binds to the target, and the laccase's reaction signals the binding event (Schmid and Urlacher 2007).

CONCLUSIONS

Laccases are ubiquitious in nature, being produced by wide variety of plants, fungi, and also bacteria. The functions of enzyme differ from organism to organism. Laccase plays an important role in the carbon cycle and could help in degrading a wide range of xenoaromatics. Laccases have become important, industrially relevant enzymes that can be used for a number of diverse applications such delignification of lignocellulosics, bioremediation applications such as waste detoxification, and textile dye transformation, food technologic uses, personal and medical care applications, and biosensor and analytical applications. Therefore, it is not surprising that this enzyme has been studied intensively and yet remains a topic of intense research today.

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