PHYSIOLOGICAL ASPECTS OF LIGNIN-DEGRADING ENZYMES FROM A MACROFUNGUS ISOLATED IN THAILAND

INTRODUCTION

Lignin-degrading enzymes such as laccase (Lacc, E.C. 1.10.3.2), manganese peroxidase (MnP, E.C. 1.11.1.13) and lignin-peroxidase (LiP, E.C. 1.11.1.14) can catalyze the degradation of lignin compounds. These enzymes could be found in several macrofungi. *Phlebia brevispora*, *Phlebia radiata*, *Pleurotus ostreatus*, *Ganoderma lucidum*, *Trametes versicolor* and *Pycnoporus cinnabarinus* have been demonstrated to produce laccases (Gianfreda *et al*., 1999). Nevertheless manganese peroxidase has been demonstrated in *Phanerochaete chrysosporium*, *Trametes hirsuta* and *Phanerochaete sordida* (Hofrichter, 2002) and lignin peroxidase could be found in *Trametes versicolor*, *Phlebia radiata* and *Phlebia brevispora* (Eggert *et al*., 1996).

It has been suggested that white-rot fungi are the most effective lignin-degrading microbes in nature (Hatakka, 2001). Laccase, MnP, and LiP are the major enzymes associated with lignin-degrading ability of the white rot fungi (Hofrichter, *et al*., 1999).

Our group previously reported on a success of using lignin-degrading enzymes in biopulping of paper mulberry, a traditional paper, and eliminated the amount of alkaline used (Poonpairoj *et al*., 2001b). The enzyme was from a macrofungi selected among 84 mushrooms and filamentous fungi isolated in Thailand that could produce lignin degrading enzymes, mostly MnP and laccase. The selected strain was designated as KU-Alk4, that exhibited high activities of laccase and MnP and the activities are exhibited when the culture were grown in liquid medium that initial pH was 8.0 as well as 7.0. Lignin-degrading enzymes from KU-Alk4 showed potential in removing lignin from paper mulberry bark in the pulping process prior alkaline used (Appendix Table A1). Electron microscopic observations of the enzyme digested bark

showed noticeably loose fibers (Appendix Figure A1). Papers from the enzyme-treated pulp followed with 7% NaOH exhibited paper brightness comparable to that from only alkaline-treated pulp which more than 15% are extremely used. The use of 6% NaOH, after the enzyme treatment, brings about optimum strength of the paper, though less brightness. The 6% alkaline used after enzyme treated meant 1-3% less than that recently use in paper mulberry industry in Thailand. Trend in using the enzyme pulping and decreasing alkaline concentration in chemical process are possible. This leaded to the improvement of pulping process that for environmental friendly.

White-rot fungi can decolorize textile dye in industrial effluents, too. Synthetic dyes are widely used in textile and dyeing industries because of the simplicity and cost-effectiveness of their synthesis, firmness, high stability to light, temperature, detergent and microbial deterioration. Large amounts of dyes, 5 to 10% of the total production are released in the industrial effluents and cause serious environmental pollution. Even small amounts of dye in water (10-50 mg/L) not only cause a high visible pollution but also can be an ecological and public health risk (Chung and Stevens, 1993). The efficiency on dye decolorization of white-rot fungi correlates with their extracellular lignin-degrading enzymes. Laccase, especially, catalyze the oxidation of wide range of synthetic dyes (Campos *et al*., 2001; Couto *et al*., 2004; Zhang *et al*., 2006).

Laccases have received much attention of researchers in last decades due to their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes. Such applications are not only the detoxification of industrial effluents, mostly from the paper and pulp and textile but also that of petrochemical industries. Laccase is used as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccase is also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics, replacing chemical agents that were previously used.

Their capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes (Durán and Esposito, 2000; Poonpairoj *et al*., 2001b; Minussi *et al*., 2002; Grönqvist *et al*., 2003; Wesenberg *et al*., 2003; Golz-Berner *et al*., 2004; Rodríguez and Luis, 2006). Recently, the inhibitory activity of *Ganoderma lucidum* laccase toward HIV-1 reverse transcriptase was also reported (Wang and Ng, 2006). Moreover, they have been used in organic synthesis, where their typical substrates are phenols and amines (Riva, 2006). The importance of these enzymes for biotechnology, results also from their considerable retention of activity in organic solvents (Luisa *et al*., 1996).

Because accumulating evidence shows that many biotechnologically and ecologically important fungi vary in lignin-degrading enzyme systems, more research is needed. It is important to carry out studies on taxonomical or functional differences of fungi, the involvement of accessory factors on the enzyme systems, i.e., small molecular weight effectors and mediators, isoenzymes or isoforms expressed under natural conditions and enzymes or factors involved in the degradation of macromolecule of native lignin.

Studying the lignin-degrading enzymes of white-rot fungi is valuable not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications. Physiology of production of lignin-degrading enzymes by white-rot fungi has been studied mostly in *Phanerochaete chrysosporium* (Venkatadri and Irvine, 1990; Kerem *et al*., 1992; Mester *et al*., 1995; Kapich *et al*., 2004) and *Trametes versicolor* (Arora and Gill, 2000; Couto *et al*., 2002; Couto *et al*., 2003).

Another genus responsible for white-rot in wood is *Ganoderma*. The biochemistry of this genus has been extensively studied because most of its species possess medicinal properties (Jong and Birmingham, 1992). Additionally one of the important properties of *Ganoderma* spp. is their ligninolytic potential. Little is known about the lignin-degrading enzymes of *Ganoderma* species.

Characteristics and potential applications of the enzymes in biopulping of paper mulberry from the macrofungus, KU-Alk4, made them of interests for this research (Poonpairoj *et al*., 2001b). In this research, some physiological aspects of KU-Alk4 on solid state and submerged cultivation production and regulation of lignin-degrading enzymes especially laccase were studied. Taxonomy of the fungus, KU-Alk4, was done using molecular techniques. Statistical experimental design was applied for optimizing laccase production. Purification and characterization of five major laccases were studied, resulted a finding of a new and novel laccases. Catalytic reactions of the enzymes against various aromatic compounds include dyes, were observed. The enzyme was immobilized for industrial use. Statistical Experimental Design was also employed to gain the best conditions of the enzyme immobilization. Efficiency in using immobilized and free form of the enzymes in the process of dye decolorization were investigated their differences for the applications into industry. A typical dye decolorization using the immobilized enzymes in large scale reactor (5 L) was carried out. Complete dye decolorization was successfully demonstrated with the immobilized lignin-degrading enzymes of KU-Alk4 under the proposed conditions.

OBJECTIVES

1. Study on some physiological aspects of a macrofungus, KU-Alk4, on regulations of lignin-degrading enzymes synthesis, especially group of laccases.

2. Production of lignin-degrading enzymes using solid state fermentation and submerged cultivation.

3. Improvement of lignin-degrading enzymes production by medium engineering.

4. Study on the catalytic reactions of the enzyme against various phenolic compounds by the enzymes.

5. Dye decolorization by using immobilized laccase.

6. Purification and characterization of laccases from KU-Alk4.

7. Molecular taxonomy of KU-Alk4 using ITS4 and 18S rRNA genes and phylogenetic comparison with reported strains in the gene bank.

LITERATURE REVIEW

Lignin-degrading enzymes

Extracellular enzymes involved in lignin degradation are laccase (Lacc, benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2), manganese peroxidase (MnP, "Mn-dependent peroxidase", E.C. 1.11.1.13) and lignin peroxidase (LiP, "ligninase", E.C. 1.11.1.14) (Hatakka, 2001). In addition, some accessory enzymes involved in hydrogen peroxide production such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO, E.C. 1.1.3.7) also belong to this group. Table 1 shows a general overview of the main cofactors or substrates and the principal effects or reactions of each enzyme.

Laccase is a multicopper-containing oxidase, a true phenoloxidase with broad substrate specificity (Tuor *et al*., 1995), which utilizes molecular oxygen as an oxidant. It oxidizes phenolic rings by one-electron abstraction with formation of phenoxy radicals that can repolymerize or lead to depolymerization (Figure 1) (Hatakka, 2001). It has also a capacity to oxidize nonphenolic compounds under certain conditions, e.g. if the reaction mixture is supplemented with ABTS or other mediating molecules (Call and Mucke, 1997).

MnP and LiP are heme-containing glycoproteins belong to the class of peroxidases which require hydrogen peroxide as an oxidant. MnP oxidizes Mn(II) to Mn(III) which then oxidizes phenolic rings to phenoxy radicals which lead to decomposition of compounds (Figure 1). Recently, it has been shown that MnP in the presence of suitable organic acids is even able to mineralize lignin and lignin model compound to considerable amounts. LiP oxidizes nonphenolic lignin substructures by abstracting one electron and generating cation radicals that are then cleaved Cα-Cβ bond, ring opening (Figure 1) (Hatakka, 2001). Ring-cleavage of aromatic rings is a key step of lignin mineralization. In contrast, oxidation of the corresponding phenolic compounds by LiP did not yield ring-opened products (Tuor *et al*., 1995).

Microorganisms that produced lignin-degrading enzymes

Lignin-degrading enzyme activity has been demonstrated in more than 60 fungal strains belonging to various classes. Table 2 lists the fungi that have been demonstrated to produce lignin-degrading enzymes. The fungi, mainly belong to the class known as white-rot fungi (i.e., Basidiomycetes), were involved in lignin degradation and could completely mineralize those substrates (Gianfreda *et al*., 1999).

The fungi that produced lignin-degrading enzymes were classified into six main groups (Table 2):

1. Fungi that expressed LiP, MnP and laccase. This group was the best known white-rot fungi: *Trametes versicolor*, *Phanerochaete chrysosporium* and *Phlebia radiata*.
2. Fungi that simultaneously produced only 2 enzymes, MnP and laccase, but were reported not to secrete any detectable levels of LiP. Nevertheless, these fungi were strong lignin degraders. *Dichomitus squalens*, *Ganoderma lucidum* and the edible fungus, *Lentinula edodes*, belonged to this group.
3. Fungi with LiP and one of either laccase or MnP. Laccase was the predominant phenoloxidase mostly produced. *Coriolus pruinosum* and *Coriolopsis occidentalis* produced MnP together with LiP.
4. Fungi that secreted only LiP.
5. Fungi that apparently did not produce peroxidases but only laccase.
6. Fungi that produced only MnP.

Table 1 Enzymes involved in lignin degradation and their main reactions.

Enzyme activity, abbreviation	Cofactor or substrate, "mediator"	Main effect or reaction
Laccase, Lacc	O_2; mediators, e.g., hydroxybenzotriazole or ABTS	phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators
Manganese peroxidase, MnP	H_2O_2, Mn, organic acid as chelator, thiols unsaturated lipids	Mn(II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Lignin peroxidase, LiP	H_2O_2, veratryl alcohol	aromatic ring oxidized to cation radical
Glyoxal oxidase, GLOX	glyoxal, methyl glyoxal	glyoxal oxidized to glyoxylic acid; H_2O_2 production
Aryl alcohol oxidase, AAO	aromatic alcohols (anisyl, veratryl alcohol)	aromatic alcohols oxidized to aldehydes; H_2O_2 production
Other H_2O_2 producing enzymes	many organic compounds	O_2 reduced to H_2O_2

Source: Hatakka (2001)

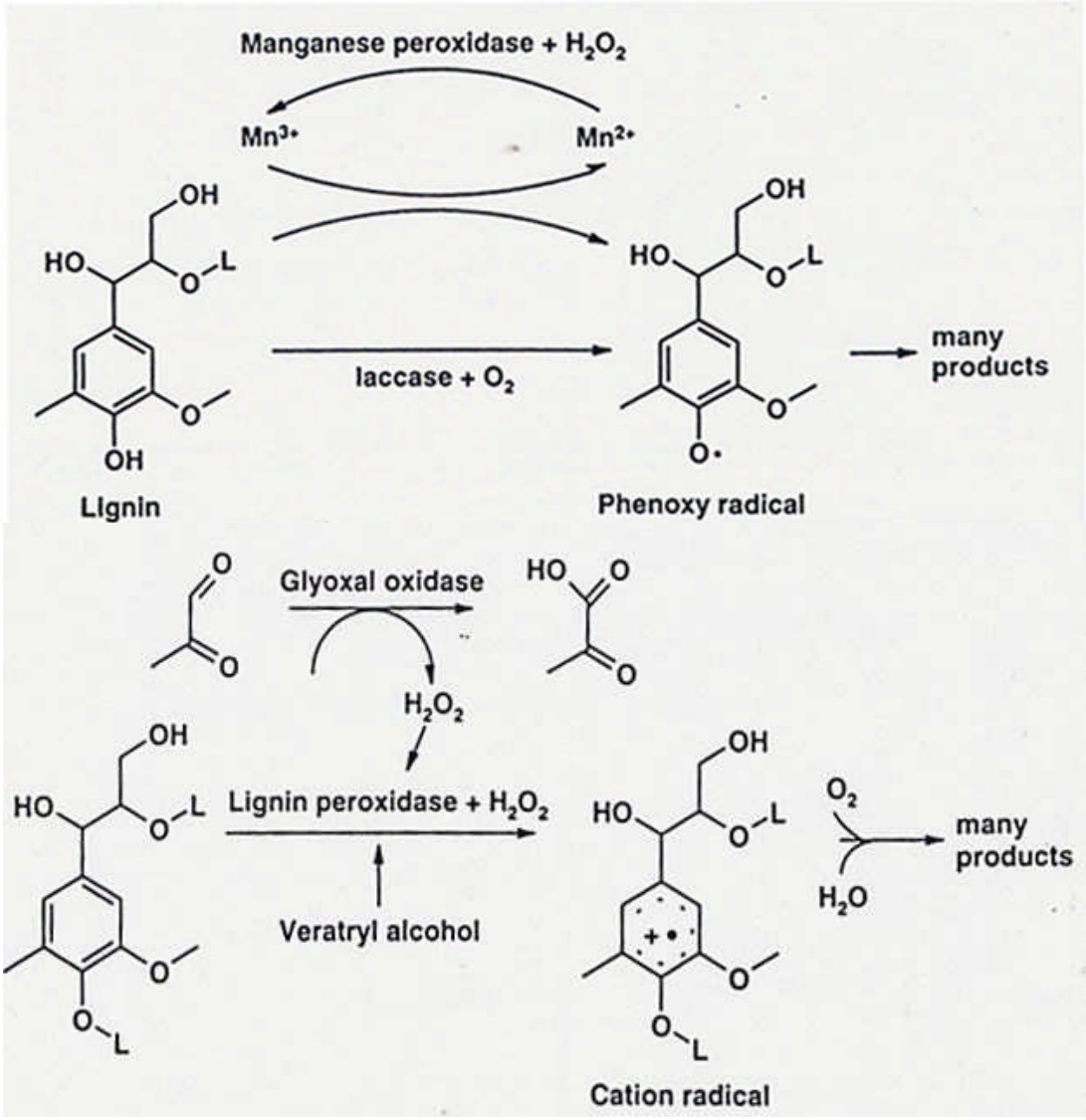

Figure 1 The enzyme mechanisms of laccase, MnP and LiP.

Source: Hatakka (2001)

Table 2 Distribution of lignin-degrading enzymes in fungi.

Microorganism	LiP	MnP	Lacc	Reference
I. *Trametes versicolor*	+	+	+	Fahraeus and Reinhammar, 1967; Jonsson *et al*., 1968; Dodson *et al*., 1987; Johansson and Nyman, 1987; Waldner, 1987; Waldner *et al*., 1988; Johansson, 1993
Phanerochaete chrysosporium	+	+	+	Glenn *et al*., 1983; Tien and Kirk, 1983; Leisola *et al*., 1987
Phlebia brevispora	+	+	+	Perez and Jeffries, 1990; Ruttimann *et al*., 1992
Phlebia radiata	+	+	+	Hatakka *et al*., 1987; Niku-Paavola *et al*., 1988; Vares *et al*., 1994
Pleurotus ostreatus	+	+	+	Sannia *et al*., 1986; Waldner, 1987: Waldner *et al*., 1988; Sannia *et al*., 1991; Beckeret *et al*., 1993
Pleurotus sajor-caju	+	+	+	Fukuzumi, 1987; Bourbonnais and Paice, 1989; Boyle *et al*., 1992
Pleurotus sapidus	+	+	+	Orth *et al*., 1993
Trametes gibbosa	+	+	+	Nerud *et al*., 1991
Trametes hirsuta	+	+	+	Nerud *et al*., 1991
II. *Ceriporiopsis subvermispora*	none	+	+	Ruttimann *et al*., 1992; Lobos *et al*., l994
Cyathus stercoreus		+	+	Orth *et al*., 1993
Dichomitus squalens	none	+	+	Petroski *et al*., 1980; Nerud *et al*., 1991; Perie and Gold, 1991; Orth *et al*., 1993

Table 2 (Continued)

Microorganism	LiP	MnP	Lac	Reference
Ganoderma colossum		+	+	Horvarth *et al*., 1993
Ganoderma lucidum		+	+	Orth *et al*., 1993
Ganoderma volesiocum		+	+	Nerud *et al*., 1991
Grifola frondosa		+	+	Kimura *et al*., 1990; Orth *et al*., 1993
Lentinula edodes	none	+	+	Leatham and Stahmann, 1981; Leatham, 1986; Forrester *et al*., 1990; Orth *et al*., 1993
Panus tigrinus		+	+	Maltseva *et al*., 1991; Golovleva *et al*., 1993
Rigidoporus lignosus	none			Geiger *et al*., 1986; Galliano *et al*., 1991
Stereum hirsutum		+	+	Jong *et al*., 1992
Trametes villosa		+	+	Jong *et al*., 1992
III. *Coriolus pruinosum*	+	+		Waldner, 1987; Waldner *et al*., 1988
Coriolopsis occidentalis	+	+		Nerud *et al*., 1989
Oudemansiella radiata	+		+	Huttermann *et al*., 1989
Phlebia ochraceofulva	+		+	Vares *et al*., 1993
Phlebia tremellosus	+		+	Biswas-Hawkes *et al*., 1987; Huttermann *et al*., 1989

Table 2 (Continued)

Microorganism	LiP	MnP	Lacc	Reference
Pleurotus florida	+		+	Huttermann *et al*., 1989
Polyporus brumalis	+		+	Huttermann *et al*., 1989
Polyporus pinsitus	+		+	Huttermann *et al*., 1989
Polyporus platensis	+		+	Huttermann *et al*., 1989
Ustulina deusta	+		+	Huttermann *et al*., 1989
Xylaria polymorpha	+		+	Huttermann *et al*., 1989
IV. *Bjerkandera adusta*	+	none		Waldner, 1987; Huttermann *et al*., 1989; Muheim *et al*., 1990; Muheim, 1991
Daedaleopsis confragosa	+			Huttermann *et al*., 1989
Phallus impudicus	+			Huttermann *et al*., 1989
Polyporus varius	+			Huttermann *et al*., 1989
V. *Abortiporus biennis*			+	Nerud and Misurcova, 1996
Agaricus bisporus			+	Wood, 1980; Sermanni *et al*., 1982; Matcham and Wood, 1992; Perry *et al*., 1993; Ratcliffe *et al*., 1994
Agaricus brunnescens			+	Fagan and Fergus, 1984
Amillaria mellea			+	Worral *et al*., 1986; Rehman and Thurston, 1992; Billal and Thurston, 1996
Aspergillus nidulans			+	Law and Timberlake, 1980; Kurtz and champe, 1982; Aramayo and Timberlake, 1990

Table 2 (Continued)

Microorganism	LiP	MnP	Lac	Reference
Botryosphaeria sp.			+	Barbousa *et al*., 1996
Botrytis cinerea			+	Marbach *et al*., 1983; Bollag and Leonowicz, 1984; Nun *et al*., 1988; Viterbo *et al*., 1993; Slomczynski *et al*., 1995; Fortina *et al*., 1996
Cerrena maxima			+	Gindilis et al., 1990
Cerrena unicolor			+	Bekker *et al*., 1990; Zakariashvill and Elisashvili, 1993; Pelaez *et al*., 1995; Gianfreda *et al*., 1998
Chaetomium thermophila			+	Ishigaml *et al*., 1988
Coriolus verilereus			+	Zhou *et al*., 1993
Cryphonectria parasitica			+	Rigling and Alfen, 1993; Slomczynski *et al*., 1995
Curvularia sp.			+	Banerjee and Vohra, 1991
Cyathus bulleri			+	Vasdev and Kulad, 1994
Daedalea flavida			+	Arora and Sandhu, 1985
Flammulina velutipes			+	Lee and Suh, 1985
Fomes annosus			+	Kaufmann and Wellendorf, 1980; Haars *et al*., 1981; Haars and Huttermann, 1983; Bollag and Leonowicz, 1984
Grifola frondosa			+	Xing *et al*., 2006
Inonus hispidus			+	Nerud and Misurcova, 1996
Phellinus noxius			+	Geiger *et al*., 1986

Table 2 (Continued)

Microorganism	LiP	MnP	Lac	Reference
Pycnoporus cinnabarinus			+	Nerud *et al*., 1991
Trametes sanguinea			+	Nishizawa *et al*., 1995
VI. *Perenniporia medulla-panis*		+		Orth *et al*., 1993
Trametes cingulata		+		Orth *et al*., 1993
Phanerochaete sordida		+		Ruttimann *et al*., 1994

+ = produce enzyme, none = does not produce enzyme,
blank = no reports on enzyme activity
Source: Tuor *et al*. (1995), Eggert *et al*. (1996), Gianfreda *et al*. (1999)

Trametes versicolor (syn. *Coriolus*, *Polyporus*, *Polystictus versicolor*) produced laccase, LiP and MnP, but primarily laccase and MnP in the lignin degradation (Hatakka, 2001). Apparently the enzyme system in which all three components, LiP, MnP and laccase, are readily produced, allowed *Phlebia radiata* be the fungus that very efficient in degrade lignin. These demonstrated in experiments where substrates were lignocelluloses (Vares *et al*., 1994).

Lignin-degrading enzymes were widespread not only in fungi but also in some bacteria. Recently, there has been increasing many evidences for the existence in bacteria which the enzymes were shown to be proteins with typical features of the multi-copper oxidase enzyme family (Claus, 2004). The first convincing data for a bacterial laccase activity was presented in *Azospirillum lipoferum* (Givaudan *et al*., 1993). Early reports of laccase in actinomycetes had been verified in *Streptomyces griseus* and *S. lavendulae* (Claus, 2004). So far, no bacteria or yeasts had been

reported to produce MnP and LiP, only limited number of basidiomycetous fungi are known to produce them.

Previous studies on physiology of lignin-degrading enzymes production

The physiology of lignin-degrading enzymes production by white-rot fungi has been studied extensively. Lignin-degrading enzymes produced by white-rot fungi during their secondary metabolism lignin oxidation and provided no net energy to the fungi (Wesenberg *et al*., 2003, Gold and Alic, 1993). As is evident from the long list of fungi reported in Table 2, several fungal strains have been investigated for their ability to produce lignin-degrading enzymes. However, the production of the enzyme is dependent not only on fungal strain but also on the growth conditions employed, i.e., the presence or absence of inducers, induction time, nature and composition of culture medium and culture conditions.

1. Induction control

Lignin-degrading enzymes were reported to be either constitutive or inducible enzymes. The presence of an inducer, its chemical nature, the amount added and the time of its addition influence lignin-degrading enzymes to the greatest extent. Several compounds were shown to be inducers that could improve lignin-degrading enzymes formation. They included mainly phenolic compounds, strictly related to lignin or lignin derivatives. Table 3 listed the main inducers of lignin-degrading enzymes production by various fungi.

Veratryl alcohol (3,4 dimethoxybenzyl alcohol), one of the most common and most often used as laccase-inducers, had important functions in the utilization of lignin. It exerted a positive effect on the production of lignin-degrading enzymes in several fungi. In the presence of 1 mM veratryl alcohol, *Trametes modesta* (Nyabhongo *et al*., 2002), *Botryosphaeria* sp. (Dekker and Barbosa, 2001), and *Phlebia radiata* (Arora and Gill, 2001) produced 100-300 more laccase than did in the cultures lacking the alcohol.

Addition of 2,5 Xylidine could stimulate laccase formation in *Volvariella volvaceae* (Chen *et al*., 2003). On contrary, laccase production by *Podospora anserine* was markedly inhibited by the compound (Rogalski *et al*., 1990). Positive responses to 2,5 Xylidine addition were reported with other fungi. Laccase formation by *Pycnoporus cinnabarinus* was enhanced to 9 fold after 24 h of addition of the compound to the shaken cultures. However, no alteration of the isoenzyme pattern was detected (Eggert *et al*., 1996).

Other aromatic compounds including substituted phenols (e.g., guaiacol, 2,6 dimethoxyphenol), and acids (e.g., ferulic acid, syringic acid) were tested for their ability to stimulate the production of laccase in fungi (Herpoël *et al*., 2000; Arora and Gill, 2001; Galhaup and Haltrich, 2001). A high production of laccase was observed when ferulic acid was added to *Pycnoporus cinnabarinus* culture (Herpoël *et al*., 2000).

Lignocellulosic substrates were essential for the production of MnP by *Phanerochaete chrysosporium* (Kapich *et al*., 2004). There was no production of MnP when lignocellulosic substrates were omitted from the medium.

Table 3 Effect of various phenolic and nonphenolic compounds on lignin-degrading enzymes production in fungi.

Inducer	Microorganism	Improvement (fold)	Reference
Veratryl alcohol	*Phanerochaete chrysosporium*	10	Kapich *et al*., 2004
	Trametes modesta	375	Nyabhongo *et al*., 2002
	Botryosphaeria sp.	100	Dekker and Barbosa, 2001
	Phlebia radiata	200	Arora and Gill, 2001
	Daedalea flavida	2-6	
	Phlebia brevispora		
	Polyporus sanguineus		
	Ganoderma lucidum	3	D'souza *et al*., 1999
	Pycnoporus cinnabarinus	2-3	Eggert *et al*.,1996
2,5-Xylidine	*Volvariella volvacea*	40	Chen *et al*., 2003
	Trametes modesta	400	Nyanhongo *et al*., 2002
	Trametes pubescens	4	Galhaup and Haltrich, 2001
	Pycnoporus cinnabarinus	9	Eggert *et al*., 1996
	Dichomitus squalens	2	Périé *et al*., 1998

Table 3 (Continued)

Inducer	Microorganism	Improvement (fold)	Reference
Ferulic acid	*Pycnoporus cinnabarinus*	3	Herpoël *et al*., 2000
	Trametes versicolor	2	Leonowicz *et al*., 1978
	Pleurotus ostreatus	5	
Guaiacol	*Phlebia radiata*	232	Arora and Gill, 2001
	Daedalea flavida	20	
	Trametes pubescens	2	Galhaup and Haltrich, 2001
Lignocellulose	*Phanerochaete chrysosporium*	40-100	Kapich *et al*., 2004

2. Limitation of nutrient

Synthesis and secretion of these enzymes were often induced by limitation of nutrient levels, mostly C or N sources. Nitrogen limitation was the most commonly used in experiments with *Phanerochaete chrysosporium* (Kapich *et al*., 2004). The effect of nitrogen limitation was as well low levels in wood (Gold and Alic, 1993). Nitrogen limited medium (1.2 mM) enhanced the production of the enzymes in *Pycnoporus cinnabarinus* (Eggert *et al*., 1996), too. By contrast, high levels of laccase were observed when *Cyathus stercoreus* (Sethuraman *et al*., 1999) and *Ganoderma lucidum* (D'souza *et al*., 1999) were grown in media with high nitrogen (24 mM).

3. Oxygen concentration

Production of LiP and MnP of a white-rot fungus were shown to be optimal at high oxygen tension but they were repressed by agitation in submerged culture, but its laccase production was often enhanced by agitation (Wesenberg *et al*., 2003). Aeration to the culture increased the production of laccase in *Botryosphaeria* sp. (Dekker and Barbosa, 2001) and *Phanerochaete chrysosporium* (Srinivasan *et al*., 1995; Gold and Alic, 1993), too.

4. Metal ion

White-rot fungi required trace amounts of essential heavy metals such as Cu, Mn or Zn for their growth, but these metals are toxic when present in excess. Low concentrations of essential heavy metals are necessary for the development of the lignin-degrading enzymes system.

Addition of low concentrations of Zn (0.006–1.8 mM) and Cu (0.0004–1.2 mM) into a metal-free synthetic cultivation medium increased the production of LiP and MnP of *Phanerochaete chrysosporium*. The metals also increased solubilization and mineralization of lignin. Two heavy metals are directly involved in the reactions catalyzed by lignin-degrading enzymes (Baldrian, 2003).

Since laccase is a copper-containing protein, copper supplementation of the culture medium enhances both laccase transcription and activity (Palmieri *et al*., 2000; Galhaup and Haltrich, 2001). Stimulation of laccase activity upon the addition of Cu^{2+}-ion (50 mM) is also reported in *Pleurotus ostreatus* (Baldrian *et al*., 2002). Positive effect of copper addition on the production of laccase was also observed in *Ceriporiopsis subvermispora*, *Marasmius quercophilus* (Baldrian, 2003), *Trametes versicolor* (Collins *et al*., 1997), *Pleurotus ostreatus* (Palmieri *et al*., 2000), *Trametes pubescens*, and *Ganoderma applanatum* (Galhaup and Haltrich, 2001).

Mn was directly involved in the catalytic reaction of MnP and it has been reported that Mn also played a regulatory role in expression of LiP, MnP and laccase as well as in the degradation of lignin (Baldrian, 2003). Moilanen *et al.* (1996) demonstrated that 180 µM of Mn(II), together with malonate, stimulated MnP production significantly but, in contrast, repressed LiP production in *Phlebia radiata*. Moreover, laccase production was also enhanced and production continued longer under such MnP-malonate conditions. Moreover, Mn (133 µM) was reported to significantly enhance MnP and laccase in *Clitocybula dusenii* and *Nematoloma frowadii* (Scheel *et al*., 2000), too.

5. Catabolite repression control

Glucose was often found to repress the production of lignin-degrading enzymes, as its catabolite caused the repression of gene responsible to the enzymes. Boominathan and Reddy (1992) concluded that the onset of lignin-degrading enzymes in *Phanerochaete chrysosporium* had been correlated with increased levels of cAMP. Studies with cAMP inhibitors suggested that LiP was inhibited and MnP production was delayed or its activity was inhibited when intracellular cAMP levels were decreased.

Glucose repression was effective in *Trametes pubescens* (Galhaup *et al*., 2002). Increasing of the glucose concentration from 10 g/L to 40 g/L resulted in a more than 5-fold increase in laccase activity, but further increasing to 60 g /L did no further increase of laccase activity, in fact, somewhat lower activities were obtained. Furthermore, this phenomenon could be found in *Botryosphaeria* sp. (Dekker and Barbosa, 2001), in which concentrations of glucose more than 10 g/L repressed the synthesis of laccase.

Improvement of the production of lignin-degrading enzymes

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1. Improvement by using solid state fermentation

Most studies on lignin biodegradation by white-rot fungi have been performed in liquid cultures, which do not reflect the natural living conditions of these microorganisms. Solid state fermentation has shown to be particularly suitable for the production of the enzymes by white-rot fungi such as *Panus tigrinus* (Fenice *et al*., 2003), *Ceriporiopsis subvermispora* (Ferraz *et al*., 2003) and *Trametes versicolor* (Couto *et al*., 2003; Ullah *et al*., 2000), because they reproduced the conditions under which these fungi grew in nature. Moreover, they offered numerous advantages over submerged fermentation such as simpler technique and lower cost (Couto *et al*., 2002). Table 4 listed lignin-degrading enzymes production on solid substrates.

Leontievsky *et al*. (1997) reported that *Panus tigrinus* and *Phlebia radiata* were shown to produce laccase during surface cultivation on wheat straw led to an increase as high as 1.5-fold of that in submerged culture.

Banana waste was the most preferable alternative substrate to the other agricultural/ agro-industrial wastes, wheat bran/straw, saw dust and bagasse, which were already in use for the production of lignin-degrading enzymes by *Pleurotus ostreatus* and *Pleurotus sajor-caju*. The maximum productions of laccases obtained on banana waste from these 2 fungi were 1.71 and 1.67 units/mg protein, respectively (Reddy *et al*. 2003).

Table 4 Lignin-degrading enzymes production on various solid substrates by microorganisms.

Microorganism	Solid substrate	Reference
Panus tigrinus	maize stalks	Fenice *et al*., 2003
Ceriporiopsis subvermispora	wood chips	Ferraz *et al*., 2003
Trametes versicolor	beech wood chip, cereal grain, wheat straw, bran straw, wheat husk and wheat bran	Couto *et al*., 2003; Couto *et al*., 2002; Ullah *et al*., 2000
Phanerochaete chrysosporium	polyurethane	Couto *et al*., 2000
Nematoloma frowardii	wheat straw	Hofrichter *et al*., 1999
Pleurotus ostreatus	Banana agricultural waste, perlite	Reddy *et al*., 2003; Ardon *et al*., 1998
Phlebia radiata	wheat straw	Vares *et al*., 1995

2. Improvement by Statistical Experimental Design

Lignin-degrading enzymes expression by fungi is found to be influenced by culture conditions such as nature of carbon source, concentration of carbon source and nitrogen source, pH, presence of inducers and presence of lignocellulose materials. For effective lignin-degrading enzymes expression, it is essential to optimize all the culture conditions and composition for production media especially those of liquid medium, which further facilitates economic design of the full-scale fermentation operation system. However, practically to optimize all the parameters and to establish the best possible conditions by interrelating all the parameters, numerous experiments have to be carried out with all possible parameter combinations, which is considered to be not economical and practical. For this type of cases, statistical tools and experimental design help to gain more information about the optimization conditions. Conventional optimization procedures involve altering of one parameter at a time keeping all other parameters constant, which enables to assess the impact of those particular parameters on the process performance. These procedures are time consuming, cumbersome, require more experimental data sets and can not provide information about the mutual interactions of the parameters (Beg *et al*., 2003).

Application of statistical methodologies is helpful in finding the effects and interactions of the physiological factors that play roles in biotechnological processes. The use of different statistical designs for medium optimization has been recently employed for laccase, lysozyme, xylanase and amylase production by fungal cultures (Ghanem *et al*., 2000; Dey *et al*., 2001; Francis *et al*., 2003; Parra *et al*., 2005a; Parra *et al*., 2005b). These strategies in using statistics had been successfully applied to the optimization of medium composition (Roseiro, 1992), conditions of enzymatic hydrolysis (Ma and Ooraikul, 1986), parameters of food preservation and fermentation processes (Rosi *et al*., 1987). Oh *et al*., (1995) used response surface designs for rapid optimization of media for the growth of *Lactobacillus casei* YIT 9018. Parra *et al*., (2005a) used an orthogonal design to optimize 13 medium components to enhance squalestatin production from *Phoma* sp. Ghanem *et al*. (2000)

used a Plackett–Burman design to evaluate nutritional requirements for xylanase production of *Aspergillus terreus*.

Table 5 Using of different statistical designs for medium optimization.

Metabolite	Microorganism	Statistical design	Reference
Protease	*Bacillus* sp. L21	Response surface method	Tari *et al*., 2006
Uricase	*Pseudomonas aeruginosa*	Plackett-Burman & Box-Behnken	Abdel-Fattah *et al*., 2005
α-amylase	*Aspergillus oryzae*	Response surface method	Francis *et al*., 2003
Xylanase	*Aspergillus terreus*	Plackett-Burman	Ghanem *et al*., 2000
Laccase	*Botryosphaeria* sp.	Response surface method	Vasconcelos *et al*., 2000
Squalestatin	*Phoma* sp.	Orthogonal & Response surface method	Parra *et al*., 2005
Exopolysaccharide	*Trametes versicolor*	Full-factorial	Tavares *et al*., 2005

Catalytic reactions of lignin-degrading enzymes

Lignin-degrading enzymes play an important role in depolymerization of lignin in nature and polymerization of lignin-related substrates.

1. Degradation of polymers

Lignin-degrading enzymes involved in the degradation of complex natural polymers, such as lignin (Figure 2) or humic acids (Claus and Filip, 1998). The reactive radicals generated lead to the cleavage of covalent bonds and to the release of monomers. When polymeric lignin is degraded, numerous aromatic fragments are generated. Those aromatic compounds were veratryl alcohol, vanillyl alcohol, veratraldehyde, benzoic acid, 2,6-dimethoxybenzoic acid, veratric acid, vanillic acid, syringic acid, *p*-hydroxycinnamic acid (*p*-coumaric acid), ferulic acid, catechol, guaiacol, and 2,6-dimethoxyphenol (Pometto and Crawford, 1988) (Figure 3).

Various xenobiotic compounds (Barr and Aust, 1994; Scheibner *et al*., 1997; Pointing, 2001) including dyes (Glenn and Gold, 1983; Paszczynski *et al*., 1992; Spadaro *et al*., 1992) could be also degraded by lignin-degrading enzymes. An example of those compounds such as 2,4,6-trichlorophenol, a highly toxic compound, found in wastewater, was transformed by laccase and MnP from *Panus tigrinus* and *Trametes versicolor* to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone (Leontievsky *et al*., 2000). Anthracene, which is carcinogenic pollutant, could be degraded by laccase from *Trametes trogii* without any accumulation of anthraquinone (Levin *et al*., 2003).

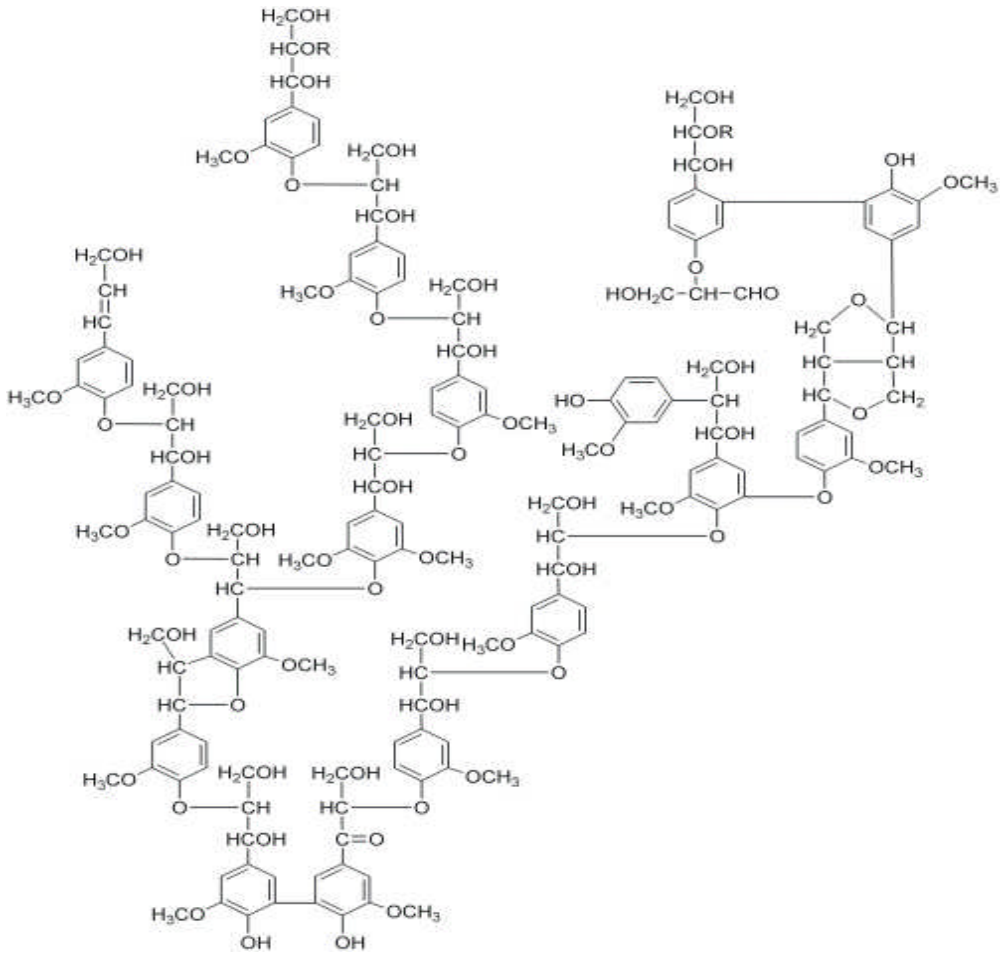

Figure 2 Structure of lignin

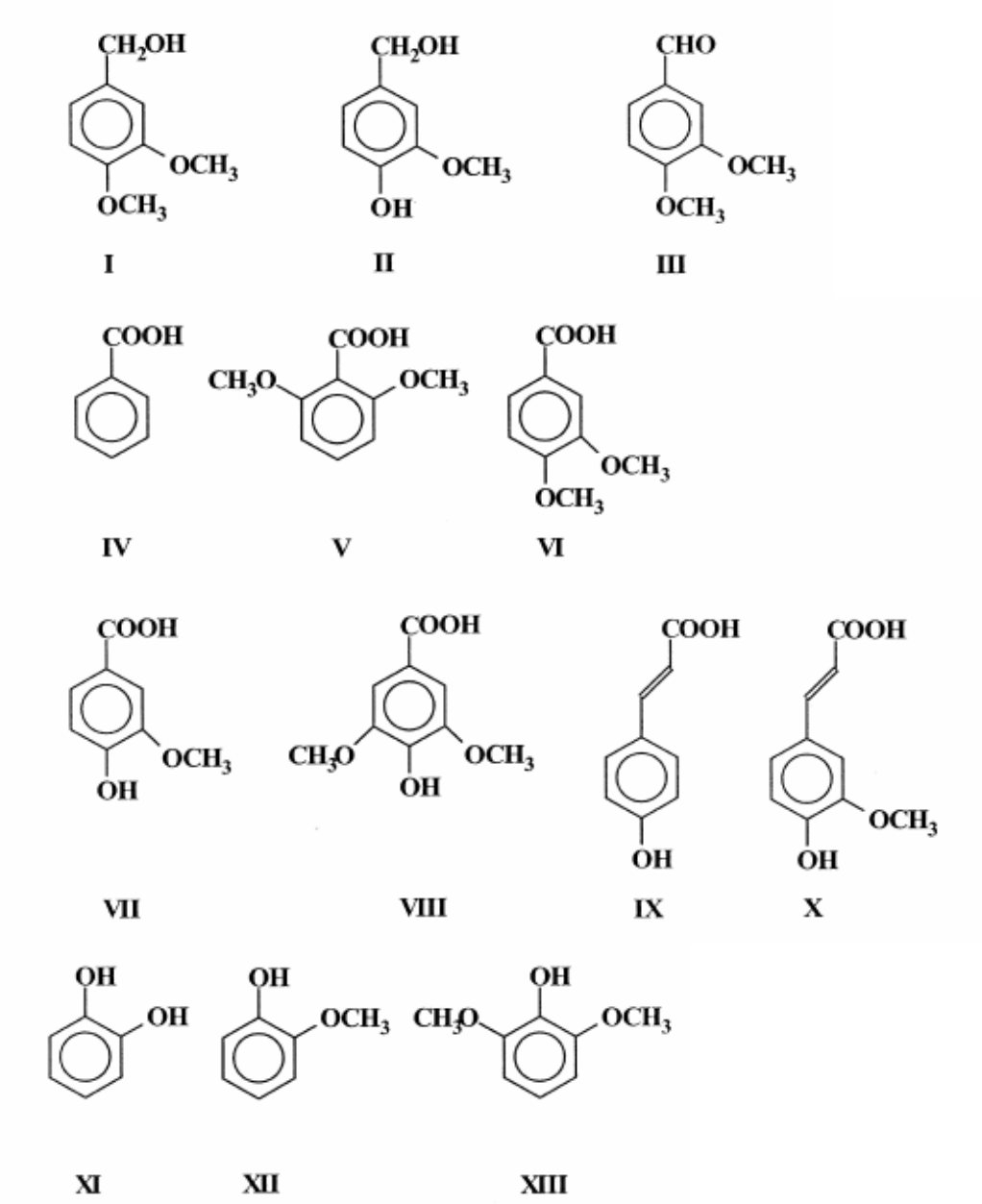

Figure 3 Chemical structures of lignin-related compounds: I–veratryl alcohol; II–vanillyl alcohol; III–veratraldehyde; IV–benzoic acid; V–2,6-dimethoxybenzoic acid; VI–veratric acid; VII–vanillic acid; VIII–syringic acid; IX–*p*-hydroxycinnamic acid (*p*-coumaric acid); X–ferulic acid; XI–catechol; XII–guaiacol; XIII–2,6-dimethoxyphenol.

Source: Dekker *et al*. (2002)

2. Cross-linking of monomers

Enzymatic oxidation of phenolic compounds and anilines by the lignin-degrading enzymes generated radicals that reacted with other molecules and formed dimers, oligomers or polymers, covalently coupled by C-C, C-O and C-N linkages (Claus, 2004). In higher plants, the cross-linking of phenolic precursors by laccases is one part in the lignification process.

Lignin, a major component of wood, second to cellulose, is a structurally complex aromatic biopolymer that is recalcitrant to degradation. The biosynthesis of lignin is the result of its formation via the oxidative polymerization of various phenolic compounds such as coniferyl, *p*-coumaryl and sinapyl alcohol precursors (Sarkanen *et al*., 1997). These aromatic compounds and their derivatives have many interesting functions which are potentially useful such as naphthoquinones, which isolated from a medicinal plant, *Rhinacanthus nasutus*, was found to have antimicrobial and cytotoxic activities (Kongkathip *et al*., 2003, 2004), as well as naphthol, a major component of pesticides, drugs, dyes, and plastics (Aktas *et al*., 2000), etc.

Roles of the lignin-degrading enzymes are not only degradation of lignin but also catalyze the oxidation of various aromatic compounds, especially phenols, leading to polymeric products with increasing complexcity. Aktas *et al*. (2000) demonstrated that laccase catalyzed α-naphthol oxidation generating α-naphthol radicals which reacted with other radicals, forming poly-naphthol molecules, a major component of pesticide napropamide. Catechol was oxidized by laccase generating dimers which subsequent polymerized (Aktas and Tanyolac, 2003).

Laccase

1. Structure

Laccase or benzenediol oxygen oxidoreductase is a cuproprotein belonging to a small group of named blue oxidase enzymes. Fungal laccase often occurs as isoenzymes that oligomerize to form multimeric complexes. An important feature is a covalently linked carbohydrate moiety (10–45%), which may contribute to the high stability of the enzymes (Claus, 2004). Laccase from *Coprinus cinereus* expressed in *Aspergillus oryzae* has been crystallized and its three-dimensional structure determined (Ducros *et al*., 2001). Three-dimensional structure of the *Coprinus cinereus* laccase is shown in Figure 4.

2. Catalytic site

In general, native laccases have four neighboring copper atoms, which are distributed among different binding sites and they are classified into three types. Copper type 1, 2 and 3 which are differentiated in their specific properties and allow them to play an important role in the catalytic mechanism of the enzyme (Figure 5) (Durán *et al*., 2002). According to Call and Mücke (1997), the copper type 1 and 2 are involved in electron capture and transfer. The Cu site of type-1 functions as the primary electron acceptor, extracting electrons from reducing phenolic substrate and delivering them to the trinuclear center that are Cu site of Type-2/Type-3. The trinuclear center involved in binding with the second substrate, dioxygen, which accepts electrons from the Type-1 site for reduction. Catalytic cycle of laccase is shown in Figure 6.

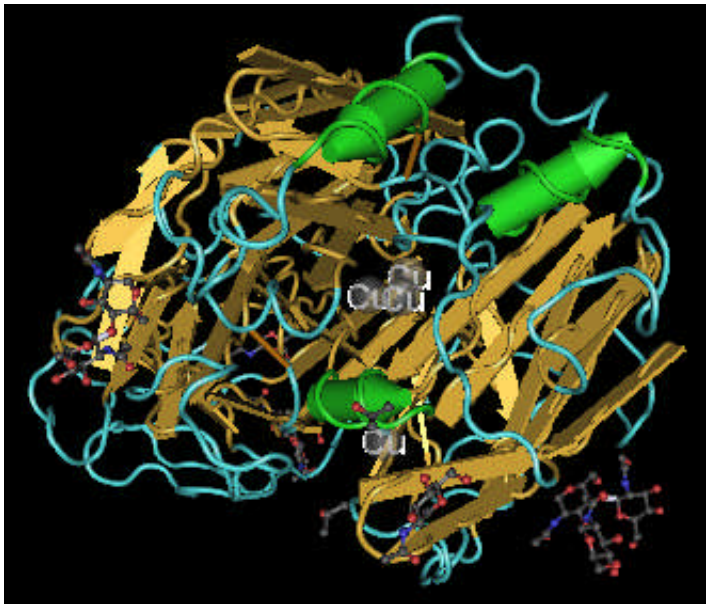

Figure 4 Three-dimensional structure of the *Trametes versicolor* laccase.
Source: Piontek *et al*. (2002)

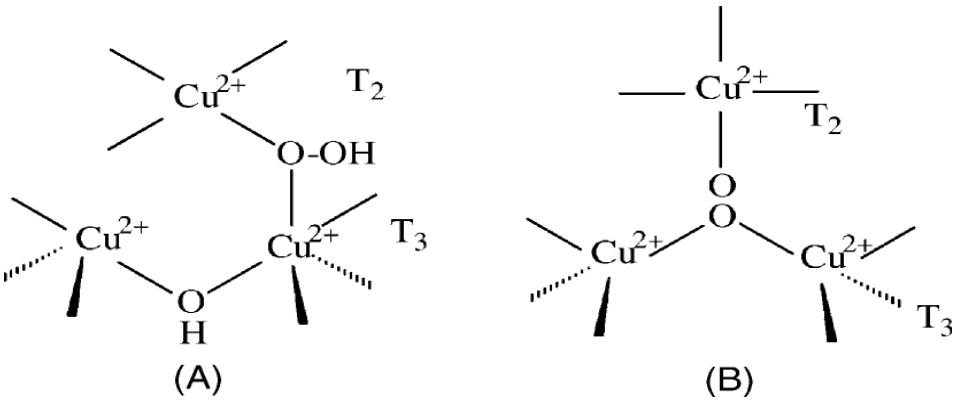

Figure 5 Two possible spectroscopically models for peroxide bridging at the trinuclear cluster site: (A) bridging between Type 2 and one of the Type 3 copper; (B) bridging all three copper.
Source: Durán *et al*. (2002)

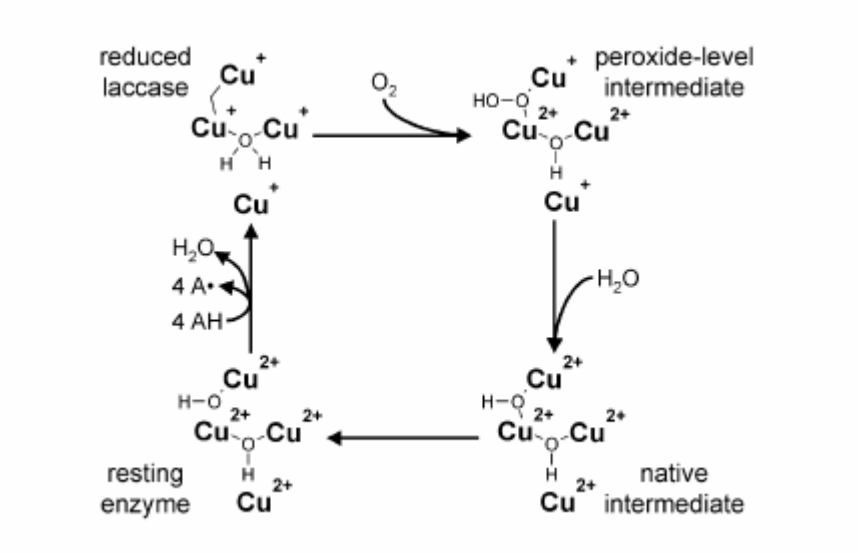

Figure 6 Catalytic cycle of laccase
Source: Wesenberg *et al*. (2003)

3. Catalytic Activity

Laccases have low substrate specificity and their catalytic competence varies widely depending on the source. Simple diphenols such as hydroquinone and catechols are good substrates for most of laccases (Shin and Lee, 2000; Durán *et al*., 2002; Robles *et al*., 2002), but guaiacol (Shin and Lee, 2000; Robles *et al*., 2002) and 2,6-dimethoxyphenol (Min *et al*., 2001; Xiao *et al*., 2003) generally are better substrates. Laccase can, also, catalyze the oxidation of other substitutent polyphenols, aromatic amines, benzenethiols and a series of other compounds. The second substrate of the enzymatic reaction is molecular oxygen. All of the known laccase can also catalyze, with equally high efficiency, the oxidation of ascorbic acid and phenolic substrates. Unlike the reactions that were catalyzed by the other oxidoreductases, oxygen is reduced directly to water by a four-electron mechanism (Yaropolov *et al*., 1994). The values of the Michaelis Constants and k_{cat} for laccase-catalyzed reactions against various substrates are shown in Table 6.

Table 6 Catalytic activities of laccases with various substrates of white-rot fungi.

Compounds	Laccase sources	K_m, μM	k_{cat}, s^{-1}	Reference
ABTS	*Trametes* sp.	25	692	Xiao *et al*., 2003
	Chalara paradoxa	77		Robles *et al*., 2002
	Phellinus ribis	207	1333	Min *et al*., 2001
	Coriolus hirsutus	56.7	260	Shin and Lee, 2000
Guaiacol	*Trametes* sp.	420	69	Xiao *et al*., 2003
	Coriolus hirsutus	10.9	2.24	Shin and Lee, 2000
2,6-dimethoxyphenol	*Trametes* sp.	25.5	81	Xiao *et al*., 2003
	Chalara paradoxa	1472		Robles *et al*., 2002
	Phellinus ribis	38	2166.67	Min *et al*., 2001
	Coriolus hirsutus	53	126	Shin and Lee, 2000
Catechol	*Chalara paradoxa*	425		Robles *et al*., 2002
	Coriolus hirsutus	39.9	7.49	Shin and Lee, 2000
Hydroquinone	*Chalara paradoxa*	287		Robles *et al*., 2002
Syringaldazine	*Phellinus ribis*	11	12000	Min *et al*., 2001
	Coriolus hirsutus	142.7	107	Shin and Lee, 2000
Ferulic acid	*Coriolus hirsutus*	16.8	83.7	Shin and Lee, 2000
p-Phenyldiamine	*Armillaria mellea*	1.7		Rehman and Thurston, 1992
Vanillic alcohol	*Trametes versicolor*	400	182	Yaropolov *et al*., 1994
Vanillin	*Trametes versicolor*	2000	90	Yaropolov *et al*., 1994
Vanillic acid	*Trametes versicolor*	1000	160	Yaropolov *et al*., 1994
Eugenol	*Trametes versicolor*	600	150	Yaropolov *et al*., 1994
Dihydroeugenol	*Trametes versicolor*	260	160	Yaropolov *et al*., 1994

ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)

4. Properties of laccase

In recent years, the extracellular fungal laccases of *Myrothecium verrucaria, Sclerotium rolfsii, Pleurotus ostreatus, Trametes* sp., *Mauginiella* sp., *Magnaporthe grisea, Chalara paradoxa, Trichophyton rubrum, Phellinus ribis, Marasmius quercophilus, Cyathus stercoreus* and *Chaetomium* sp. were purified using various methods and partially characterized.

Several standard protein purification methods were used to purify laccases. They were reported to consist of four to five steps. The concentration steps of the crude enzyme were by means of either ultrafiltration or salting out. Two to three purification steps were followed, using ion exchange, gel filtration, or affinity chromatography. The enzyme could be recovered at a relatively low yield such as 29%. A purified enzyme could be obtained in a highly purified form (purification factor 443), and its homogeneity was demonstrated by SDS-PAGE analysis (Gianfreda *et al*., 1999).

Most fungal laccases had molecular mass ranging from 59-110 kDa (Wesenberg *et al*., 2003). The most frequent molecular mass value found was 66 kDa (Luisa *et al*., 1996). Optimum pH for the laccases activity was in general situated in the acidic region, between 3.0 and 5.0 depending on the substrates. However, there were some exceptions. The enzymes of some soil inhabiting basidiomycetes had neutral pH optima, about 7.0 such as that of *Rhizoctonia praticola* (Bollag and Leonowicz, 1984); *Rigidoporus lignosus* (Grazillo *et al*., 2001); and *Coprinus cinereus* (Schneider *et al*., 1999). On the other hand, laccase of *Myrothecium verrucaria* showed higher optimum reaction pH of 9.0 (Sulistyaningdyah *et al*., 2004). Isoelectric points of the fungal laccases varied from 2.9 (*Pleurotus ostreatus*) to 4.8 (*Ceriporiopsis subvermispora*). Optimum temperature for the enzymatic reactions was usually between 50 and 60°C. In *Marasmius quercophilus* strain (Farnet *et al*., 2000), a litter decomposing fungus, had a laccase with its optimal temperature was as high as 75°C. Table 7 shows some chemical properties of laccases from

various white-rot fungi. Comparison of the properties of laccase, MnP and LiP from white-rot fungi were shown in Table 8.

N-terminal amino acid sequences of laccases showed the close similarity within the group of white-rot fungi (Table 9) such as N-terminal protein sequence of the *Pycnoporus cinnabarinus* laccase which showed the closest similarity to that of *Trametes versicolor* (86% similarity). Moreover it was 76% similarity to the laccase of *Coriolus hirsuta* and 64% similarity to that of *Phlebia radiata*. In contrast, when compared with N-terminal sequences of laccase of non-wood-rotting fungi, such as *Neurospora crassa* and the yeast like fungus, *Cryptococcus neoformans*, they were significantly different, with the similarities of 18 and 0%, respectively.

5. Mediator

The co-presence of various “mediator” molecules was found to expand the oxidation potential of laccase to nonphenolic of lignin (Bourbonnais and Paice, 1990; Kawai *et al*., 1999). This led to re-evaluation of the significance of laccase not only on natural lignin degradation, but also especially in industrial applications, e.g., bleaching of wood pulp and textiles. Certain small molecular weight mediators enhanced laccase activity (Bourbonnais *et al*., 1997; Call and Mucke, 1997; Li *et al*., 1999). The mediators so far be studied were ABTS [2,2'azinobis-(3-ethylthiazoline-6-sulfonate](Bourbonnais *et al*., 1995) and 1-hydroxybenzotriazole (HBT) (Call and Mucke, 1997).

Table 7 Chemical properties of laccases from white-rot fungi

Microorganism	Optimum		Stability		Reference
	pH	Temp (°C)	pH	Temp (°C)	
Myrothecium verrucaria	9.0	70	8-11.5	50	Sulistyaningdyah *et al*., 2004
Sclerotium rolfsii	2.4	62	4.5	18	Ryan *et al*., 2003
Pleurotus ostreatus	3.6				Palmieri *et al*., 2003
Trametes sp.	4.5	50	4.2-8.0		Xiao *et al*., 2003
Mauginiella sp.	3.5		6-8	40	Palonen *et al*., 2003
Magnaporthe grisea	6	30	6-8	40	Iyer and Chattoo, 2003
Chalara paradoxa	6.5	30			Robles *et al*., 2002
Trichophyton rubrum	3-4		5-7	50	Jung *et al*., 2002
Phellinus ribis	4-6	65	55	50	Min *et al*., 2001
Marasmius quercophilus	5.0	80		40-60	Farnet *et al*., 2000
Cyathus stercoreus	4.8			<25	Sethuraman *et al*., 1999
Chaetomium sp.		60	<6	40-60	Chefetz *et al*., 1998

Table 8 Comparison of the properties of laccase, MnP and LiP from white-rot fungi

E.C.	Lac 1.10.3.2	MnP 1.11.1.13	LiP 1.11.1.14
	p-benzendiol: O_2-oxidoreductases	Mn(II): H_2O_2 oxidoreductases	diarylpropan O_2, H_2O_2 oxidoreductases
Prosthetic group	1 type-1-Cu, 1 type-2-Cu, 2 coupled type-3-Cu,	Heme	Heme
MW (kDa)	59–110 (tetramersV390[a])	32[c]– 62.5[b] (122[c])	38– 47
Glycosylation	N–	N–	N–
Isoforms	mono-, di-, tetramers; several	monomers; up to 11[d]	monomers; up to 15
pI	2.9–4.8	2.8[e] –7.2[f]	3.2– 4.7
pH range	2.0–8.5	2.6[g]– 4.5[h]	2.0– 5.0
E_0 (mV)	500–800[i]	1510[j]	1450[k]
C–C cleavage	no	yes	yes
H_2O_2-regulated	no	yes	yes
Stability	+ + +	+ + +	+
Native mediators	3-HAA[l]	Mn^{2+}; Mn^{3+}	VA?[m], 2Cl-14DMB[n]
Specificity	broad, phenolics	Mn^{2+}	broad, aromatics, and nonphenolics
Secondary and synthetic mediators	ABTS[o], HBT[o,] syringaldazine	Thiols, unsaturated fatty acids	No

[a] (Thurston, 1994). [b] *Ceriporiopsis subvermispora* in SSF (Lobos *et al*., 1994).

[c] Basidiomycete strain RBS k1 (Willmann and Fakoussa, 1997).

[d] *Ceriporiopsis subvermispora* (Urzúa *et al*., 1995).

[e] *Nematoloma frowardii* (Schneegaß *et al*., 1997). [f] *Panaeolus sphinctrinus* (Heinzkill *et al*., 1998). [g] *Panus tigrinus* (Maltseva *et al*., 1991). [h] *Pleurotus ostreatus* (Sarkar *et al*., 1997). [i] (Messerschmidt, 1997). [j] Chelator H_2O (Cui and Dolphin, 1990).

[k] (Schoemaker and Leisola, 1990). VA: Veratryl alcohol. [l] (Eggert *et al*., 1995). 3-HAA:3-hydroxyanthranilic acid. [m] (Farrell *et al*., 1989; Tien and Kirk, 1983).

[n] (Teunissen and Field, 1998; Teunissen *et al*., 1998b). 2Cl-14DMB:2-chloro-1,4-dimethoxybenzene.[o] ABTS: 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate); HBT:1-hydroxybenzotriazole (Bourbonnais *et al*., 1996).

Source: Wasenberg *et al.* (2003).

Table 9 Comparison of N-terminal amino acid sequences of laccases, MnP and LiP

Microorganism	N-terminal amino acid sequences	Reference
Laccase		
Myrothecium verrucaria	APQISPQYPMFTVPLPI	Sulistyaningdyah *et al*., 2004
Trametes sp.	AIGPTADLTISNAEV	Xiao *et al*., 2003
Trichophyton rubrum	AIGPVADLHITDDTIAP	Jung *et al*., 2002
Dichomitus squalens	GIGPVTDLTITNADIAP	Périé *et al*., 1998
Pycnoporus cinnabarinus	AIGPVADLTLTNAAVSP	Eggert *et al*., 1996
Trametes villosa	AIGPVADLVVANAPVSP	Yaver *et al*., 1996
Coriolus hirsutus	AIGPTADLTISNAEVSP	Eggert *et al*., 1996
Phlebia radiata	SIGPVTDFHIVNAAVSP	Eggert *et al*., 1996
Agaricus bisporus	DTXKTFNFDLVNTRLAP	Eggert *et al*., 1996
Neurospora crassa	GGGGGCNSPTNRQCWSP	Jung *et al*., 2002
Cryptococcus neoformans	XKTDESPEAVSDNYMPK	Eggert *et al*., 1996
Trametes versicolor	GIGPVADLTISDAEV	Bourbonnais *et al*., 1995
Phellinus ribis	AIVSTPLLIPNANCL	Min *et al*., 2001
Pleurotus ostreatus	AIGPTGDMYIVNEDV	Palmieri *et al*., 1997
Ceriporiopsis subvermispora	AIGPVTDLEITDAFV	Fukushima *et al*., 1995
MnP		
Phanerochaete flavido-alba	ATCPDGTVVSNEACCAFIPLAQD	Rubia *et al*., 2002
Pleurotus ostreatus MnP	VTCATGQTTANEACCALFPILED	Giardina *et al*., 2000
Trametes versicolor MnP	VAXPDGVNTATNAAXXQLFD	Johansson *et al*., 1993
LiP		
Bjerkander adusta LiP	VAXPDGVNTATNAAXXALFA	Wang *et al*., 2002
Trametes versicolor LiP	VTCPDGVNTATNAAXXQLFH	Johansson *et al*., 1993

6. Laccase immobilization

Many methods are available for enzyme immobilization. Since the methods used the immobilization procedures greatly influence the properties of the resulting biocatalyst, the selection of an immobilization strategy determines the process specifications for the catalyst. They include several parameters such as overall catalytic activity, effectiveness of catalyst utilization, deactivation and regeneration kinetics, and cost. Also, toxicity of immobilization reagents should be considered in connection with the immobilization process, waste disposal and final application of the immobilized enzyme catalyst.

There are five principal methods for immobilization of enzymes: adsorption, covalent binding, entrapment, encapsulation and cross linking (Bickerstaff, 1997).

6.1 Adsorption

Immobilization by adsorption is the simplest method and involves reversible surface interactions between enzyme and support material. The forces involved are mostly electrostatic such as van der Waals forces, ionic and hydrogen bonding interactions. Hydrophobic bonding though very weak can be significant.

6.2 Covalent Binding

This method of immobilization involves the formation of a covalent bond between the enzyme and a support material. The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme. Those functional groups are mostly amino group (NH_2) of lysine or arginine, the carboxyl group (CO_2H) of aspartic acid or glutamic acid, the hydroxyl group (OH) of serine or threonine or tyrosine and the thiol group (SH) of cysteine.

6.3 Entrapment

Immobilization by entrapment differs from the two former methods, adsorption and covalent binding, in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel. The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of enzyme, yet at the same time allows free in/out movement of substrate and product. The support will act as a barrier to mass transfer, which can have serious implications to be kinetic reaction, but its advantage is that the enzymes are not harmed by interaction between the immobilized biocatalyst and substrates or products. Another advantage is that the immobilized enzyme can be employed in biphasic system which organic solvent is used to dissolve hydrophobic substrate and products and the encapsulated biocatalyst can be prevented from the damage by organic solvent phase.

6.4 Encapsulation

Encapsulation of enzymes can be achieved by enveloping the biological components within various forms of semipermeable membranes. It is similar to the entrapment method that the enzymes are free in solution, but restricted in space. Large enzymes cannot pass out or into the capsule, but small substrates and products are allowed to pass freely across the semipermeable membrane.

6.5 Crosslinking

This type of immobilization is support-free immobilization which involves joining the enzymes to each other to form a large, 3-D dimensional complex structure, and can be achieved by chemical or physical methods.

Scheme of methods of enzyme immobilization was shown in Figure 7. Examples of microbial laccases immobilized on different supports were reported in Table 10. The type of support, the method of immobilization, and the substrate used

in the catalytic process are specified. Some comments on the main features of each example are also included.

A crude laccase of *Pleurotus ostreatus* was immobilized by entrapment in copper alginate bead attaining 65% yield of laccase activity. Remazol Brilliant Blue R decolorization efficiency was about 70% even after 20 cycles of stepwise dye addition in batch operations, though decolorization time exponentially increased after the 10^{th} cycle. Decolorization processs in fixed-bed bioreactors was successful by decreasing the amount of enzyme loaded and by improving laccase retention using chitosan-coated alginate beads (Palmieri *et al*., 2005).

Phlebia radiata laccase was immobilized on APTES-CPG (3-aminopropyltriethoxysilane-controlled porosity glass) using GLUTAL (glutaraldehyde), with good yield, 96%. A decrease of the enzyme affinity for guaiacol resulted Km values of 1.76 and 4.78 mM for the free and immobilized forms, respectively. A remarkable increase of both storage stability and resistance to inhibitors, such as Cu-chelators and quinone, was evident. After 180 days storage at 4ºC the immobilized enzyme lost only 4% of its initial activity against more than 93% loss shown by the free form (Rogalski *et al*., 1995).

Pleurotus eryngii laccase was immobilized by covalent attachment to chemically-activated gels. Aldehyde, amino and amino-GLUTAL sepharose CL-6B derivatives were used and gave rise to 95, 70 and 55% coupling yields, respectively. Covalent immobilization onto aldehyde gels markedly increased the laccase stability in 60% N,N-dimethylformamide. The immobilized enzyme was inefficient in the treatment of a paper industry effluent, but *P*. *eryngii* laccase immobilized by entrapment within calcium alginate gels was very effective on this effluent (Munõz *et al*., 1996).

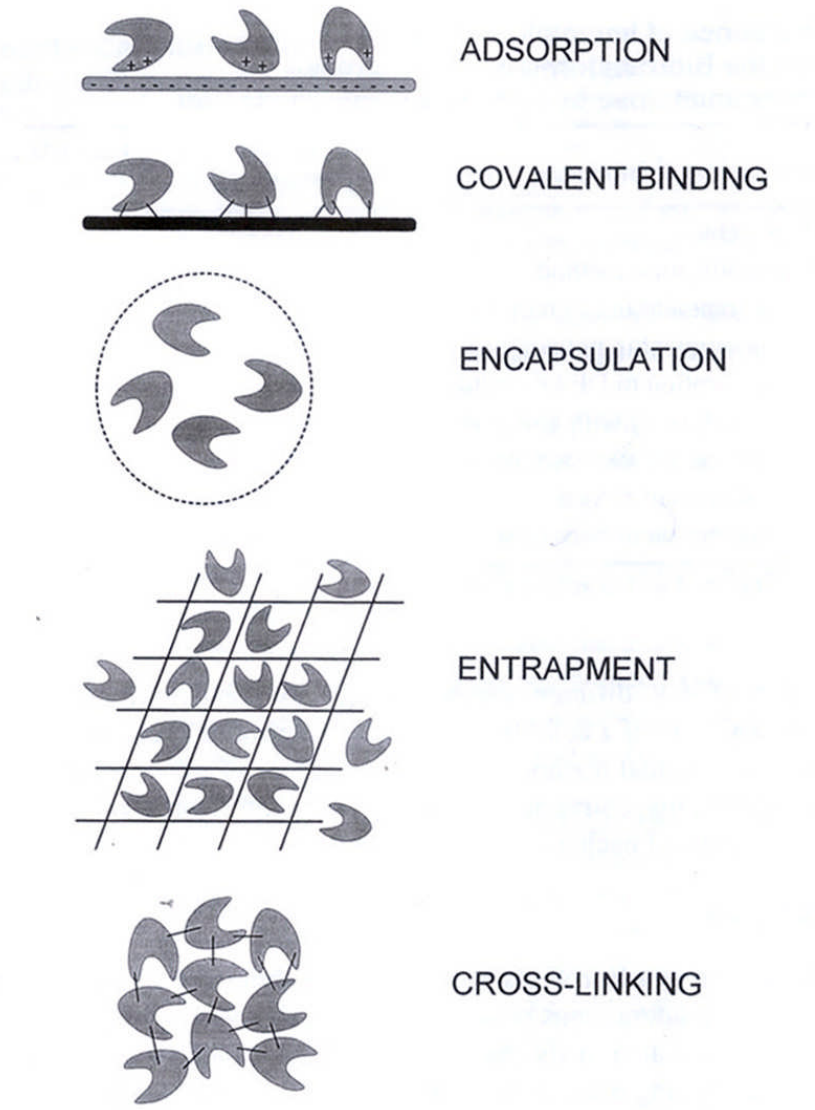

Figure 7 Scheme of principal methods of enzyme immobilization

Source: Bickerstaff (1997)