ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PURIFICATION AND CHARACTERIZATION OF LACCASE FROM *CORIOLOPSIS POLYZONA*

M.Sc. Thesis by

Pınar HÜNER, B.Sc.

Department : Advanced Technologies

Programme : Molecular Biology-Genetics and Biotechnology

FEBRUARY 2007

ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PURIFICATION AND CHARACTERIZATION OF LACCASE FROM *Coriolopsis polyzona*

M.Sc. Thesis by

Pınar HÜNER

521041212

Date of submission : 4 January 2007

Date of defence examination: 30 January 2007

Supervisors (Chairman): Assoc. Prof. Dr. Hakan BERMEK

Assoc. Prof. Dr. Candan Tamerler BEHAR

Members of the Examining Committee: Assoc. Prof. Dr. Z. Petek Çakar ÖZTEMEL (I.T.U.)

Assoc. Prof. Dr. Orhan GÜNEY (I.T.U.)

Prof.Dr. Dilek KAZAN (Marmara Uni.)

FEBRUARY 2007

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Coriolopsis polyzona'nın ÜRETMİŞ OLDUĞU LAKKAZ ENZİMİNİN SAFLAŞTIRILMASI VE ÖZELLİKLERİNİN BELİRLENMESİ

YÜKSEK LİSANS TEZİ

Pınar HÜNER
521041212

Tezin Enstitüye Verildiği Tarih : 4 Ocak 2007
Tezin Savunulduğu Tarih : 30 Ocak 2007

Tez Danışmanları: **Doç. Dr. Hakan BERMEK**

Diğer Jüri Üyeleri:
Doç. Dr. Candan Tamerler BEHAR
Doç. Dr. Z. Petek Çakar ÖZTEMEL (İ.T.Ü.)
Doç. Dr. Orhan GÜNEY (İ.T.Ü.)
Prof. Dr. Dilek KAZAN (Marmara Üni.)

ŞUBAT 2007

ACKNOWLEDGEMENTS

I am grateful to my supervisors Assoc. Prof. Dr. Hakan Bermek and Assoc. Prof. Dr. Candan Tamerler Behar for giving the chance to me to study in this project which is one of the most interesting and sophisticated project among others. I would like to thank for their advice, support and guidance throughout the study.

I would like to thank to Koray Yeşiladalı and Hande Asımgil. I have learnt lots of things from them. I'll never forget the nights which we studied with Hande in front of the chromatography columns.

I would like to thank to my dear friends Aslı Ergun and Firuze Başar. They helped both in my experiments and in moral support. They always made laboratory studies more enjoyable. I always feel their support. I think that we are a good team.

I would like to thank to my dear friends Anıl Cebeci and Deniz Şahin for passing with me happy, serious, and stressfull moments.

I am also thankful to Ceren Alkım for helping during writing this thesis.

I would like to thank to K. Semih Karabulut for his energy and strength by his existence, although he is far away.

I would like to thank to my family for their continuous support and reliance for all the time. My farther for supporting to carry out my academic career, emotionally as well as financially. To my mother, for being emotional crutch-for always being at hand to listen and support. Also to my brother for believing in me.

Lastly, I would like to thank to Vincenza Faraco and Giovanni Sannia at Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II.
This study is supported by EU 6th Frame Integrated Project (IP No: 505899), 'SOPHIED-Novel sustainable bioprocesses for the European colour industries'.

Pınar HÜNER February, 2007

CONTENTS

ABBREVIATIONS

ABTS	: 2,2'-azino-bis-(3-thylbenzothiazoline-6-sulphonic acid)
BCA	: Bicinchoninic acid
DMP	: 2.6-dimethoxyphenol
HIC	: Hydrophobic interaction chromatography
IEX	: Ion exchange chromatography
Lac	: Laccase
LMS	: Laccase mediator system
LiP	: Lignin peroxidase
MnP	: Manganese peroxidase
MWCO	: Molecular weight cut-off
Native-PAGE	: Native-polyacrylamide gel electrophoresis
SDS-PAGE	: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
UF	: Ultrafiltration
VA	: Veratryl alcohol

LIST OF TABLES

LIST OF FIGURES

PURIFICATION AND CHARACTERIZATION OF LACCASE FROM *CORIOLOPSIS POLYZONA*

SUMMARY

Laccases are included in multicopper oxidase family and the mostly distributed of all the blue copper-containing proteins. They catalyze the oxidation of a wide variety of compounds including polymeric lignin, aromatic amines, benzenthiols. Oxidation of phenolic compounds and reduction of molecular oxygen to water by laccases have led to intensive study of them. Laccases are produced by a wide range of higher plants, bacteria and fungi. Fungal laccases have a large variety of applications, such as pulp delignification, textile dye decolorization-detoxification, effluent detoxification, and bioremediation. Among fungi, white-rot fungi are the most important producers of laccases.

In this study, white-rot fungus *Coriolopsis polyzona* MUCL 38443 was cultured for laccase production. A medium containing 50g/l glucose, 17 g/l bacteriological peptone, 2.5 g/l KH_2PO_4, 1.027 g/l $MgSO_4.7H_2O$, 0.03127 g/l $CuSO_4.5H_2O$, 0.0559 g/l $MnSO_4.H_2O$, 10 mg/l Thiamine–HCl supported high levels of laccase production by *C. polyzona*. When the enzyme activity reached to a maximum value, fungal mycelia were removed and the supernatant was purified to homogeneity by ultrafiltration. The purification protocol consisted of anion exchange chromatography with two different columns and hydrophobic interaction chromatography. The molecular weight of laccase was estimated by SDS-PAGE analysis and activity staining was determined by Native-PAGE analysis. Effects of pH, temperature, substrates and inhibitors on the activity of the purified laccase were further studied. Kinetic properties, spectrum analysis, and N-terminal aminoacid sequences were done for the purified enzyme.

CORIOLOPSIS POLYZONA TARAFINDAN ÜRETİLEN LAKKAZ ENZİMİNİN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Lakkazlar birden fazla bakır taşıyan oksidaz ailesinin içinde yer almakta ve mavi bakır içeren proteinler arasında en yaygın olarak bulunmaktadır. Polimerik lignin, aromatik amin ve benzentiyol gibi birçok bileşiğin yükseltgenmesini katalizlerler. Lakkaz enzimleri tarafından gerçekleştirilen fenolik bileşiklerin yükseltgenmesi ve moleküler oksijenin suya indirgenmesi reaksiyonları bu enzimin yoğun olarak çalışılmasını sağlamıştır. Lakkazlar yüksek bitkiler, bakteriler ve mantarlar tarafından üretilmektedir. Mantarlar tarafından üretilen lakkazların kağıt hamuru endüstrisi, tekstil boyalarının renksizleştirilmesi ve zehirinin giderimi, atık zehirlerinin giderimi ve biyolojik arıtma gibi birçok alanda uygulamaları bulunmaktadır. Mantarlar arasında beyaz çürükçül küf mantarları lakkazların en önemli üreticileridir.

Bu çalışmada, beyaz çürükçül küf mantarı olan *Coriolopsis polyzona* MUCL 38443 lakkaz üretimi için yetiştirilmiştir. 50g/l glikoz, 17 g/l bakteriyolojik pepton, 2.5 g/l KH_2PO_4, 1.027 g/l $MgSO_4.7H_2O$, 0.03127 g/l $CuSO_4.5H_2O$, 0.0559 g/l $MnSO_4.H_2O$, 10 mg/l Tiamin–HCl içeren besiyeri *C. polyzona*' dan yüksek miktarlarda lakkaz üretimini sağlamıştır. Enzim aktivitesi en yüksek değerine ulaştığında, organizma uzaklaştırılmış ve enzimi içeren sıvı ultrafiltrasyon tekniği ile homojen bir hale getirilmiştir. Saflaştırma protokolü iki farklı kolonun kullanıldığı anyon değişim kromatografisi ile hidrofobik etkileşim kromatografisinden oluşmaktadır. Lakkazın moleküler ağırlığı SDS-PAGE analizi ile hesaplanmış olup, aktivite tayini Native-PAGE analizi ile belirlenmiştir. Saflaştırılan lakkaz enzim aktivitesi üzerinde pH, sıcaklık, substrat ve inhibitor maddelerin etkileri incelenmiştir. Ayrıca saflaştırılmış lakkaz enziminin kinetik özellikleri, dalga boyu ve N-bölgesinde yer alan aminoasitlerin tayini yapılmıştır.

1. INTRODUCTION

1.1. Wood Composition

Wood is composed of severely spindle-shaped cells. Figure 1.1 shows a general representation of the organization of wood cell wall structure and ultrastructure.

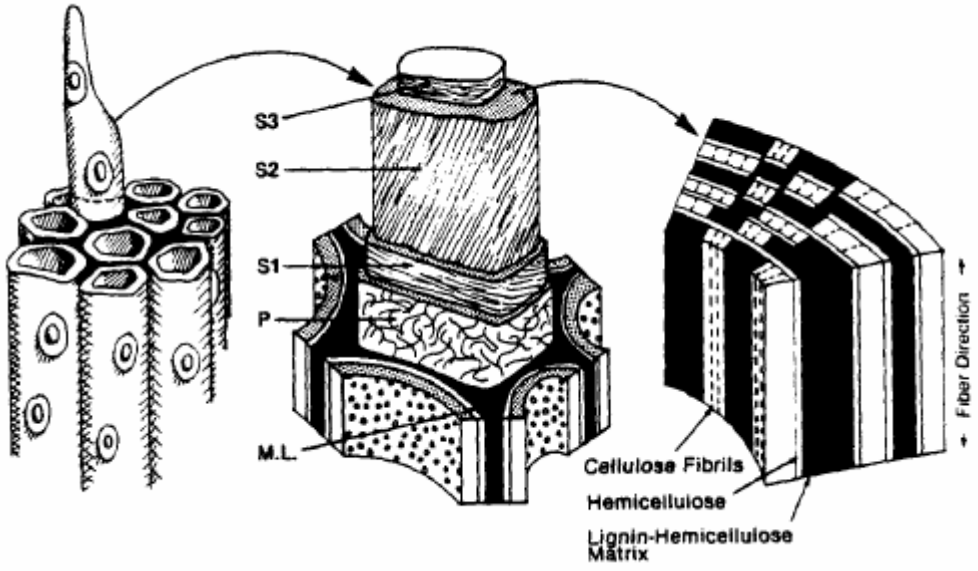

Figure 1.1 Organization of wood tissue: the relationship of adjacent cells *(left),* cutaway view of the cell wall layers *(middle),* and relationship between the lignin, hemicelluloses, and cellulose in the secondary wall. S1-S2-S3, secondary cell wall layers; P, primary wall; M.L., middle lamella. (Kirk et al., 1998).

The cell wall consists of microfibrils having different orientations in primary wall and secondary cell wall layers. In the primary wall, microfibrils are located in the matrix. The matrix contains xyloglucan and pectic substances at the cell surface. In the secondary wall, three layers are observed: S1, S2, S3 layers. Microfibrils included in these layers have different parallel arrangements in respect to the cell axis.

Cellulose which makes up approximately 45% of the weight of wood determines the basic structure of wood cell walls. It is a linear polymer of anhydrocellobiose units linked by β-1.4-glycosidic bonds. Van der Waals forces and hydrogen bonding interactions hold cellulose molecules together. These interactions make natural cellulose structurally complex; the individual cellulose molecules are ordered in bundles known as microfibrils, each microfibril contains approximately 40 individual cellulose molecules (Kirk et al., 1998). A matrix of hemicelluloses and lignin surrounds the cellulose microfibrils; a coating on the cellulose microfibrils is formed by the hemicelluloses (Figure 1.1).

Hemicelluloses make up 25 to 30% of the weight of wood. Hemicellulose backbones are linear β-1.4-linked monosaccharide polymers. In contrast to cellulose, the hemicellulose molecules have a random, amorphous structure with little strength (Kirk et al., 1998). The main sugar residue in the backbone (e.g. xylans, mannans, galactans and glucans) helps classifying hemicelluloses. Hemicelluloses are chemically connected with or cross-linked to other polysaccharides, proteins or lignin (Palonen, 2004). It has been suggested that the hemicellulose and cellulose affect the organization of lignin.

The structure of lignin is an entirely different from cellulose and hemicellulose. Lignin is a complex, hydrophobic, cross-linked aromatic polymer of substituted phenylpropane units joined by C-C and C-O-C linkages (Kirk et al., 1998). The polymer consists of aromatic compounds of three ρ-hydroxycinnamyl alcohols; ρ-coumaryl (7, below), coniferyl (1, below) and sinapyl alcohol (13, below) (Figure 1.2).

Lignin is made up of phenyl-propanoid units which are linked through a variety of different bonds of which the most abundant linkage is guaiacylglycerol-β-arylether (β-0-4), shown linking units 1 and 2. Other linkages contain diaryipropane (β-2) between units 14 and 15, pinoresinol (β-β') between units 10 and 11, and diphenyl ether (4-0-5') between units 8 and 10 (Youn et al., 1995). Cellulose and hemicellulose are physically protected from enzymatic hydrolysis owing to lignin. Due to the heterogeneous nature of lignin, it is resistant to degradation (Palonen, 2004).

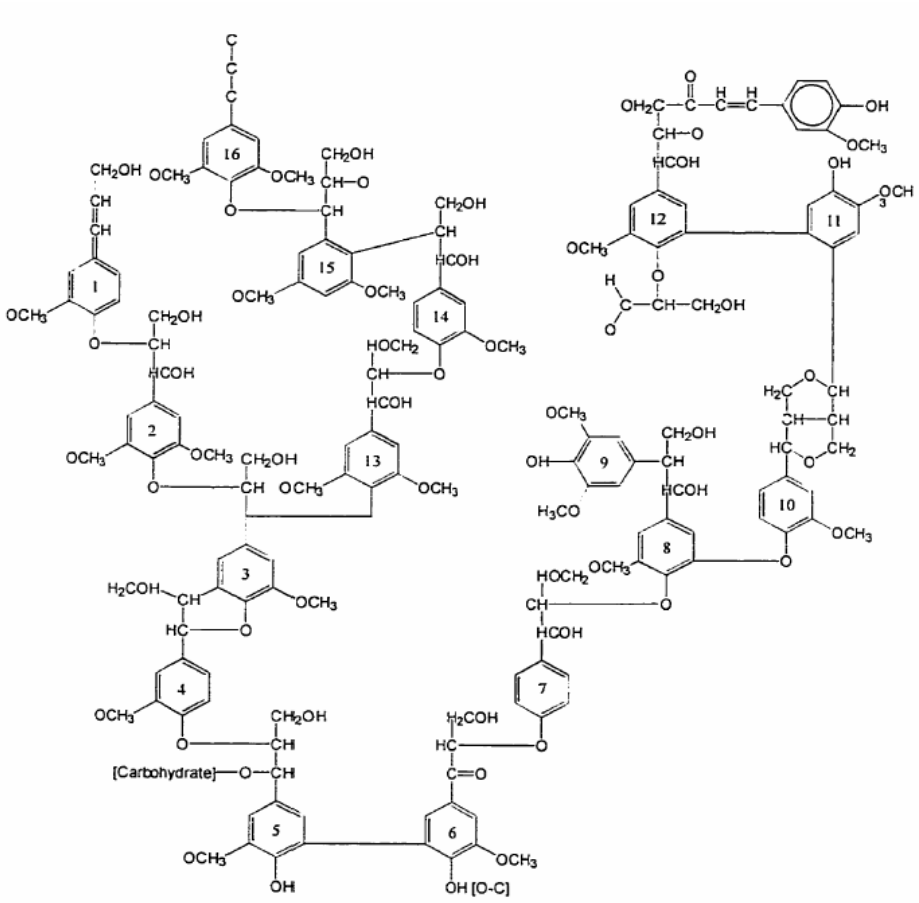

Figure 1.2 Lignin structure (Palonen, 2004)

1.2. White-rot Fungi

1.2.1. Physiology of white rot fungi

Wood is a remarkably difficult substrate to degrade due to its physical and chemical properties (Kirk et al., 1998). Extracellular enzymes that are able to break down the woody cell wall are produced by different types of microbes. Growth characteristics of the microorganism in wood and the type of degradative system cause different decay models. According to the type of decay, different physical, chemical and morphological changes occur in wood (Blanchette, 2000). Fungi those are able to degrade wood fall into three types according to their components utilized and qualities of the decayed wood. These categories include soft rot fungi, brown rot fungi and white rot fungi. Soft

rot fungi contain several Ascomycota and mitosporic species, for instance *Chaetomium* and *Ceratocystis* in terrestrial environments and species of *Lulworthia, Halosphaeria* and *Pleospora* in aquatic and estuarine environments. Soft rot fungi are able to decompose cellulose and hemicellulose, while lignin is partially degraded. The soft rots are mainly predominant at the early stages of wood decay when the environment includes high moisture and increased nitrogen content (Blanchette, 2000). Brown rot fungi are able to degrade cellulose and hemicellulose but not able to digest the lignin component. At the end of the degradation, a brown, amorphous, crumbly residue is produced. The brown-rots include Basidiomycota (the mushrooms) (Blanchette, 2000; Leonowicz et al., 2001). White rot fungi need assimilable carbohydrate constituents of the lignocellulosic complex for their metabolism. Therefore, they penetrate wood tissues to come into contact with these constituents. Structural elements of the lignocellulosic complex (polysaccharides and lignin) are degraded by a multi-enzyme system included by the white-rots. These "feed back" types enzymes cooperate with certain secondary metabolites of fungi and are able to attack the lignin barrier efficiently. Ascomycota, such as *Xylaria* spp. and Basidiomycota (e.g. *Armillariella mellea*) are examples for white-rot fungi (Leonowicz et al., 2001).

Lignin-degrading fungi are widely distributed in a range of environments from tropical to temperate (Cullen, 2002). Microorganisms included in this group are mostly saprophytes that belong to the division Eumycota, subdivision Basidiomycotina. The white-rot fungi are included in the division Eumycota, subdivision Basidiomycotina, class Hymenomycetes, subclass Holobasidiomycetidae. All of the wood-decay fungi are contained in this subclass (Burdsall, 1998). Lignin-degrading fungi produce extracellular peroxidases and oxidases that act non-specifically using the generation of unstable lignin free radicals (Aust, 1995). Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are the main enzymes acting directly or indirectly on lignin (Orth et al., 1993).

Phanerochaete chrysosporium and *Trametes versicolor* are two of the best examined lignin-degrading fungi (Leonowicz et al., 2001). The physiological requirements of an organism for lignin biodegradation (ligninolysis) are extensively studied in *P. chrysosporium* cultures. It has been found that ligninolytic peroxidases (LiP and MnP) are produced in *P. chrysosporium* cultures only in response to nutrient depletion (Erwin

et al., 1993). For a long time, it was believed that *P. chrysosporium* did not produce laccase. The studies showed that laccase activity in *P. chrysosporium* is repressed by glucose. During the studies on regulation of expression of *lip* and *mnp* genes in cellulose and wood-grown cultures of *P. chrysosporium*, low but consistent levels of laccase were produced by this fungus. Laccase activity has been detected in low-nitrogen (2.4 mM) or high-nitrogen (24 mM) defined media containing cellulose as the carbon source (Srinivasan et al., 1995).

T. versicolor is another organism that is well studied for secreting substantial amounts of laccase. It is also known as *Coriolus versicolor* or *Polyporus versicolor. T. versicolor* secretes both LiP and MnP similar to *P. chrysosporium* (Han et al., 2005). *T. versicolor* differs from *P. chrysosporium* because of secreting substantial levels of laccase. *T. versicolor* is the most studied laccase-producing fungus and most of the knowledge of laccase has been determined from these studies (Call and Mucke, 1997).

1.2.2. Microscopic features of the white rot process

White-rot fungi exist primarily as branching threads termed *hyphae*. They are usually 1 to 2 μ in diameter and grow from the tips. They make depolymerization of the hemicelluloses and cellulose and fragmentation of the lignin possible by secreting the battery of enzymes and metabolites (Kirk et al., 1998).

The white-rot fungi show two different patterns of decay: (1) In simultaneous decay, the cellulose, hemicellulose, and lignin are removed more or less simultaneously. Erosion channels are observed beneath the hyphae. The structure of cell wall changes and becomes gradually thinner, often in a nonuniform manner, with holes appearing between cells as decay advances. From cell to cell, the degree of decay varies. For example, some heavily attacked cells take place adjacent to relatively sound cells. (2) In the delignification pattern, lignin and hemicelluloses are eliminated before the removal of cellulose. Cell walls do not lose their morphology. On the other hand, they gradually become unreactive to lignin stains and increasingly reactive to cellulose stains because of the removal of the protective lignin coating (Kirk et al., 1998).

1.3. White-rot Enzymes

White rot fungi (WRF) are key regulators of the global carbon cycle. The major enzymes produced by WRF are oxidoreductases, i.e., two types of peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) and a phenoloxidase, laccase (Lac) (Fig. 1.3).

The reduction of peroxide such as hydrogen peroxide (H_2O_2) and the oxidation of a variety of organic and inorganic substrates are catalyzed by peroxidases. Laccase is one of the few enzymes that were investigated as early as the end of the 19th century (Ikehata et al., 2004). Laccases contain multiple copper atoms at their active sites and use molecular oxygen as a substrate for the oxidation of phenols and other aromatic compounds to corresponding reactive quinines (Wesenberg et al., 2003). These enzymes are directly involved both in lignin degradation in their natural lignocellulosic substrates and in various xenobiotic compound degradation (Cullen, 2002).

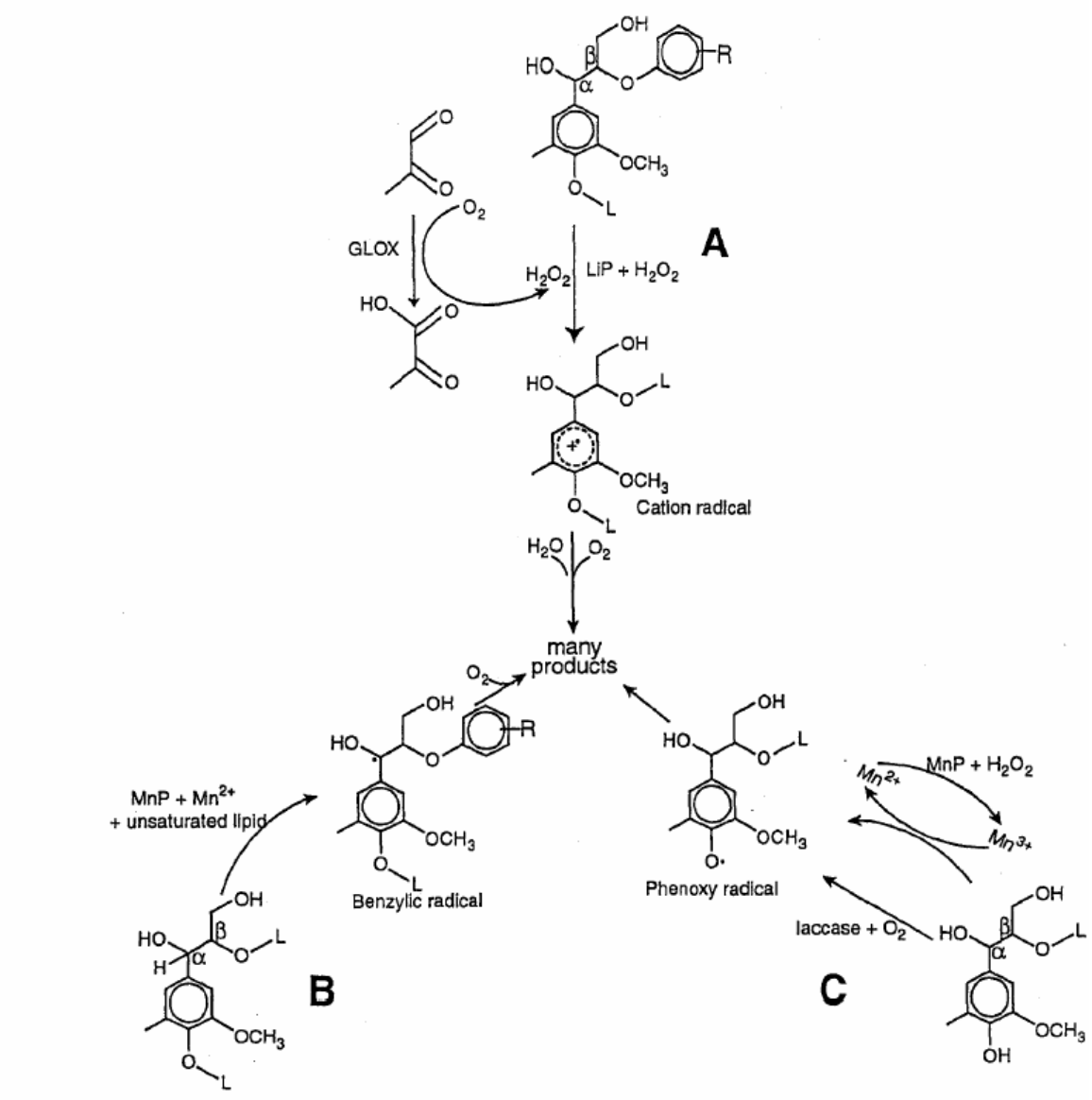

Figure 1.3 Extracellular oxidative enzyme systems of *P. chrysosporium* and related white-rot fungi (Cullen, 2002)

1.3.1. Lignin peroxidase

Lignin peroxidase (LiP) (at first named as "ligninase") was discovered in cultures of *Phanerochaete chrysosporium*. Two American teams and a Japanese group identified and described this enzyme when the corticoid basidiomycete *P. chrysosporium* cultures were grown on nitrogen and carbon-limited media. LiP was also found in other wood colonizing, corticoid and polyporous basidiomycetes, e.g., *Phlebia radiata, Phlebia tremellosa, Trametes versicolor* (Leonowicz et al., 2001).

Lignin peroxidases catalyze a variety of oxidations, e.g. nonphenolic aromatic lignin moieties and similar compounds. The molecular weight of LiP varies between 38 and 46 kDa (Wesenberg et al., 2003). The enzyme, which comprises hem in the active site, necessarily requires hydrogen peroxide (H_2O_2) generated by other enzymes (e.g., oxidases) to be active. It has been suggested that veratryl alcohol mediates the reactions of lignin peroxidase. Veratryl alcohol (VA), a low-molecular-mass fungal metabolite, is oxidized by LiP to veratraldehyde through an aryl cation radical. Veratraldehyde is highly reactive and should, in turn, oxidize the lignin polymer (Leonowicz et al., 2001).

The lignin peroxidase's redox cycle is schematically illustrated in Figure 1.4. The cycle includes two-electron oxidation of LiP by hydroperoxidases (ROOH) to compound I. Then, compound I is reduced to compound II and native enzyme, producing two oxidations of the electron donor. The electron donor for LiP can be aromatic compounds (AH) (Ruiz-Dueñas et al., 1999).

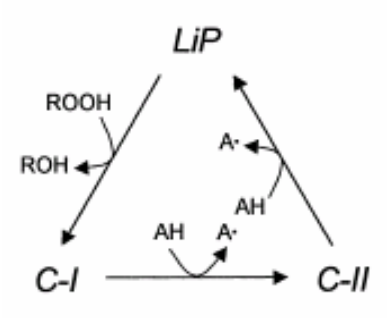

Figure 1.4 The redox cycle of *P. chrysosporium* LiP (Ruiz-Dueñas et al.,1999)

LiP is not an essential enzyme for the attack on lignin, e.g. highly active white-rot and litter-decaying fungi (e.g., *Ceriopsis subvermispora*, *Dichomitus squalens*, *Panus tigrinus*, *Rigidosporus lignosus*) do not excrete this enzyme (Cullen, 2002; Wesenberg et al., 2003).

1.3.2. Manganese peroxidase

Manganese peroxidase (MnP) is secreted by several fungi including *P. chrysosporium*, *P. tigrinus*, *P. radiata*, *L. edodes*, *Phanerochaete sordida*, and *T. versicolor*. This wide distribution among ligninolytic fungi proposes that MnP also plays a key role in the process of lignin degradation. Certain efficient lignin degraders, such as *Ceriporiopsis subvermispora, Phanerochaete sordida,* and *Dichomitus squalens* produce MnP while they are not able to secrete LiP (Cullen, 2002).

MnPs provide low molecular weight diffusible oxidants. The molecular weight of MnP varies between 36 and 60 kDa. They contain a ferric iron protoporphyrin IX heme cofactor and require H_20_2 to oxidize Mn(II) to Mn(III) for its activity (Ruiz-Dueñas et al.,1999). Figure 1.5 shows the catalytic cycle of MnP. The Mn(II) must be chelated by organic acid chelators such as glycolate or oxalate. The product Mn(III) is stabilized by these chelators and promoted releasing from the enzyme. The resulting Mn(III) chelates are diffusible oxidants, so that they can work at some distance from the enzyme. Nevertheless, they are not strong oxidants and not able to attack the non-phenolic units of lignin. As an alternative, they can oxidize phenolic structures, which make up about 10% of the units in lignin (Cullen, 2002).

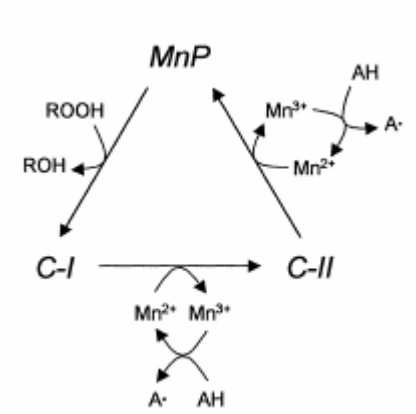

Figure 1.5 The catalytic cycle of *P. chrysosporium* MnP (Ruiz-Dueñas et al., 1999)

1.3.3. Laccase

Laccase is the most widely distributed of all the large blue copper-containing proteins. It is not only found in a wide range of higher plants and fungi but also found in bacteria. Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi produce laccases (Leonowicz et al., 2001). In the fungi, the white-rot Basidiomycetes have been a focus for lignin degradation studies rather than Ascomycetes and Deuteromycetes. *Monocillium indicum* is the first ascomycete that secretes laccase showing peroxidative activity (Baldrian, 2006). Other ascomycetes that produce laccase are *A. nidulans*, *N. crassa*, and *P. anserina*, and wine fungus *B. cinerea* (Bollag and Leonowicz, 1984). Yeasts are a physiologically specific group of both ascomycetes and basidiomycetes. *Cryptococcus* (Filobasidiella) *neoformans* is the only human pathogen yeast which is able to produce laccase (Baldrian, 2006).

Laccases in plants appear to be far more limited than in fungi. All laccases described to date have been shown to be glycoproteins (D'Souza et al., 1996). The laccase in *Rhus vernicifera* has been well documented and characterized in great detail. Additionally, laccase is contained by the entire family of the Anacardiaceae in the resin ducts and in the secreted resin. The cell cultures of *Acer pseudoplatanus* are able to produce and secrete laccase. *Pinus taeda* tissue also contains eight laccases, all expressed predominantly in xylem tissue (Mayer et al., 2002).

Laccase isolated from *Sinorhizobium meliloti* is the best characterized bacterial laccase. *Sinorhizobium meliloti* laccase is a 45-kDa periplasmic protein with isoelectric point at pH 6.2 and able to oxidize syringaldazine (Rosconi et al., 2005).

Lentinula edodes is a white-rot basidiomycete that is able to secrete laccase. It has different morphological characteristics, such as having gills that are serrate on the edges. Nevertheless, the biology of *Lentinus* is significantly different. All of the species cause a brown-rot of wood, not a white-rot as with *Lentinula edodes* (Burdsall, 1998). *Ganoderma lucidum* is a medicinal white rot basidiomycete known by the common name "zizhi" or "ling chi" in China and "reishi" among other less known names in Japan (Wang and Nq, 2006).

Collybia velutipes, *Fomes annosus*, *Fomes fomentarius*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Pholiota mutabilis*, *Pleurotus ostreatus*, *Poria subacida*, *Sporotrichum pulverulentum*, *Trametes sanguinea* and *Trametes versicolor* are included in laccase-rich basidiomycetes and are known as lignin degraders (Baldrian, 2006).

Both extracellular and intracellular laccases were detected in *T. versicolor* cultures grown on media containing glucose, wheat straw and beech leaves (Baldrian, 2006). Intracellular as well as extracellular laccases were identified for *N. crassa*. Due to similarities between intracellular and extracellular laccases, it has been suggested that the intracellular laccase functioned as a precursor for extracellular laccase (Froehner et al., 1974).

1.4. Properties of Laccases

1.4.1. Physical properties

Molecular oxygen (dioxygen) is used as substrate (co-substrate) by more than 200 enzymes divided into oxygenases and oxidases. Besides cytochrome-*c* oxidase, a heme: Cu containing enzyme, only the blue oxidases (laccase, L-ascorbate oxidase, L-ascorbate: oxygen oxidoreductase; ascor-base), ceruloplasmin (Fe(II): oxygen oxidoreductase; ferrooxidase) are able to reduce dioxygen to two molecules of water. All these 'blue' copper containing enzymes are similar in their three dimensional structure

while differing in their primary amino acid sequence and their biological function (Solomon et al., 1996).

UV/visible and electroparamagnetic resonance (EPR) spectroscopy can be used for distinguishing three copper centers of laccase. Laccases which containing four copper atoms per monomer bound to three redox sites generally show acidic pIs. They have been isolated as monomeric, dimeric or even as tetrameric proteins in their active holoenzyme form. Copper centers are differentiated as type 1 (T1), or blue copper center, type 2 (T2) or normal copper, and type 3 (T3) or coupled binuclear copper centers based on spectroscopic analysis (Solomon et al., 1996). Figure 1.6 shows copper centers in the laccase. Type 1 copper is responsible for the deep blue colour of the protein at an absorbance of approximately 600 nm. Enzymes lacking the T1 copper are not called as true laccases by some authors. Others use the term 'yellow laccases' because these enzymes lack the characteristic absorption band around 600 nm. Type 2 copper does not give colour. Type 3 copper consists of a pair of copper atoms in a binuclear conformation that gives a weak absorbance in the near UV region. It is spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) (Baldrian, 2006). The occurrence of laccase enzymes which lack the typical absorption around 600 nm has been reported. For instance, a ''white laccase'' (containing 1Cu, 1Fe, 2Zn atoms) has been purified from *Pleurotus ostreatus*, while ''yellow laccases'' (containing copper but in an altered oxidation state) have also been reported (Palmieri et al., 1997).

Two nitrogens from two histidines and sulphur from cysteine are linked with Type 1 Cu (Figure 1.6). The characteristic blue colour of typical laccase enzymes is defined by the bond between the Type 1 Cu and sulphur. The substrates are oxidized at the T1 copper site. Strongly conserved His-Cys-His tripeptide motif functions during the extracted electron transferring to the T2/T3 site (Baldrian, 2006).

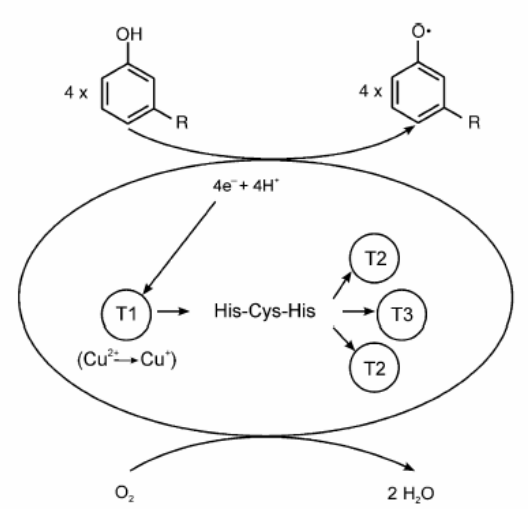

Figure 1.6 Catalytic cycle of laccase (Baldrian, 2006).

The crystallographic structure of copper-2 depleted laccase from *Coprinus cinereus* is illustrated in Figure 1.7. The tertiary structure of laccases isolated from *Melanocarpus albomyce*s, an ascomycete, and *Trametes versicolor* were demonstrated in Figure 1.8.

Figure 1.7 Crystallographic structure of the Cu-2 depleted laccase for *Coprinus cinereus* (Bertrand et al., 2002)

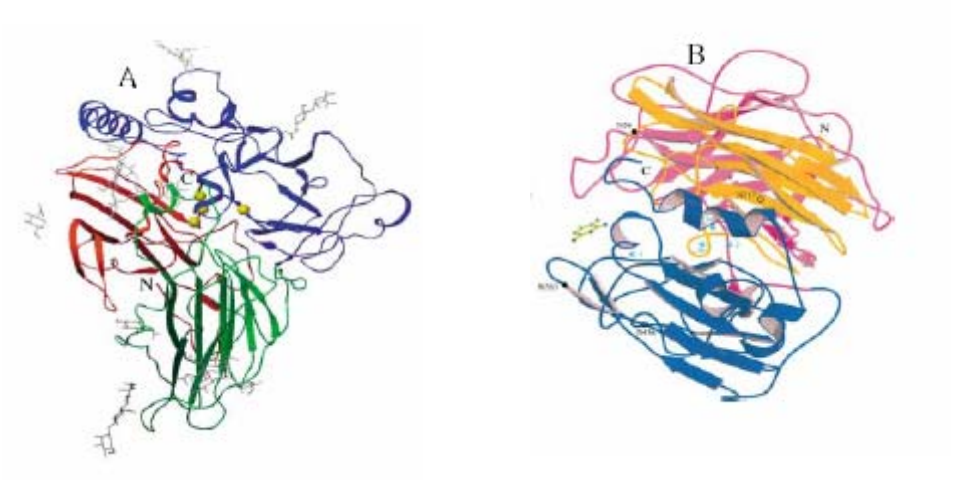

Figure 1.8 Tertiary structure of laccases. A) *Melanocarpus albomyce*s laccase crystal structure (Hakulinen et al., 2002); b) *Trametes versicolor* laccase crystal structure (Bertrand et al., 2002).

Current data about the structure and physico-chemical properties of fungal laccase proteins is derived from the study of purified proteins. Purified laccase enzymes demonstrate considerable heterogeneity. Growth medium composition affects glycosylation content and composition of fungal glycoproteins. For this reason the reports of molecular weights, pH and temperature optima and substrate specificity are extremely heterogeneous (Baldrian, 2006).

Fungal laccases work in a pH range between 3.0- 7.5. The pH optima are usually lower than 4.0 for the oxidation of ABTS, whereas phenolic compounds like DMP, guaiacol and syringaldazine show different values of between 4.0 and 7.0 (Baldrian, 2006).

Laccase activity is achieved at temperature optima between 25^0 and 70^0C. Few enzymes with optima below 35^0C have been described (Baldrian, 2006). For instance, *G. lucidum* laccase shows the highest activity at 25^0C. The temperature stability varies significantly. The half life at 50^0C ranges from minutes in *B. cinerea*, to over 2-3 h in *L. edodes* and *A. bisporus*, to up to 50-70 h in *Trametes* sp. (Bonnen et al., 1994). While *G. lucidum* laccase was immediately inactivated at 60^0C, the thermostable laccase from *M. albomyces* still exhibited a half life of over 5 h and thus a very high potential for selected biotechnological applications (Lalitha Kumari et al., 1972; Kiiskinen et al., 2002).

Molecular weight of a typical fungal laccase is approximately 60–70 kDa with isoelectric point between 2.6-7.6 (Baldrian, 2006). On the other hand, a catalytic polypeptide chain with low molecular mass (16.3 kDa) and one or two regulatory/structural chains with high molecular mass (81.5 kDa) form the first bacterial laccase from rhizospheric bacterium *Azospirillum lipoferum* (Diamantidis et al., 2000).

A wide variety of isoenzymes is produced by several species. The studies revealed that laccase isoenzymes are up to five, with the exception of white-rot fungus *P. ostreatus*. It produces at least eight different laccase isoenzymes, which six of them have been isolated and characterized. The POXC is the major protein present in *P. ostreatus* cultures with molecular weight 59 kDa and pI 2.7. The molecular weights of the POXA2, POXB1 and POXB2 isoenzymes are around 67 kDa, while POXA1b and POXA1 are smaller (61 kDa) (Giardina et al., 1999).

1.4.2. Substrate specificity of laccase

Aromatic rings substituted with electron donating groups, such as phenolics and aromatic amines, are oxidized by laccase. These are the preferred electron rich substrates of laccase. Defining laccase by its reducing substrate has some difficulties. Laccase does not catalyze the oxidation of tyrosine, which is a unique substrate for tyrosinase (another type of Cu-containing oxidase). Therefore, laccases are non-specific towards their reducing substrate, and the range of oxidized substrates differs from one laccase to another. The substrate of ligninolytic enzymes is defined by structure and redox potential of a compound, including laccase. LiPs can oxidize substrates of extremely high electropotential, up to $E_{1/2}$= 1.49 V, while MnPs can oxidize substrates up to $E_{1/2}$ = 1.12 V. Laccases cannot oxidize non-phenolics when electropotential is higher than $E_{1/2}$= 1.06 V (Call and Mucke, 1997).

Fungal laccases oxidize a very wide range of substrates but the catalytic constants have been reported mostly for a small group of substrates (e.g. the non-natural test substrate ABTS and the phenolic compounds 2.6-dimethoxyphenol (DMP), guaiacol and syringaldazine) (Leonowicz et al., 2001). While the oxidations of guaiacol and DMP are significantly slower, ABTS and syringaldazine have high catalytic constant with high affinity by laccases. The substrates such as sinapic acid, hydroquinone and syringic acid

have low K_m values than para-substituted phenols, vanillic acid or its aldehyde. Laccases can be classified into three types according to their preference; ortho-, meta- or para-substituted phenols. Guaiacol, caffeic acid, catechol, gallic acid and pyrogallol are included in ortho-substituted compounds. These are better substrates than para-substituted compounds, including hydroquinone. Meta-substituted compounds, including m-phenylenediamine, orcinol, resorcinol and phloroglucinol have the lowest rates (Baldrian, 2006).

1.4.3. Laccase catalyzed reactions

Substrate oxidation by laccase is a one-electron reaction producing a free radical. In a typical laccase-catalyzed reaction (Figure 1.9), a phenolic substrate is subjected to a one-electron oxidation giving rise to an aryloxyradical. In the second stage of the oxidation, this active species can be changed into quinone. The quinone undergoes non-enzymatic coupling reactions leading to polymerisation. The bonds of the natural substrate, lignin, that are cleaved by laccase include, C_α - oxidation, C_α-C_β cleavage and aryl-alkyl cleavage. Laccases have low substrate specificity and their catalytic competence changes widely depending on the source. Although simple diphenols are good substrates for the majority of laccases, guaiacol and 2.6–dimethoxyphenol are generally better substrates. While laccase is able to catalyse the oxidation of other substituted polyphenols, aromatic amines, benzenthiols and a series of other compounds, it is unable to oxidase tyrosine, unlike tyrosinases (Chiacchierini et al., 2004).

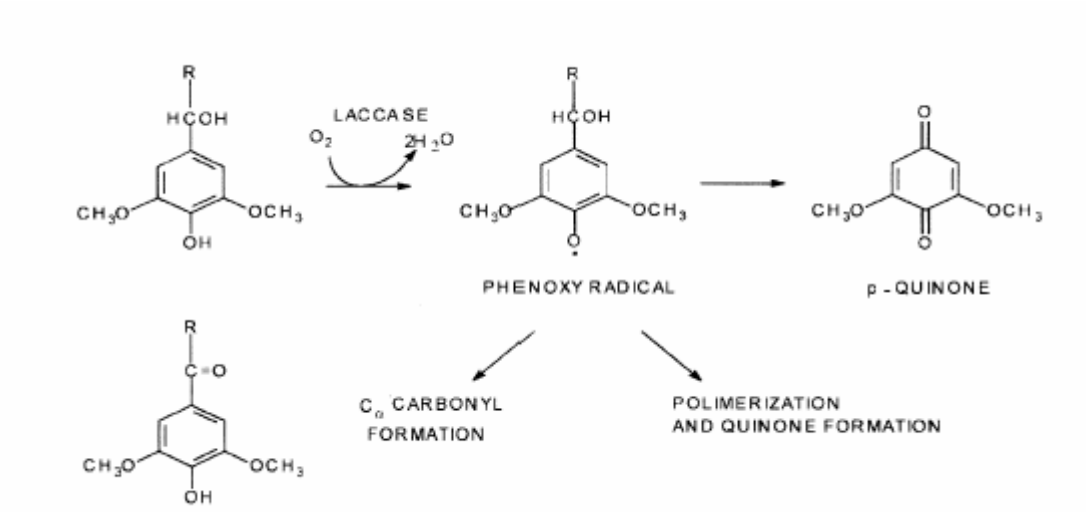

Figure 1.9 Laccase-catalysed oxidation of phenolic groups of lignin (Chiacchierini et al., 2004)

1.4.4. Laccase mediator system

The laccase mediator system (LMS) was first described in 1990 with the use of ABTS (2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) as the first mediator to solve problems in bio-bleaching of wood pulps (Bourbonnais and Paice, 1990). In the biodegradation of lignin, laccases were thought to play a key role. On the other hand, it was restricted to phenolic compounds due to the low oxidation potentials of laccases. The oxidation of nonphenolic lignin model compounds resulted from application of these enzymes in the presence of mediator compounds (Archibald et al., 1997).

Two main factors affect the activity of a LMS towards lignin. Firstly, the redox potential of the enzyme and, secondly, the stability and reactivity of the radical resulting from the oxidation of the mediator. Laccases isolated from different organisms react variably with different mediators and different substrates. Although approximately hundred different mediator compounds have been described, ABTS and HBT (1-Hydroxybenzotriazole) are the most common mediators for the LMS.

ABTS is oxidized by laccase to form a stable cation radical (Fig. 1.10). The function of ABTS in pulp delignification is not yet fully understood. On the other hand, it has been proposed that the ABTS cation radical acts as an electron carrier between residual lignin

in the kraft pulp fibre wall and the large laccase molecule that is not able to enter the fibre wall (Paice et al., 1995; Archibald et al., 1997).

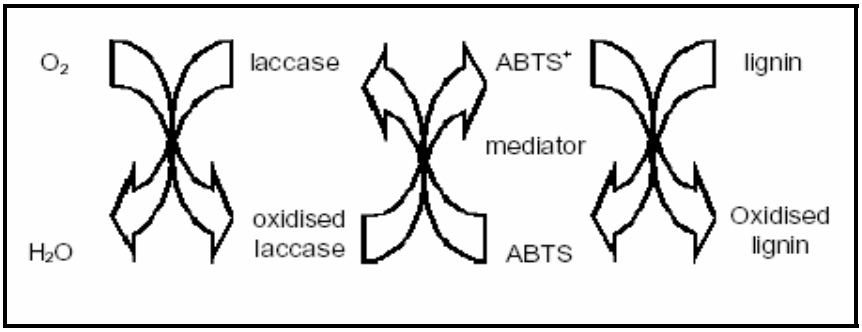

Figure 1.10 The oxidative catalytic action of laccase on lignin (Paice et al., 1995)

1-hydroxybenzotriazole (HBT) is oxidized by laccase to nitroxide radical (Figure 1.11). HBT nitroxide cation radical is able to oxidize a range of aromatic compounds only if HBT is used with laccase. The experiments with lignin model compounds revealed that laccase/HBT system depolymerized not only the phenolic polymer but also nophenolic lignin structures (Srebotnik and Hammel, 2000). Oxidation of HBT by laccase cannot be detected with spectrophotometric analysis. On the other hand, an O_2 consumption method was described for the oxidation of HBT (Ander and Messner, 1998).

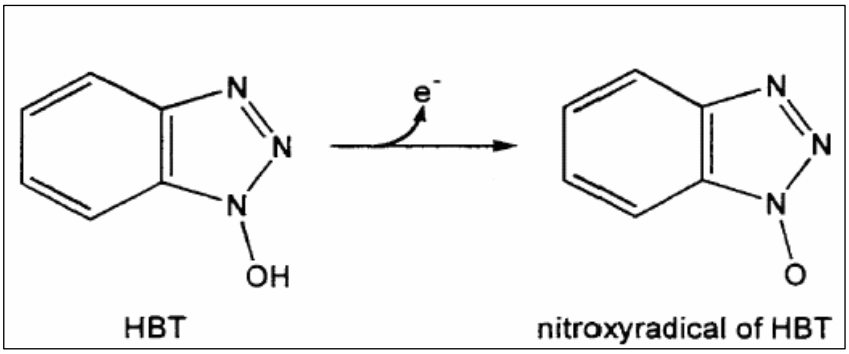

Figure 1.11 Nitroxyradical formation of HBT by laccase

1.4.5. Inhibition of laccase

A very wide range of compounds can inhibit laccase. Different laccases have differing tolerance toward inhibition by halides, indicating differential halide accessibility (or the opening size of the channel) of the trinuclear Cu cluster. Halides (excluding iodide), azide, cyanide, and hydroxide bind to the type 2 and type 3 Cu causing an interruption of the internal electron transfer and activity inhibition (Gianfreda et al., 1999). Tyrosinases can be inhibited by carbon monoxide, 4-hexylresorcinol or salicylhydroxamic acid. On the other hand, these inhibitors do not affect laccases. Dithiothreitol, thioglycolic acid, cysteine and diethyldithiocarbamic acid are reported as laccase inhibitors, which do not reduce the oxygen consumption by laccase during the catalysis, contrary to sodium azide (Baldrian, 2006).

Under alkaline pH conditions, laccase inhibition by OH^- can dominate catalysis. Other inhibitors, such as metal ions (e.g., Hg^{2+}), fatty acids, sulfhydryl reagents, hydroxyglycine, Kojic acid, desferal, and cationic quaternary ammonium detergents, may result in amino acid residue modification, Cu chelation, or conformational change (Gianfreda et al., 1999).

Due to the natural occurrence of laccases in soil, the inhibition by heavy metals must be taken into account. Some laccases exhibit sensitivity towards heavy metals, e.g. *G. lucidum* laccase. Many different substrates of laccase can competitively inhibit the transformation of other compounds in the complex environment of soil or decaying lignocellulosic material (Baldrian, 2006).

1.5. Production of Laccases

Laccase activity was identified in the cultures of a wide range of fungi, such as ascomycetes, basidiomycetes, wood and litter decomposing fungi, ectomycorrhizal fungi (Ikehata et al., 2004). Large amounts of readily available crude and purified laccases are required for biotechnological and environmental applications. Many studies have been devoted to identifying the most efficient laccase-producing source; to selecting the most suitable culture medium; to developing appropriate, reproducible, and inexpensive isolation procedures; and mainly to optimizing the enzyme production (Gianfreda et al., 1999).

1.5.1. Improvement of yield

It has been investigated that laccase was produced by several fungal strains and their diverse mutants. However, the production of laccase is dependent not only on the type of fungal strain but also on the composition of growth medium, pH, carbon/nitrogen ratio, temperature, and aeration rate (Ikehata et al., 2004). Among these factors, laccase production is affected by the presence of an inducer, its chemical structure, its amount, and the time of its addition. It has been reported that laccase formation is improved by several compounds, including mainly phenolic compounds, related to lignin or lignin derivatives. Nevertheless, nonphenolic compounds and extracts were found to be effective inducers of laccase production (Gianfreda et al., 1999).

2,5-xylidine which is the most effective inducer in fungi was first described in 1958 for the white-rot basidiomycete *Trametes (Coriolus) versicolor* (Ikehata et al., 2004). When this aromatic compound was added to fungal cultures, an over 160-fold stimulation of laccase activity was observed. Additionally, it was shown that not only in *Trametes versicolor*, but also in other lignin degrading fungi such as *Fomes annosum*, *Pholiota mutabilis*, *Pleurotus ostreatus* and *Phlebia radiata*, laccase levels are enhanced in the presence of 2,5- xylidine, whereas enzyme activities of *Botrytis cinerea* and *Rhizoctonia praticola* remained almost the same (Gianfreda et al., 1999). On the contrary, the compound markedly inhibited the laccase production in *Podospora anserina* cultures. When the compound was added after about 24 h to shaken cultures of *Pycnoporus cinnabarinus*, laccase formation was enhanced about 9-fold (Gianfreda et al., 1999). A surfactant, Tween 80, was also tested for its inducer properties in *Pycnoporus sanguineus* CY788 cultures. However, it was found to be less effective than 2,5-xylidine. More recently, ethidium bromide, a DNA intercalating agent, was used to induce *C. bulleri* Brodie 195062 laccase. It has been found that 1.5 μgmL^{-1} of ethidium bromide provided six-fold increase in laccase production compared to control culture (Ikehata et al., 2004).

It has been reported that laccase was detected in *P. chrysosporium* cultures when copper as copper sulfate ($CuSO_4$) was added to the culture medium, thus indicating the role of copper ion as an inducer (Gianfreda et al., 1999). The laccase production in *T. pubescens*

MB 89 cultures during the exponential growth phase was increased by the addition of copper. The cultures of two other *Trametes* species, *T. multicolour* MB 49 and *T. trogii* BAFC 463A showed the similar results with an optimum Cu concentration ranging from 0.5 mM to 2.0 mM (Ikehata et al., 2004).

The positive effect of ethanol was reported by Lee *et al*. in 1999. Ethanol is a preferable material than other inducers owing to its less expensive and less toxic properties. It has been reported that ethanol increased laccase production by a factor of 20 in the cultures of *T. versicolor* ATCC 20869 and in the cultures of *Botryosphaeria* sp. MAMB-5. On the other hand, it was also referred that the laccase production was decreased by addition of ethanol to the semi-solid-state culture of *T. versicolor* CBS100.29 (Ikehata et al., 2004).

Veratryl alcohol (3,4-dimethoxybenzyl alcohol) has important functions in the degradation of lignin and positive effects on the production of laccase in several fungi. When 1 mM veratryl alcohol was added to fungal cultures, *P. radiata, P. cinnabarinus*, and *T. versicolor* produced two to three times more laccase than did cultures lacking the alcohol (Gianfreda et al., 1999).

The production of the enzyme in both *T. versicolor* and *P. ostreatus* cultures was doubled by the addition of ferulic acid. It also provided a 3- to 4-fold increase in *P. mutabilis* (Gianfreda et al., 1999). *Marasmius quercophilus*, a predominant basidiomycete in typical Mediterranean ecosystems such as evergreen (*Quercus ilex* L.) or cork oak (*Quercus suber* L.) litters, is able to produce laccase. When the organism is induced with ferulic acid, it exhibits interesting enzymatic properties, such as great thermal stability at 30 and 40^{o}C (24 h), no inhibition with EDTA, Cystein or SDS (Farnet et al., 2004).

1.6. Purification of Laccases

In the cell-free supernatant, the amount of enzyme is generally low, so that the removal of excess water is an important process. Recently, membrane separation processes have been widely used for downstream processing. Ultrafiltration (UF) is the most commonly used membrane process in order to separate a solution that has a mixture of some

desirable components and some that are not desirable. The technique is dependent on both charge and size of the particle. The driving force for transport across the membrane is a pressure differential. UF provides both purification and concentration, diafiltration, removal of salt, changing the salt composition. UF membranes are able to retain species in the range of 300-500.000 daltons of molecular weight.

Proteins differ from each other according to their size, shape, charge, hydrophobicity, solubility, and biological activity. Proteins can be purified using different purification techniques which separate according to differences in their inherent similarities and differences (Table 1.1). Therefore, they can be purified using these properties by means of chromatographic techniques.

Table 1.1 Protein properties used during purification

Protein property	**Technique**
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic Interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)
Charge, hydrophobicity	Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC

1.7. Industrial Applications

1.7.1. Pulp and paper industry

Separation and degradation of lignin in woody tissues have attracted a great deal of research, especially because of its importance in the pulp and paper industry. Conventional and polluting chlorine-based delignification/bleaching procedures should

be replaced because of environmental concerns (Couto et al., 2006). Pre-treatments of wood pulp with ligninolytic enzymes might provide softer and cleaner strategies of delignification that also respect the integrity of cellulose (Mayer et al., 2002).

Few enzymatic treatments exhibit the delignification/brightening capabilities of modern chemical bleaching technologies, whereas extensive studies have been performed to develop alternative biobleaching systems. One of the few exceptions to this generalisation is the development of laccase mediator system (LMS) delignification technologies for kraft pulps (Call and Mucke, 1997). Additionally, laccase is more readily available and easier to manipulate compared to LiP and MnP. It has been reported that laccase is produced during bleaching by the fungus *Trametes versicolor* (Couto et al., 2006).

1.7.2. Food industry

Laccase can be used in bioremediation, elimination of unwanted phenolics, responsible for the browning, haze formation and turbidity in fresh fruit juice, beer and wine, ascorbic acid determination, sugar beet pectin gelation, baking, and as biosensor and to improve food sensory parameters. Productivity, efficiency and quality of food products can be increased by laccases without a costly investment. Laccases are able to cross-link biopolymers. Therefore, they are especially included in baking industry (Couto et al., 2006).

1.7.3. Textile industry

The textile industry has an important position in the total dyestuff market. During wet processing of textiles, large volumes of water and chemicals are consumed. Chemical compositions of chemical reagents used in the textile industry are diverse, ranging from inorganic compounds to organic compounds (Couto et al., 2006). Commercially available dyes are too resistant to exposure to light, water and different chemicals because of their chemical structures. Therefore, it is difficult to decolourise them due to their synthetic origin.

In developed countries, government rules are stricter about the removal of sytnthetic dyes from industrial effluents because of carcinogenic components, such as benzidine

and aromatic compounds (O'Neill et al., 1999). Due to uneconomical and insufficient processes for treating dye wastewater, laccases seems an attractive solution in degrading chemical structures of dyes (Couto et al., 2006). For example, several synthetic dyes like Azure B, Brilliant green, Congo red, Crystal violet, and Remazol Brilliant Blue R were decolourized by *F. flavus* in low nitrogen medium. *Trametes hirsuta* laccase degraded triarylmethane, indigoid, azo, and athraquinonic dyes sufficiently used in dyeing textiles (Mayer et al., 2002).

In 1996, Novozyme (Novo Nordisk, Denmark) started a new industrial application of laccases in denim fishing: DeniLite®. It was the first industrial laccase and the first bleaching enzyme acting with the help of a mediator molecule. Also, in 2001 the company Zytex (Zytex Pvt. Ltd., India) developed a new technique based on laccase-mediator system and named the new product as Zylite (Couto et al., 2006).

1.7.4. Other applications

Improving fuel ethanol production from renewable raw materials is another application of laccase in industry (Mayer et al., 2002). It has been experimented that *Trametes versicolor* laccase was expressed under control of the PGK1 promoter in *S. cerevisiae*. The aim was to increase its resistance to phenolic inhibitors in lignocellulose hydrolysates. The results showed that using laccase-expressing yeast strains for producing ethanol from lignocellulose was advantageous (Larsson et al., 2001).

Laccase is used for a new enzymatic method based on differentiate morphine from codeine in drug samples injected into a flow detection system. In this method, laccase and glucose dehydrogenase were immobilized at a Clark oxygen electrode, called an enzyme sensor. Morphine is oxidized by laccase with consumption of oxygen, while glucose dehydrogenase regenerates it. Laccase is not able to oxidize codeine. Therefore, the sensor is selective for morphine (Mayer et al., 2002).

Laccases in two different fungi, *Phanerochaete chrysosporium* and *T. versicolor*, were included in herbicide degradation studies. For example, isoxaflutole is an herbicide. It is activated in soils and plants to its diketonitrile derivative, the active form of the herbicide. This active form undergoes cleavage to the inactive form, benzoic acid analogue. Laccases from these organisms were capable of converting the diketonitrile to

the acid, in the presence of 2 mM ABTS acting as a redox mediator at pH 3 (Mougin et al., 2000).

1.8. The Aim of the Study

The aim of this project was to purify and characterize laccase from white-rot fungus *C. polyzona*. Purification and characterization of the laccase from different organisms not only might help for a better understanding of the mechanism and function of the enzyme, but could also open an avenue in quest of more effective enzymes with better physical and chemical properties. The importance of enzyme purification was also described by Dixon and Webb (1979) as follows: "Enzymes are found in nature in complex mixtures, usually in cells which perhaps contain a hundred or more different enzymes, and in order to study a given enzyme properly it must be purified.".

Properties investigated in this study include: molecular mass, the effect of pH and temperature on laccase activity, substrate specificity, effects of various inhibitors, spectrum analysis and N-terminal aminoacid sequence of laccase enzyme. By studying these various properties it will be possible to compare *C. polyzona* laccase with other characterized laccases reported in the literature.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Fungal culture and media

The white rot fungus *Coriolopsis polyzona* MUCL 38443 were provided by Tajalli Keshavarz from Westminster University London, UK. The production media studied were listed below.

2.1.1.1. Nutrient agar medium

39 g potato dextrose agar was dissolved in distilled water and completed to a total volume of 1 l. The solution was sterilized for 15 min. under 1.5 atm pressure at 121^{o}C. After cooling the solution to room temperature, it was poured on the plates.

2.1.1.2. Nutrient broth medium

Medium composition for cultivation of *C. polyzona* is shown in Table 2.1.

Table 2.1 Medium composition for cultivation of *C. polyzona* (in 1 liter distilled water)

Glucose	50 g
Bacteriological peptone	17 g
KH_2PO_4	2.5 g
$MgSO_4.7H_2O$	1.027 g
$CuSO_4.5H_2O$	0.03127 g
$MnSO_4.H_2O$	0.0559 g
Thiamine – HCl	10 mg

2.1.2. Chemicals

Acetic acid was obtained from Merck (Germany).

Acrylamide was obtained from Sigma –Aldrich (Germany).

4-aminobenzoic acid was obtained from Fluka (Switzerland).

Ammonium heptamolybdate tetrahydrate was obtained from Riedel-de Haen (Germany).

Ammonium persulphate (APS) was obtained from Merck (Germany).

Ammonium sulfate was obtained from Riedel-de Haen (Germany).

2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was obtained from Sigma-Aldrich (Germany).

L-asparagine monohydrate was obtained from Merck (Germany).

Bacteriological peptone was obtained from Acumedia (USA).

B-Mercaptoethanol was obtained from Merck (Germany).

Bovine serum albumine (BSA) was obtained from Sigma-Aldrich (Germany).

Bromophenol Blue was obtained from Sigma-Aldrich (Germany).

2,2'-Bichinolyl-4,4'-dicarbonic acid (BCA) was obtained from Fluka (Switzerland).

D-biotin was obtained from Fluka (Switzerland).

Bisacrylamide was obtained from Sigma-Aldrich (Germany).

Boric acid was obtained from Merck (Germany).

Calcium chloride dihydrate was obtained from Merck (Germany).

Catechol was obtained from Sigma-Aldrich (Germany).

Citric acid was obtained from Riedel-de Haen (Germany).

Cobalt chloride hexahydrate was obtained from Riedel-de Haen (Germany).

Coomassie Brilliant Blue was obtained fromBio-Rad (USA).

Copper sulfate pentahydrate was obtained from Merck (Germany).

L-cysteine was obtained from Merck (Germany).

2,6-dimethoxyphenol (DMP) was obtained from Fluka (Switzerland).

Diammonium tartarate was obtained from Sigma-Aldrich (Germany).

Disodium hydrogen phosphate was obtained from Riedel-de Haen (Germany).

Disodium tartarate was obtained from Merck (Germany).

Dithiothreitol was obtained from Merck (Germany).

Ferulic acid was obtained from Sigma-Aldrich (Germany).

Folic acid was obtained from Fluka (Switzerland).

Glucose was obtained from Riedel-de Haen (Germany).

Glycine was obtained from Merck (Germany).

Glycerol was obtained from Fluka (Switzerland).

Guaiacol was obtained from Merck (Germany).

Hydrochloric acid was obtained from Merck (Germany).

Hydroquinone was obtained from Sigma-Aldrich (Germany).

Iron chloride was obtained from Riedel-de Haen (Germany).

Iron sulfate heptahydrate was obtained from Riedel-de Haen (Germany).

Malt extract was obtained from Merck (Germany).

Manganese chloride tetrahydrate was obtained from Merck (Germany).

Manganese sulfate monohydrate was obtained from Merck (Germany).

Magnesium sulfate heptahydrate was obtained from Riedel-de Haen (Germany).

Maltose was obtained from Merck (Germany).

Methanol was obtained from Riedel-de Haen (Germany).

Niacinamide was obtained from Fluka (Switzerland).

Nickel chloride hexahydrate was obtained from Lachema (Czech Republic).

D-pantothenic acid hemicalcium salt was obtained from Fluka (Switzerland).

PDA was obtained from Acumedia (USA).

Potassium dihydrogen phosphate was obtained from Fluka (Switzerland).

Di-potassium hydrogen phosphate was obtained from Merck (Germany).

Protein molecular weight marker was obtained from Fermentas (USA).

Riboflavin was obtained from Fluka (Switzerland).

Rice used at spore formation was Reis (Turkey) or Sezon (Turkey) brand.

Sodium acetate was obtained from J. T. Baker (USA).

Sodium azide was obtained from Merck (Germany).

Sodium bicarbonate was obtained from Riedel-de Haen (Germany).

Sodium carbonate was obtained from Merck (Germany).

Sodium dodecyl sulphate was obtained from Sigma-Aldrich (Germany).

Sodium fluoride was obtained from Merck (Germany).

Sodium hydroxide was obtained from Riedel-de Haen (Germany).

Sodium dihydrogen phosphate was obtained from Riedel-de Haen (Germany).

Sodium molybdate dihydrate was obtained from Merck (Germany).

Sodium tartarate dihydrate was obtained from Panreac (Spain).

Soytone peptone was obtained from DIFCO (USA).

Tartaric acid was obtained from Riedel-de Haen (Germany).

TEMED was obtained from Carlo Erba (Italy).

Thiamine-HCl was obtained from Fluka (Switzerland).

Tri-sodium citrate dihydrate was obtained from Riedel-de Haen (Germany).

Tris-HCl was obtained from Merck (Germany).

Tyrosine was obtained from Merck (Germany).

Yeast extract was obtained from Lab M (UK).

Zinc chloride was obtained from Merck (Germany).

Zinc sulfate heptahydrate was obtained from Riedel-de Haen (Germany).

2.1.3. Solutions and buffers

2.1.3.1. Enzyme assay solutions and buffers

Table 2.2 shows the concentrations of enzyme assay solutions and buffers used during purification and characterization of *C. polyzona* laccase.

Table 2.2 Enzyme assay solution and buffer concentrations

ABTS solution in Na-tartrate buffer	2.5 M
Acetate buffer (pH 4.5)	20 mM
HCl	0.1 M
NaOH	10 M
Na-tartrate buffer (pH 3.0)	0.1 M
Phosphate buffer (pH 6.0 to 7.0)	50 mM
Tris-HCl buffer (pH 7.0)	20 mM
Veratryl alcohol	500 µl

2.1.3.2. Bicinchoninic acid (BCA) assay solutions

Two different solutions (Solution A and B) were prepared for detection and quantitation of total protein. To prepare working reagent, 50 ml of solution A and 1 ml of solution B were mixed. The concentrations of solutions were shown in Table 2.3.

Table 2.3 BCA assay solution concentrations

Solution A	BCA	1 g
	Sodium carbonate	2 g
	Sodium tartrate	0.16 g
	Sodium bicarbonate	0.95 g
	pH was adjusted to 11.25 with 10 M NaOH and dissolved in 100 ml of distilled water.	
Solution B	$CuSO_4$	4 g
	dissolved in 100 ml of distilled water.	
Working reagent	50 ml of solution A and 1 ml of solution B were mixed.	

2.1.3.3. SDS-PAGE and Native-PAGE solutions and buffers

- Monomer solution for SDS-PAGE (30% T/2.7% C_{bis})

  58.4 g acrylamide

  1.6 g bisacrylamide

  dissolved in 200 ml of distilled water and stored at 4^0C in the dark.

- 4X Running (Separating) gel buffer for SDS-PAGE

  1.5 M Tris-HCl (pH 8.8)

  dissolved in 200 ml of distilled water.

- 4X stacking gel buffer for SDS-PAGE

  0.5 M Tris-HCl (pH 6.8)

  dissolved in 50 ml of distilled water.

- 2X sample buffer for SDS-PAGE

  2.5 ml 4X Stacking buffer

  4 ml SDS solution (10%)

  2 ml Glycerol

  1 ml β-Mercaptoethanol

  0.05 % (w/v) Bromophenol blue

  dissolved in 10 ml of distilled water.

- Tank buffer for SDS-PAGE

  3 g Tris

  14.4 g Glycine

  10 ml SDS (0.1%)

  dissolved in 1 l of distilled water.

- Coomassie Blue Stain for SDS-PAGE

  0.1 g CBB R-250

  50 ml Methanol

  10 ml Acetic acid

  dissolved in 100 ml of distilled water.

- Gel Destain Solution for SDS-PAGE

  5 ml Methanol

  10 ml Acetic acid

  dissolved in 100 ml of distilled water.

- Ammonium persulphate (APS) concentration

  10% (w/v)

- Sodium dodecyl sulphate (SDS) concentration

  10% (w/v)

- 2X Sample Buffer for Native-PAGE

  2.5 ml 4X Stacking gel buffer

  2 ml Glycerol

  1 ml β-Mercaptoethanol

  0.05% (w/v) Bromophenol blue

  dissolved in 10 ml of distilled water.

- Tank buffer for Native-PAGE

  3 g Tris

  14.4 g Glycine

  dissolved in 1 l of distilled water.

- Native Gel substrate solution

  2 mM Guaiacol

  50 mM Acetate buffer (pH 4.5)

  stored in the dark.

2.1.3.4. Chemical solutions

Concentrations of chemical solutions used in the experiment were shown in Table 2.4.

Table 2.4 Concentrations of chemical solutions

ABTS solution	5 mM
Catechol	5 mM
2.6 dimethoxyphenol (DMP)	5 mM
DTT stock solution	10 mM
Ferulic acid	5 mM
Guaiacol	5 mM
Hydroquinone	5 mM
L-cysteine stock solution	10 mM
Sodium azide stock solution	1 mM
Sodium fluoride stock solution	10 mM
Tyrosine	3 mM

2.1.4. Laboratory equipments

Autoclaves	Nüve OT 4060 Steam Sterilizer (Turkey) Tuttnauer 2540 ML (Switzerland) Tuttnauer 3870 ELVC (Switzerland)
Balances	Precisa XB620C (Germany) Precisa 125A SCS (Germany)
Cellulose nitrate filter	0.45 μm cellulose nitrate membrane filter, Sartorius (USA)
Centrifuges	Microfuge 18, Beckman Coulter (Germany) Allegra™ 25R Centrifuge, Beckman Coulter (Germany)
Chromatography columns	Q sepharose, Amersham Biosci. (USA) Phenyl sepharose™ 6 Fast Flow (high sub.), Amersham Biosci.(USA) Mono Q™ 4.6/100 PE, Amersham Biosci.(USA)
Deep Freezers	- 20 ºC Arçelik (Turkey) - 80 ºC New Brunswick Scientific U410 Premium (England)
Electrophoresis equipment	Bio-Rad (USA)
FPLC	BioLogic Duo-Flow, Bio-Rad (USA)
Fraction collector	Model 2128, Bio-Rad (USA)
Ice machine	AF 10, Scotsman (UK)
Incubators	Memmert UM400 (Germany) Nüve EN400 (Turkey)
Laminar flow cabinet	Biolab Faster BH-EN2003 (Italy)
Magnetic stirrers	Labworld (Germany) Velp Scientifica (Italy)

Microplate reader	Model 3559 UV Microplate, Bio-Rad
Micropipettes	Eppendorf AG (Germany), 5000µl, 1000µl, 200µl, 100µl, 10µl
Microscope	Olympus (USA)
Orbital Shakers	Shell lab 1575R-2E (USA) Thermo Electron Corporation (USA)
pH-meter	Mettler Toledo MP220 (Switzerland)
Pipettes	10, 20, 200, 1000 µl, Eppendorf
Refrigerator	+ 4 ºC Arçelik (Turkey)
Ultracentrifugal filters	Vivascience
Ultrafiltration membrane	Millipore, Amicon Bioseperations
UV-Visible Spectrophotometers	Perkin Elmer Inst. Lambda 25 (USA) Shimadzu UV-Pharmaspec 1700 (Japan)
Vortex apparatus	Heidolph Reax top (Germany)

2.2. Methods

2.2.1. Cultivation conditions for *C. polyzona*

C. polyzona was grown on Potato Dextrose Agar (PDA) plates for 5 days at 28 ^{o}C. The agar blocks of 0.5 cm in diameter were taken from the mycelial edge in the PDA plates and used as inoculums for the spore production. For the spore production, long-grain rice was washed with deionized water for 30 min. in a buchner funnel, and autoclaved for 15 min in Fernbach Erlenmeyer flasks (130 g of rice per 2 l - Fernbach Erlenmeyer flask), and inoculated with the mycelial block. Humidity kept high in order to maintain rice moist for spore formation during incubation. After a 8-day incubation at 28°C, the fungal spores were harvested aseptically by extraction with 0.9 % NaCl solution and filtered through six layers of cheesecloth. After washing with 0.9 % NaCl, the spore suspension was adjusted to a concentration of about 1 x 10^8 spores/ml and was stored in 0.9% NaCl in a brown glass bottle at 4°C. 1.0 ml of the spore suspension was added to 100 ml of a culture medium in 500-ml Erlenmeyer flasks. The cultures were incubated at

28°C for 10 days on a rotary shaker (150 rpm). When laccase activity reached a maximum value, mycelium was removed from the culture by filtration.

2.2.2. Medium optimization studies of *C. polyzona*

Nine different media compositions were examined for medium optimization studies of *C. polyzona*.

Medium #1 : 10 g yeast extract, 0.25 g citric acid, 5.0 g $(NH_4)_2SO_4$, 5.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 0.02 g $CaCl_2.2H_2O$ per liter of distilled water.

Medium #2 : 10 g glucose, 3.0 g L-asparagine monohydrate, 0.5 g $MgSO_4.7H_2O$, 0.5 g KH_2PO_4, 0.6 g K_2HPO_4, 0.4 mg $CuSO_4.5H_2O$, 0.09 mg $MnCl_2.4H_2O$, 0.07 mg H_3BO_3, 0.02 mg $Na_2MoO_4.2H_2O$, 1.0 mg $FeCl_3$, 3.5 mg $ZnCl_2$, 0.1 mg Thiamine -HCl and 5.0 mg Biotin per liter of distilled water.

Medium #3 : 55 g glucose monohydrate, 17 g bacteriological peptone, 2.5 g K_2HPO_4, 1.027 g $MgSO_4.7H_2O$, 31.27 mg $CuSO_4.5H_2O$, 55.90 mg $MnSO_4.H_2O$ and 10 mg Thiamine - HCl per liter of distilled water.

Medium #4 : 15 g malt extract, 4.0 g yeast extract and 4.0 g glucose per liter of distilled water.

Medium #5 : 20 g malt extract, 1.0 g peptone and 15 g glucose per liter of distilled water.

Medium #6 : 15 g malt extract, 4.0 g yeast extract and 40 g glucose per liter of distilled water.

Medium #7 : 20 g maltose, 1.84 g diammonium tartarate, 2.3 g disodium tartarate, 1.33 g KH_2PO_4, 0.1 g $CaCl_2.H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.07 g $FeSO_4.7H_2O$, 0.046 g $ZnSO_4.7H_2O$, 0.035 g $MnSO_4.H_2O$, 0.007 g $CuSO_4.5H_2O$, 1.0 g yeast extract and vitamin solution (D-biotin 2 mg, D-pantothenic acid hemicalcium salt 0.2 mg, folic acid 0.2 mg, niacinamide 40 mg, thiamine- HCl 40 mg, p-aminobenzoic acid 20 mg and riboflavin 20 mg) per liter of

distilled water.

Medium #8 : 200 g Boiled potato, 20 g dextrose (glucose) per liter of distilled water.

Medium #9 : 10 g glucose, 10 g soytone peptone, 1 ml trace metals solution, 0.2 mM $MnSO_4$ per liter of distilled water and pH adjust with acetic acid at 5.0 [Trace metals solution: $FeSO_4.7H_2O$ 20mM, $C_6H_5Na_3O_7.2H_2O$ 40mM, $CuSO_4.5H_2O$ 1mM, $ZnCl_2$ 5mM, $MnSO_4.H_2O$ 20mM, $MgCl_2.6H_2O$ 50mM, $CoCl_2.6H_2O$ 5mM, $NiCl_2.6H_2O$ 0.1mM, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.5mM (pH 2 - adjust with HCl)].

These media were examined for laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) activities in order to select the most suitable medium for shake flask cultivations of *C. polyzona*. A concentration of 10^8spores/ml of *C. polyzona* was used to inoculate 100 ml medium in 500 ml shake flasks. The fungal cultures were grown in Thermo Electron Corporation (USA) orbital shaker incubator at 150 rpm and 28^{o}C. The experiment was done in triplicate.

2.2.3. Experimental methods

2.2.3.1. Laccase assay

The non-phenolic dye 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase to a cation radical. The concentration of the cation radical responsible for the intense blue-green colour can be correlated to the enzyme activity and is most often read between 414 nm and 420 nm. After the mycelium was removed by centrifugation (30 min at 12,500 x *g*), laccase activities in the culture supernatants were routinely determined by measuring the oxidation of ABTS (2.5 mM) in a sodium tartrate buffer (0.1 M, pH 3.0) at 414 nm (ε = 36 $mM^{-1}cm^{-1}$). One unit (U) of laccase activity was defined as 1.0 μmol of product formed per minute under the assay conditions. Laccase activities were monitored on a Perkin-Elmer Lambda 25 UV-Vis Spectrophotometer (Shelton, CT, USA). The reaction mixture which contained 950 μl

sodium tartrate buffer, 200 µl substrate and 50 µl enzyme, loaded into spectrophotometer and measured at 414 nm for 3 minutes of reaction time at 28°C (Eggert et al., 1996).

The laccase activity was calculated as follows:

$$U/l = [\ (\Delta A/t) / \varepsilon.d\ ] \ .\ (1 \times 10^6\ \mu mol/mol)\ .\ (V/v) \qquad \textbf{(2.1)}$$

ΔA = Absorbance change at 414 nm

ε = Extinction coefficient of ABTS at 414 nm = 36000 M^{-1} cm^{-1}

d = Light path of cuvette (cm)

V = Total reaction volume (ml)

v = Sample volume (ml)

2.2.3.2. Lignin peroxidase (LiP) assay

LiP activity in the culture supernatant was determined by measuring the oxidation of veratryl alcohol in glycine-HCl buffer (50 mM, pH 3.0) at 310 nm (ε= 9.3 mM^{-1} cm^{-1}). Glycine-HCl buffer was used as blank in spectrophotometer measurements. During LiP activity determination assay, the reaction mixture contained 500 µl veratryl alcohol, 390 µl glycine-HCl buffer and 100 µl centrifuged enzyme sample. Finally, 10 µl H_2O_2 (10 mM) was added to total 1000 µl final volume in quartz cuvvette, loaded into spectrophotometer and measured at 310 nm for 2 minutes of reaction time at 28°C (Tien et al., 1984). Equation 2.1 was used to calculate LiP activity.

2.2.3.3. Manganese peroxidase (MnP) assay

MnP activity was determined by the oxidation of 25 mM DMP in sodium tartrate buffer (50mM, pH 5.0). Sodium tartrate buffer was used as blank in spectrophotometer measurements. In MnP activity determination assay, the reaction mixture contained 100 µl 2,6-dimethoxyphenol, 690 µl sodium tartrate buffer and 100 µl centrifuged enzyme sample. Finally, 10 µl H_2O_2 (10 mM) was added for initiation of enzyme reaction to total 1000 µl final volume in quartz cuvvette, loaded into spectrophotometer and

measured at 469 nm (ε = 49.6 mM^{-1} $cm^{-}1$) and 30^{o}C for 2 minutes of reaction time (Wariishi et al, 1992). Equation 2.1 was used to calculate MnP activity.

2.2.3.4. Protein determination with bicinchoninic acid (BCA) assay

Protein concentration was determined with 2,2'-Bichinchonlyl-4,4'-dicarbonic acid (BCA), Na_2-salt at 562 nm in a Bio-Rad Model 3550 UV Microplate Reader (Hercules CA, USA). Bovine serum albumin was used as a standart.

BCA assay measures the formation of Cu^{+1} from Cu^{+2} by the Biuret complex in alkaline solutions of protein. There are two distinct reactions that take place with copper ions unique to the BCA assay. The first reaction occurs as the result of the interaction of copper and BCA with the following residues: cysteine, cystine, tryptophan and tyrosine at lower temperatures. At elevated temperatures it has been shown that peptide bond itself is responsible for colour development. This is why performing the assay at 37^{0}C or 60^{0}C increases the sensitivity and reduces the variation in the response of the assay to protein composition. The BCA reagent replaces the Folin-Ciocalteu reagent used in the Lowry assay with bicinchoninic acid (BCA). The BCA reagent forms a complex with Cu^{+1}, which has a strong absorbance at 562 nm.

BCA is advantageous in that it does not interact with as many substances as the Folin-Ciocalteu reagent, especially detergents and buffers. BCA method has sensitivity in the range of 0.5 μg to 20 μg of protein. The BCA assay is limited in that it interacts with most reducing agents and copper chelators. In general, these are not critical components of buffers and can be easily eliminated prior to the assay. The BCA assay has many advantages over other protein determination techniques: the color complex is stable; there is less susceptibility to detergents; it is applicable over a broad range of protein concentrations.

To prepare working reagent, 50 parts of Reagent A was mixed with 1 part of Reagent B. 25 μl of each standart, blank or unknown sample were put into the appropriate microtitre plate wells. 200 μl of working reagent was added to each well. The microtitre plate was covered and incubated at 37^{0}C for 30 minutes for colour development. Absorbance values were read at 562 nm with microtitre plate reader. All readings were carried out in

triplicate. Using BSA standart curve, protein concentration for each sample was determined.

2.2.4. Purification of the enzyme

2.2.4.1. Ultrafiltration

Ten-day-old cultures were filtered through Whatman filter papers no.1 to remove fungal mycelia. The supernatant was frozen (-80°C), thawed, and filtered through 0.45 µ filter to remove the insoluble material and then ultrafiltered through 10 kDa MWCO membrane (Millipore). The volume of the retentate was 150 ml.

2.2.4.2. Q sepharose anion exchange chromatography

After ultrafiltration, 30 ml of the extract was loaded onto the Q sepharose anion exchange column (ca. 67.5 ml resin volume) (Amersham Biosciences) equilibrated with 20 mM acetate buffer (pH 4.5). The column was then developed with a linear gradient of 0.5 M NaCl in 20 mM acetate buffer (pH 4.5). The flow rate was 2 ml/min and 5 ml fractions were collected with a fraction collector (Model 2128, Bio-Rad). Laccase-positive fractions were pooled and desalted by using MWCO 10 kDa ultracentrifugal filter devices. The buffer was changed at least three times. The solution was concentrated to 3 ml and adjusted to 1.7 M ammonium sulphate.

2.2.4.3. Phenyl sepharose hydrophobic interaction chromatography

After concentration of the enzyme solution, a hydrophobic interaction chromatography step was performed on a Phenyl Sepharose Fast Flow column (ca. 27 ml resin volume) (Amersham Biosciences) equilibrated with 1.7 M ammonium sulphate in 0.02 M acetate buffer (pH 4.5). The enzyme was eluted with a decreasing linear gradient of 0.02 M acetate buffer pH 4.5. Fractions of 3 ml were collected at a flow rate 3 ml/min. Laccase-positive fractions were pooled and buffer-exchanged by using MWCO 10 kDa ultracentrifugal filter devices. The enzyme solution was concentrated to 2 ml in 20 mM Tris-HCl (pH 7.0).

2.2.4.4. Mono Q anion exchange chromatography

After concentrating of the HIC fractions, the pooled sample was applied to Mono Q 4.6/100 anion exchange column (Amersham Biosciences) pre-equilibrated with 0.02 M Tris-HCl pH 7.0. Proteins were eluted with a linear gradient of 0.4 M NaCl in 0.02 M Tris-HCl (pH 7.0). 2 ml fractions were collected with a flow rate 1 ml/min. The active-fractions were pooled and desalted by using MWCO 10 kDa ultracentrifugal filter devices. The buffer was changed at least three times. The enzyme solution was concentrated to 2 ml in 20 mM Tris-HCl (pH 7.0) and upon determining its purity, was used for characterization studies.

2.2.5. Characterization of laccase

2.2.5.1. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), or denaturing gel electrophoresis, was used to monitor the development of the purification process, to determine homogeneity and to determine the relative molecular mass of the laccase enzymes. SDS-PAGE was carried on a 12.5 % separating gel and a 5 % stacking gel. Electrophoresis was run two hours with a voltage of 120 mV. Protein bands were stained with Coomassie brilliant blue R250. Gels were then destained with a mixture of acetic acid and ethanol (5%; 10%).

The Protein Molecular Weight Marker mix (SM #0431) included; β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), endonuclease Bsp 981 (25 kDa), β-lactoglobin (18.4 kDa) and lysosym (14.4 kDa).

2.2.5.2. Native-polyacrylamide gel electrophoresis

Native PAGE was accomplished by utilizing the same protocol used for SDS-PAGE with the exception that the anionic detergent, SDS, was omitted from gels and samples and the samples were not incubated at boiling temperatures prior to the loading of the gel. For the detection of the laccase activity, the gel was immersed in 2 mM guaiacol dissolved in 50 mM acetate buffer at pH 4.5.

2.2.5.3. Effect of temperature on the enzyme activity

The effect of temperature on laccase acitivity was determined by incubating the laccase in Na-tartarate buffer (0.1 M, pH 3.0) for 2 minutes at various temperatures ranging 20^0C to 80^0C and then determining the laccase activity with the ABTS assay method.

2.2.5.4. Effect of pH on the enzyme activity

The influence of pH on laccase activity was studied spectrophotometrically. The pH dependence was determined with ABTS (2.5 mM) as the substrate in 50 mM glycine-HCl buffers (pH 2.0 to 3.0), 50 mM sodium acetate buffers (pH 4.0 to 5.0) and 50 mM sodium phosphate buffers (pH 6.0 to 7.0). The assay was done in duplicate.

2.2.5.5. Effect of substrates on the enzyme activity

The oxidation of substrates was determined spectrophotometrically at the specific wavelength of each substrate (except ferulic acid which was dissolved in absolute ethanol) in Na-tartrate buffer (0.1 M, pH 3.0). The reaction mixtures contained 50 µl of enzyme that was added to 200 µl of substrate to start the reaction. The oxidation of the substrates was followed during a wavelength scan on the Lambda 25 UV-Vis Spectrometer (Perkin Elmer Instruments).

2.2.5.6. Effect of inhibitors on the enzyme activity

Four potential inhibitors (sodium azide, sodium fluoride, L-cysteine, dithiothreitol) were evaluated to test the inhibition properties of laccase. The effect of inhibitors on laccase activity was determined using ABTS (2.5 mM) as the substrate in Na-tartrate buffer (0.1 M, pH 3.0) in the presence of an inhibitor. The percentage of inhibition was then calculated.

2.2.5.7. Kinetic properties of the enzyme

Kinetic studies were conducted for ABTS as the substrate that could be oxidized by the laccase at various concentrations ranging 0.1 mM to 0.5 mM at pH 3.0. The data was subjected using the Michaelis-Menten equation and the binding affinity (K_m) was determined.

2.2.5.8. Spectral analysis

Laccase was tested as spectrophotometrically using Shimadzu UV-Pharmaspec 1700 spectrophotometer for giving absorbance at which wavelength.

2.2.5.9. N-terminal aminoacid sequencing

Purified laccase was buffer-exchanged to distilled water using MWCO 10 kDa ultracentrifugal filter devices, and sequence determination was kindly carried out by Vincenza Faraco at Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II.

3. RESULTS

3.1. Laccase Activities in Different Media Compositions

Culture conditions and medium composition can play a major role in the level of enzyme production. Studies were performed to choose a medium supporting maximum enzyme production by *C. polyzona.*

Nine media were examined for laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) activities and it was observed that *C. polyzona* produced high amounts of laccase in some media compositions. The organism did not appear to produce noticeable amounts of the other phenol-oxidases.

Laccase activity was detected in three different media; medium #1, medium #3 and medium #6. Laccase activities in medium #1 and medium #6 were detected on the 2nd day of cultivation which was early for a secondary metabolite activity (Figure 3.1 and 3.3). The third medium where laccase activity was detected was medium #3. Although first laccase activity was detected on the 5th day of the cultivation, later than medium #1 and #6, the level of activity was very high (75000 U.l^{-1}) compared to others (Figure 3.2). Therefore, medium #3 containing 50g/l glucose, 17 g/l bacteriological peptone, 2.5 g/l KH_2PO_4, 1.027 g/l $MgSO_4.7H_2O$, 0.03127 g/l $CuSO_4.5H_2O$, 0.0559 g/l $MnSO_4.H_2O$, 10 mg/l Thiamine–HCl was chosen for further laccase experiments.

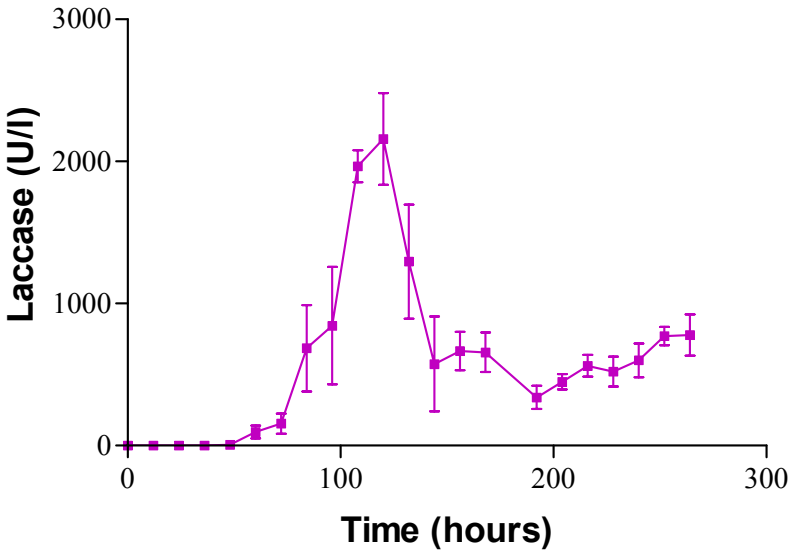

Figure 3.1 Laccase production by *C. polyzona* in medium #1

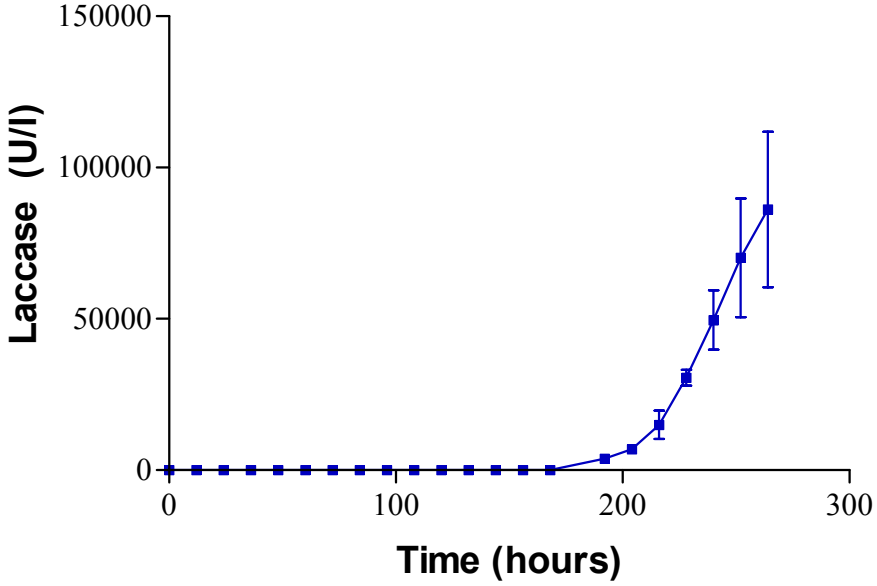

Figure 3.2 Laccase production by *C. polyzona* in medium #3

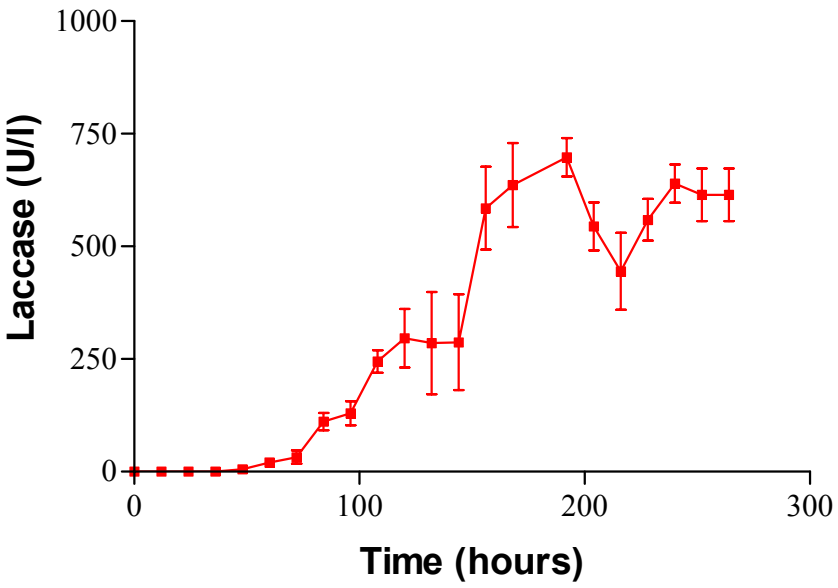

Figure 3.3 Laccase production by *C. polyzona* in medium #6

3.2. Purification of Laccase

The laccase activity in the medium #3 reached a maximum after ten days of cultivation. Ten-day-old cultures were filtered through Whatmann filter papers to remove fungal mycelia. The supernatant was frozen (-80°C), thawed, and filtered through 0.45 µ filter to remove the insoluble material and then ultrafiltered through 10 kDa MWCO membrane (Millipore) to remove polysaccharides from the supernatant, which were produced during growth of the fungus.

3.2.1. Elution profile of laccase on Q sepharose anion exchange resin

The Q sepharose anion exchange chromatography resin successfully separated the laccase enzyme from the contaminating dark brown colour present in the supernatant (Figure 3.4). The chromatography step resulted in the separation of several protein peaks in addition to the laccase enzyme.

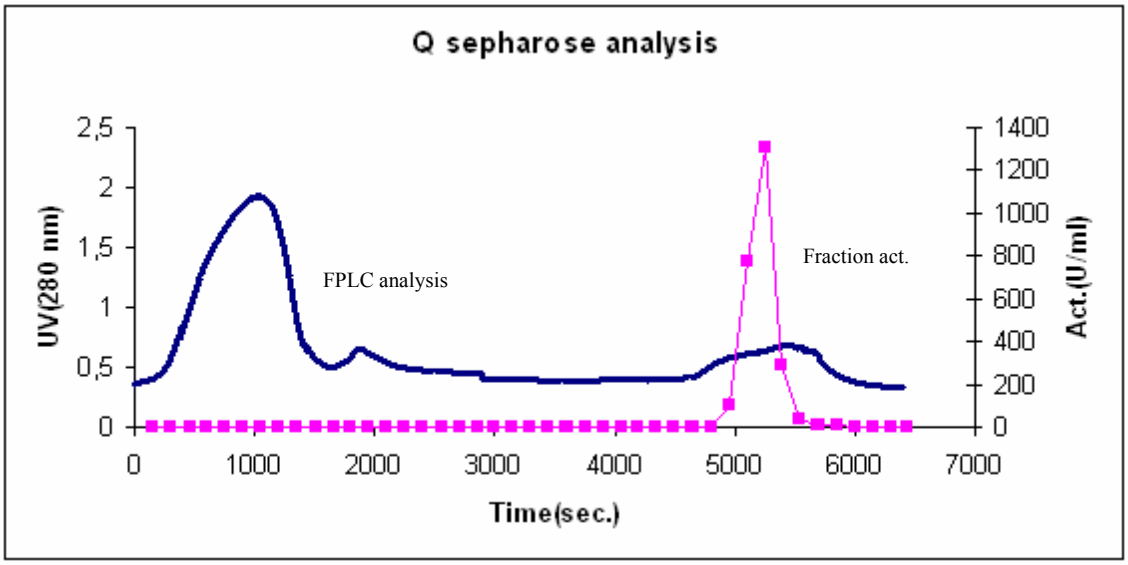

Figure 3.4 An elution profile of laccase on Q sepharose anion exchange resin. Blue line corresponds to the UV trace of the elution, while the enzyme activity was demonstrated in pink.

3.2.2. Elution profile of laccase on phenyl sepharose hydrophobic interaction resin

The phenyl sepharose hydrophobic interaction resin served to further separate the laccase enzyme, free of contaminating brown colour, from other proteins present in the fraction (Figure 3.5).

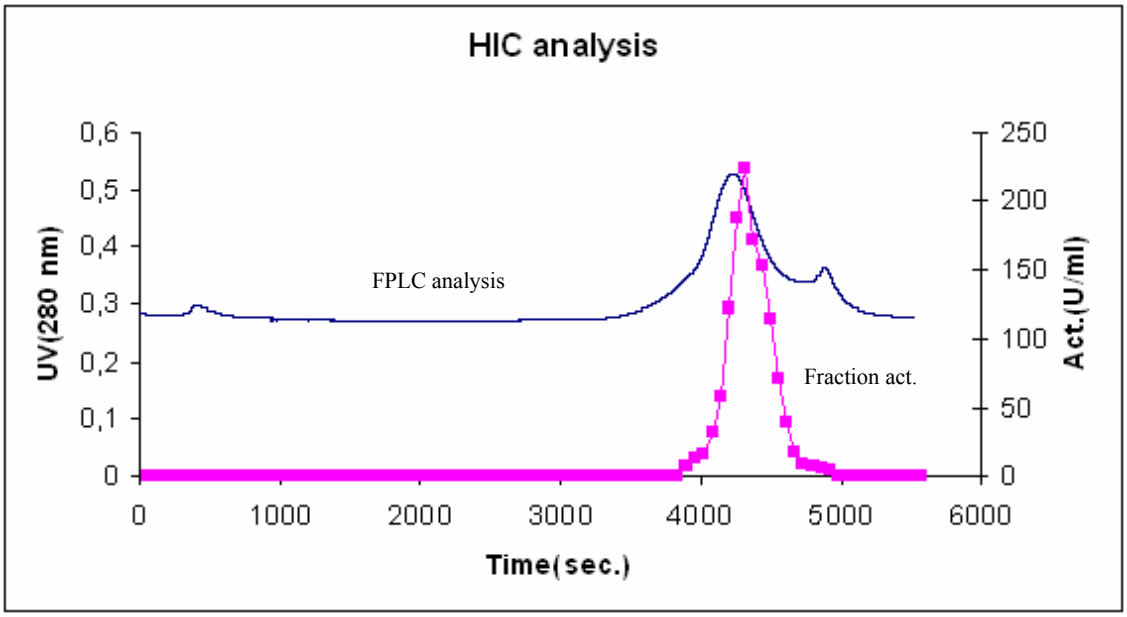

Figure 3.5 An elution profile of laccase on phenyl sepharose hydrophobic interaction resin. Blue line corresponds to the UV trace of the elution, while the enzyme activity was demonstrated in pink.

3.2.3. Elution profile of laccase on Mono Q anion exchange resin

For the final column chromatography step, Mono Q anion exchange chromatography resin was used. It provided isolation of laccase, free of contaminating proteins (Figure 3.6). The purification factor for laccase by *C. polyzona* was about 87 fold. The obtained purified laccase enzyme appeared as a bright blue fraction.

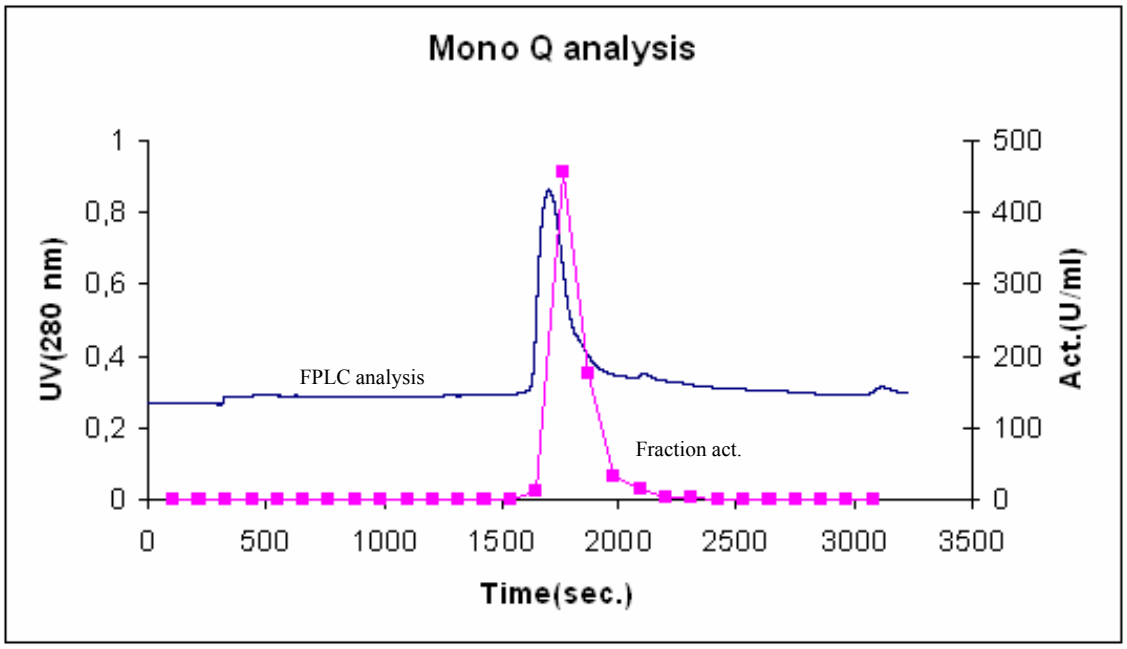

Figure 3.6 An elution profile of laccase on Mono Q anion exchange resin. Blue line corresponds to the UV trace of the elution, while the enzyme activity was demonstrated in pink.

- Laccase activity was routinely determined after each step by measuring the oxidation of 2.5 mM ABTS buffered in 0.1 M sodium tartrate buffer (pH 3.0). The measurements were monitored at 414 nm (Table 3.1).

Table 3.1 Laccase activities after each step

	Activity (U/ml)
Crude sup.	45
Concentrated ultrafiltrate	252
Q sepharose eluate	4106
HIC eluate	890
Mono Q eluate	434

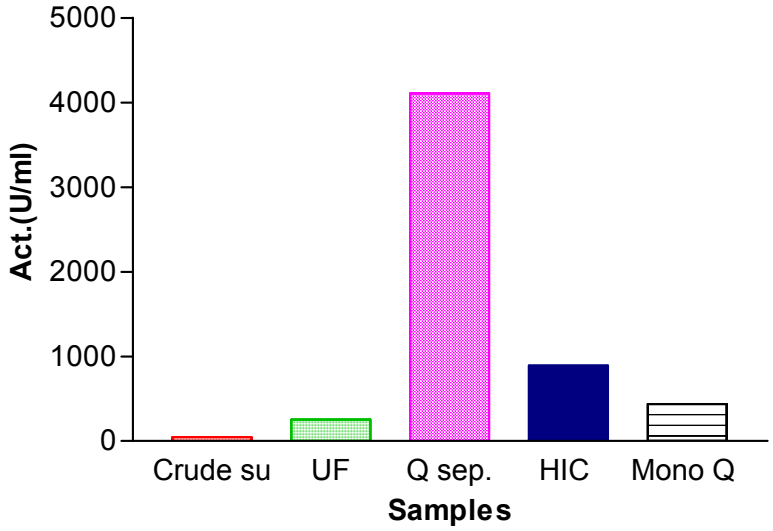

Figure 3.7 Enzyme activities after each step

- The purification yield for *C. polyzona* laccase was about 87 fold with a specific activity of 435 U/mg (Table 3.2).

Table 3.2 Purification table of *C. polyzona* laccase

Samples	Volume(ml)	Act.(U/ml)	mg/ml Protein	Total act.(U)	Total prot.(mg)	Specific act.(U/mg)	Purif.fold
Crude sup.	1800	45	9	81689	15709	5	1
Concent. ultrafiltrate	150	252	45	37760	6735	6	1
Q sep.eluate	2	4106	56	8212	112	73	15
HIC eluate	3	890	3	2669	8	334	67
Mono Q eluate	2	434	1	869	2	435	87

3.3. Characterization of Laccase

3.3.1. Electrophoretic analysis

Laccase produced by *C. polyzona* was purified to homogeneity according to the SDS-PAGE (Figure 3.8). The relative molecular mass of the protein was determined to be approximately 59 kDa relative to the molecular mass marker. Activity staining of the laccase, with guaiacol as substrate, revealed that the single protein band corresponded with the position of the laccase activity (Figure 3.9).

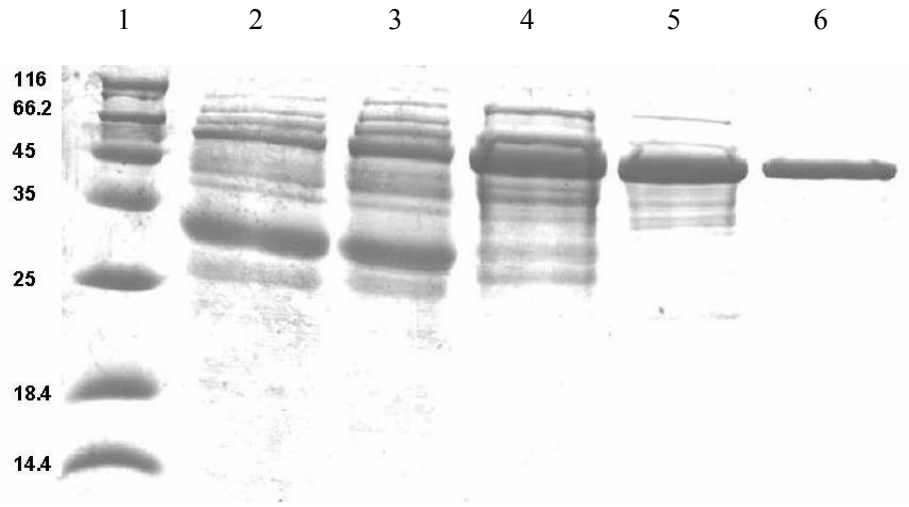

Figure 3.8 Results of SDS-PAGE analysis (Lane 1: Molecular weight marker (kDa)**,** Lane 2: Crude supernatant, Lane 3: Ultrafiltration retantate, Lane 4: Q sepharose eluate, Lane 5: HIC eluate, Lane 6: Mono Q eluate)

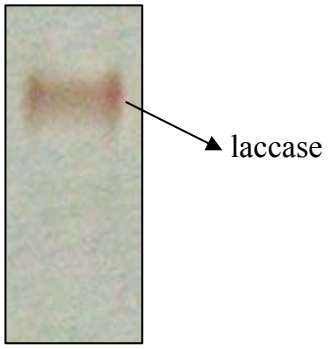

Figure 3.9 Zymogram staining of Native-PAGE gel for laccase with guaiacol as substrate

3.3.2. Effect of pH on the enzyme activity

The enzyme was tested for its characteristics under different pH conditions. The results showed that the enzyme had an optimal pH value at pH 3.0 with ABTS as the substrate. It did not exhibit any activity at pH 7.0 (Figure 3.10).

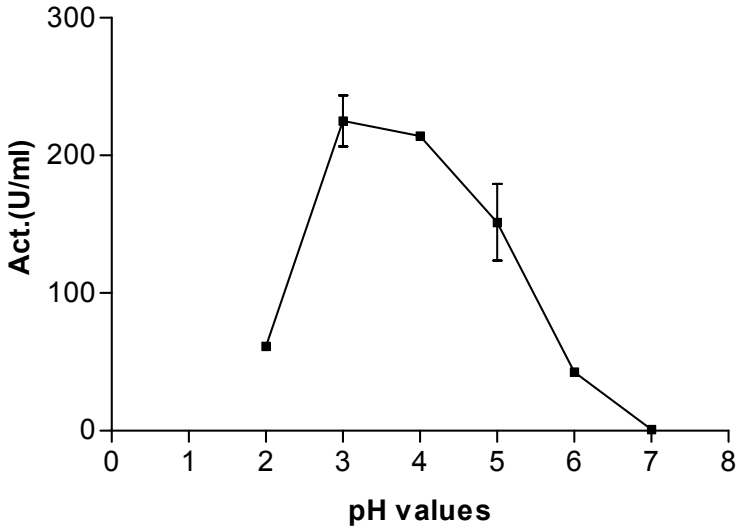

Figure 3.10 Effect of pH on laccase activity

3.3.3. Effect of temperature on the enzyme activity

The results showed that the optimum temperature for laccase was 40^0C. The enzyme rapidly started to lose its activity after 60^0C. It did not show any activity at 80^0C and was completely inactivated (Figure 3.11).

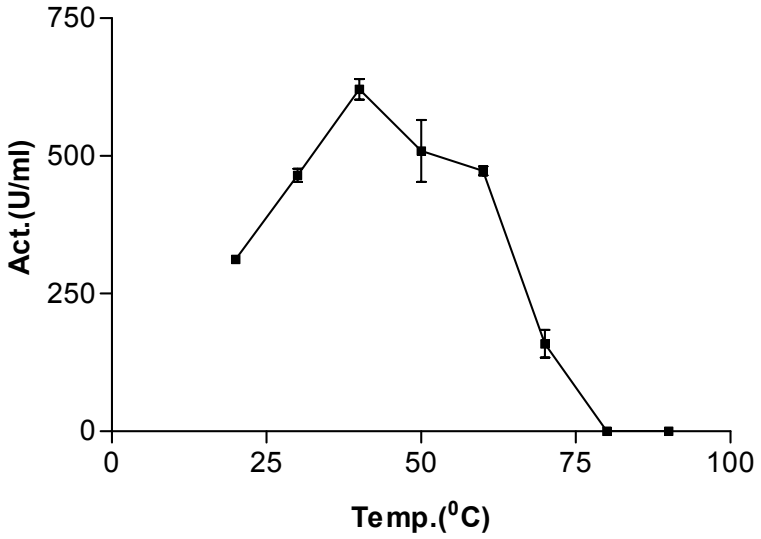

Figure 3.11 Effect of temperature on laccase activity

3.3.4. Effect of substrates on the enzyme activity

The substrate specificity of *C. polyzona* laccase was tested with a range of substrates (Table 3.3). Laccase oxidized hydroquinone much faster than other phenols. Oxidation of catechol was very low and oxidations of ferulic acid and tyrosine were not detectable.

Table 3.3 Effect of substrates on laccase activity

Substrate	Conc.(mM)	ε_{max} (M^{-1} cm^{-1})	Wavelength (nm)	Laccase act.(U/ml)
Hydroquinone	5	17542	248	1038
ABTS	5	36000	420	361
2,6 DMP	5	35645	470	56
Guaiacol	5	6400	436	31
Catechol	5	2211	450	12
Ferulic acid	5	12483	287	0
Tyrosine	3	0	280	0

3.3.5. Effect of inhibitors on the enzyme activity

The effects of several laccase inhibitors were examined with ABTS as substrate at pH 3.0 (Table 3.4). DTT is a reducing agent so it will give an electron to laccase and therefore it cannot oxidize substrates to acquire an electron for O_2 reduction. L-cysteine, another reducing agent like DTT, is able to bind to the enzyme to stop catalysis via the type 1 Cu center. Laccase activity was reduced by a variety of inhibitors and completely inhibited by 0.1 mM sodium azide and 5 mM L-cysteine. 1 mM L-cysteine and 2 mM DTT exhibited 95% inhibition on laccase while sodium fluoride caused 98% inhibition.

Table 3.4 Effect of inhibitors on laccase activity

Compounds	Conc. (mM)	Inhibition (%)
Sodium azide	0,1	100
Sodium fluoride	1	98
L-Cysteine	1	95
	5	100
Dithiothreitol (DTT)	1	90
	2	95

3.3.6. Kinetic properties of the enzyme

The results showed that the binding affinity (K_m value) of laccase for ABTS was 0.1703 (Figure 3.12).

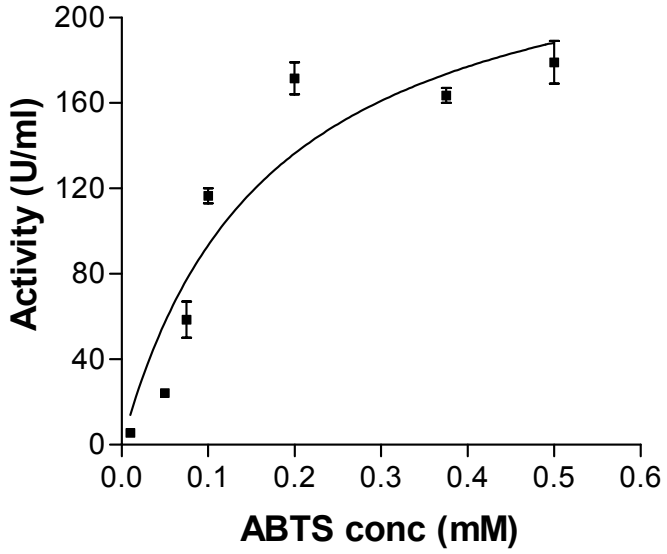

Figure 3.12 Kinetic properties of laccase for ABTS

3.3.7. Spectral analysis

The blue colour of laccase is due to its Cu content. Cu atoms are an important cofactor and require consideration when studying the features of laccase. This laccase seems to be typical. Spectral analysis of purified *C. polyzona* laccase showed a peak at 610 nm, corresponding to a type 1 blue Cu atom and a protein peak at 280 nm (Figure 3.13).

The 280 nm/610 nm absorbance ratio was 11. The ratio of A_{280} to A_{610} is indicative of the purity of the enzyme preparation. As the value gets smaller there is more Cu for protein. The smaller the number is calculated, the purer the enzyme preparation will be get.

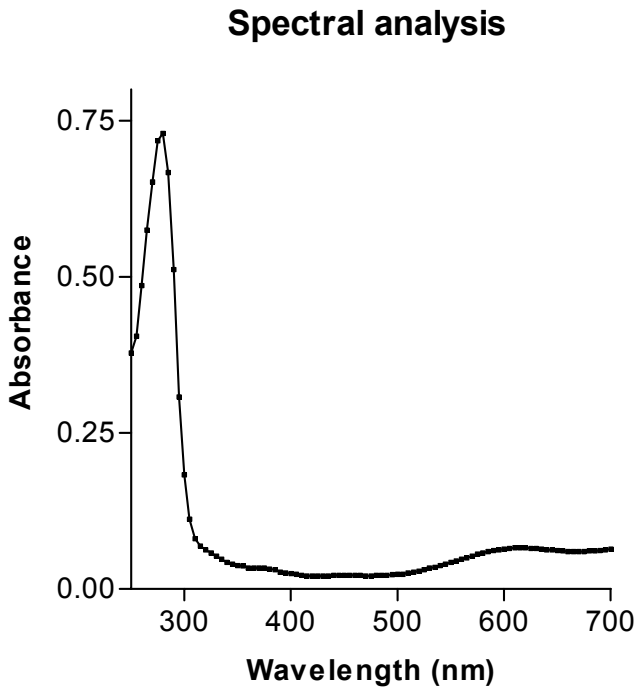

Figure 3.13 UV-Vis spectra of laccase

3.3.8. N-Terminal aminoacid sequence

The N-terminal sequence of *C. polyzona* laccase was determined to be n-Ala-Val-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile and exhibited the closest similarity to *P. cinnabarinus*, *C. hirsutus* and *T. rubrum* laccases (80%), *T. villosa* and *C. gallica* laccases (70%) and *P. radiata* laccase (50%) (Table 3.5).

Table 3.5 Comparison of N-terminal aminoacid sequences of *C. polyzona* laccase and other fungal laccases

Organism	N-terminal aminoacid sequence
Coriolopsis polyzona	**A V G P V A D L T I**
Pycnoporus cinnabarinus[a]	**A** I **G P V A D L T** L T N A A V S P D G F S
Coriolus hirsutus[b]	**A** I **G P** T **A D L T I** S N A E V S P D G F A
Phlebia radiata[c]	S I **G P V** T **D** F H **I** V N A A V S P
Coriolopsis gallica[d]	S I **G P V A** - **L T I** S N G - V - P
Trametes villosa[e]	**A** I **G P V A D L** V V A N A P V S P
Trichophyton rubrum[f]	**A** I **G P V A D L** H **I** T D D T I A P

[a] Eggert et al. (1996)
[b] Kojima et al. (1990)
[c] Saloheimo et al. (1991)
[d] Calvo et al.(1998)
[e] Yaver et al. (1999)
[f] Jung et al. (2002)

4. DISCUSSION AND CONCLUSION

Nowadays, most industries utilize chemical processes that cannot be considered as environmentally benign. These kinds of processes not only pollute the air, but also the water bodies in a very wide area. Particularly in the recent years, the concerns about the environmental pollution sped up the search for more environmentally friendly, in other words, biological processes. Therefore, studies about industrial enzyme technologies are in growing interest (Couto et al., 2006). Enzymatic processes are usually not only environmentally friendly, but also due to the substrate specificity, they may also increase the product or the application quality as well.

For use in large industries, mostly preffered enzymes to be studied are those oxidases and hydrolyses. Laccases are able to oxidize both phenolic and nonphenolic lignin related compounds (Mayer et al., 2002). They have high capacity to remove xenobiotic substances and produce polymeric products. Therefore, they are widely used in several biotechnological processes. These applications include the degradation and removal of lignin from woody tissues for paper and pulp industries (Call and Mucke, 1997), use for herbicide and pesticide degradation as a bioremediation agent (Mougin et al., 2000). They are even used as ingredients in cosmetics (Golz-Berner et al., 2004).

In this study, the initial aim was the optimization of culture conditions for *Coriolopsis polyzona* MUCL 38443 to get better enzyme production. The following target was to purify and characterize of *C. polyzona* laccase in order to understand enzyme properties.

The data obtained proved that sources of nutrients to create a medium are important to determine the type and amount of enzyme produced by fungi (Kachlishvili et al., 2005). Therefore, nine different media were examined for LiP, MnP and laccase production in *C. polyzona* cultures. The organism did not appear to produce noticeable amounts of LiP and MnP. On the other hand, laccase was produced in three different media compositions. The laccase activity was detected in medium #1 and medium #6 on the 2^{nd} day of cultivation, whereas the activity was detected in medium #3 on the 5^{th} day of

cultivation. Medium #1 was a complex medium that contained yeast extract as the carbon source, ammonium salt as the nitrogen source and citric acid as the chelator for elimination of excess metal phosphates (Muzariri et al., 2001). Although using complex medium in the process is much cheaper than pure substrates, it causes unpredictable biomass and product yield. Therefore, complex media obtained from cheap sources such as side products of some industries must be considered in the future studies when industrial scale production is in progress. Medium #6 contained only carbon sources, malt extract, yeast extract and glucose (Cifuentes et al., 1989). Adding a combination of carbon sources to the *C. polyzona* cultures did not support laccase production, and it is difficult to know which nutrient was efficiently used by the organism. Medium #3 contained glucose as the carbon source, bacteriological peptone as the nitrogen source, potassium, magnesium, manganese, $CuSO_4$ as minerals, and Thiamin-HCl as the vitamin source (Bermek et al., 2004). Due to problems in metabolism of complex sugars, glucose was chosen as the carbon source for *C. polyzona* cultures. Earlier studies suggested that the nature and concentration of nitrogen sources are important for regulating laccase production by fungi. While *L. edodes*, *R. lignosus* and *T. pubescens* cultures gave the highest laccase activity in high nitrogen media, *P. cinnabarinus*, *P. sanguineus* and *P. radiata* produced laccase in the nitrogen-limited cultures (Mikiashvili et al., 2006). Medium #3 contained bacteriological peptone as the nitrogen source in higher amounts, and it resulted in an increase in the enzyme production. It has been reported that addition of copper as copper sulfate ($CuSO_4$) to the culture medium induced laccase production in *P. chrysosporium* (Gianfreda et al., 1999), *T. pubescens* MB 89, *T. multicolour* MB 49 and *T. trogii* BAFC 463A cultures (Ikehata et al., 2004). Like other fungi, adding $CuSO_4$ to *C. polyzona* cultures provided the enhancement of laccase production. Due to the detection of high levels of laccase production in medium #3, it was chosen for further studies.

Following the production of high levels of laccase, purification procedures were performed.

Most purification protocols require more than one step to get the desired level of enzyme purity. The purification level needed is correlated to the instance where the protein preparation is needed. For most protein characterization studies, 85-95% purity is considered sufficient. In the current study, three chromatographic steps were studied for purification of laccase. Ion exchange chromatography provides high resolution, high capacity and high speed primary purification. It offers different selectivities using either anion or cation exchangers. The pH and the type of the buffer used during the step may be modified according to the charge properties of the sample, enabling the user to elute the same molecule in different time and affinity profiles. Therefore, it is possible, and most of the times, preferred to use ion exchange chromatography more than once during purification procedure. In the current study, Q sepharose anion exchange column was chosen for the first primary purification step. Q sepharose anion exchange resin was also used for the purification of laccases produced by *Aspergillus oryzae* (Berka et al., 1997), *Pleurotus eryngii* (Wang et al., 2005), *Poliporus pinsitus* (D'Acunzo et al., 2002), *Cerrena unicolor* strain 137 (Michniewicz et al., 2005), *Hericium erinaceum* (Wang et al., 2004), *Cyathus bulleri* (Vasdev et al., 2005), *Sclerotium rolfsii* (Ryan et al., 2003), *Trichophyton rubrum* LKY-7 (Jung et al., 2002) cultures and the purification of LiP and MnP produced by *Bjerkandera adusta* (Heinfling et al., 1998). In our study the technique provided the removal of the dark brown colored contaminating compounds from the supernatant.

As the second chromatography step, phenyl sepharose hydrophobic interaction resin was selected. Phenyl sepharose earlier also proved successful in purification of various laccases, i.e., *Melanocarpus albomyces* (Kiiskinen et al., 2002), *Trametes versicolor* (Brown et al., 2002), *Sclerotium rolfsii* (Ryan et al., 2003), *Trichophyton rubrum* LKY-7 (Jung et al., 2002), *Acer pseudoplatanus* (Sterjiades et al., 1992), *Trametes hirsuta* (Schroeder et al., 2006). The resin has high physical and chemical stability, very high batch-to-batch reproducibility and high capacity. While adsorption takes places in high salt concentrations, desorption takes place in low salt concentrations. These properties make this technique advantageous for bridgening between other steps. For example, *Rhus vernicifera* laccase was purified using phenyl sepharose hydrophobic interaction resin after ammonium sulphate precipitation (Johnson et al., 2003). The enzyme was

then eluted from phenyl hydrophobic interaction resin in low strength buffer, so that it was applied to an ion exchange column without an extra desalting step.

As the last step, Mono Q anion exchange column was used. It is a high resolution column that was prepacked for use with moderate pressure chromatography systems such as FPLC. The most advantageous properties of the column are its reproducibility, extremely high resolution and high capacity which make it indispensable as an ion exchange media (Périé et al., 1998, Saparrat et al., 2002, Xiao et al., 2003, Michniewicz et al., 2005, Cambria et al., 2000, Farnet et al., 2002). The column is generally used for the polishing steps of purification protocols. In our study, the column provided a selective isolation of laccase enzyme, with a bright blue color.

The purification procedure of *C. polyzona* laccase provided a 87-fold purification with a specific activity of 435 U/mg. Specific activity shows the ratio of the activity of the enzyme to the total amount of protein. During the procedure, total protein decreases as a consequence of desire to remove as much impurities as possible. In the study, the loss of nonspecific protein was much greater than the loss of activity; therefore specific activity increased step by step.

Following the confirmation of purity by SDS-PAGE, *C. polyzona* laccase was characterized. Characterization studies provide better understanding laccase reaction mechanisms, gathering information about the function of the enzyme for a given white-rot fungus and identifying better and more efficient enzymes by studying laccases from organisms that were not studied before, for potential industrial applications of the enzyme.

Previous studies revealed that fungi might produce several isozymes of laccases. For instance, *P. ostreatus* produces at least eight different laccase isoenzymes, six of which have been isolated and characterized (Giardina et al., 1999). In the current study, spectrophotometric analysis of the chromatographic fractions as well as zymogram staining of Native-PAGE gel with guaiacol revealed that *C. polyzona* produced a single isozyme with a molecular weight of approximately 59 kDa.

Optimum pH in fungal laccases is dependent on the substrate being studied. Generally, laccases are active in a pH range between 3.0-7.5 (Shleev et al., 2004). *C. polyzona* showed optimal laccase production at acidic pH (pH 3), when ABTS was used as the substrate. It was suggested that at higher pH, laccase activity is reduced due to the binding of a hydroxide anion to the T2/T3 copper centers of the enzyme that disrupts the internal electron transfer from T1 copper center to T2/T3 centers (Baldrian, 2006). It was shown that decolorization studies depend on initial pH. If the environment is not basic (between pH 5.5 and 7), the fungus reduces it to 4-5.5. If pH is too high (>7), decolorization will not occur, or it will only occur when pH is reduced to an optimal value (Gadd, 2001).

Laccase activity is achieved at temperatures ranging from 25^0C to 70^0C. Laccases produced by *Azospirillum lipoferum*, a bacteria, and *Albatrella dispansus*, an edible mushroom, show maximum activity at 70^0C. (Diamantidis et al., 2000; Wang et al., 2004, respectively). On the other hand, most WRF are mesophiles with optimum living temperatures at 25-30^0C. For example, *G. lucidum* laccase (Bonnen et al., 1994) and *T. rubrum* LKY-7 laccase (Jung et al., 2002) work between 25-30^0C. However, studies with *P. chrysosporium* showed that it has an unusual optima 37-40^0C. Although this organism needs higher temperatures, there is no evidence to suggest that decolorization rates faster than other white-rots performing at their own temperature optima (Gadd, 2001). In the study, *C. polyzona* laccase showed the maximum activity at 40^0C and was completely inhibited at 80^0C.

Laccases are able to oxidize phenolic and aromatic amines. *C. hirsutus* laccase highly oxidizes hydroquinone, guaiacol, ABTS, but not oxidize tyrosine (Shin et al., 2000). *P. cinnabarinus* laccase oxidized substrates in the order of ABTS>guaiacol>DMP>ferulic acid. Like *C. hirsutus*, tyrosine was not oxidized by *P. cinnabarinus* and *T. rubrum* LKY-7 (Eggert et al., 1996; Jung et al., 2002; respectively) laccases. ABTS was the first oxidized substrate by *T. rubrum* LKY-7 and *C. thermophilium* (Jung et al., 2002; Chefetz et al., 1998) laccases. *C. polyzona* laccase preferentially oxidized the substrates in the order of hydroquinone>ABTS>DMP>guaiacol>catechol. However, oxidation of tyrosine, which is a unique substrate for tyrosinase, was not detected. Like tyrosine, ferulic acid was not oxidized by laccase.

Laccases are inhibited by variety of compounds such as halides, heavy metals in the soil. The inhibitors may exhibit their activity on the enzyme in different concentrations. For example, *C. polyzona* laccase was completely inhibited by 0.1 mM sodium azide and 5 mM L-cysteine. Even 1 mM L-cysteine caused 95% loss of enzyme activity. Sodium fluoride caused 98% inhibition. 1 mM and 2 mM DTT caused 90% and 95% inhibition of laccase activity, respectively. These inhibition capabilities are comparable to those of other laccases reported before. For example, *P. cinnabarinus* laccase was completely inhibited by 0.1 mM sodium azide, 1 mM L-cysteine and 1 mM DTT (Eggert et al., 1996). Inhibitor concentrations were different for *C. hirsutus* laccase. It was completely inhibited by 0.1 mM L-cysteine, 0.1 mM sodium azide (Shin et al., 2000). Laccase enzyme by *T. rubrum* LKY-7 was completely inhibited by 0.1 mM sodium azide, 1 mM L-cysteine and 2 mM DTT. On the other hand, 1 mM L-cysteine caused 50% loss of enzyme activity (Jung et al., 2002). While 0.1 mM sodium azide caused 96% loss of enzyme activity in *C. thermophilium* laccase, 0.01 mM sodium azide caused 12% inhibition of laccase activity (Chefetz et al., 1998).

The Michaelis constant (K_m) of an enzyme is a measure of the affinity of the enzyme for its substrate. The value of K_m varies according to fungi that produce laccase and substrates being studied. For example, when ABTS was used as the substrate, K_m values for *T. rubrum* LKY-7 (Jung et al., 2002), *P. ostreatus* (Giardina et al., 1999), *P. sanguineus* (Litthauer et al., 2006) were calculated as 0.045 mM, 0.37 mM, 0.13 mM, respectively. In the study, the binding affinity (K_m value) for *C. polyzona* laccase was calculated using Michaelis-Menten kinetics and calculated as 0.1703 for ABTS as the substrate.

Laccases contain four copper atoms per monomer and three copper centers. Type 1 copper is responsible for the deep blue colour of the protein at an absorbance of approximately 600 nm. The purified laccase by T. *rubrum* LKY-7 has the distinct blue colour and gives absorption at 278 nm and 610 nm and a shoulder at 338 nm (Jung et al., 2002). Enzymes lacking the T1 copper are not called as true laccases by some authors. Others use the term ‘white laccases’ because these enzymes lack the characteristic absorption maximum at around 600 nm, such as *P. ostreatus* (Palmieri et al., 1997). *C.*

polyzona laccase was found to be a typical laccase with blue colour, giving absorbance optima at 280 nm and 610 nm.

Further studies must include medium optimizations (including investigations for ideal laccase inducers for this organism) for industrial applications followed by immobilization applications for further improve industrial application possibilities. More work might be undertaken to improve enzyme tolerance of towards inhibitors and thermal stabilities, etc.

REFERENCES

Ander, P., Messner, K., 1998. Oxidation of 1-hydroxybenzotriazole by laccase and lignin peroxidase. *Biotechnology Techniques*, **12**(3), 191-195

Archibald, F.S., Bourbonnais, R., Jurasek, L., Paice, M.G., Reid, I.D., 1997. Kraft pulp bleaching and delignification by *Trametes versicolor*. *Journal of Biotechnology*, **53**, 215-336

Aust, S. D., 1995. Mechanisms of degradation by white rot fungi. *Environ. Health Perspect*, **103**(5), 59-61

Baldrian, P., 2006. Fungal laccases-occurrence and properties. *FEMS Microbiol Rev.*, **30**, 215–242

Berka, R., Schneider, P., Golightly, E., Brown, S., Madden, M., Brown, K., Halkier, T., Mondorf, K., Xu, F., 1997. Characterization of the Gene Encoding an Extracellular Laccase of *Myceliophthora thermophila* and Analysis of the Recombinant Enzyme Expressed in *Aspergillus oryzae*. *Applied and Environmental Microbiology*, **63**(8), 3151-3157

Bermek, H., Yazıcı, H., Öztürk, H., Tamerler, C., Jung, H., Li, K., Brown, K., Ding, H., Xu, F., 2004. Purification and characterization of manganese peroxidase from wood-degrading fungus *Trichophyton rubrum* LSK-27. *Enzyme and Microbial Technology*, **35**, 87-92

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., Mougin, C., 2002. Crystal Structure of a Four-Copper Laccase Complexed with an Arylamine: Insights into Substrate Recognition and Correlation with Kinetics. *Biochemistry*, **41**, 7325-7333

Blanchette, R. A., 2000. A review of microbial deterioration found in archaeological wood from different environments. *International Biodeterioration & Biodegradation*, **46,** 189-204

Brown, M. A., Zhao, Z., Mauk, A. G., 2002. Expression and characterization of a recombinant multi-copper oxidase: laccase IV from *Trametes versicolor*. *Inorganica Chimica Acta*, **331**, 232-238

Bollag, J.-M., Leonowicz, A., 1984. Comparative Studies of Extracellular Fungal Laccases. *Applied and Environmental Microbiology*, **48**(4), 849-854

Bonnen, A. M., Anton, L. H., Orth, A. B., 1994. Lignin-Degrading Enzymes of the Commercial Button Mushroom, *Agaricus bisporus*. *Applied and Environmental Microbiology*, **60**(3), 960-965

Bourbonnais, R., and Paice, M.G., 1990. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of ABTS. *Applied Microbial Biotechnology*, **36**, 823-827.

Burdsall, Jr., 1998. Taxonomy of industrially important white rot fungi. In: Young, E., Akhtar, M., editors. Environmentally friendly technologies for the pulp and paper industry. John Wiley and Sons, Inc. New York. 481-504

Call, H., Mucke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (lignozym-process). *J. Biotechnol.*, **53**, 163-202

Calvo, A., Copa-Patino, J., Alonso, O., Gonzalez, A., 1998. Studies of the production and characterization of laccase activity in the basidiomycete *Coriolopsis gallica*, an efficient decolorizer of alkaline effluents. *Arch. Microbiol.*, **171**, 31-36.

Cambria, M. T., Cambria, A., Ragusa, S., Rizzarelli, E., 2000. Production, Purification, and Properties of an Extracellular Laccase from *Rigidoporus lignosus*. *Protein Expression and Purification*, **18**, 141-147

Chefetz, B., Chen, Y., Hadar, Y., 1998. Purification and Characterization of Laccase from *Chaetomium thermophilium* and Its Role in Humification. *Applied and Environmental Microbiology*, **64**(9), 3175-3179

Chiacchierini, E., Restuccia, D., Vinci, G., 2004. Bioremediation of Food Industry Effluents: Recent Applications of Free and Immobilised Polyphenoloxidases. *Food Sci. Tech. Int.*, **10**(6), 373–382

Cifuentes, V., Martínez, C., Pincheira G., 1989. Screening for lignin peroxidase genes in natural isolates of white rot fungi.- Laboratorio de Genética, Dept. de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Casilla 653, Santiago Chile

Couto, S., Herrera, J.-L., 2006. Industrial and biotechnological applications of laccases: A review. *Biotechnology Advances*, **24,** 500–513

Cullen, D., 2002. Molecular Genetics of Lignin-Degrading Fungi and Their Applications in Organopollutant Degradation. Springer-Verlag Berlin Heidelberg

D'Acunzo, F., Galli, C., Masci, B., 2002. Oxidation of phenols by laccase and laccase-mediator systems: Solubility and steric issues. *Eur. J. Biochem.*, **269**, 5330-5335

D'Souza, T. M., Boominathan, K., Reddy, C. A., 1996. Isolation of Laccase Gene-Specific Sequences from White Rot and Brown Rot Fungi by PCR. *Applied and Environmental Microbiology*, **62**(10), 3739-3744

Diamantidis, G., Effosse, A., Potier, P., Bally, R., 2000. Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*. *Soil Biology & Biochemistry*, **32**, 919-927

Dixon, M., Webb, E.C., 1979. Enzymes, third edition. New York: Academic Press, Inc.

Eggert, C., Temp, U., Eriksson, K.-E., 1996. The Ligninolytic System of the White Rot Fungus *Pycnoporus cinnabarinus*: Purification and Characterization of the Laccase. *Applied and Environmental Microbiology*, **62**(4), 1151–1158

Erwin, E. J. , Jong, ed De, Field, J. A., 1993. Stimulation of Ligninolytic Peroxidase Activity by Nitrogen Nutrients in the White Rot Fungus Bjerkandera sp. Strain BOS55. *Applied and Environmental Microbiology*, **59**(12), 4031-4036

Farnet, A.-M., Criquet, S., Pocachard, E., Gil, G., Ferré, E., 2002. Purification of a new isoform of laccase from a *Marasmius quercophilus* strain isolated from a cork oak litter (*Quercus suber* L.). *Mycologia*, **94**(5), 735-740

Farnet, A.-M., Criquet, S., Cigna, M., Gil, G., Ferré, E., 2004. Purification of a laccase from *Marasmius quercophilus* induced with ferulic acid: reactivity towards natural and xenobiotic aromatic compounds. *Enzyme and Microbial Technology*, **34**, 549–554

Froehner, S.C., Eriksson, K.E.L., 1974. Purification and properties of *Neurospora crassa* laccase. *Journal of Biotechnology*, **120**(1), 458-465

Gadd, G. M., 2001. Fungi in bioremediation. British Society symposium series; 23. ISBN 0 521 78119 1

Gianfreda, L., Xu, F., Bollag, J., 1999. Laccases: A Useful Group of Oxidoreductive Enzymes. *Bioremediation J.*, **3,** 4-25

Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G., Sannia, G., 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem. J.*, **341**, 655-663

Golz-Berner, K., Walzel, B., Zastrow, L., Doucet, O., 2004. Cosmetic and dermatological preparation containing copper-binding proteins for skin lightening. Int. Pat. Appl. WO2004017931

Hakulinen, N., Kiiskinen, L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., Rouvinen, J., 2002. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Natural Structural Biology*, **9**, 601-605

Han, M.-J., Choi, H.-T., Song, H.-G., 2005. Purification and Characterization of laccase from the White Rot Fungus *Trametes versicolor*. *The Journal of Microbiology*, **43**(6), 555-560

Heinfling, A., Martínez, M.-J., Martínez, A.-T., Bergbauer, M., Szewzyk, U., 1998. Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*. *FEMS Microbiology Letters*, **165**, 43-50

Ikehata, K., Buchanan, I. D., Smith, D. W., 2004. Recent developments in the production of extracellular fungal peroxidases and laccases for waste treatment. *J. Environ. Eng. Sci.*, **3,** 1–19

Johnson, D., Thompson, J., Brinkmann S., Schuller, K., Martin, L., 2003. Electrochemical Characterization of Purified *Rhus* V*ernicifera* Laccase: Voltammetric Evidence for a Sequential Four-Electron Transfer. *Biochemistry*, **42**, 10229-10237

Jung, H., Xu, F., Li, K., 2002. Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. *Enzyme and Microbiol Technology*, **30**, 161-168

Kachlishvili, E., Penninckx, M., Tsiklauri, N., Elisashvili, V., 2005. Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World Journal of Microbiology & Biotechnology,* DOI 10.1007/s11274-005-9046-8

Kiiskinen, LL., Viikari, L., Kruus, K., 2002. Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. *Appl. Microbiol. Biotechnol.*, 59, 198–204.

Kirk, T. K., Cullen, D., 1998. Enzymology and Molecular Genetics of Wood Degradation by White-Rot Fungi. In: Raymond A., Akhtar M., editors. Environmentally Friendly Technologies for the Pulp and Paper lndustry. John Wiley and Sons, Inc. New York. 273-307

Kojima, Y., 1990. Cloning, sequence analysis and expression of ligninolytic phenoloxidase genes of the white rot basidiomycete *Coriolus hirsutus*. *J. Biol. Chem.*, **266**, 19867–19870

Kumar, S. V. S., Phale, P. S., Durani, S., Wangikar, P. P., 2003. Combined Sequence and Structure Analysis of the Fungal Laccase Family. *Biotechnology and Bioengineering*, **83**, 386-384

Lalitha Kumari, H., Sirsi, M., 1972. Purification and properties of laccase from *Ganoderma lucidum*. *Arch Microbiol*., **84**, 350–357.

Larsson, S., Cassland, P., Jönsson, L. J., 2001. Development of a *Saccharomyces cerevisiae* Strain with Enhanced Resistance to Phenolic Fermentation Inhibitors in Lignocellulose Hydrolysates by Heterologous Expression of Laccase. *Applied and Environmental Microbiology*, **67**(3), 1163-1170

Lee, I.-Y., Jung, K.-H., Lee, C.-H., Park, Y.-H., 1999. Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. *Biotechnol. Lett.*, **21**, 965–968

Leonowicz, A., Cho, N-S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D., Rogalski, J., 2001. Fungal laccase: properties and activity on lignin. *J. Basic Microbiol.*, **41**(3-4), 185–227

Litthauer, D., van Vuuren, M. J., van Tonder, A., Wolfaardt, F. W., 2006. Purification and kinetics of a thermostable laccase from *Pycnoporus sanguineus* (SCC 108). *Enzyme and Microbiol Technology*, Article in Press

Mayer, A., Staples, R. C., 2002. Laccase: new functions for an old enzyme. *Phytohemistry*, **60**, 551-565

Michniewicz, A., Ulrich, R., Ledakowicz, S., Hofrichter, M., 2005. The white-rot fungus *Cerrena unicolor* strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. *Appl. Microbiol. Biotechnol*. DOI 10.1007/s00253-005-0015-9

Mikiashvili, N., Wasser, S., Nevo, E., Elisashvili, V., 2006. Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. *World Journal of Microbiology & Biotechnology*, **22**, 999-1002

Mougin, C., Boyer, F.D., Caminade, E., Rama, R., 2000. Cleavage of the diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases. *J. Agric. Food Chem.*, **48**, 4529–4534

Muzariri, CC., Mapingire, J., Mazorodze, J., Mandikutse, L., 2001. Isolation and screening of microorganisms for potential application in remediation of effluent water from the pulp and paper industry 2nd WARFSA/WaterNet Symposium: Intergrated Water Resources Management: Theory, Practice, Cases; Cape Town

Nagai, M., Kawata, M., Watanabe, H., Ogawa, M., Saito, K., Takesawa, T., Kanda, K., Sato, T., 2003. Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. *Microbiology*, **149**, 2455-2462

O'Neill, C., Hawkes, F.R., Hawkes, D.L., Lourenco, N.D., Pinheiro, H.M., Delee, W.,,1999. Colour in textile effluents-sources, measurement, discharge consents and simulation: a review. *J. Chem. Technol. Biotechnol.*, **74**, 1009–18

Orth, A. B., Royse, D. J., Tien, M., 1993. Ubiquity of Lignin-Degrading Peroxidases among Various Wood-Degrading Fungi. *Applied and Environmental Microbiology*, **59**(12), 4017-4023

Paice, M.G., Bourbonnais, R., Reid, I.D., Archibald, F.S., Jurasek, L., 1995. Oxidative bleaching enzymes: A review. *Journal of pulp and paper science*, **21**(8), 280-284

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A., Sannia, G., 1997. A Novel Laccase from *Pleurotus ostreatus*. *The Journal of Biology Chemistry*, **272**(50), 31301-31307

Palonen, H., 2004. Role of lignin in the enzymatic hydrolysis of lignocellulose. *VTT publications* 520

Périé, F. H., Reddy, G. V. B., Blackburn, N. J., Gold, M. H., 1998. Purification and Characterization of Laccases from the White-Rot Basidiomycete *Dichomitus squalens*. *Archives of Biochemistry and Biophysics*, **353**(2), 349-355

Piontek, K., Antorini, M., Choinowski, T., 2002. Crystal Structure of a Laccase from the Fungus *Trametes versicolor* at 1.90-Å Resolution Containing a Full Complement of Coppers. *The Journal of Biological Chemistry*, **277,** 37663-37669

Rosa, L. H., Machado, K. M., Jacob, C. C., Capelari, M., Rosa, C. A., Zani, C. L., 2003. Screening of Brazilian Basidiomycetes for Antimicrobial Activity. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, **98**(7), 967-974

Rosconi, F., Fraguas, L. F., Martinez-Drets, G., Castro-Sowinski, S., 2005. Purification and characterization of a periplasmic laccase produced by *Sinorhizobium meliloti*. *Enzyme and Microbial Technology*, 36, 800-807

Ruiz-Dueñas, F., Martines M. J., Martinez A. T., 1999. Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. *Molecular Microbiology*, **31**(1), 223-235

Ryan, S., Schnitzhofer, W., Tzanov, T., Cavaco-Paulo, A., Gübitz, G. M., 2003. An acid-stable laccase from *Sclerotium rolfsii* with potential for wool dye decolourization. *Enzyme and Microbiol Technology*, 33, 766-774

Saloheimo, M., Niku-Paavola, ML., Knowles, JKC., 1991. Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. *J. Gen. Microbiol*., **137**, 1537– 1544

Saparrat, M. C. N., Guillén, F., Arambarri, A. M., Martínez, A. T., Martínez, M. J., 2002. Induction, Isolation, and Characterization of Two Laccases from the White Rot Basidiomycete *Coriolopsis rigida*. *Applied and Environmental Microbiology*, **68**(4), 1534-1540

Schoemaker, H. E., Piontek, K., 1996. On the interaction of lignin peroxidase with lignin. *Pure & Appl. Chem.*, **68**(11), 2089-2096

Schroeder, M., Heumann, S., Silva, C. J. S. M., Cavaco-Paulo, A., Guebitz, G. M., 2006. Specificities of a chemically modified laccase from *Trametes hirsuta* on soluble and cellulose-bound substrates. *Biotechnol Lett.*, **28**, 741–747

Shin, K.-S., Lee, Y.-J., 2000. Purification and Characterization of a New Member of the Laccase Family from the White-Rot Basidiomycete *Coriolus hirsutus*. *Archives of Biochemistry and Biophysics*, **384**(1), 109-115

Shleev, SV., Morozova, O., Nikitina, O., Gorshina, ES., Rusinova, T., Serezhenkov, VA., Burbaev, DS., Gazaryan, IG, Yaropolov, AI., 2004. Comparison of physico-chemical characteristics of four laccases from different basidiomycetes. *Biochimie*, **86**, 693-703

Solomon, E. I., Sundaram, U. M., Machonkin, T. E., 1996. Multicopper Oxidases and Oxygenases. *Chem. Rev.*, **96**, 2563-2605

Srebotnik, E., Hammel, K. E., 2000. Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system. *Journal of Biotechnology*, **81**, 179-188

Srinivasan, C., D'Souza, T. M., Boominathan, K., Reddy, C. A., 1995. Demonstration of Laccase in the White Rot Basidiomycete *Phanerochaete chrysosporium* BKM-F1767. *Applied and Environmental Microbiology*, **61**(12), 4274–4277

Sterjiades, R., Dean, J. F., Eriksson, K.-E., 1992. Laccase from Sycamore Maple (*Acer pseudoplatanus*) Polymerizes Monolignols. *Plant Physiol.*, **99**, 1162-1168

Tien, M., Kirk, T. K., 1984. Lignin-Degrading Enzyme from *Phanerochaete chrysosporium*: Purification, Characterization, and Catalytic Properties of a Unique H_2O_2-Requiring Oxygenase. *Biochemistry*, **81**, 2280-2284

Wang, HX., Ng, TB., 2004. A new laccase from dried fruiting bodies of the monkey head mushroom *Hericium erinaceum*. *Biochemical and Biophysical Research Communications*, **322**, 17-21

Wang, HX., Ng, TB., 2004. A novel laccase with fair thermostability from the edible wild mushroom (*Albatrella dispansus*). *Biochemical and Biophysical Research Communications*, **319**, 381–385

Wang, HX., Ng, TB., 2005. Purification of a laccase from fruiting bodies of the mushroom *Pleurotus eryngii*. *Appl. Microbiol. Biotechnol.* DOI 10.1007/s00253-005-0086-7

Wang, HX., Ng, TB., 2006. A laccase from the medicinal mushroom *Ganoderma lucidum*. *Appl. Microbiol. Biotechnol.*, **72**(3), 508-13

Wariishi, H., Vallis, K., Gold, M. H., 1992. Manganese(II) Oxidation by Manganese Peroxidase from the Basidiomycete *Phanerochaete chrysosporium*. *The Journal of Biological Chemistry*, **267**(33), 23689-23695

Wesenberg, D., Kyriakides, I., Spiros N. Agathos S. N., 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*, **22**, 161–187

Xiao, Y. Z., Tu, X. M., Wang, J., Zhang, M., Cheng, Q., Zeng, W. Y., Shi, Y. Y., 2003. Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. *Appl. Microbiol. Biotechnol.*, **60**, 700-707

Vasdev, K., Dhawan, S., Kapoor, R.-K., Kuhad, R.-C., 2005. Biochemical characterization and molecular evidence of a laccase from the bird's nest fungus *Cyathus bulleri*. *Fungal Genetics and Biology*, **42**, 684-693

Yaver, DS., Xu, F., Golightly, EJ., Brown, KM., Brown, SH., Rey, MW., Schneider, P., Halkier, T., Mondorf, K., Dalboge, H., 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa. Appl. Environ. Microbiol.*, **62**, 834–41

Youn, H., Hah, Y., and Kang, S., 1995. Role of laccase in lignin degradation by white-rot fungi. *FEMS Microbiol. Let.*, **132**, 183-188

BIOGRAPHY

Pınar Hüner was born in Istanbul in 1982. She was graduated from Lüleburgaz High School in 2000. She received her B.Sc. degree in Istanbul University, Cerrahpasa Medical Faculty, Medical Biological Sciences, in 2004. She started Molecular Biology-Genetics and Biotechnology M.Sc. Programme at Istanbul Technical University in 2004.