ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CLONING OF THE LACCASE cDNAs FROM *PYCNOPORUS SANGUINEUS* MUCL 38531, EXPRESSION IN *PICHIA PASTORIS* AND CHARACTERIZATION OF RECOMBINANT LACCASES

Ph.D. Thesis by
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Department : Advanced Technologies

Programme : Molecular Biology-Genetics and Biotechnology

NOVEMBER 2010

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

*PYCNOPORUS SANGUINEUS'*TAN LAKKAZ cDNALARININ KLONLANMASI, *PICHIA PASTORIS*TE EKSPRESYONU VE REKOMBİNANT LAKKAZLARIN KARAKTERİZASYONU

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FOREWORD

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ABBREVIATIONS

2,6-DMP **:** 2,6-dimethoxyphenol
3-HAA **:** 3-hydroxyanthranilic acid
ABTS **:** 2-2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)
BLAST **:** Basic local alignment search tool
bp **:** base pair
CA **:** Cinnabarinic acid
cDNA **:** Complementary Deoxyribonucleicacid
dNTP **:** deoxynucleosidetriphosphate
DTT **:** Dithiothreitol
EDTA **:** Ethylene diamine tetra-acetic acid
FPLC **:** Fast protein liquid chromatography
gDNA **:** Genomic Deoxyribonucleicacid
HPTLC **:** High performance thin layer chromatography
k_{cat} **:** Turnover number
kDa **:** Kilodalton
K_m **:** Michaelis-Menten constant
MUCL **:** Mycotheque de l'Universite catholique de Louvain
NCBI **:** National Center for Biotechnology Information Database
PCR **:** Polymerase chain reaction
RACE **:** Rapid amplification of cDNA ends
Rf : Retention factor
RT-PCR **:** Reverse transcriptase-polymerase chain reaction
SDS-PAGE **:** Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
UV **:** Ultraviolet

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CLONING OF THE LACCASE cDNAs FROM *PYCNOPORUS SANGUINEUS* MUCL 38531, EXPRESSION IN *PICHIA PASTORIS* AND CHARACTERIZATION OF RECOMBINANT LACCASES

SUMMARY

Laccases are blue multi copper oxidases catalyzing the reduction of oxygen to water coupled with oxidation of aromatic compounds. Laccases are currently accepted as highly interesting industrial enzymes because of their useful applications for several biotechnological processes such as the detoxification of the industrial effluents from the paper, pulp and textile industries, the bioremediation of the herbicides and pesticides, the processing of the beverages (wine, fruit juice and beer), the determination of the ascorbic acid, the gelation of the sugar beet pectin, baking, being as ingredients in cosmetics and as biosensor.

The present research aimed at identification, heterologous expression and biochemical characterization of two novel laccases, *lcc1* and *lcc2*, from white rot fungus *Pycnoporus sanguineus* MUCL 38531. Principally, the laccase specific genes within the genome of the white-rot fungi *Pycnoporus sanguineus* were screened and this study revealed two different laccase genes, designated as *lcc1* and *lcc2*. Full-length *lcc1* and *lcc2* genes were isolated and the corresponding open reading frame for full length *lcc1* cDNA is 1557 bp coding for 518 amino acids with a putative 20-residue signal sequence. *lcc2* gene is 2296 bp in length, contains an open reading frame of 1713 bp and codes for 570 amino acids with a signal sequence of 27-residues. The genomic DNA sequence of both *lcc1* and *lcc2* genes revealed the 10 introns.

The full-length *lcc1* and *lcc2* cDNAs were successfully cloned into the yeast shuttle expression vector pPICZB with their own secretion signal sequence and expressed in methylotrophic yeast *Pichia pastoris*. Factors influencing the expression such as nutrients, methanol and copper concentrations were,then, investigated. Recombinant laccases were purified to electrophoretically homogenous state by ammonium sulphate precipitation, Q-sepharose anion exchange and Sephadex G-100 size exclusion chromatography. The molecular weight of purified laccases have been estimated about 56 kDa and 62 kDa for R-LCC1 and R-LCC2 respectively and both laccases were categorized as a yellow laccase due to the lack of typical absorbance at 600 nm. Among tested compounds for substrate specificity, the maximum activity was observed for ABTS with pH optima 3.0. Although optimum temperatures were found as 60ºC and 30ºC, the thermostability was better at 30ºC with a half-life of more than 7 hours for LCC1. Sodium azide, L-cysteine and SDS were found as usual inhibitors for the purifed enzyme. The catalytic properties of the recombinant enzymes were also determined for both ABTS and DMP substrates.

As a result, this work allowed obtaining the isolation of laccase genes from *Pycnoporus sanguienus* MUCL 38531, heterologous expression of two laccases in yeast *P. pastoris*, their purification and characterisation. Moreover, this research work revealed the potentiality of the developed expression system in industrial

biosynthesis of textile dyes and antibiotic cinnabarin pigment. Promising performances of constructed novel laccase host, *P. pastoris*, will lead to further investigations on structure-function relationships and also protein engineering studies.

*PYCNOPORUS SANGUINEUS'*TAN LAKKAZ cDNALARININ KLONLANMASI, *PICHIA PASTORIS*TE EKSPRESYONU VE REKOMBİNANT LAKKAZLARIN KARAKTERİZASYONU

ÖZET

Lakkazlar aromatik bileşiklerin oksidasyonunu, oksijenin suya indirgenmesiyle katalizleyen çoklu bakır oksidazlardır. Çeşitli substratları okside edebilme özellikleri ile lakkazlar farklı biyoteknolojik uygulamalarda rol oynayan enzimler olarak son yıllarda ön plana çıkmaktadır. Lakkazların kağıt ve tekstil endüstrilerinden gelen atıksuların detoksifikasyonu, herbisitlerin ve pestisitlerin temizlenmesi, şarap, meyve suyu ve bira gibi içeceklerin işlenmesi, şeker pancarı pektininden jel oluşturulması, fırıncılıkta kullanılabilme özelliklerinin yanısıra kozmetik malzemesi ve biosensör yapımında da kullanılabildiği bildirilmektedir.

Bu çalışmada beyaz çürükçül küf mantarı *Pycnoporus sanguineus* MUCL 38531'te lakkaz kodlayıcı *lcc1* ve *lcc2* genlerinin izolasyonu ve bu genlerin heterolog ekspresyonları ile karakterizasyonu amaçlanmıştır. Öncelikle *Pycnoporus sanguineus* genomunun taranması sonucunda iki farklı lakkaz kodlayıcı genin varlığı belirlenmiş ve bu genler *lcc1* ve *lcc2* olarak adlandırılmıştır. Tam boyda *lcc1* ve *lcc2* genleri izole edilmiş ve *lcc1* cDNA'sının 1557 bç uzunluğunda olup, teorik olarak 20 amino asitlik sekresyon sinyali taşıyan 518 amino asitlik bir polipeptiti kodlayabildiği görülmüştür. *lcc1* ve *lcc2* genlerinin 10 intron içerdiği belirlenmiş olup, *lcc2* geninin 2296 bç uzunluğunda ve 570 aminoasit kodlayan 1713 bçlik bir açık okuma çerçevesi içerdiği tespit edilmiştir.

Lakkaz genlerinin klonlanmasının ardından heterolog ekspresyon yoluyla üretilebilmeleri sayesinde istenilen özellikleri taşıyan proteinlerin endüstriyel uygulamalarda kullanılabilecek düzeyde elde edilebilmesi mükün olabilmektedir. İzole edilen tam boyda lakkaz cDNA'ları maya mekik ekspresyon vektörü pPICZB'ye kendi sekresyon sinyal dizileri ile klonlanmış ve metilotrofik maya *Pichia pastoris*'te eksprese edilmiştir. Besiyeri, metanol ve bakır konsantrasyonlarının ekspresyon düzeyi üzerindeki etkisi belirlendikten sonra lakkazlar amonyum sülfat çöktürmesi, iyon değiştirme ve jel filtrasyon kromatografisi teknikleriyle saflaştırılmıştır. Saflaştırılan proteinlerin ağırlıkları R-LCC1 ve R-LCC2 için sırasıyla 56 kDa ve 62 kDa olarak bulunmuş ve bu enzimler 600 nm'de lakkazlara özgü absorbansı vermedikleri için sarı lakkazlar olarak tanımlanmıştır. Aktivite üzerinde substrat etkisi incelendiğinde maksimum aktivitenin ABTS için izlendiği ve bu substrat kullanıldığında optimum pH 3.0 olarak belirlenmiştir. Optimum sıcaklıklar R-LCC1 ve R-LCC2 için sırasıyla 60ºC ve 30ºC olarak belirlenmiş olup, LCC1'in termal stabilitesinin 30ºC'de daha iyi olduğu ve yarılanma ömrünün de 7 saatten fazla olduğu saptanmıştır. Saflaştırılan enzimler üzerinde sodyum azid, L-sistein ve SDS'nin inhibe edici etkisi olduğu görülmüş olup, enzimlerin katalitik özellikleri de hem ABTS hem de DMP substratları kullanılarak tespit edilmiştir.

Sonuç olarak, bu tez çalışması ile beyaz küf mantarı *Pycnoporus sanguienus* MUCL 38531'tan iki adet lakkaz geninin izole edilmesi, *P. pastoris* mayasında heterolog

ekspresyonu, saflaştırılması ve biyokimyasal karakterizasyonu çalışmaları başarıyla gerçekleştirilmiştir. Ayrıca bu çalışma sayesinde kurulan ekspresyon sistemi aracılığıyla bazı tekstil boyalarının ve turuncu renkli antibiyotik pigment "cinnabarin" biyosentezi başarıyla gerçekleştirilmiştir. Bu çalışma sonucunda lakkaz yapı-fonksiyonunun araştırılması ve protein mühendisliği çalışmalarında kullanılabilme potansiyeli olan yeni bir lakkaz üreticisi *P. pastoris* suşu elde edilmiştir.

1. INTRODUCTION

1.1 White-Rot Fungi

Carbon recycling needs lignocellulose degradation as a central step in land ecosystems and wood degradation is mainly performed by fungi in nature. Lignocelluloses, such as wood or straw, are continously produced and recent estimations showed that 140 billion tons of biomass comes from plant primary metabolism annually (Bouws et al. 2008, Martinez et al. 2005).

Basidiomycetes are the only known group of microorganisms that can degrade lignin via an enzymatic process. The phylum Basidimycota comprises of more than 30000 species and most familiar Basidiomycetes are fruiting body producing and sexually reproductive ones. The degradative ability of the fungus is due to the hyphal organization, allowing the penetration. Basidiomycete fungus have several ecological roles, such as symbionts as in lichens, leaves and needles, plant and animal parasites or pathogens and saphrotrophs for conversion of lignocelluloses into the simple sugars, oligosaccharides and humic substances in soil (Arora and Sharma, 2010, Martinez et al. 2005, Lundell et al. 2010).

Fungi are classified into three categories upon their mode of attack, soft-rot fungi, which causes softening the tissues in wood decomposition, brown-rot fungi, that preferentially degrades cellulose and hemicellulose and white-rot fungi, which is the most efficient lignin degraders and leave white-powdery material behind. The white-rot Basidiomycetes can decompose all wood lignocellulosic compounds in coniferous softwood and angiosperm hardwood and are the unique organisms that are completely mineralize lignin to CO_2 and H_2O. They are not only use lignin as a source of carbon and energy, but also degrades cellulose as the energy providing substrate. Two white-rot pattern, selective delignification and simultaneous rot, have been described in different wood types. In selective delignification, fungi remove lignin selectively from wood without appreciable loss of cellulose and those fungi are attractive for industrial applications, including biological pulping processes,

through this characteristic. However, some white rotters may simultaneously degrade lignin with hemicellulose and cellulose components of wood. Degradation capability of white rot fungi is based on its capacity to produce one or more extracellular lignin-modifying enzymes (LME), such as manganese peroxidase, lignin peroxidases and laccases. Some white rot fungi expresses all of the LMEs, the others can produce one or two of them. Lignin oxidation does not provide net energy to the fungus and LME are synthesized and secreted during the secondary metabolism of white rot fungi, especially in the limited carbon and nitrogen levels (Arora and Sharma, 2010, Martinez et al. 2005, Lundell et al. 2010, Wesenberg et al. 2003, Luna et al. 2004).

White Rot Basidiomycetes are not only involved in the degradation of lignocellulosic compounds, but also degrade various environmentally persistent pollutants, such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, various dyes and xenobiotic compounds due to the low substrate specifity and strong oxidative activity of their ligninolytic enzymes. Therefore, the white rot fungi and their enzymes are thought to have potential in industrial applications, where removal or modification of lignin and other phenolic substrates are required. Gained information on physiology and characteristics of biocatalysis and stability is necessary for designing treatment of those processes (Wesenberg et al. 2003, Ohkuma et al. 2001, Chi et al. 2007, Luna et al. 2004).

1.1.1 Pycnoporus sanguineus

Pycnoporus sanguineus is a slow growing saprophytic fungus belonging to the Basidiomycetes of the Polyporaceae family. Polyporale group of fungi causes wood decay and they are the most important representatives of saprophytic basidiomycetes with their high lignocellulolytic potential (Figure 1.1).

The white-rot fungi from the genus Pycnoporus are characterized by orange-red color of the pileus surface and pores and divided into four species. *Pycnoporus cinnabarinus* is found in the North Temperate Zone, *Pycnoporus coccineus* occurs in countries on the coastal part of the Indian and Pacific Oceans, *Pycnoporus puniceus* is in Africa and India and *Pycnoporus sanguineus* is found in the forests of tropical and subtropical areas of the Northern and Southern hemispheres (Correa et al. 2006, Uzan et al. 2010).

Figure 1.1 : *Pycnoporus sanguineus* growing out of dead hardwood (Url-1).

Various pigments, with a basic ring phenoxazinone structure, are synthesized by *Pycnoporus sanguineus* and its unique color helps to identify it. One of the produced pigment is antibiotic cinnabarin that has been shown to be active against gram positive and gram negative bacteria from clinical samples and this feature of *P. sanguineus* made this fungus a popular medicine, used for healing several ilnesses by African and American indigenous people (Smania et al. 2003, Smania et al. 1998, Smania et al. 1997). Moreover, this fungus produce several enzymes that can be used in industrial applications, such as invertase, tyrosinase, α-amylase, β-glucosidase and laccase. Produced laccase is the most interesting enzyme among the others because of the effectiveness in wide range of biotechnological applications (Pointing and Vrijmoed, 2000, Lu et al. 2007). However, the most studied white-rot fungi are generally *Trametes versicolor*, *Pleurotus ostreatus* and limited number of studies have been reported from the laccases of *Pycnoporus sanguineus* compared to those species. Several *P. sanguineus* strains and their laccases have been reported by different research groups so far. Applications, such as decolorization and detoxification of azo, triphenylmethane and anthraquinone dyes from textile industry and soil bioremediation have been reported by different *Pycnoporus sanguineus* laccases so far (Trovaslet et al. 2007, Litthauer et al. 2007). Furthermore immobilization of whole *P. sanguineus* cells onto the various surfaces for different purposes as biotransformation of synthetic compounds and removal of heavy metals from environment (Zulfadhly et al. 2001). Recently Uzan et al. (2010) reported three different *P. sanguineus* strains and their characteristics for polycyclic dye decolorization and oxidation of nonphenolic compounds, Lu et al. (2009) have

reported azoi triphenylmethane, anthraquioneone and indigo dye decolorization capacity of recombinant *Pycnoporus sanguineus* laccase.

1.2 Laccases

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are cuproproteins, exhibiting four copper atoms to catalyze the oxidation of wide range of aromatic substrates as hydrogen donors with concominant reduction of molecular oxygen to water. This group of enzymes represent the largest subgroup of blue multicopper oxidases and six classes of enzymes (cytochrome-c oxidase, laccases, L-ascorbate oxidase, ceruloplasmin, bilirubin oxidase, phenoxazinone synthase) are capable of catalyzing this kind of oxygen reactions among more than 200 kinds of oxidases and oxygenases. Those broad range of reducing substrates include ortho- and para-diphenols, aminophenols, polyphenols, polyamines and lignin. Typically, laccases do not oxidize tyrosine as tyrosinases do (Giardina et al., 2010, Michniewicz et al., 2008, Baldrian, 2006, Mikolasch and Schaur, 2009, Thurston, 1994).

The four single electron oxidation of the reducing substrate to the four electron cleavage of the dioxygen bond occurs by means of the four copper atoms distributed in the three binding sites. The phenolic substrate is firstly subjected to one-electron oxidation giving rise to an aryloxyradical and subsequently this is converted to a quinone in a typical laccase reaction. Both quinone and the free radical product undergo non-enzymatic coupling reactions resulting in polymerization. Furthermore laccases do not only oxidize phenolic compounds, but also decarboxylate and demethylate them (Wesenberg et al., 2003, Giardina et al. 2010, Duran et al. 2002). The active site of laccases usually contain four copper atoms and those atoms are organized into the mononuclear T1 site and trinuclear copper cluster, containing the one type 2 and two type 3 copper atoms. The oxidation of the substrates takes place in the type 1 copper site, internal electron transfer occurs from type 1 to type 2 and type 3 coppers and the reduction of oxygen to water happens at these sites (Wong 2009; Morozova et al. 2007; Baldrian, 2006; Mayer and Staples, 2002). Copper atoms in the type 1 site of the enzyme gives the blue color of the laccase. However, T1-type copper atoms lacking laccases are not blue and designated as “yellow” or “white” laccases. Yellow laccase from *Pleurotus ostreatus* (Palmieri et al. 1997), *Trametes hirsuta* (Haibo et al. 2009) and also different strains of *Pycnoporus*

sanguineus (Litthauer et al. 2007, Garcia et al. 2007, Trovaslet et al. 2007) has been purified and characterized so far.

There are two types of laccases depending on the structure and properties of copper center, the low-redox potential and the high-redox potential laccases. While white-rot fungi have high-redox potential laccases, molds, bacteria, insects and plants have the other type of laccases. Although laccases have low redox potential allowing the direct oxidation of phenolic units, oxidation of non-phenolic substrates with high redox potential needs the presence of small molecules acting as electron transfer mediators, such as synthethic ABTS or native 3-HAA. In the presence of the mediators, especially with the synthetic ones, oxidation of non-phenolic compounds have been performed and it caused an interest for biotechnological applications, like depolymerization of polyphenolics, lignin, degradation of xenobiotic compounds and chlorine-free bleaching of paper pulp. In contrast to other ligninolytic enzymes LiP and MnP, the laccases have been oxidized by molecular oxygen and hydrogen peroxide which has not been required (Antorini et al. 2002, Martinez et al. 2005, Bulter et al. 2003, Burton, 2003, Piontek et al. 2002, Couto and Toca-Herrera, 2006a, Mikolasch and Schaur, 2009).

Laccases are very ancient enzymes and were first discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera* at the end of the 19th century (Yoshida et al. 1883). Detection of the first fungal laccase had followed this discovery a few years later (Baldrian 2006). Although Ascomycetes and Deuteromycetes fungi can produce laccase, white-rot fungi from Basidiomycetes class are the most efficient laccase producer group and those laccases are the most important representatives of multi-copper oxidase family (Arora and Sharma 2010; Giardina et al. 2010). Laccases have been also isolated from some bacteria and insects so far (Enguita et al. 2003; Dittmer et al. 2009). Different physiological functions have been proposed for laccases. In fungi, laccases participate not only in lignin degradation (Hatakka 1994; Bermek et al. 1998) but also in pigment formation by polymerization reactions (Eggert et al 1995), fruiting body development (Wood 1980) and pathogenesis (plant-pathogen/host interaction and stress defense) (Binz et al., 1996). Laccase was also involved in lignin biosynthesis in plants, pigment formation and protection against UV light in bacterial spore coat (Sharma et al. 2007) and sclerotization of the cuticle of insects (Dittmer et al. 2004). Fungal laccases are often extracellular and are

produced as highly glycosylated forms to increase hydrophilicity and molecular weights vary between 40 to 140 kDa. They function in various environments by means of their characteristics, such as to be resistant against high temperatures, high or low pH and various harsh conditions (Burton, 2003; Nakamura and Go, 2005). Great variability have been observed in the induction mechanism and physico-chemical characteristics, such as molecular weight, pH and temperature optima, carbohydrate content, isoelectric point and also kinetic properties among different laccases (Duran et al. 2002). Many fungal species examined secrete more than one laccase isoenzyme depend on the different growth conditions and multiplicity of fungal laccase genes is a common feature (Ng, 2004).

Laccases become favourite tools for industrial biocatalysis thanks to its versatility and broad substrate range. The textile dye decolorization, purification of coloured wastes waters, transformation and inactivation of toxic pollutants, beverage and food treatment, dye bleaching and delignification of lignocellulosics are among the biotechnological applications of laccases (Widsten and Kandelbauer, 2008; Couto and Toca-Herrera, 2006b).

1.2.1 Three dimensional structure and the mechanism of catalysis

1.2.1.1 Molecular structure

A number of the three-dimensional structures have been determined for fungal laccases from basidiomycete *Coprinus cinereus* (with the T2 copper removed), *Trametes versicolor, Rigidoporus lignosus, Lentinus tigrinus, Tratemetes trogii* and Ascomycete *M. albomyces*, as well as the structure of laccase CotA from endospores of *Bacillus subtilis.* Although these laccases have structural homology, there are also some differences, in particular in the organization of loops and formation of the substrate-binding pocket. A three dimensional homology model of the laccase from *Pycnoporus sanguienus* was constructed upon the *T. versicolor* laccase crystallographic structure and is given in Figure 1.2 (Morozova et al. 2007; Wong, 2009; Hilden et al. 2009).

Most monomeric fungal laccases shows a globular protein formed from three sequentially arranged cupredoxin-like domains consisting of antiparallel β-barrels, domain A, domain B and domain C (Figure 1.3). The size of the protein globule of

laccases from *C. cinereus* and *T. versicolor* is 70 × 50 × 45 Å and 65 × 55 × 45 Å, respectively.

Figure 1.2 : Three-dimensional model of the *P. sanguineus* laccase constructed over the *T. versicolor* crystallographic structure (PDB Q12718). Copper atoms are indicated by stars and *N*-glycans are depicted as black spheres (Vite-Vallejo et al. 2009).

Protein is also stabilized by two disulfide bridges connecting domain A and B and A and C, respectively. The T1 copper site is located in the third domain and three nuclear cluster (TNC) T2/T3 is embedded between the first and third domains and has ligand residues for copper coordination. Amino acid residues of the second and third domains are involved in the formation of the substrate-binding pocket (the binding site of electron donor substrate). Based on the 3D structure superimposition, four loop regions (I-IV) involved in substrate binding have been identified.

Structures of laccases isolated from different sources are very similar and based on the multiple sequence alignments of more than 100 laccases, four ungapped conservative regions, L1-L4, were revealed which are specific for all laccases. One cysteine and ten histidine residues are housed within these four identified conserved regions and form a ligand environment of copper ions of the laccase active site. L2 and L4 are in line with the sequences of other reported multi copper oxidases, whereas L1 and L3 are distinctive to the laccases (Giardina et al. 2010; Morozova et al. 2007).

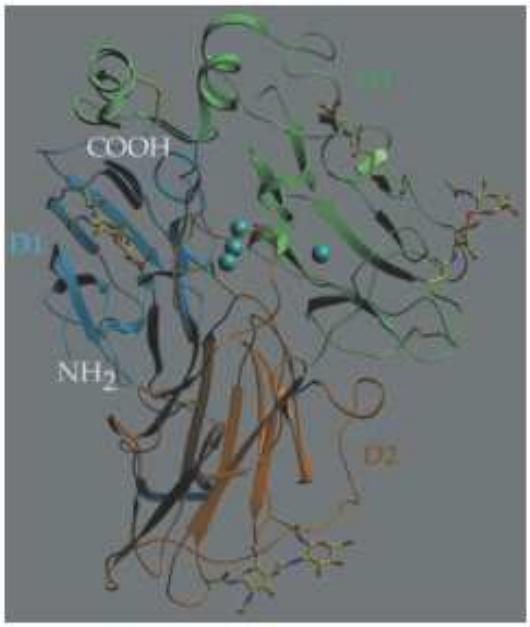


Figure 1.3 : Ribbon diagram of laccase from *Trametes versicolor*. Domain structures are depicted in different color coding (D1–D3). Blue spheres are copper ions and carbohydrates and disulfide bonds are included as stick models (Piontek et al. 2002).

1.2.1.2 Active site of laccases

The active site of laccases contains four copper ions that have been classified based on their electron paramagnetic resonance (EPR) features, one Type 1 or blue copper ion, one Type 2 or normal copper ion and two Type 3 coppers (Fig 1.3). The distance between the T2 and T3 sites of the enzyme is 4 Å and the T1 copper ion is located at the distance of about 12 Å from trinuclear copper center (Morozova et al. 2007). Physical methodology such as circular dichroism (CD) and electron paramagnetic resonance (EPR) have been used to characterize metal centers of laccases and copper ions of the laccase active site are classified by their spectroscopic and magnetic properties (Mayer and Staples, 2002; Morozova et al. 2007). The Type 1 copper shows an intense absorption at 600 nm ($\varepsilon \sim 5000 M^{-1} \cdot cm^{-1}$) and EPR detectable. It gives a light blue color to the enzyme solutions and acts as electron acceptor from substituted phenols or amines. The T1 site has two histidine imidazoles and the sulfhydryl group of cysteine as ligands, which form a trigonal structure and this copper ion can be replaced by mercury or cobalt ions. The type 1 copper is not necessary for reactivity with dioxygen and in the absence of type 1 copper an intermediate sharing some properties with the oxygen intermediate is formed. Apart from two histidine an done cysteine residues T1 site contains weakly coordinating

one methionine or non-coordinating 1Phe/1Leu as axial ligands. The T2 copper does not give strong absorption an 600-700 nm of the enzyme, which is invisible in electron absorption spectra and is also EPR-detectable. The T2 site can be selectively removed from the enzyme molecule, and this is accompanied by a significant loss of the enzyme activity. Type 2 copper ion transfers electrons to the final acceptor, dioxygen, which is reduced to water. The T3 site of laccases is a binuclear copper site with copper ions paired antiferromagnetically through a hydroxide bridge that makes this site diamagnetic and prevents its detection in the EPR spectra. In the UV-visible spectrum, this site can be identified by the presence of a shoulder at 330 nm. The two type 3 coppers act as intermediates in the electron transfer pathway and eight imidazoles of histidines are ligands of the T2/T3 cluster (Morozova et al. 2007; Martinez et al. 2005; Mayer and Staples, 2002; Sakuari and Kataoka, 2007; Nakamura and Go, 2005). Figure 1.4 displays the active site of laccase CotA from *B. subtilis* and positions of ligands of the T1, T2, and T3 sites, with indications of the interatomic distances is given.

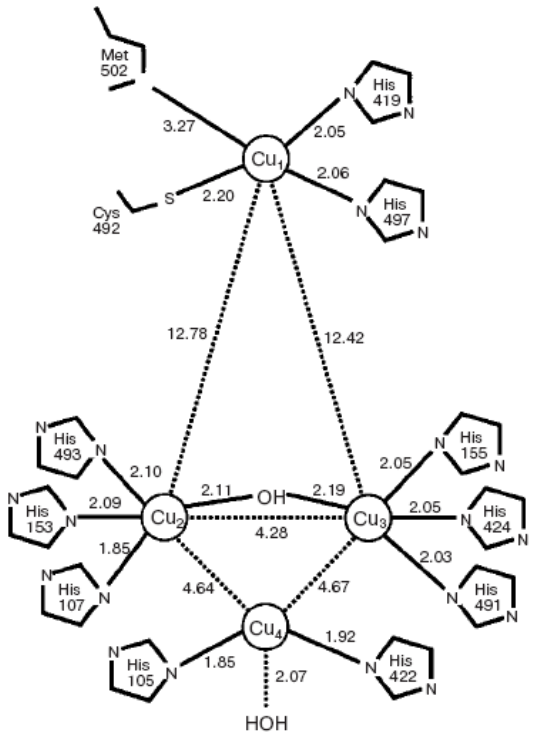


Figure 1.4 : Scheme of T1 (Cu1) and T2/T3 (Cu4/Cu2_Cu3) copper sites of laccase CotA from *Bacillus subtilis*, with indicated distances (Å) between the most important atoms (Morozova et al. 2007).

1.2.1.3 Catalytic cycle

The laccase catalytic cycle involves binding of the reducing substrate, followed by binding of oxygen and it allows intramolecular electron transfer resulting in oxygen reduction and then proton transfer which leads to release of the reaction products. Laccases catalyze four 1 e^- oxidation of a reducing substrate with two concominant 2 e^- reduction of dioxygen to water. The first step of catalysis is reduction of the reducing substrate by the copper (Cu^{2+} to Cu^{+}) at the primary electron acceptor T1 site. The electrons extracted from the reducing substrate are transferred to the T2/T3 trinuclear site and results in the conversion of the resting form (fully oxidized) of the enzyme to a fully reduced state. From four substrate molecules, successive $4e^-$ oxidation is required to fully reduce the enzyme (Fig. 1.5). The rate-limiting step is the intramolecular electron transfer from T1 to the trinuclear copper site. Reduction of dioxygen occur in two steps via the formation of bound oxygen intermediates. The dioxygen molecule first binds to the T2/T3 site, and two electrons are rapidly transferred from the T3 coppers, resulting in the formation of a peroxide intermediate. The diffusion of dioxygen to the trinuclear site is rate limiting, followed by a rapid $1e^-$ transfer from T1. The peroxide intermediate decays to an oxy radical and undergoes a $2e^-$ reductive cleavage of the O–O bond with the release of a water molecule. The peroxide intermediate decays to an oxy radical and undergoes a $2e^-$ reductive cleavage of the O–O bond with the release of a water molecule. Decay of the intermediate is facilitated by the final electron transfer from the T2 copper, and is accelerated with decreasing pH, with protonation from a carboxylic acid residue near the active site. In the last step, all four copper centers are oxidized, and O^{2-} is released as a second water molecule. The reoxidation of the T2 copper correlates with the decay of the intermediate in which the first water is released and the second water molecule remains bound and slowly exchanged with the bulk (Wood, 2009; Mikolash and Schaur, 2009).

Despite their different origins, the overall structure of laccases is similar. Crystalline structures demonstrated that, the substrate cavity of laccases is wide enough to allow accommodations of wide array of substrates with various sizes. Small aromatic molecules, for example 2,6-DMP or 2,5-xylidine, are buried into the cavity by hydrophobic interactions with apolar amino acid residues. Besides larger ligands, like ABTS places in the cavity with non-linear structure, while one part of the

molecule is embedded in the cavity, the other part interacts with the amino acids around the substrate binding cavity. The inner part of the hydrophobic substrate cavity of laccases includes a highly conserved aspartic acid residue very close to the active histidine residue. The positively charged histidine residue is thought to initiate the catalytic cycle by subtracting an electron from the reducing substrate, whereas negatively charged aspartic acid residue can stabilize the formed radical cation. Not only these polar contacts takes place in the cavity, but also a number of hydrophobic protein-ligand interactions occur between the aromatic rings of the substrates and the lateral chains of apolar residues of laccase (Giardina et al. 2010; Colao et al. 2009; Ng, 2004).

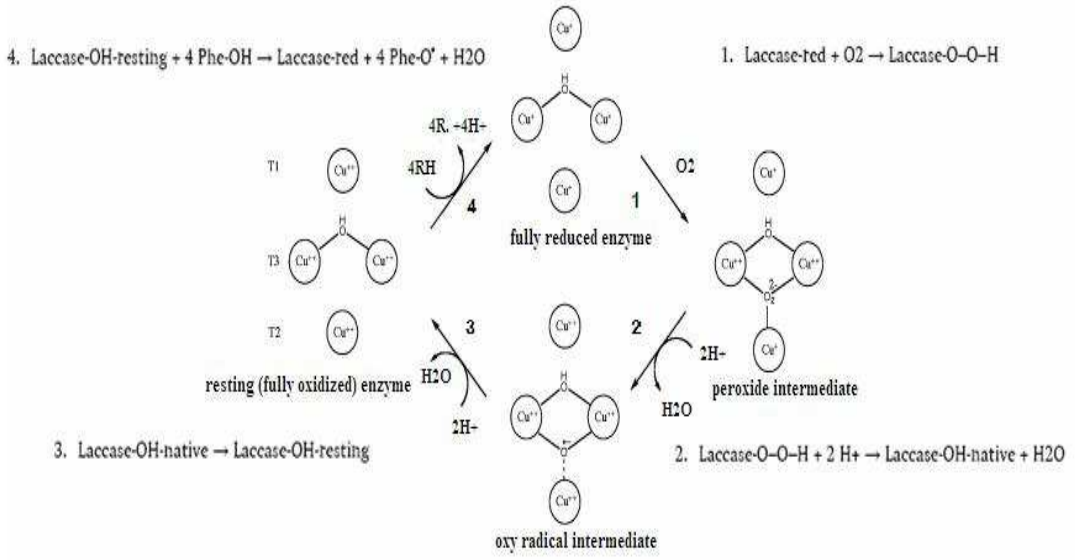


Figure 1.5 : General catalytic cycle of laccases (modified from Lundell et al. 2010 and Wong 2009).

1.2.1.4 Oxidation of phenolic compounds

Typical laccase substrates are phenolic compounds because their redox potentials, ranging from 0.5 to 1.0 V are low enough to permit electron subtraction by the type 1 copper. Most common phenolic substrates DMP, guaiacol, and syringaldazine are oxidized to phenoxy radicals, which generally undergo polymerization via radical coupling. Moreover phenoxy radicals can rearrange themselves leading to quinone formation through disproportionation, alkyl-aryl cleavage, Cα oxidation, cleavage of Cα-Cβ bond or aromatic ring. The catalytic effieciency of oxidation of phenols, anilines and benzenethiols correlates to the structure of the substrate and to the one-

electron redox potential difference between T1 copper site of laccase and the substrate. The relative contribution of steric and redox properties of a substrate determines its susceptibility to laccase oxidation and the bulky substituents cause decreased reactivity since they impose steric interference with substrate binding (Wong, 2009 and Giardina et al. 2010).

1.2.1.5 Oxidation of non-phenolic compounds

Although lignin peroxidase and manganase peroxidase/mediator systems are known to be able to oxidize non-phenolic models, some white –rot fungi (*Pycnoporus cinnabarinus*), which have only laccase predominantly, can degrade lignin efficiently. Non-phenolic substrates are oxidized by laccases with the aid of mediator compounds. More studied mediators are ABTS, 1-hydroxybenzotriazole (HBT) and 3-hydroxyanthranilic acid (HAA).The oxidation of ABTS and HBT are different as they involve a di-cation and a benzotriazolyl-1-oxide radical respectively. In the ABTS-mediated oxidation of non-phenolic substrates, ABTS is first oxidized to the radical cation ($ABTS^{+}$) and then to the di-cation ($ABTS^{2+}$), which is the active intermediate for the oxidation of the non-phenolic compounds. HBT-mediated oxidation of non-phenolic substrates starts wth initial oxidation of HBT to HBT^{+} by laccase and it is followed by deprotonation to form N-oxy radical. HBT-mediated oxidation is more common than the ABTS mediated way especially for the degradation of non-phenolic lignin compounds (Wong, 2009).

1.2.1.6 Laccases with unusual spectral properties

Although most of the laccases has four copper atoms and show typical absorbance at 600 nm, some laccases does not contain the type I copper and not display the typical blue color. These laccases have been called “yellow” or “white” laccases and can be defined as true laccases since they are able to oxidize phenolic and non-phenolic substrates but not tyrosine as the other laccases do. Yellow laccases have been purified from phytopathogenic Ascomycete *Gaeumannomyces graminis var. tritici.*, Basidiomycetes *Agaricus bisporus*, *Schizophyllum commune*, *Panus tigrinus*, *Phlebia radiata*, *Phellinus ribis*, *Pleurotus ostreatus*, P*ycnoporus cinnabarinus*, *Pycnoporus sanguineus*, *Rigidoporus lignosus*, *Trametes trogii*, and *Coriolus hirsutus* (Giardina et al. 2010; Nakamura and Go, 2005; Mayer and Staples, 2002). It has been supposed that yellow laccases are formed as a result of binding aromatic

compounds of lignin degradation to the blue laccase during fungal growth in solid-state culture conditions. This modification may result in the reduction of Cu1 and Cu2 in the active center and disappearance of the blue color. Furthermore, the modifier molecule bound to the apoenzyme of the yellow laccase performs the function of mediator and they can oxidize non-phenolic compounds without mediators (Giardina et al. 2010). Instead of the regular four copper atoms per chain, different transition metals can exist in the active site of the yellow laccases. For example, the white laccase from *P. ostreatus* contains two zinc, one iron and one copper atoms, although of the copper-binding residues of laccases are conserved. Laccase extracted from *P. ribis* binds two zinc, one manganese and one copper ion, whereas the laccase of T. hirsuta has the copper and manganase in 3:1 ratio. It has been speculated that the type 1 copper is replaced with Fe/Mn and the type 3 coppers are replaced with zinc ions whereas the type 2 copper remains (Giardina et al. 2010; Nakamura and Go, 2005).

1.2.2 Natural occurrence of laccases

Laccases were first discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera* at the end of the 19th century (Yoshida et al. 1883). Detection of the first fungal laccase had followed this discovery a few years later (Baldrian, 2006). Although laccases were firstly detected in the plants, the occurrence of laccases in higher plants are much more limited than in fungi. Because of the crude plant extracts contain a large number of other oxidative enzymes with broad substrate specificities, plant laccases have not been detected or purified easily (Sharma et al. 2007). The plant laccases have been detected in lacquer, mango, mung, bean, peach, prune, sycamore and the experiments on the laccase expression in the crop plants have also been reported (Arora and Sharma, 2010). Furthermore, cell culture of *Acer pseudoplatanus* and the xylem tissue of *Pinus taeda* have been reported to contain laccases (Mayer and Staples, 2002).

To date, most of the laccases have been isolated and characterized from plants and fungi, and only fungal laccases are currently used in biotechnological applications. Although little is known about bacterial laccases, whole genome analysis techniques suggested that laccases are also widespread in bacteria. The occurrence of laccases is generally within the genera *Aquifex, Pyrobaculum, Azospirillum, Sinorhizobium, Marinomonas, Ralstonia, Streptomyces* and *Bacillus*

(Mikolasch and Schaur, 2009). The best-studied bacterial laccase is the CotA, the endospore coat component of *Bacillus subtilis* and participates in the biosynthesis of the melanin-like brown spore pigment and seems to be responsible for the protection against UV light and hydrogen peroxide (Driks, 2004; Martins et al. 2002). Moreover, most of the bacterial laccases so far studied are located intracellularly or in periplasmic protoplast differing from the most fungal laccases. Until now, only three bacterial laccases have been completely purified and characterized from *Azospirillum lipoferum, Marinomonas mediterranea* and *Pseudomonas syringae* and it has been shown to participate in cell pigmentation and plant phenolic compounds' utilization (Sharma et al. 2007). Recently Palanisami et al. (2010) reported the presence of constitutive laccases (LACs) and polyphenol oxidases (PPOs) in the ten tested strains of marine cyanobacteria and this is the first report on the cyanobacterial laccases.

Laccases are widely distributed in fungi. Although Ascomycetes and Deuteromycetes fungi can produce laccase, white-rot fungi from Basidiomycetes class are the most efficient laccase producer group and those laccases are the most important representatives of multi-copper oxidase family (Arora and Sharma 2010; Giardina et al. 2010; Call and Mücke, 1997). White-rot fungi *Agaricus bisporus, Armillaria mellea, Ceriporiopsis subvermispora, Coriolopsis polyzona, Cyathus bulleri, Daedela quercina, Fomes annosus, Ganoderma lucidum, Lentinula edodes, Marasmius quercophilus, Panus tigrinus, Phlebia radiata, Pleurotus eryngii, Pleurotus ostreatus, Pycnoporus sanguineus, Pycnoporus cinnabarinus, Rigidoporus lignosus, Trametes villosa, Volvariella volvacea, Ascomycetes Cryphonectria parasitica, Melanocarpus albomyces, Nurospora sp., Podospora anserina, İmperfect fungi Aspergillus nidulans, Botrytis cinerea, Ophiostoma ulmi, Rhizoctonia solani, Trichoderma atroviride* and a number of brown rot fungi *Gleophyllum trabeum, Postia placenta, Antrodia vaillantii, Fomitopsis pinicola* and *Coniophora puteana* have been shown to produce different level of laccases (Arora and Sharma, 2010). Laccase activity has not been indicated in lower fungi Zygomycetes and Chytridiomycetes.

A variety of physiological functions have been proposed for laccases. In fungi, laccases participate not only in lignin degradation but also in pigmentation fruiting body development and pathogenesis (plant-pathogen/host interaction and stress

defense). Laccase was also involved in lignin biosynthesis in plants, pigment formation and protection against UV light in bacterial spore coat and sclerotization of the cuticle of insects (Arora and Sharma, 2010; Giardina et al. 2010).

1.2.2.1 Morphogenesis and pigmentation

Laccases play important role in morphogenesis especially in terms of pigmentation. Temp and Eggert (1999) reported that glucose-grown culture of *Pycnoporus cinnabarinus* has produced laccase, associated with ligninolysis as well as formation of pigment, cinnabarinic acid. This pigment gives the characteristic orange-red color of the fruiting body of that fungus and it has also been defined as antimicrobial agent. Fruiting body formation may involve synthesis of extracellular pigments connected with oxidative polymerization of cell wall components for strengthening cell-to-cell adhesion by laccase-catalyzed reaction. In *Aspergillus nidulans*, *Aspergillus fumigatus*, *Daldinia concentrica* and some other fungi laccases are involved in oxidative polymerizations to produce pigments, giving the color of conidium or to produce polyphenolic glues that stick hyphae together. Laccase level is regulated in relation to fruiting body development as observed in the example of *Agaricus bisporus*, where abundant laccases are secreted into the medium during vegetative growth and loss of activity occurs during the fruiting body formation (Call and Mücke 1997; Arora and Sharma, 2010).

1.2.2.2 Phenoxazinone dye cinnabarin synthesis by use of laccase

The o-aminophenol 3-hydroxyanthranilic acid (3-HAA) is one of the tryptophan metabolites along kynurenine pathway and is the precursor of the phenoxazinone derivative, cinnabarinic acid (CA). 3-HAA is found in different group of organisms, bacteria, yeast, fungi, plants and mammals (Eggert et al. 1995; Li et al. 2001). 3-HAA is converted into CA through the active oxygen species and transition metals by non-enzymatically. Oxidation is also be mediated enzimatically by horseradish peroxidase, myeloperoxidase, catalase, tyrosinase, glucose oxidase and also laccase. The formation of phenoxazinone ring from anthranilate precursors starts with 6 electron oxidation of precursor and is followed by any of several reaction sequences all of which proceed reactive intermediates such as quinone imines The oxidation mechanism of 3-HAA and formation of cinnabarinic acid is shown in Figure 1.6(Hiramatsu et al., 2008; Eggert et al. 1996). Oxidation of 3-HAA is related to

carcinogenic and antioxidative activities and this conversion has attracted attention in clinical studies because 3-HAA and CA induce apoptosis in T cells, act as powerful scavenger of reactive oxygen species and also are known carcinogen for bladder and breast carcinomas (Hiramatsu et al., 2008; Iwahashi, 1999). 3-HAA is also the precursor for other phenoxizanone derivatives such as actinomycin class of antibiotics synthesized by the *Streptomycetes*, which is used in the treatment of certain forms of cancer (Smania et al. 2003).

Cinnabarinic acid and other two derivatives, cinnabarin and tramesanguin are orange-red compounds and antibiotic substance produced by the members of *Pycnoporus* genus of white-rot fungi. These phenoxazinone chromophores give orange-red color of fruiting bodies of fungus and also used as natural pigments by insects and also Australian marsupials (Eggert et al. 1995; Le Roes-Hill et al., 2009).

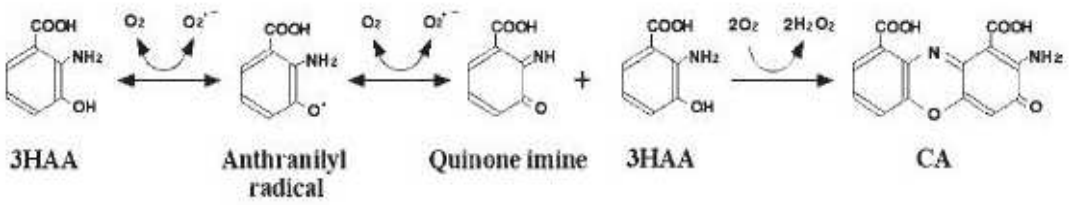


Figure 1.6 : The oxidation of 3-hydroxyanthranilic acid into the cinnabarinic acid (Hiramatsu et al. 2008).

Cinnabarin produced by *Pycnoporus sanguineus* has a basic phenoxazine-3-one structure with a carbonyl group at C-1, an amino group at C-2 and an hydroxyl group at C-9. This pigment is produced between 18th and 23rd days of culture and increased significantly when the pH of the growth media is 9.0 and growth temperature was 25°C under light (Smania et al. 1997). Cinnabarin is known with its antimicrobial properties and Smania et al. (1998) reported the antimicrobial activity of cinnabarin produced from three different strains of *Pycnoporus sanguineus* against 11 species of bacteria from foods as this chromophore can be used in food products as a colorant. Toxicity of cinnabarin was also investigated and this substance had no toxic effect on mice neuroblastoma cells (Smania et al. 2003). Temp and Eggert (1999) reported a characteristic red pigment, cinnabarinic acid, synthesized by laccase-catalyzed oxidation of the precursor 3-hydroxyanthranilic acid when glucose is the carbon source in white-rot fungus *Pycnoporus cinnabarinus* culture.

Besides their physiological roles in nature, phenoxazinone dyes attracted interests for several industrial purposes. Using phenoxazinone chromophores for the development of fluorescent probes detecting hydroxyl radicals has been reported and Bruyneel et al. (2008) investigated regioselective synthesis of 3-hydroxyorthanilic acid and sulfonated compound was biotransformed into a novel phenoxazinone dye mimicking the cinnabarinic acid by using laccase. Meldola Blue used in textiles, paper, and paints, mainly as a pigment and Nile Red dyes are also reported as industrially interesting phenoxazinone dyes especially for fluorescent probe synthesis for the detection of biological and organic molecules (Jose and Burgess, 2006).

1.2.2.3 Pathogenesis

Extracellular laccases has also been employed in pathogenesis. Laccase is the major virulence factor of the human pathogenic fungus *Cryptococcus neoformans*. This organism has emerged as a major fungal pathogen especially in patients with AIDS, organ transplant recipients and those receiving high doses of corticosteroid treatment. Laccase is responsible for brown pigment produced by the organism and is expressed as a cell wall enzyme, oxidizing polyphenolic compounds and iron. Fungus *Botyrtis cinerea* causes soft rot of crop plants and produces extracellular laccases involved in the pathogenic process (Call and Mücke, 1997; Zhu and Williamson, 2004). Laccase from *G. graminis* var. *tritici* which is considered as important wheat pathogen and is expressed only in planta or in the presence of plant homogenate (Giardina et al. 2010).

1.2.2.4 Lignin degradation

Lignocellulose is the most abundant biomass on earth and is the predominant component of woody plants and dead plant materials. Lignin is an irregular and heterogenous arrangement of phenylpropanoid polymer and protects cellulose with its resistant structure to chemical and enzymatic degradation. Because of this fact, lignin degradation is a rate-limiting step of carbon recycling. The major enzymes involved in lignin degradation are lignin peroxidase, manganase peroxidase, versatile peroxidase and laccase. Laccase plays a major role in lignin degradation and most of the lignin degrading fungi produce extracellular laccases. Laccase catalyze one-electron subtraction from phenolic hydroxyl group of lignin to give phenoxy radicals. Although laccases have low redox potential compared to the other ligninolytic

enzymes, their potential can be increased by mediators, that can be derived from oxidized lignin units (external) or directly from fungal metabolism (internal). For an efficient degradation laccase must be in contact with the substrate, but the compact structure of plant cell walls and the enzyme size prevent direct contact. However mediators can be migrate far away from the fungal mycelium into the tight lignocellulose complex which is inaccessible to the laccase itself (Ohkuma et al. 2001; Theurl and Buscot, 2010; Arora and Sharma, 2010).

1.2.2.5 Detoxification

Laccases have roles in detoxification via their polymerizing activity. Low molecular weight phenolic compounds are potentially toxic and are polymerized to reaction products of sufficient size hindered in penetration of cells. Laccases are considered as efficient radical scavengers and this property is widespread among proteins, especially in blue copper proteins. The radicals interact with the limited number of amino acid side chains (only one or two amongst histidine, tryptophan or tyrosine) to form adducts with OH group$^{\cdot}$. As a result of the electron transfer from the protein site to the copper type-1 site, copper reduction can be observed (Call and Mücke, 1997).

1.2.2.6 Lignin biosynthesis

Lignin is a cementing constituent integrated into xylem cell walls of plants. The xylem tissue conduct water and solutes over great distances without significant evaporative loss by means of water insoluble lignin molecule. The lignin monomers, *p*-coumaryl alcohol (forming H-units), coniferyl alcohol (forming G-units), and sinapyl alcohol (forming S-units) are end products of the phenylpropanoid pathway that is initiated by deamination of phenylalanine. Monolignols are polymerized into lignin from freeradical intermediates to form lignin lattices and formation of a monolignol radical is catalysed by laccases (Gavnholt and Larsen, 2002; O'Malley et al. 1993; Caparro's-Ruiz et al. 2006).

Laccase was the first enzyme shown to be able to polymerize lignin monomers both in vitro and in vivo. It has been indicated that, laccase and laccase-like activities are closely correlated with lignin deposition in developing xylem. Since laccase operates in the absence of toxic H_2O_2, it has been proposed that laccases could play a role in the early stages of lignification in living cells and it would be the case during the

formation of lignified seed coats near sensitive developing embryos. Furthermore, laccase might be the main lignification enzyme when lignin concentration has reached a level where the middle lamella has become so hydrophobic that most water and H_2O_2 is excluded, whereas O_2 is still available (Gavnholt and Larsen, 2002).

1.2.3 Industrial applications of laccases

Oxidation reactions are essential in several industries but most of the conventional oxidation technologies have several drawbacks such as, non-specific or undesirable side-reactions and the use of environmentally hazardous chemicals. Those drawbacks caused to the search for new oxidation technologies based on the biological systems such as enzymatic oxidation. Enzymes are specific, biodegradable catalysts and reactions are carried out in mild conditions. Potential applications of enzymatic oxidation techniques are employed in various industrial fields including the pulp and paper, textile and food industries. Laccases are particularly promising enzymes for the above-mentioned purposes, because of the most interesting enzymes are the enzymes recycling on molecular oxygen as an electron acceptor (Couto and Toca-Herrera, 2006a). Laccase is a promising enzyme with a great potential application in several areas of industry. Laccase production, purification and immobilization techniques at lower costs are needed to improve the industrial application of this enzyme and recombinant DNA methods offers new possibilities of raw materials and microorganisms improvement for use in the different areas of industry (Minussi et al. 2002; Arora and Sharma, 2010). Industrial applications of laccases are summarized below.

1.2.3.1 Applications in food industry

Potential applications of laccase exist in different aspects of food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar–beet pectin gelation, baking, etc. Laccase is a promising enzyme with a great potential application in several areas of food industry (Minussi et al. 2002; Arora and Sharma, 2010).

Bioremediation of food industry wastewater

Laccase is a well-known enzyme in bioremediation because of its ability to degrade phenolic compounds. Aromatic compounds, including phenols and aromatic amines,

are one of the major classes of pollutants and the presence of these compounds in drinking and irrigation water or in cultivated land represent a significant health hazard. Some fractions of beer-factory wastewaters represent an important environmental concern due to their high content of polyphenols and dark-brown colour. Laccases were degraded high-tannin-containing wastewater and effluent from fermentation of sugar-cane molasses. Laccases were also studied for bioremediation of olive mill wastewaters and phenolic compounds were drastically reduced (Minussi et al. 2002).

Removal of phenolics from must and wine

Color and taste of must and wine are dependent on particular phenolic compounds present in different kinds of wine. Laccase treatments have been proposed as a specific and mild technology for the removal of phenolics responsible for discoloration, haze, and flavor changes, clouding,. The use of immobilized laccase might be a suitable method to overcome legal barriers, since such an enzyme is not yet allowed as a food additive, and laccase could find application in preparation of must and wine and in fruit juice stabilization (Arora and Sharma, 2010; Minussi et al. 2002). Some fungal laccases were immobilized on different inorganic and organic supports and tested for phenolic removal in must and wine. Laccase immobilized on silica gel and glutaraldehyde resulted in a decrease of the catechin concentration in solution and was reusable for up to 5 times. Browning, is one of the major faults in beverages. Various pre- and post treatments to avoid post-turbidity and discoloration of fruit juices are available and utilization of laccase for the fruit juice stabilization have been performed especially for apple juice (Minussi et al. 2002).

Beer stabilization

One of the problems faced in the brewing industry is the tendency for hazes to develop in beers during long-term storage. Protein precipitation that is stimulated by small quantities of naturally occurring polyphenols causes haze formation in beers and traditional removal methods constitutes serious environmental and health hazards. Laccases could be used as an alternative technology and could be added at the end of the process, because oxygen is unwanted in the finished beer. Furthermore, laccase may remove some of the polyphenols and any excess oxygen to enhance storage life (Minussi et al. 2002).

Sugar beet pectin gelation

Sugar beet pectin is a food ingredient with specific functional properties and it may form gels by an oxidative cross-linking of ferulic acid. It has been shown to cross-linking of the beet pectin by the oxidative coupling catalyzed with laccase (Minussi et al. 2002).

Baking

Addition of bread and/or dough-improvement additives to the bread dough causes improved machinability of the dough and also improved texture, volume, flavour and freshness of the bread. Enzymes have been known to use as dough and/or bread improving agents and when a laccase enzyme is added to dough , it may exert an oxidizing effect on the dough constituents and serves to improve the strength of gluten structures. In particular, the use of laccase results in an increased volume, and softness of the baked product, as well as increased resistance, stability and reduced stickness and improved machinability of the dough. (Minussi et al. 2002; Couto and Toca-Herrera 2006a).

1.2.3.2 Pulp and paper industry

Cellulose and lignin are rigid organic polymers which have been`invented and optimized' by nature for constructive and long term preservation purposes. Paper manufacturers are mainly interested in cellulose as a primer source for paper industry. Woody substrates like eucalyptus, bamboo and agricultural residues are used for manufacturing paper depending on the type and quality of paper. The industrial preparation of paper requires separation and degradation of lignin in wood pulp and it is an important step in processing of wood for manufacturing of paper pulp. Microbial or enzyme-based delignification systems can overcome the drawbacks of conventional methods involve chlorine-, sulfite-, or oxygen- based chemical oxidants which impose loss of cellulose fiber strength. Laccase can degrade natural or synthetic lignin polymers by breaking aromatic and aliphatic C–C bonds and depolymerizing lignin and provide milder and cleaner strategies of delignification that are also respectful of the integrity of cellulose.

Two main steps for paper manufacturing are pulping and bleaching of woody substrates. Using mechanical and chemical methods the raw materials are reduced to

the fibrous state during pulping. Bleaching which involves the consumption of enormous amounts of chemicals follows pulping and those chemicals causes serious environmental hazards. Few enzymatic treatments exhibit the delignification/brightening capabilities of modern chemical bleaching technologies and the use of laccases as biological processes provides environmentally safe technology. The treatment of wood chips prior to mechanical or chemical pulping by white rot fungi is called biopulping and laccases from the white-rot fungi are used as biopulping agents since they partially degrade lignin and loosen lignin structures before pulping. By using laccases strength of the paper increases, effluent toxicity is reduced and also electrical energy is saved. Chlorination is used for removing the residual lignin in conventional bleaching while Laccase Mediator System (LMS) has been shown to substitute chlorine-containing reagents thus reducing the pollution load caused by chloroorganics. Laccase can also be applied as biobleaching agent as it degrades the residual lignin in pulp and decolorize it and it is an ideal oxidative enzyme for pulp bleaching because it is readily available and use atmospheric oxygen as its electron acceptor. Laccase of *Trametes versicolor* has been studied widely for biobleaching of paper pulp, treatment of effluents, and various other industrial applications (Arora and Sharma, 2010; Call and Mücke, 1997; Couto and Toca-Herrera, 2006b; Widsten and Kandelbauer, 2007).

1.2.3.3 Textile industry

The textile industry accounts for two-thirds of the total dyestuff market and the chemical reagents used are very diverse in their compositions, ranging from inorganic compounds to polymers and organic products. There are more than 100,000 commercially available dyes and approximately 7×10^5 t of dyestuff is produced annually. Most of the dyes are decolorized hardly because of their resistance to fading on exposure to light, water and different chemicals. The use of laccase in the textile industry as an alternative method is growing very fast, since their ability to decolourise textile effluents, bleach textiles and even to synthesize dyes (Couto and Toca-Herrera, 2006 b).

Textile dye transformation

Textile dye degradation by laccases of different fungi has been reported. Laccase from *Phlebia tremellosa* decolorized eight synthetic textile dyes under stationary

conditions, cell-free enzyme extracts obtained from Phlebia species have been successfully employed for biodecolorization of many synthetic and industrial dyes and malachite green was successfully transformed by laccase from *Ganoderma lucidum* (Arora and Sharma, 2010). Moreover, immobilized and *Pleurotus ostreatus* on polyurethane foam cubes in bioreactors have been reported to efficiently decolorized three industrial dyes even at the high concentrations up to 2000 ppm (Casieri et al. 2008).

Textile dye biosynthesis

The textile industry produces and uses approximately 1.3 million tones of dyes, pigments and dye precursors, valued around 23 billion dollars per year and almost all of the produced dyes is obtained synthetically. Until the ends of the 19th century, all of the dyes used for textiles were naturally derived. The synthetic dye industry has grown enormously since Perkin had synthesized mauvine in 1856 and are widely used for textile dying and other industrial applications (Sengupta and Singh, 2003; Chander and Arora, 2007). Although natural dyes were available for centuries, synthetic dyes became so popular because of the production in large quantities, manufacturing at a reasonable price (10-100 $ per kg), variety of colors demanded by customers and endurance of colors on fabric after repeated washing cycles. However, synthetic dye manufacturing have some limitations, such as their production requires environmentally unfriendly chemicals creating worker safety problems like strong acids and alkalis, they needs very toxic and hazardous chemicals generating hazardous wastes and causes economical challenge for their disposing. In order to minimize the damage to the environment caused by the process and effluents of the dyestuff industry, a proposal for a new EU regulatory framework for chemicals was adopted by the European Commission on December 2006 and this new system called REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) requires that enterprises manufacturing or importing more than 1 ton/ year of a chemical substance would register it in a central database. These stringent environmental standards accepted by many countries because of the toxic and allergic reactions associated with the synthetic dyes and also limitations mentioned above caused exploration of cheaper and more environmentally friendly routes, such as natural “green” dye production methods. Natural dyes have higher compatibility with the environment and can exhibit biodegradability, which makes them friender to

the environment than synthetic dyes (Duran et al. 2002; Sengupta and Singh, 2003; Nagia and El-Mohamedy, 2007). Bioprocesses usually consume less energy, require fewer raw materials and no extra reactants, allow achievement of more selective higher yield. Natural dye production at a comparable price provides benefits for waste disposal and occupational safety by reducing toxic chemical usage as starting materials, saving in transportation costs by decentralizing production, energy recycling by using biomass after extraction of dye and prevention of waste generation by recycling growth media (Sengupta and Singh, 2003).

Fungi are the ecological source of pigments, since they are rich in stable colorants such as anthraquinone and a revival interest in the use of natural dyes in textile coloration has been growing recently. Nagia and El-Mohamady (2007) reported the natural anthraquinone dyes isolated from the fungus *Fusarium oxysporum*, their evaluation for dyeing wool and the factors affecting dyeability and fastness properties. Because of the high color strength values and good color fastness properties of dyed fabric made these anthraquinone dyes the candidate source of raw materials in the future. Moreover some natural dyes have antimicrobial activity on the wool fibers and shown to be able to use in developing clothing for infants, elderly and infirm people to protect them against common infections (Singh et al. 2005).

Enzymes have been used as biocatalysts and it is possible to use different classes of enzymes for the biosynthesis of many compounds especially for the production of pharmaceutical and agrochemical precursors. Although hydrolases represent approximately 80% of industrial enzymes, laccases show high potential as industrial biocatalysts through its advantages. Laccases are cheaply available because of the high level secretion from fungi upon induction and also molecular oxygen as co-substrate can be easily renewed by running experiment in aerated vessels. Colorization of fungi by means of polymerization reactions is among the physiological functions of laccases and several studies have aimed at determination of coloring capacity of laccase produced colored products and synthesis of colorants by laccase-catalyzed polymerization reactions (Enaud et al. 2010; Mustafa et al. 2005).

One of the applications of laccase in the textile industry is dying of cotton fibers. Conventional methods used in the dying processes needs a wide range of dyes and auxillaries and their usage at elevated pH and temperature values. Laccases can be

applied instead of oxidant agents initiating coupling of dye precursors and formation of coloured compounds that remains fixed in the fiber structure. Hadzhiyska et al. (2006) reported in-situ dyeing of cotton cellulose with a polymeric dye formed by oxidative coupling of colourless 2,5-diaminobenzenesulfonic acid and 1-hydroxyphenol (catechol) with laccase assistance. Both mild reaction conditions instead of very high temperatures and higher fixation of dyes onto the fibers up to 70 % has been achieved. Recently, several investigations on the biological production of indigo from indole (so-called bio-indigo) using recombinant microorganisms expressing mono- or dioxygenase have been reported and produced bacterial indigo dye was applied for dying cotton fabrics. The enzyme system responsible for indigo formation generally consists of one or more enzymes, typically monooxygenases, dioxygenases or hydroxylases and compared with the chemical synthesis, microbial biosynthesis has some advantages, such as lower cost, lower energy consumption, and being eco-friendly (Han et al. 2008; Bhushan et al. 2000; Pathak and Madamwar, 2010).

Waste effluent treatment

During manufacturing and usage, approx. 10-15% of total dyes are released into the environment and water soluble azo dyes can cause highly colored waste streams even at low concentrations. Those azo dyes and their biotransformation products have been shown to be toxic, mutagenic and even carsinogenic. Many microorganisms from different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolorize azo dyes. White-rot fungi are reported to be the most efficient in detoxification and decolorization of such effluents by their lignin-degrading enzymes, especially laccases (Moosvi et al. 2007; Arora and Sharma, 2010; Couto and Toca Herrera, 2006b). Furthermore, a major source of phenolic wastes is alkaline extraction stage effluent comes from bleaching of wood or pulp and it contains over 50% of color load. Instead of the expensive chemical and physical treatments methods, alternative biotreatment processes are now being considered. The development of processes based on laccases seems an attractive solution due to their potential in degradation of dyes with diverse chemical structures. Laccase oxidizes phenolics to aryl-oxy radical insoluble complexes and enzyme-mediated bioremediation processes include polymerization of pollutants among themselves or copolymerization with other nontoxic substances

such as humic materials to facilitate easy removal by adsorption, sedimentation, or filtration (Husain 2006, Robinson et al. 2001). The employment of laccase with the combination of mediators and cellobiose dehydrogenase for the decolorization of azo textile dyes proved to be a valid alternative expensive and less environmentally friendly chemical treatments of textile dye wastes (Arora and Sharma, 2010). In 2001, the company Zytex (Zytex Pvt. Ltd., Mumbai, India) developed a formulation based on laccase mediator system, capable of degrading indigo in a very specific way. The trade name of the product is Zylite. (Couto and Toca Herrera, 2006b).

Textile dye bleaching

Laccase has been reported to quickly bleach released dye stuff as part of the washing solution and prevent back staining of dyed or printed textiles. Laccase-catalyzed textile dye bleaching may also be useful in finishing steps of cotton fabric dying. A laccase-based system has been shown to be capable of bleaching indigo dye in denim by replacing conventional chemical oxidants (e.g., hypochlorite). This process, thus, results in the reduction of processing time, energy, and water needed to achieve satisfactory quality of the textile (Arora and Sharma, 2010). In 1996, Novozyme (Novo Nordisk, Denmark) developed a new industrial application of laccase enzyme in denim finishing: DeniLite®, as the first industrial laccase and the first bleaching enzyme acting with the help of a mediator molecule (Couto and Toca-Herrera, 2006b).

1.2.3.4 Nanobiotechnology

Bioelectrochemistry has received increased attention during the past two decades and has been integrated into analytical applications, such as in biosensors working as clinical and environmental analysis detectors. Laccase containing biosensors have been developed to detect various phenolic compounds, oxygen, glucose, aromatic amines or azides, since laccases are able to catalyse electron transfer reactions without additional cofactors (Couto and Toca-Herrera, 2006a). Two types of laccase-based O_2 sensors are widely used. One type monitors visible spectral changes (at 600 nm) of laccase resulting from the reoxidation of the type 1 copper in laccase by O_2. Another type monitors current or voltage change from a modified oxygen electrode on which O_2 reduction is enhanced under the electrocatalysis of immobilized laccase. Three types of laccase-based sensors have been reported for detection of phenols,

anilines, or other reducing substrates. One type detects the photometric change resulting from the oxidation of a chromogenic substrate; the second type monitors the O_2 concentration change that is coupled to the substrate oxidation, and the third type uses an electrode that replace O_2 as the acceptor for the electrons drawn from the substrate (through laccase). Laccase-based biosensors can be used for different electrochemical measurements. Smaller and more efficient biosensors through controlled deposition and specific adsorption of biomolecules on different types of surfaces could be developed by using nanotechnology (Arora and Sharma, 2010; Couto and Toca-Herrera, 2006a).

Immobilisation of laccases has an important effect on the biosensor sensitivity and micropatterning has been shown to be an efficient method for the immobilisation of laccases on a solid surface in order to develop a multi-functional biosensor. Recently, the fabrication of an optical biosensor by using stacked films where 3-methyl-2-benzothiazolinone hydrazone (MBTH) was immobilized in a hybrid nafion/sol-gel silicate film and laccase in a chitosan film for the detection of phenolic compounds (Abdullah et al. 2007).

Laccase can also be immobilised on the cathode of biofuel cells that could provide power, for example, for small transmitter systems (Chen et al.,2001). Biofuel cells are extremely attractive from an environmental point of view because electrical energy is generated without combusting fuel, thus, providing a cleaner source of energy. (Couto and Toca-Herrera, 2006a).

1.2.3.5 Other applications

Soil bioremediation

Polycyclic aromatic hydrocarbons (PAHs) together with other xenobiotics are major contaminants of soil, and their degradation is important for the environment, since the aromatic compounds are the major classes of pollutants and those toxic phenolics enter the environment in wastewater streams, released by numerous industries. The catalytic properties of laccases can be used to degrade such compounds. Laccases are able to mediate the coupling of reduced 2,4,6-trinitrotoluene (TNT) metabolites to an organic soil matrix, and results in detoxification of the munition residue. Moreover, PAHs arising from natural oil deposits were also found to be degraded by laccases (Couto and Toca-Herrera, 2006a, Chiacchierini et al. 2004).

Hydroxylated metabolites, appeared as phenolic compounds, are formed in soil behaves like substrates of laccase and it has been reported that laccase from white-rot fungus T. versicolor has the ability to transform the herbicide N,N′-dimethyl- N-(hydroxyphenyl) urea into insoluble purple phenolic compounds, p-benzoquinone, at pH 3, which can be easily metabolized by other fungi (Arora and Sharma, 2010).

Fossil fuel desulfurization

Fossil fuels emit harmful sulfur-containing compounds and various chemical and physical desulfurization methods reduce the emission of such harmful chemicals in extreme conditions like high temperature and pressure and with high maintenance costs. However, desulfurization by laccase can be performed under milder conditions and remove organic sulfur more efficiently (Arora and Sharma, 2010).

Cosmetics

Laccase-based hair dyes are less irritant and easier to handle than current hair dyes, since laccases replace H_2O_2 as an oxidising agent in the dye formulation. Recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed (Couto and Toca-Herrera, 2006a).

Medicinal applications

Synthesis of pharmaceutical products by laccases has also been reported. The capability of laccase to synthesize new compounds might also be used to generate new therapeutic compounds for treatment of microbiol infection or cancer. For example, laccase can oxidize iodide to produce iodine which is a reagent widely used as disinfectant (Arora and Sharma, 2010).

Immunochemical applications

Laccase catalysis can be used to assay other enzymes, such as amylase (alanine, cysteine, or leucine specific), amino-peptidases, alkaline phosphatase, γ-glutamyl transpeptidase, arylamidase, cellobiose oxidase, chymotrypsin, glucosidase, kallikrein, plasmin, and thrombin. Laccase that is covalently conjugated to an antibody or antigen can be used as a marker enzyme for immunochemical assay. In this immunochemical detection application, through modulation of laccase activity, binding of the antibody (or antigen) to its immunological counterpart is detected by

localized laccase activity on a gel or a blot membrane, much like the conventional peroxidase or phosphatase-assisted immunochemical assays. (Arora and Sharma, 2010).

1.2.4 Molecular biological properties

1.2.4.1 Laccase isozymes

Laccases of many fungal species are secreted as more than one isozymes (or isoforms) that may differ in their amino acid sequences and in characteristics with regard to pH optima, subsrate specifity, inducibility, etc. Although diversity in laccase isozymes within a single species was firstly thought to be only the result of the post translational modifications of the same gene product or allelic gene variants, characterization of laccase gene families revealed that diversity was also the result of the existence of multiple laccase genes in the fungal genomes (Arora and Sharma, 2010; Majeau et al. 2010; Madhavi and Lele, 2009; Rodgers et al., 2009).

During the life cycle of fungus different laccase isoenzymes occurs due to the induction or repression of laccase genes at different growth levels to fullfill various physiological roles (Theuerl and Buscot, 2010; Giardina et al. 2010). Fungi are able to produce several laccase isozymes and the proportion of the enzymes produced depends upon the culture composition, operational conditions employed, and also induction by ferulic acids, vanillic acid, veratric acids and copper (Arora and Sharma, 2010; Ng, 2004).

The isoenzymes has revealed diverse molecular weight, glycosylation degree and type of carbohydrate, pH, pI values, inducibility, and substrate specifity and those properties has suggested variable physiological roles or catalytic properties under different environmental conditions. For example, the isoenzymes from a particular microorganism usually possess different kinetic properties resulting in broadened substrate specificity and they may have an adaptive value for rotting fungi that grow on complex substrates such as hard wood, and in changing environments (Majeau et al., 2010; Theuerl and Buscot, 2010). Furthermore, different properties of laccases purified from different strains of the same species can be observed as a result of both the production of different laccase isoenzymes and different laccase properties in different strains. On the contrary, isozymes that are closely related both structurally and in their catalytic properties can be produced in different fungus e.g. *Coriolopsis*

rigida, *Dichomitus squalens*, *Physisporinus rivulosus* and *Trametes gallica* (Baldrian, 2006).

Numerous gene and protein sequences of Basidio- and Ascomycetes have been characterized so far (Arora and Sharma, 2010; Theurl and Buscot, 2010). Several laccase genes either genomic or cDNA clones have been isolated and characterized including those from *T. versicolor*, *Phlebia radiata*, *Trametes villosa*, *Ganoderma lucidum*, *Trametes sp*. AH 28-2, *Pycnoporus cinnabarinus*, *Pleurotus sapidus*, *Rhizoctonia solani*, etc. The copy number of laccase gene varies among fungi. To our knowledge, among 2628 fungal laccase genes, 1440 entry are belong to the Basidiomycete and only 14 complete laccase sequences from *Pycnoporus* genus among Basidiomycetes laccase genes are available in the NCBI database. Two laccase genes in the same chromosome of the Basidiomycetes *Agaricus bisporus* reports the first example of a laccase gene family in fungi. Thirty-one putative laccase genes from *Trametes versicolor* genomic sequences are known in the NCBI database. In the saprophytic fungus *Coprinus cinerea* genome contains 17 non-allelic laccase genes, clustered at seven different loci and nine of which were active. Five distinct laccase genes have been characterized from *Trametes villosa*, four from *Rhizoctonia solani*, three from *Trametes sp.* I-62, *Trametes sp.* AH 28-2. Laccase gene families have also been described in *Pleurotus genera* with four isolated members in *P. sajor-caju*, two in *P. eryngii*, and twelve members in *P. ostreatus*. The ectomycorrhizal fungus *Laccaria bicolor* have 11 laccase genes (Giardina et al. 2010; Majeau et al. 2010; Sarnthima and Khammuang, 2008; Rodgers et al. 2009). Laccase genes submitted to the NCBI database and their encoded protein molecular weights are given in Table 1.1.

Isolated laccase genes typically display a high degree of identity with one another and one cysteine and ten histidine residues involved in the binding of the four copper atoms are conserved in most of laccases, (Sarnthima and Khammuang, 2008).

1.2.4.2 Transcriptional regulation of laccase genes

Various environmental and cellular factors regulate the expression of the genes encoding the enzymes and some of those factors are common while others are more unique to either a certain fungus or a class of enzymes (Aro et al. 2005).

Table 1.1: Some laccase genes that have been characterized from different organisms.

	Laccase Gene	Encoded Protein		
Organism	Accession number	Length (aa)	MW (kDa)	Reference
Bacillus subtilis	Cot A, U51115	513	65	Martins et al. 2002
Myceliophthora thermophila	Lcc1, AR023901	623	80	Berka et al. 1997
Trametes versicolor *UAMH 8272*	Lac2, AB212732	519	53.6	Fujihiro et al. 2009
Pycnoporus sanguineus	FJ513077	518	68	Dantan-Gonzalez et al. 2008
Trametes versicolor	laccase I U44430	519	67	Ong et al. 1997
Trametes villosa	Lcc1, AY249052	520	63	Yaver et al. 1996
Trametes villosa	Lcc2, L49377	519	63	Yaver et al. 1996
Trametes pubescens	Lap2, AF414807	523	65	Galhaup et al. 2002
Marasmius quercophilus	Lac1, AF414807	517	62	Dedeyan et al. 2000
Trametes sp. C30	Lac1, AF491759	517	75	Klonowska et al. 2005
Melanocarpus albomyces	Lac1, AJ571698	623	80	Kiiskinen and Saloheimo, 2004
Pleurotus ostreatus	Poxa1b, AJ005017	533	62	Giardina et al. 1999
Pleurotus ostreatus	Poxc, Z49075	533	67	Palmieri et al. 1993
Pycnoporus coccineus	Lcc1, AB072703	518		Hoshida et al. 2001
Pleurotus sajor-caju	Lac 4	533	54	Soden et al. 2002
Gaemannomyces graminis var. tritici	Lac2, AJ417686	577	70	Litvintseva and Henson, 2002
Pycnoporus cinnabarinus	Lcc3-2	521	58,4	Temp et al. 1999
Trametes sp. 420	LacD, AY839942	521	53,3	Hong et al. 2007
Coriolus versicolor	CVLG1	526	53,6	Mikuni et al. 1997
Volvariella volvacea	Lac4	540	58	Chen et al. 2004
Ceriporiopsis subvermispora	Lcs1	520		Karahanian et al. 1998

Table 1.1 (continued): Some laccase genes that have been characterized from different organisms.

Populus euramericana	Lac90, Y13772	574	90	Ranocha et al. 1999
Rhizoctonia solani	Lcc4 Z54277	530	66	Wahleithner et al. 1996
Phoma sp. UHH 5-1-03	Lac1 EU267173	607	75.6	Junghanns et al. 2009
Pleurotus eryngii	Ery3 AM773999	531	56.6	Bleve et al. 2008
Aspergillus niger	Pel3 AY686700	532	54	Rodriguez et al. 2008

The nutrient levels, culture conditions, and developmental stage as well as by the addition of different inducers to cultural media influence the synthesis and secretion of laccases. In many fungal species, the effect of these factors at the level of laccase gene transcription has been demonstrated and laccase expression is generally regulated by some factors, such as metals, aromatic compounds and nitrogen and carbon sources (Giardina et al. 2010). Regulation of laccase expression by metals is widespread in fungi. In *T. versicolor, C. subvermispora, P. ostreatus, P. sajor-caju* and *Trametes pubescens* the effect of copper on the regulation of laccase transcript level have been demonstrated. Moreover, $Cd^{+2,}$ Ag^{+2}, Mn^{+2} ions are strong modulators of laccase transcript level. The activity and stability of laccase from *Pleurotus ostreatus* are enhanced by copper but reduced by mercury. The presence of putative metal-responsive elements (MRE) in the laccase promoter regions is associated with the metal effect. Although the presence of several putative ‘‘activation of *cup1* expression’’ (ACE) responsive elements in some laccase promoters have been indicated it has been observed that the copper induction has occurred in laccase genes whose promoters lack ACE or MRE elements and a totally different mechanism could be hypothesized (Giardina et al. 2010; Aro et al. 2005).

In order to increase laccase production, aromatic compounds, such as xylidine (XYL), ferulic acid (FA), or veratric acid (VA), are routinely added to fungal cultures and different white-rot fungi may respond different aromatic compounds.

Laccase induction by phenolic substances may be a response of fungi against highly reactive aromatic compounds. Laccases may play a defensive role by catalyzing their polymerization, since a reduction in the oxidative stress caused by oxygen radicals of these molecules. In the upstream regions of several induced laccase genes, such as those from *T. Versicolor* , *P. sajor caju* , *P. ostreatus* , and *Trametes* sp. AH28-2 putative xenobiotic response elements (XRE) have been found and the induction occurs at the transcriptional level (Giardina et al. 2010).

Concentration, ratio and and nature of carbon and nitrogen sources have been shown to affect laccase activity. Change in laccase activity in response to nitrogen concentration is a controversial issue, since activity increases under both limiting and nonlimiting conditions. For example, two isozymes of four laccase genes from *P. sajor-caju* are induced by nitrogen sources, whereas others are unaffected at the transcriptional level. In addition to this, laccase expression is subjected to catabolite repression and high glucose levels inhibit laccase transcription in some Basidiomycete. The existence of a carbon catabolite repressor CreA binding sites have been determined in the promoter regions of these repressed laccase genes (Giardina et al. 2010).

1.2.4.3 Structural and biochemical characteristics of laccases

Until now more than hundred laccases have been purified and very few have been structurally and biochemically characterized so far. Although, most of the fungal laccases are produced extracellularly most of the white-rot fungi produce both extracellular and intracellular laccases with isoenzymes showing similar activity patterns. Laccases are often produced as highly glycosylated forms and they are more stable in the extracellular environment since carbohydrates increase their hydrophilicity. Most of the laccases are monomeric proteins, but some of them exhibit homodimeric structure, which is composed of two identical subunits. Laccases are copper containing glycoproteins with molecular weight between 50 and 110 kDa with 10- 20% carbohydrates and usually contain four copper atoms except unusual laccases containing one, two and three coppers. Purified laccases exhibit characteristic blue color from their absorption around 600 nm due to type I copper and 320 nm (type III copper) and catalyze four electron oxidation of mostly phenolic compounds such as 2,6- dimethoxyphenol, syringaldazine, catechol, hydroquinone with redox potential up to 0.8 V. The optimum pH of fungal laccases generally lies in

the acidic range and it can changed towards different substrates. Different laccases have different catalytic preferences and they can be grouped as ortho-, meta- and para- substituted phenols. Studies showed that ortho-substituted compounds were better substrate than para-substituted compounds (Arora and Sharma, 2010).

1.3 Recombinant Expression of Laccases

The industrial enzyme market has been estimated to be worth over $1.6 billion in 1998 for the food, 45%; detergents, 34%; textiles, 11%; leather, 3%; pulp and paper 1.2% application areas and none of these areas are pharmaceutical enzymes. Those non-pharmaceutical protein market reached $2 billion in 2000 and over 60% of the enzymes used in the food, detergent and starch-processing industries are recombinant proteins. Furthermore many recombinant proteins are of fungal origin due to the low yield of non-fungal proteins. *Aspergillus niger*, as a filamentous fungi is an attractive model organism with its high secretion capacity, whereas high production yields can only be obtained for homologous proteins and non-fungal mammalian, bacterial and plant derived proteins are expressed only a few tens of miligrams per liter of growth medium (Sharma et al. 2009).

Industrial scale production of most fungal laccases could not be performed in the host fungi because of the low amount of the produced protein and the requirement of the toxic and expensive inducers to improve the yield. Although some laccases are employed in the industrial processes, there is not any natural laccase containing all desired characteristics, such as stability over a range of temperatures and pH with high reduction potential, halide/hydroxyl tolerance and suitability for cost-effective production. Instead of the protein production in the fungi itself, cloning and heterologous expression of the laccase genes have been performed as an alternative technology. Currently heterologous protein products are about one-third of all industrial applications. Several potential advantages of heterologous laccase expression exist compared to the purification of the enzyme from the culture broth. Firstly, single protein of known sequence is obtained by heterologous expression, as opposed to the fungal culture, where the production of laccases occurs in several isoforms. Secondly, recombinant production can result in higher protein yields, and the last advantage is the purification facility obtained by recombinant protein

production if recombinant proteins are secreted (Ranieri et al. 2009; Rodgers et al. 2009).

Microoorganisms are widely used host organisms for heterologous protein expression, whereas recombinant fungal laccase expression in maize seed has also been performed and maize-produced laccase has been demonstrated as a polymerization agent (Bailey et al. 2004). Although bacteria seems the easiest and quickest way to obtain heterologous proteins, they are insufficient for most of the eukaryotic proteins because of the lack of post-translational processing. Heterologous protein production is not only a protein synthesis but also involves co- or post-translational translocation of newly synthesized proteins into the endoplasmic reticulum (ER), protein folding in the ER, post-translational glycosylation in the ER and golgi apparatus, intracellular protein trafficking and sorting, proteolytic degradation and stress response for misfolding or overexpression. Besides, correct expression of the fungal laccases is depend on the selection of the appropriate host, whose codon usage and folding apparatus are suitable for functional enzyme production. Eukaryotic yeasts are the ideal hosts for fungal laccase expression and can perform post-translational modifications (proteolytic processing of secretion si,gnal sequences, disulfide bond formation, acylation, prenylation, phosphorylation, certain types of O- and N-linked glycosylation, which is essential for activity and correct folding) and secretion of the laccase in its native and biologically active form (Idiris et al. 2010; Freigassner et al. 2009).

Yeast expression system allows extracellular laccase production and secretion of heterologously expressed proteins presents several benefits, such as prevention of toxic heterologus proteins inside the cell, purification of the desired protein easily without breaking the cells and get rid of removal of intracellular proteins, which is an expensive step, and lastly obtaining the high level of native protein by using fermentation technology (Sharma et al. 2009). Most commonly used yeasts as heterologous hosts for laccase expression are *Pichia pastoris, Saccharomyces cerevisiae, Kluyveromyces lactis* and *Yarrowia lipolytica*. Different laccases expressed in yeast, bacteria and filamentous fungi are summarized in the Table 1.2.

Methylotrophic yeasts, such as *Pichia pastoris*, are attractive alternatives to *Saccharomyces cerevisiae* due to the less hypermannosylation of the overexpressed protein and characteristics of the *Pichia pastoris* is explained below.

1.3.1 Pichia pastoris

Pichia pastoris is a methylotrophic yeast that can metabolize methanol as a sole carbon source and can be genetically engineered for heterologous expression of proteins for scientific research and industrial purposes. During the past 25 years, the methylotrophic yeast *Pichia pastoris* has become a popular host organism for the production of heterologous proteins. More than 400 proteins from human endostatin to spider dragline silk protein have been successfully expressed in this yeast (Cereghino et al. 2002).

Bacterial expression systems, especially *Escherichia coli*., is the easiest and quickest way to express heterologous proteins. However, expression of larger proteins, for example S–S rich proteins and proteins requiring post translational modifications can not be performed in this organism, because of the lack of post translational processing system associated with higher eukaryotes, such as processing of signal sequences, folding, lipid addition, O- and N-linked glycosylation, disulfide bridge formation. In that situation, larger proteins are often expressed in yeasts, fungi, mammalian cells or the baculovirus system. *Saccharomyces cerevisiae* and *Pichia pastoris* are mostly preferred yeasts and are much more easily cultivated to high cell densities and efficiently secrete heterologous proteins compared to filamentous fungi, which are good hosts for protein secretion and are more time consuming to work. Furthermore, some proteins that can not be produced in mammalian cells, *Saccharomyces cerevisiae*, and baciloviruses were efficiently expressed in the easily manipulated *P. pastoris* cells (Liu et al. 2003; Demain and Vaishnav, 2009; Cereghino et al. 2002; Cereghino and Cregg, 2000; Zhu et al. 2009).

Several factors caused to increment of the popularity of *Pichia pastoris*: the simplicity of molecular genetic manipulations similar to those in *Saccharomyces cerevisiae* (DNA transformation, gene targeting and cloning by functional complementation), ability of high level intracellular or extracellular foreign protein production with secretion of low level of endogenous protein, providing the potential for expression of the soluble, correctly folded recombinant proteins that had all post-translational modifications for functionality. Moreover, foreign gene expression in *Pichia pastoris* is driven by tightly regulated promoter of alcohol oxidase I gene (AOX1) and this strong promoter is repressed in the growth medium containing glucose and other carbon sources and induced when cells were transferred into the

methanol containing medium. Recently, dual promoter system in the shuttle expression vector for *Pichia pastoris* has been reported by Duan et al. 2009. They constructed a *P. pastoris* expression vector containing two inducible promoters (an alcohol oxidase 1 promoter and a formaldehyde dehydrogenase 1 promoter) based on pPIC9k and at least two proteins from one vector has been induced. Foreign genes can be integrated into the chromosomal DNA by homologous recombination, since *P. pastoris* has no stable episomal plasmids, and more stable cell lines can be obtained by elimination of segregational instability relating to plasmids Another reason of using *P. pastoris* as an expression host is the respiratory preference of the organism rather than the fermentative mode of growth, where toxic levels of ethanol and acetic acid are obtained quickly in high cell density production. Additionally, this expression system is available as a commercial kit (Cereghino and Cregg, 2000; Demain and Vaishnav, 2009; Cereghino et al. 2002; Freigassner et al. 2009).

Pichia pastoris expression system provides useful and cost-effective experimental tool for protein engineering studies and high cell densities during cultivation guarantees the efficient and economically sustainable protein production required for functional, physiological and structural studies (Cereghino et al. 2002).

Table 1.2: Several laccase genes of fungi expressed in different heterologous hosts.

Laccase gene	Heterologous host	Explanation	Reference
P. sajor-caju LAC4	*Pichia pastoris*	4.85 mg l^{-1} of active laccase was produced and the enzyme was purified and partially characterized.	Soden *et al.*, 2002
Trametes sp. AH28-2 *lacb*	*P.pastoris*	1012 U/mg	Li et al_2007
Trametes versicolor lcc1	*P. pastoris*	Secreted laccase activity was optimized	Jönnson et al., 1997, O'Callaghan et al., 2002, Hong et al., 2002
P. cinnabarinus lac1	*P. pastoris*	8.0 mg l^{-1} of hyperglycosylated active laccase was secreted	Otterbein et al_2000
Trametes trogii lcc1	*P.pastoris*	The highest production level obtained in fed-batch culture was 2520 U/l. 17 mg/l	Colao et al., 2006
Trametes trogii lcc2	*P.pastoris*	Secreted laccase activity was obtained and the highest activity was 340 U/l	Colao et al., 2009
T. versicolor lcc1	*P. pastoris*	Active laccase was secreted in the medium. Both active laccase and its truncated version (LCCIa) were purified and partially characterized.	Gelo-Pujic *et al.*, 1999

Table 1.2 (continued): Several laccase genes of fungi expressed in different heterologous hosts.

Fome lignosus laccase	*P. pastoris*	Active secreted laccase activity value reached 9.03 Uml^{-1}	Liu et al., 2003
T. versicolor LCCIV	*P. pastoris*	Secreted laccase activity of 1.5 U/ml. The enzyme was purified and partially characterized.	Brown *et al.*, 2002
T. villosa lcc1	*Aspergillus oryzae*	Secreted laccase was purified and partially characterized.	Yaver *et al.*, 1996
C. cinereus lcc1	*A. oryzae*	8.0 to 135 mg/l of active laccase was secreted. The enzyme was purified and partially characterized.	Yaver *et al.*, 1999
Pleurotus ostreatus POXA1B	*S.cerevisiae*	Effect of growth conditions on laccase activity was observed. The highest laccase activity (200 U/l) was produced in the presence of copper sulphate and glucose containing medium	Piscitelli et al., 2005
Pleurotus ostreatus POXA1B	*Kluyveromyces lactis*	Expression conditions were optimised, highest laccase activity obtained was 3900 U/l and copper concentration did not affect laccase activity	Piscitelli et al., 2005
Pleurotus ostreatus POXC	*S.cerevisiae*	Reliable activity values were not measured due to the low amount of recombinant protein produced.	Piscitelli et al., 2005
Pleurotus ostreatus POXC	*Kluyveromyces lactis*	Expression conditions were optimised and copper concentration affected laccase activity (100 U/l)	Piscitelli et al., 2005
Thermus thermophilus HB27 laccase	*E.coli*	10 mg/l Enzyme was expressed as apoprotein and dialyzed against copper-containing buffer to yield a holoprotein	Miyazaki et al_2005
Pycnoporus cinnabarinus lac1	*Yarrowia lipolytica*	20 mg/l extracellular laccase was expressed in a bioreactor.	Madzak et al., 2005

1.4 Aim of the Thesis

Efficient and green oxidation technologies using enzymes has been searched to replace the conventional non-biological methods. Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2), among the different existing oxidant enzymes, generally exhibit a considerable level of stability in the extracellular environment and the inducible expression of laccases in most fungal species contributes to their applicability in biotechnological processes. Thus they have been become the subject of intensive research in the last decades. Employment of the laccases in diverse biotechnological applications requires the enlightment of the detailed molecular aspects of the enzyme by much more studies. Furthermore, laccase commercialization needs sufficient enzyme stocks, but there are some limitations for natural large scale production in white-rot fungi in terms of the selection of fungus,

their cultivation and the optimisation of enzyme production. Thus, gene cloning and heterologous expression of the cloned genes in suitable hosts are applied and these techniques allow to obtain greater quantities of enzymes which are weakly produced by the fungus itself. Moreover, those approaches are important for the study of structure-function relationships in laccase which can explain the differences observed between these enzymes in their biochemical properties (kinetic parameters, redox potential, stability, etc...).

The white-rot fungus *Pycnoporus sanguineus* produce several enzymes that can be used in industrial applications and produced laccase is the most interesting enzyme among the others because of the effectiveness in wide range of biotechnological applications, such as dye decolorization of azo, triphenylmethane and anthraquinonic dyes. To our knowledge, only limited number of complete laccase gene sequences from genus *Pycnoporus* among Basidiomycetes have been submitted to the NCBI database so far and this number of sequences are very limited for extensively studied and biotechnologically important fungal laccases.

In this thesis, isolation of full-length cDNAs coding for the laccase isozymes of *Pycnoporus sanguineus* MUCL 38531, heterologous expression in the yeast *Pichia pastoris* under the control of alcohol oxidase promoter and purification and biochemical characterization of the recombinant laccases have been aimed. Furthermore, demonstration of the functionality of the produced recombinant laccases for pigment and textile dye biosynthesis has also been aimed in this dissertation.

2. MATERIALS AND METHODS

2.1 Materials

Materials used in this study is listed below.

2.1.1 Strains and plasmids

Strains and their genotypes that are used in this project are listed in Table 2.1.

White-rot fungus *Pycnoporus sanguineus* MUCL 38531 was kindly provided from Sophie Vanhulle, Université Catholique de Louvain, Belgium. This fungus was grown in Nutrient broth and maltose medium at 28-30ºC in this study.

E.coli Top 10F' bacteria were used for subcloning studies and it was grown on Luria-Bertani medium at 37 ºC.

Methylotrophic yeast *Pichia pastoris* X-33 is the wild-type Pichia strain and it is useful for selection on Zeocin™ antibiotic and also large-scale growth. It grows in YPD and in minimal media at 28-30 ºC and used as heterologous host in this study.

pDrive vector for cloning of PCR products was obtained from QIAGEN and the expression vector pPICZB was obtained from Invitrogen.

Table 2.1: Strains and their genotypes used in the thesis.

Strain	Genotype	Source
Pycnoporus sanguienus MUCL 38531		
E.coli Top 10F'	*lacIq Tn10*(*Tet*^{r}), *mcrA Δ*(*mrr-hsdRMS-mcrBC*), *f80lacZΔM15 ΔlacX74, deoR, recA1, araD139 Δ*(*ara-leu*)*7697,galU, galK, rpsL* (*Strr*), *endA1, nupG*	M.A.Marahiel
Pichia pastoris X-33	wild-type	Invitrogen

2.1.1.1 Cloning Vector

Direct-cloning of PCR products that were generated by non-proofreading DNA polymerases, like *Taq* Polymerase or by enzyme blends, containing *Taq* DNA polymerase and proof-reading DNA polymerases, were performed into the pDrive Cloning Vector (Qiagen), which is supplied in a linear form with a U overhang at each end. Genomic map of the pDrive cloning vector is given in Figure 2.1. This vector has *amp* and *kan* resistance genes and blue/white colony screening is available while using this vector for cloning purposes. Restriction analysis of recombinant plasmids are easily performed through the several unique restriction endonuclease recognition sites around the cloning site of pDrive. Either sides of the cloning site contains T7 and SP6 promoters for both transcription of cloned PCR products and sequence analysis (Url-2).

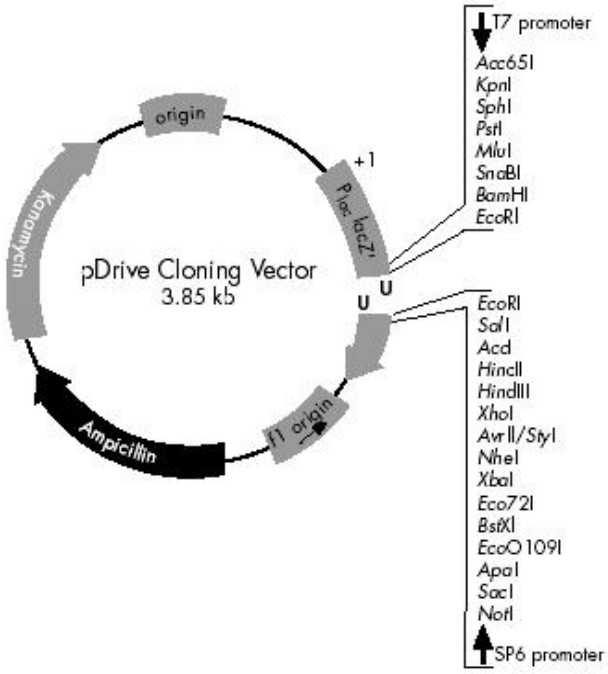


Figure 2.1 : Genomic map of pDrive cloning vector including restriction map and functional genes (Url-2).

2.1.1.2 Expression Vector

pPICZB expression vector was used in this thesis work (Figure 2.2). This vector is 3328 bp in legnth and contains strong and highly-inducible P*AOX1* promoter. Components of the vector is listed in Table 2.2. Yeast origin of replication does not exist in the *Pichia* expression vectors and zeocin-resistant transformants can be isolated, if recombination occurs between the plasmid and the *Pichia* genome.

Positive selection of transformants in *E. coli* and *Pichia* is performed by zeocin resistance-gene in the vector. Zeocin is an antibiotic isolated from Streptomyces, belongs to family of bleomycin/phleomycin-type antibiotics and has strong toxicity against bacteria, fungi, yeast, plants and mammalian cells. Zeocin ($C_{55}H_{83}N_{19}O_{21}S_2Cu$) is a copper-chelated glycoprotein, basic and water soluble. When zeocin enters the cell, the copper is reduced from Cu^{2+} to Cu^{+} and is removed by sulfhydryl compounds in the cell and zeocin is activated to bind DNA and cleavage of DNA causes cell death (Manual of Methods for Expression of Recombinant Proteins Using pPICZ and pPICZα in *Pichia pastoris*. Invitrogen Corporation, Carlsbad, CA, USA).

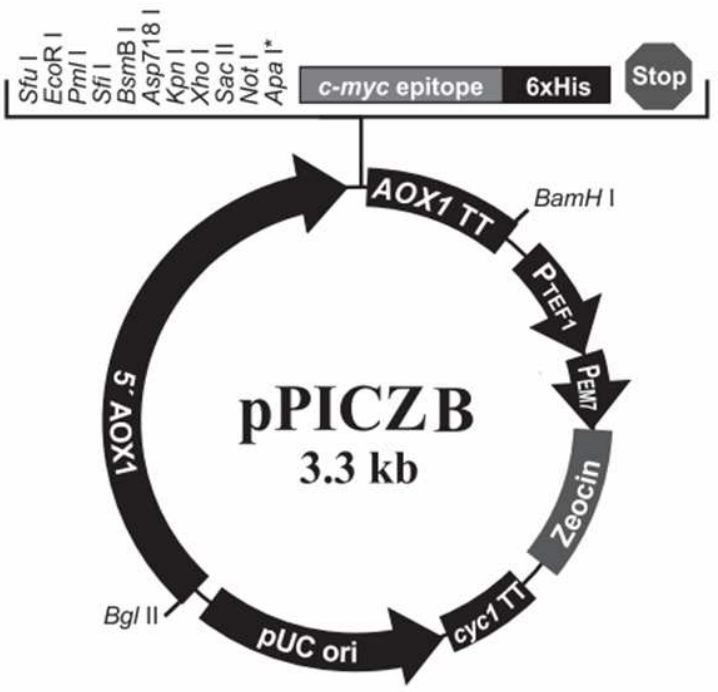


Figure 2.2 : Genomic map of pPICZB expression vector including restriction map and functional genes (Url-3).

Table 2.2: Components in the pPICZB expression vector.

Component	Function
5'-*AOX*	A fragment (942 bp) containing the AOX1 promoter, allows methanol-inducible expression in Pichia and also targets plasmid integration into the AOX1 locus of Pichia genome
Multiple cloning site with 10 restriction sites	Cloning of gene of interest into the expression vector
C-terminal *myc* epitope tag	Detection of fusion protein by Anti-myc Antibody or Anti-HRP antibody
C-terminal polyhistidine tag	Purification of recombinant fusion protein on metal chelating resin
AOX1 Transcription termination	Native transcription termination and polyadenylation signal of AOX1 gene (260bp) allows 3' mRNA processing, including polyadenylation for increased mRNA stability
TEF1 promoter	Transcription elongation factor ! gene promoter of *S. cerevisiae* driving *Sh ble* gene expression in Pichia, conferring Zeocin resistance
EM7(synthetic prokaryotic promoter)	Constitutive promoter driving expression of Sh ble gene in *E.coli* to provide Zeocin resistance
Sh ble gene (Streptoalloteichus hindustanus ble gene)	Zeocin resistance genes for selection
CYC1 transcription termination region	3'-end of the *S.cerevisiae* CYC1 gene allowing 3'-mRNA processing of the Sh *ble* gene for increased stability
pUC origin	Allows replication and maintenance of plasmid in *E.coli*
*Sac*I, *Pme*I, *Bst*XI	Unique restriction sites permitting linearization of vectors at *AOX1* locus for integration into the *Pichia* genome

2.1.2 Culture Media

The compositions and preparation of culture media are given in Appendix A1.

2.1.3 Buffers and Solutions

The compositions and preparation of buffers and solutions are given in Appendix A2.

2.1.4 Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are given in Appendix A3.

2.1.5 Laboratory Equipment

The laboratory equipment used during the project is listed in Appendix A4.

2.1.6 Cultivation of bacteria and yeast strains

White-rot fungus *Pycnoporus sanguineus* MUCL 38531 was kindly provided from Sophie Vanhulle, Université Catholique de Louvain, Belgium. This fungus was grown in solid Nutrient broth and cultivated in liquid maltose medium on rotary shaker at 150 rpm at 28 C in this study.

E.coli Top 10F' strain was grown in Luria-Bertani (LB) liquid medium at 37^0C and kept on Luria-Bertani (LB) agar plates. All cultures were stored at 4^0C and subcultured monthly. Storage of the bacteria for longer times was performed by preparation of 10 % glycerol stock and it was kept at -80^0C. Ampicillin (*Amp*) (100 µg/ml) was used as the selective antibiotic for *E.coli* Top 10F' strain. *Pichia pastoris* wild-type strain X-33 was selected on Zeocin and it has grown in YPD and in minimal media. The growth temperature of *Pichia pastoris* is 28-30°C for liquid cultures and plates. Cultures were kept on solid media at 4^0C and agar plates were refreshed monthly. Glycerol stocks of the cultures of OD_{600} of 50-100 was prepared in YPD containing 15% glycerol and stored at -80^0C. Doubling time of log phase Mut^+ phenotype (methanol utilization positive) Pichia in YPD is ~2 hours and is 4-6 hours in methanol medium. One OD_{600} = ~5 x 10^7 cells/ml for *Pichia pastoris*. Methanol is added every day to methanol containing growth media, to compensate for loss because of evaporation or consumption and also to induce expression. Zeocin™ was used for the selection of recombinant colonies for the expression. Bacteria were selected in the low salt LB plates supplemented with 25 µgml^{-1} Zeo, whereas yeast cells were selected in the YPDS plate with 100 µgml^{-1} Zeo.

2.2 Methods

Nucleic acid manipulation techniques, transformation of bacteria and methylotrophic yeast *Pichia pastoris*, heterologous expression of the full-length laccase cDNAs in yeast Pichia pastoris, purification of recombinant laccases, biochemical characterization of recombinant laccase and finally functionality of the recombinant laccases were explained in this part of the thesis.

2.2.1 Nucleic acid manipulation techniques

Basic nucleic acid manipulation techniques, including genomic DNA, total RNA and plasmid DNA isolation from *Pycnoporus sanguienus*, visualization of nucleic acids, elution of the fragments from agarose gel and also polymerase chain reaction (PCR), rapid amplification of cDNA ends (RACE), full-length cDNA synthesis and cloning of the fragments into the relevant vectors is given below.

2.2.1.1 Genomic DNA isolation

Genomic DNA isolation from *Pycnoporus sanguineus* MUCL 38531 was performed by the procedure of "DNeasy Plant Mini Kit"(Qiagen). Fungus was grown in maltose medium at 30ºC for 4 days and cells were pelleted by centrifugation at 10000 xg for 30 minutes. Supernatant was completely removed and biomass was stored at -80°C before isolation. Each genomic DNA isolation was performed from the same biomass. Maximum of 100 mg (wet weight) fungal tissue was ground to a fine powder with liquid nitrogen using a mortar and pestle. Tissue powder with liquid nitrogen was transferred to an appropriately 1,5 ml microcentrifuge tube and liquid nitrogen had evaporated. Without allowing the sample to thaw, 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) were added to disrupted fungal tissue and vortexed vigorously not to allow tissue clumps formation. The mixture was

incubated for 10 min at 65°C to lyse the cells and mixed 3 times by inverting tube. 130 µl Buffer AP2 was added to the lysate, mixed and incubated for 5 min on ice and centrifuged for 5 min at 14000 rpm to precipitate detergent, proteins, and polysaccharides. Supernatant was applied to QIAshredder Mini spin column and centrifuged for 2 min at 14,000 rpm. The lysate, it was usually 450 µl, was transferred into a new tube without disturbing the cell-debris pellet and 1.5 volumes of Buffer AP3/E (675 µl) was added to the cleared lysate, and mixed by pipetting. 650 µl of the mixture was pipetted into the DNeasy Mini spin column and centrifuged at 8000 rpm for 1 min. Remaning sample was also centrifuged and the flow-through was discarded. DNeasy Mini spin column was placed into a new 2 ml collection tube, 500 µl Buffer AW was added and centrifuged for 2 min at 14000 rpm to dry the membrane and prevent ethanol contamination. Flow-through was discarded and spin column was transferred to a 1,5 ml microcentrifuge tube. 50 µl

Buffer AE was added onto the spin column, incubated for 5 min at room temperature and genomic DNA was eluted by centrifugation for 1 min at 8000 rpm.

2.2.1.2 Total RNA isolation

Mycelia of *Pycnoporus sanguineus* grown in maltose medium at 30ºC for 4 days, in which laccase activity and red pigmentation in the culture is in maximum level, were collected and total RNA was immediately isolated using "Qiagen RNeasy Plant Mini Kit ". Procedure was carried out as quickly as possible to prevent RNA degradation and all steps were performed at room temperature. 10 µl β-Mercaptoethanol (β-ME) was added to lysis buffer RLC, containing guanidine hydrochloride, just before use. Cells were pelleted by centrifugation at 10000 xg for 30 minutes. Supernatant was completely removed and biomass was stored at -80°C before isolation. Maximum of 100 mg (wet weight) fungal tissue was ground to a fine powder with liquid nitrogen using a mortar and pestle. Tissue powder was transferred to an appropriately 2 ml microcentrifuge tube and liquid nitrogen had evaporated. Without allowing the sample to thaw, 450 µl Buffer RLC was added and vortex vigorously. The tissue was disrupted with 3 min incubation at 56°C, the lysate was transferred to a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. The supernatant of the flow-through was transferred to a new microcentrifuge tube carefully. 0.5 volume of ethanol (96–100%) was added to the cleared lysate, and mixed by pipetting. Sample was transferred to the RNeasy spin column and centrifuged for 15 s at 8000 x g. Flow-through was discarded, 700 µl Buffer RW1 was added to the spin column and centrifuged for 15 s at 8000 x g. Spin column was placed into a new collection tube carefully without contacting the flow-through. 500 µl Buffer RPE was added to the RNeasy spin column, was centrifuged for 15 s at 8000 x g to wash the spin column membrane and the flow-through was discarded. Spin column was washed again with 500 µl Buffer RPE and centrifuged for 2 min at 8000 x g. Spin column was placed into a new collection tube and centrifuged at full speed for 1 min to eliminate any ethanol interference. RNeasy spin column was transferred to a 1,5 ml microcentrifuge tube 30 µl RNase-free water directly to the spin column membrane, centrifuged for 1 min at 8000 x g to elute the RNA.

2.2.1.3 Plasmid DNA isolation

Plasmid DNA isolation of *E.coli* clones was carried out with the modified procedure of the "QIAquick Plasmid DNA Isolation Kit" (QIAGEN). All buffers and solutions were prepared according to the kit manual and described in the Appendix B.

The bacterial cells were harvested by centrifugation at 13000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 300 µl P1 buffer. 300 µl P2 buffer was added and the solution was then incubated at room temperature for 5 minutes. 300 µl P3 buffer was added and mixed through inverting the tube until the lysate is no longer viscous. The sample was incubated for 15 minutes on ice and then centrifuged at 13000 rpm for 15 minutes. Supernatant was transferred to a new 1.5 ml eppendorf tube and plasmid DNA was precipitated by adding 0.7 volume of isopropanol and collected by centrifugation at 13000 rpm for 30 minutes. The pellet was washed with 1 ml of 70% ethanol by centrifugation at 13000 rpm for 5 minutes. The supernatant was removed and ethanol was dried out at 37°C for 15 minutes. The pellet was then dissolved in 15 µl of elution buffer (EB) and stored at -20^0C. The isolated DNA was run on 1 % agarose gel.

2.2.1.4 Agarose gel electrophoresis

Neutral agarose gel system, composed of 1% agarose gel containing 1xTAE buffer (Appendix B) and ethidium bromide of a 0.2 mg/ml final concentration was used for electrophoresis of the sample DNAs. The gel was run in 1x TAE at 4V/cm and the gels were visualized under UV transillumination. Agarose gel concentration depends on the purpose of the electrophoresis and the size of the nucleic acid molecule.

2.2.1.5 Formaldehyde denaturing gel

The integrity of purified total RNA was checked by denaturing agarose gel electrophoresis. Gel was run at 5–7 V/cm in 1x FA gel running buffer. 28S and 18S ribosomal RNAs should appear as sharp bands or peaks. The ratio of 28S rRNA to 18S RNA should be approximately 2:1 and degradation of RNA can be understood if the ribosomal bands are not sharp and appear as a smear towards smaller sized RNAs.

2.2.1.6 Polymerase chain reaction (PCR)

Polymerase chain reaction was carried out in different steps of this dissertation. Screening the genome of *Pycnoporus sanguineus* with degenerate primers, cDNA cloning of laccase encoding genes in terms of RACE-PCRs and the full-length cDNA synthesis were performed with PCR using different primer sets in different amplification conditions. Conditions of RACE-PCRs will be explained in the relevant part below. The annealing temperatures depends on the primers used, and the time of the final elongation depends on the size of the fragment to be amplified (1 min for 1 kb fragment).

Screening of the genome was performed with amplification of conserved region with degenerate primers specific to the sequence of copper binding domain I (LAC-N1) and domain IV(LAC-C1). 50 ng of genomic DNA, isolated from *Pycnoporus sanguineus* was used as the template. PCR was performed using Platinum Taq polymerase (Invitrogen). PCR program was initiated at 94 °C for 4 min, followed by 5 cycles of 94°C for 1 min, 50 °C for 1 min, 72 °C for 2.5 min and 25 cycles of 94°C for 1 min, 55 ºC for 1 min, 72 ºC for 2.5 min and a final extension at 72 ºC for 10 min. The PCR reaction was carried out in the presence of 0.2 mM of each dNTP, 0.2 pmol of each primer and 2.5 units of Platinum Taq polymerase.

Screening of the laccase specific sequences at the cDNA level was also carried out by PCR with degenerate primers. Following the first strand cDNA synthesis, PCR was performed using Taq polymerase (İnvitrogen) and 2µl of the first strand mixture was used as the template. PCR was initiated with denaturation at 94 °C for 3 min, followed by 35 cycles of 94°C for 1 min, 50 ºC for 1 min, 72 ºC for 2.5 min and final extension at 72 ºC for 10 min. The PCR reaction mix (50 µl) contained 1x Taq buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 pmol of each primer, PCR-grade water and 2.5 units of Taq polymerase.

The full-length laccase cDNAs and gDNAs were amplified with Expand High Fidelity ^{PLUS} PCR System from Roche Applied Biosciences. This system contains an enzyme blend of Taq DNA polymerase and a proofreading enzyme that lacks polymerase activity and prevents incorporation of incorrect nucleotides into the newly synthesized strand in the 5'- to 3'- direction. Using this enzyme gets 6-fold higher fidelity compared to Taq DNA polymerase. For the amplification of full-

length laccase cDNAs, 50 µl of reaction mix contained appropriate amount of PCR-grade water, 1x Expand High Fidelity Buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 pmol of forward and reverse primers, 2 µl of first strand cDNA as template, 2.5 units of Expand HiFi^{PLUS} Enzyme Blend. PCR program was initiated at 94 °C for 2 min followed by 5 cycles of 94°C for 30 sec, 55 °C for 30 sec, 72 °C for 2.5 min, 25 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 2.5 min and a final extension at 72 ºC for 7 min. Amplification of laccase genes were also carried out by using the same PCR system and reaction mix was described above. 50 ng of gDNA was used as template and PCR was performed according to the following thermal profile; initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94°C for 30 sec, 56 ºC for 30 sec, 72 ºC for 2.5 min and a final elongation at 72 ºC for 7 min.

2.2.1.7 Rapid amplification of cDNA ends (RACE) technique for isolation of laccase cDNAs

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and either the 3´ or the 5´ end of the mRNA. This simple, rapid and efficient cDNA cloning strategy is developed based on the PCR and overcomes difficulties in obtaining full-length cDNA clones of low-abundance mRNAs (Frohman et al. 1988). Although PCR requires two sequence-specific primers flanking the sequence to be amplified, amplification of unknown sequences causes a limitation for PCR and those limitations can be achieved by using 3'- and 5'-RACE procedures. RACE has been used for amplification and cloning of rare mRNAs , and also applied to existing cDNA libraries (Frohman et al. 1988, Frohman 1993). RACE products can be used to prepare probes (Harvey and Darlison, 1991) and in conjunction with exon-trapping methods amplification of unknown coding sequences can be performed with the RACE procedures (Adams and Blakesley, 1991). Additionaly RACE procedures have advantage to characterize genes identified by different methods, such as cDNA subtraction, differential display, RNA fingerprinting, etc.

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and either the 3´ or the 5´ end of the mRNA. In this thesis, two

different RACE Kits were purchased from Invitrogen [3'-RACE System for Rapid Amplification of cDNA Ends (cat. no. 18373-019), 5'-RACE System for Rapid Amplification of cDNA Ends (cat. no. 18374-058)] and Clontech [BD SMART™ RACE cDNA Amplification Kit (cat. no. 634914]. 5' and 3'- ends of *lcc1* cDNA was tried to amplify by 5'- and 3'- RACE Systems from Invitrogen, whereas ends of *lcc2* cDNA was obtained by Clontech BD SMART RACE Kit. Gene-specific primers needed in the amplification of the cDNA ends were designed based on the sequence of the conserved copper binding region of laccases and strategy used in the RACE experiments of the thesis is summarized in the Figure 2.3.

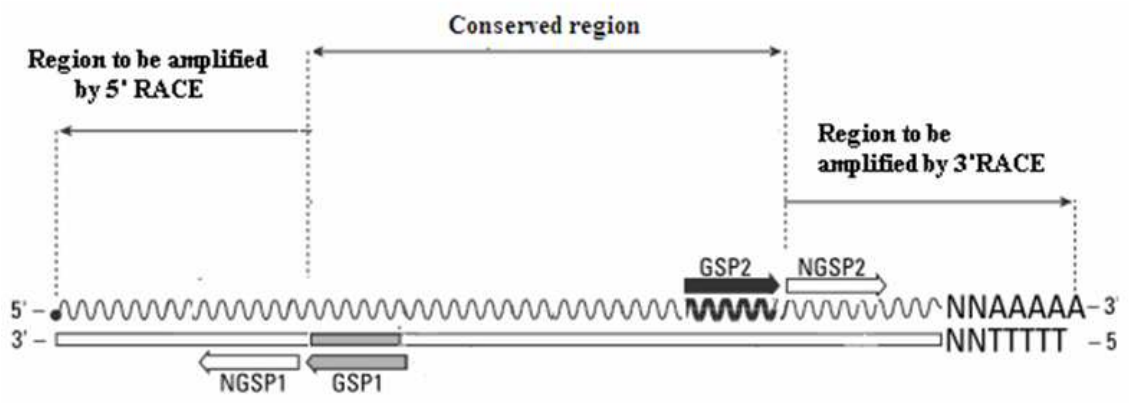


Figure 2.3 : Schematic representation of the 5'- and 3'-RACE PCR for laccase cDNA amplification, the relationship of gene-specific primers to the cDNA template is shown. The gene-specific primers were designed based on the sequence of the conserved copper binding region.

Gene-specific primer design

RACE protocols require two gene specific primers (GSP), an antisense primer for the 5'-RACE PCR and a sense primer for the 3'-RACE PCR. Since the RACE-PCRs are carried out using only one GSP, specific and efficient PCR is dependent on the correct primer design. Primers should be 23–28 nt long and should have a GC content of 50–70% with a Tm of at least 65°C; whenever possible the Tm should be greater than 70°C. Additionally, 3'-terminal complementarity should be minimized since PCR efficiency is significantly reduced by primer- dimer artifacts. The nested amplification primer should be examined for dimer formation with the commercial primer, as well as itself.

RACE-PCRs are set up with gene-specific primers and also commercial primers and list of the used commercial primers is given in Table.2.3.

Table 2.3: Commercial primers used in the first strand synthesis and RACE reactions.

Primer	Sequence
Adapter primer (AP)	5’-GGCCACGCGTCGACTAGTAC$(T)_{17}$ -3’
Abridged universal amplification primer (AUAP)	5’-GGCCACGCGTCGACTAGTAC-3’
Abridged anchor primer (AAP)	5’-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG -3’
BD SMART II™ A Oligonucleotide	5'–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3'
3'-RACE CDS Primer A	5'–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N–3' (N = A, C, G, ya da T; V = A, G, ya da C)
5'-RACE CDS Primer	5'–$(T)_{25}$V N–3' (N = A, C, G, ya da T; V = A, G, ya da C)
Universal Primer A Mix(UPM)	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'
Nested Universal Primer A (NUP)	5'–AAGCAGTGGTATCAACGCAGAGT–3'

5’-Rapid amplification of cDNA ends (5’-RACE)

Determination of the 5’ end of the laccase cDNAs was performed by 5’RACE reaction. Following to the first strand synthesis of cDNA, 5’RACE PCR was set up and RACE-PCR products were characterized by cloning and sequencing the fragment. Two different 5’-RACE Kits were purchased from Invitrogen (5’-RACE System) and Clontech (BD SMART-RACE). While Lcc1-5’ cDNA was tried to amplify by 5’-RACE System from Invitrogen, 5’-end of lcc2 cDNA was obtained by Clontech BD SMART RACE Kit.

Invitrogen 5´ RACE System uses an gene specific reverse primer for the synthesis of specific cDNA by reverse transcriptase. An adapter sequence is attached to the unknown 5´ sequences of the cDNA by TdT-tailing step prior to PCR. Specific cDNA is then amplified by PCR using a GSP-reverse primer specific to known exon sequences and an adapter primer targetting the 5´ end (İnvitrogen 5’ RACE system

instruction manual). The first strand lcc1 cDNA was synthesized from 3µg total RNA using a gene-specific Lcc1-5'RACE-reverse primer and Superscript II reverse transcriptase, a derivative of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) with reduced RNase H activity (Invitrogen 5'-RACE System). After the first strand cDNA synthesis, the original RNA template was removed by treatment with the RNase mix. Unincorporated dNTPs, gene specific primer and proteins were seperated from cDNA by using S.N.A.P. Column. After S.N.A.P. purification, a homopolymeric tail is added to the 3'-end of the cDNA using TdT (Terminal deoxy nucleotdyl transferase) and dCTP. After dC-tailed cDNA was synthesized, PCR amplification of this molecule was accomplished by using gene specific Lcc1-5'RACE-nested reverse primer, annealing to a site located in the cDNA molecule, and deoxyinosine containing Abridged Anchor Primer (AAP). PCR program was initiated at 94 °C for 3 min, followed by 35 cycles of 94°C for 1 min, 52 ºC for 1 min, 72 ºC for 2.5 min and a final extension at 72 ºC for 10 min. Following to this reaction another PCR reaction set up with product of the first PCR reaction as template, 0.2 mM of each dNTP, 10 pmol/µl of AUAP and Lcc1-5'RACE-nested-reverse primer and 2.5 units of Taq polymerase to increase the specifity of amplification.

On the contrary to the Invitrogen's procedure requiring the second-strand synthesis and adaptor ligation, the first-strand cDNA is directly used in 5'- and 3'-RACE PCR reactions following to the reverse transcription by using SMART RACE cDNA amplification kit. In this procedure, PowerScript™ Reverse Transcriptase (RT), a variant of MMLV RT and SMART II™ A Oligonucleotide are used. PowerScript RT exhibiting terminal transferase activity, reaches the end of an RNA template and adds 3–5 residues (generally dC) to the 3' end of the first-strand cDNA. SMART II A oligonucleotide, containing a terminal stretch of guanine nucleotides, anneals to the dC-rich cDNA tail and provides an extended template for RT. By means of this faster and less complex way of cDNA synthesis, complete first strand synthesis is guarenteed without performing any external adaptor ligation (SMART-RACE Kit İnstruction Manual). cDNA synthesis mechanism of SMART RACE cDNA synthesis is given in Figure 2.4.

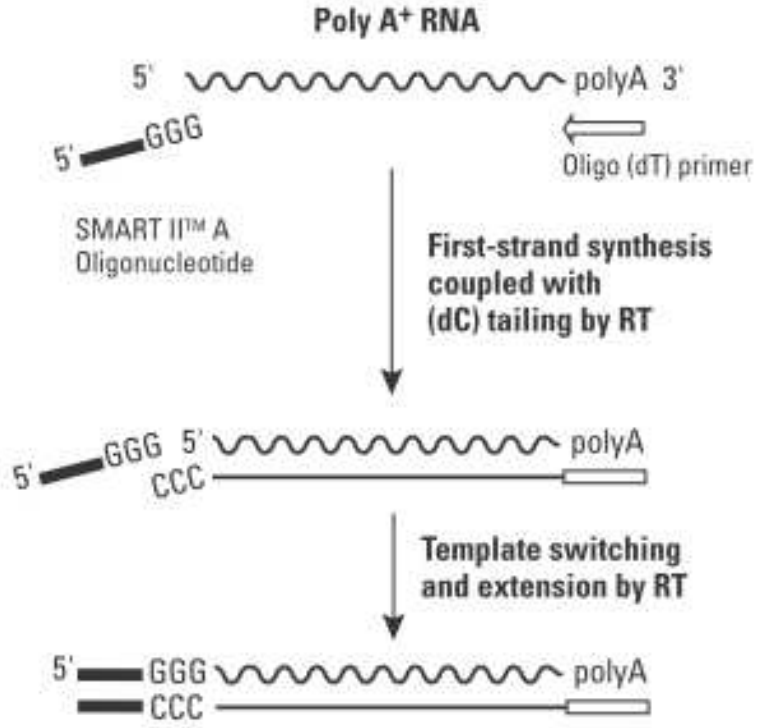


Figure 2.4 : Mechanism of SMART™ cDNA synthesis. First-strand synthesis primed with modified oligo (dT) primer and dC tailing activity of reverse transcriptase to the end of the mRNA template (SMART RACE cDNA synthesis kit manual).

The cDNA for 5'-RACE is synthesized using a modified oligo(dT) primer, termed the 5'-RACE CDS Primer A (5'-CDS) and the SMART II A oligo. The modified oligo(dT) primer has two degenerate nucleotide positions at the 3' end and the primer is positioned at the start of the poly A+ tail and the 3' heterogeneity, caused by conventional oligo(dT) priming is eliminated. Determination of the 5'-end of the *lcc2* cDNA was performed by 5'-RACE procedure as described above. The first strand cDNA was sythesized with 1 µg of total RNA, 0.6 µM of 5'-RACE CDS Primer A, 0.6 µM of BD SMART II™ A Oligonucleotide and PowerScript™ Reverse Transcriptase. 5'-RACE PCRs was set up with synthesized 5'-RACE ready cDNA, 0.4 µM of Universal Primer Mix (UPM), 1 µM of gene specific reverse primers, *Lcc2*-5'RACE-reverse-I and *Lcc2*-5'RACE-reverse-II, designed based on the 5'sequence of the conserved copper binding region sequence and Advantage 2 Polymerase(Clontech. Amplified fragment was cloned into the pDrive Cloning Vector (Qiagen) and sequenced.

3’- Rapid amplification of cDNA ends (3’-RACE)

3’-end of lcc1 cDNA was determined using 3’-RACE System from Invitrogen. In the 3’-RACE procedure using Invitrogen 3’-RACE system, natural poly(A) tail of the mRNA is used as a priming site for PCR. mRNAs are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer. The unknown 3’-region between known exon sequence and poly(A) tail is determined by amplifying specific cDNA with PCR using gene-specific forward primer, annealing a region of known exon sequences and the adapter primer (AP) targeting poly(A) tail of mRNA (İnvitrogen 3’-RACE System Instruction manual).

Lcc1-3’RACE -forward primer and Lcc1-3’RACE-nested forward primers were designed from the lcc1 partial sequence to determine the 3’end of the laccase cDNA. The sequences of the commercial oligonucleotide primers used for cDNA amplification are as in the Table 2.3 and gene specific primers are listed in the Results and Discussions chapter. In this study, 3 μg total RNA was converted into the first strand cDNA by using 10μM oligo (dT)-anchor primer (Adapter primer) and Superscript II reverse transcriptase and after the first strand cDNA synthesis, amplification of specific cDNA was performed by PCR using a gene specific , Lcc1-3’RACE -forward primer and an abridged universal amplification primer (AUAP). 2μl of the first strand mixture was used as the template and PCR was performed using Taq polymerase (İnvitrogen). PCR program was initiated at 94 °C for 3 min, followed by 35 cycles of 94°C for 1 min, 55 ºC for 1 min, 72 ºC for 2.5 min and a final extension at 72 ºC for 10 min. The PCR reaction was carried out in the presence of 0.2 mM of each dNTP, 10 pmol/μl of each primer and 2.5 units of Taq polymerase. Amplified sample was analyzed by using agarose gel electrophoresis. Amplification specificity was increased by nested amplification using a nested forward primer (Lcc1-3’RACE-nested forward), which was again based on the homology to the known laccase genes (lcc1), and the AUAP. Initial denaturation was performed at 94 °C for 3 min, followed by 35 cycles of 94°C for 1 min, 53 ºC for 1 min, 72 ºC for 2.5 min and a final extension at 72 ºC for 10 min. The PCR reaction was again carried out in the presence of 0.2 mM of each dNTP, 10 pmol/μl of each primer and 2.5 units of Taq polymerase.

3’-end of lcc2 cDNA was obtained by Clontech BD SMART RACE Kit. The 3’-RACE cDNA was synthesized by traditional reverse transcription procedure with a special oligo(dT) primer. This 3'-RACE CDS Primer A (3'-CDS) primer includes the lock-docking nucleotide positions as in the 5'-CDS primer and also has a portion of the SMART sequence at its 5' end. Incorporating the SMART sequence into both the 5'- and 3'-RACE-Ready cDNA populations, both RACE PCR reactions can be primed using the Universal Primer A Mix (UPM) recognizing the SMART sequence and the gene-specific primers. To find out the sequence of the 3’-end of the lcc2 cDNA 3’-RACE procedure was applied. The first strand cDNA was synthesized using 1 µg of total RNA, 0.6 µM of 3’-RACE CDS Primer A and PowerScript™ Reverse Transcriptase 1 µM of gene-specific Lcc2-3’RACE-forward, designed as indicated above, 0.4 µM of Universal Primer A Mix, UPM and Advantage 2 Polymerase (Clontech) was used to set up 3’-RACE PCR. Amplified fragment was cloned into the pDrive Cloning Vector (Qiagen) and sequenced.

2.2.1.8 Full-length cDNA synthesis

Following to the determination of 5’- and 3’-ends of the laccase cDNAs by RACE protocols, gene-specific forward and reverse primers were designed specific to the both ends of the cDNAs and the full length *lcc1* and *lcc2* cDNAs were obtained The first strand of the cDNA was synthesized with Roche Transcriptor First Strand Synthesis kit (Roche) according to the manufacturer’s instructions. In a thin walled, nuclease-free and sterile PCR tube, primer-template mixture, containing 3 µg of total RNA and 2.5 pmol of oligo (dT) primer was prepared on ice and PCR-grade water was added to make volume 13 µl. To denature the secondary structures of RNA, mixture was incubated in a thermal cycler with a heated lid at 65 ˚C for 10 minutes and tube was cooled on ice. 4 µl of 5x transcriptor reverse transcriptase reaction buffer, 0.5 µl of protector RNase inhibitor, 2 µl of deoxynucleotide mix and 0.5 µl of transcriptor reverse transcriptase were added to the mix and incubated at 25 ˚C for 10 minutes followed by 55 ˚C 30 minutes. Reverse transcriptase was inactivated by heating the mix to 85 ˚C for 5 minutes and reaction was stopped by placing the tube on ice. PCR was carried out in the presence of the 2 µl of the first strand cDNA, 0.2 mM of each dNTP, 0.4 pmol of each forward and reverse primers and 2.5 units of Expand High Fidelity Polymerase Plus System enzyme blend (Roche). Amplified PCR product was cloned into the pDrive cloning vector, transferred into the *E.coli*

Top10F' cells. Following to the plasmid isolation and confirmation with restriction enzyme digestion, plasmids containing the insert were sequenced.

2.2.1.9 Gel extraction

PCR products and linear plasmids were extracted and purified from agarose gel with the "QIAquick Gel Extraction Kit" from Qiagen according to the protocol. The DNA fragment was excised from the gel, 3 volumes of buffer QG was added depending on the weight of the fragment and the solution was incubated for 10 minutes at 50°C by vortexing every 2-3 minutes until the gel was dissolved completely. 1 volume of isopropanol was added, the sample was applied to the QIAquick column and centrifuged at 13000 rpm for 1 minute. The flow through was discarded, 500 µl buffer QG was added to the column and centrifuged at 13000 rpm for 1 minute. To wash the column, 750 µl buffer PE was added, standed for 2-5 minutes and then centrfiuged at 13000 rpm for 1 minute which was followed with an additional 1 minute at 13000 rpm. The column was placed into a clean 1.5 ml microfuge tube, 30 µl EB buffer was applied to the center of the QIAquick membrane, standed for 1 minute and was centrifuged for 1 minute. Eluted DNA was run on 1 % agarose gel and stored at -20°C.

2.2.1.10 Enzymatic digestion

Digestion reactions with restrictions enzymes were carried out as specified in the manufacturer's protocols. The PCR products, cloning and expression vectors were cut with a reaction mix using 5U of enzyme for each µg of DNA and 1x digestion buffer at 37°C for 1 to 2.5 hours.

2.2.1.11 Ligation of the PCR products into pDrive cloning vector

Purified PCR fragments were ligated into pDrive T/A Cloning vector (Qiagen) according to the PCR Cloning Kit (Qiagen) protocol. pDrive cloning vector, distilled water and the ligation master mix were thawed completely before use and placed on ice. The ligation reaction was set up in 10 µl of total volume by adding following components in the written order in Table 2.4.

Table 2.4: Components of the cloning reaction set up with pDrive vector.

Component	Volume
Distilled water	2 µl (variable)
pDrive Cloning Vector (50 ng/µl)	1 µl
PCR product	2 µl (1-4 µl)
2x Ligation Master Mix	5 µl

Reaction mixture was briefly mixed and incubated at 16^{o}C for 1.5 hours then the ligase was inhibited at 70^{o}C for 10 minutes. After ligation was completed, the mixture was directly used for transformation of *E.coli* Top10F'.

2.2.1.12 Cloning into expression vector

Laccase cDNAs were cloned into yeast shuttle expression vector pPICZB (Invitrogen) with laccases' own secretion signal sequence. The pDrive cloning vector having the PCR product and the pPICZB expression vector were digested with suitable restricition enzymes, while lcc1/pDrive construct was cut with *Eco*RI and *Not*I, lcc2/pDrive was digested with *Eco*RI. Resulting fragments were recovered from the gel. . pPICZB vector, distilled water and the ligation buffer were thawed completely before use and placed on ice and ligation reaction of 3.3 kb from pPICZB with laccase cDNA was set up in 20 µl of total volume by adding following components in the written order (Table 2.5).

Table 2.5: Ligation reaction of expression vector and laccase cDNA by using T4 DNA Ligase.

Component	Volume
Distilled water	6 µl
pPICZB expression vector (50 ng/µl)	8 µl (9.5-7 µl)
cDNA	2 µl (0.5-3 µl)
10x Ligation Buffer	2 µl
T4 DNA Ligase (1 Uμl^{-1})	2 µl

2.2.2 Transformation of bacteria

Recombinant DNA molecules were transferred into *Escherichia coli* Top 10 F' strains via electroporation method.

2.2.2.1 Preparation of *E.coli* electrocompetent cells and transformation of electrocomponent *E.coli* Top 10F' cells

E.coli Top10F' overnight inoculum was diluted 1:100 fold into 100 ml 2xYT medium containing Tetracyclin (20 μg/ml) and incubated at 37^0C by shaking at 250 rpm until OD_{600} reached to 0,6. Culture was stand on for 30 minutes and cells were centrifuged at 5000 rpm for 5 minutes. Supernatant was discarded, pellet was resuspended in 40 ml of cold distilled water and centrifuged at 5000 rpm for 15 minutes. Supernatant was removed, pellet was resuspended in 20 ml cold distilled water and centrifuged at 5000 rpm for 15 minutes. Then supernatant was discarded and pellet was resuspended in 1 ml of cold sterile 10 % glycerol and dispensed into aliquots of 40 μl into 1.5 ml eppendorf tubes. Aliquots were frozen immediately by immersing within liquid nitrogen and stored at -80^{o}C.

2.2.2.2 Transformation of *E.coli* Top 10F' cells

Recombinant DNA molecules obtained from ligation reactions were transferred into *E.coli* Top10F' by electroporation. Electrocompetent *E.coli* cells were thawed on ice, ligation reaction was mixed with bacteria and mixture was transferred into the electroporation cuvette with 0.1 cm gap. The sample was placed onto the electroporator (Eppendorf Electroporator 2510) and the process was carried out at 1800V. After addition of 1 ml LB medium, the mixture was transferred to a 1.5 ml tube and was incubated for 1 hour at 37°C with 250 rpm shaking. Bacteria were centrifuged at 5000 rpm for 10 minutes, the supernatant was discarded and the pellet was resuspended in 100 μl of 0.85% NaCl. 100 μl of culture was spread out for each Amp/IPTG/X-gal/LB plate or Zeo/LB plate and incubated overnight at 37°C.

2.2.2.3 DNA sequencing

The cloned fragments into cloning and expression vectors were sequenced with sequencing primers listed in Table 2.6 and also some forward and reverse gene specific primers. Sequencing reactions were carried out by the chain termination

method (Sanger et al, 1997) with dye-labeled dideoxy terminators by ABI Prism 3100 Avant automated sequencer.

Table 2.6: Primers used in the vector sequencing.

Primer	Sequence	Vector
M13-reverse	5'-AACAGCTATGACCATG-3'	pDrive
M13 forward (-40)	5'-GTTTTCCCAGTCACGAC-3'	pDrive
5´ *AOX1* sequencing primer	5´-GACTGGTTCCAATTGACAAGC-3´	pPICZB
3´ *AOX1* sequencing primer	5´-GCAAATGGCATTCTGACATCC-3´	pPICZB

All obtained cDNA and gDNA sequences were compared with National Center for Biotechnology Information (NCBI) database using the BLAST search at the web site (Url-4). To obtain the full-length sequences, reverse sequences were complemented, and aligned to forward sequences using ClustalX version 1.81 (Thompson and Jeanmougin, 2000). The alignments were manually verified and edited.

2.2.3 Transformation of methylotrophic yeast *Pichia pastoris*

Following to cloning of laccase cDNA behind the AOX1 promoter, the recombinant pPICZB vector was linearized within the 5'*AOX1* region with suitable restriction endunuclase (*Sac*I for lcc1 and *Pme*I for lcc2) to stimulate the integration by gene insertion at the 5' *AOX1* locus of host's genome. Linearized recombinant plasmids were eluted from agarose gel and chemically competent *Pichia pastoris* strain X-33 cells were transformed with 3 μg vector using the "EasyComp™ Transformation Kit" from Invitrogen according to the protocol.

2.2.3.1 Preparation of chemically competent *Pichia pastoris* cells

10 ml of YPD medium was inoculated with single colony of *Pichia pastoris* X-33 strain and cells were grown overnight at 30°C in a shaking incubator (250–300 rpm). Cells were diluted in 10 ml YPD from the overnight culture to an OD_{600} of 0.2. Cells were grown at 30°C until the OD_{600} is 0.6–1.0 and it took approximately 4-6 hours. Cells were precipitated by centrifugation at 500 x g for 5 minutes at room temperature and the supernatant was discarded. Pellet was resuspend in 10 ml of Solution I (Sorbitol solution containing ethylene glycol and DMSO for the preparation of competent cells). Cells were sedimented by centrifugation at 500 x g for 5 minutes at room temperature without incubation with Solution I, supernatant

discarded and pellet was suspended in 1 ml of Solution I. The cells became competent at this stage and aliquoted 50 µl of competent cells into labeled 1.5 ml sterile microcentrifuge tubes. The tubes were placed a styrofoam box to freeze cells slowly and kept at –80°C freezer.

2.2.3.2 Transformation of *P. pastoris* X-33 strain with laccase cDNA

Solution II and Solution III were equilibrated in room temperature before transformation. One tube of competent cells for each transformation was thawed at room temperature. 3 µg of linearized Pichia expression vector DNA was added to the competent cells and 1 ml of Solution II (PEG solution for the transformation of competent cells) was added to the DNA/cell mixture and mixed by vortexing. Transformation reactions were incubated for 1 hour at 30°C in thermomixer and were mixed every 15 minutes by vortexing to increase transformation efficiency. Mix was placed in 42°C heat block for 10 minutes to apply heat shock and cells were splited into 2 microcentrifuge tubes (approximately 525 µl per tube). 1 ml of YPD medium was added to each tube and cells were incubated at 30°C for 1 hour to allow expression of Zeocin ™ resistance. Cells were sedimented centrifugation at 3,000 x g for 5 minutes at room temperature. Pellets were resuspended in 500 µl of Solution III (Salt solution for washing and plating transformed cells) and cells were combined into one tube. Following to last sedimentation at 3,000 x g for 5 minutes at room temperature, pellet were dissolved in 100-150 µl of Solution III. Plate the entire transformation on yeast extract peptone dextrose agar (YPDS) containing 100 $\mu g.ml^{-1}$ Zeocin using a sterile spreader and plates were incubated at 30˚C for 4 days.

Subsequently, all colonies were plated onto minimal methanol plates supplemented with 0.2 mM of substrate ABTS to detect laccase-producing transformants, and plates were incubated for 3 days at 30˚C.

2.2.4 Heterologous expression of the full-length laccase cDNAs in yeast *Pichia pastoris*

For heterologous expression of laccase in *Pichia pastoris*, BMGY/BMMY (buffered complex glycerol or methanol medium), and BMG/BMM (buffered minimal glycerol or minimal methanol medium) (Recipes are given in Appendix A1). Buffered media with phosphate buffer, are usually used for secreted protein expression if pH-controlled growth conditions is necessary for the activity of the protein. Secreted

proteins were stabilized using complex media, BMGY and BMMY containing yeast extract and peptone. Proteolysis of secreted proteins were also decreased or prevented by cultivation in these media. To obtain efficient expression, cultures were always 20% of total flask volume and incubation temperature did not exceed 30°C.

In order to select best laccase producer clone, positive transformants were screened for laccase production by cultivating them in 5 ml of minimal methanol medium supplemented with 1 % methanol at 28˚C on rotary shaker (250 rpm) for 3 days. 1 % methanol added daily because of the loss by evaporation. 500 µl of culture sample was collected every 24 hours and cells were removed by centrifugation at 10000 x g for 15 minutes.

2.2.4.1 Laccase assay

Laccase activity of the supernatant was determined by measuring the oxidation of 2.5 mM ABTS at 414 nm for 3 minutes in the presence of 100 mM sodium citrate buffer, pH 3.0 at 25˚C. The non-phenolic substrate 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized to a cation radical and depending on the enzyme concentration, blue-green color is formed by cation radical. Laccase activity is measured by reading green color between 414 nm and 420 nm. The reaction mixture contained 8.5 µl of culture supernatant and one unit of laccase activity was defined as 1.0 µmol of oxidized ABTS per minute under the assay conditions. Laccase activities were monitored on a Benchmark Plus Microplate reader (Bio-Rad), calculated using equation displayed below and explained in unit per liter .

$$U/l = [\ (\Delta A/t) / \varepsilon.d\ ] \ .\ (1 \times 10^6\ \mu mol/mol)\ .\ (V/v) \qquad \textbf{(3.1)}$$

where ΔA = Absorbance change at 414 nm; ε = Extinction coefficient of substrate; d = Light path (cm); V = total reaction volume (ml); v = sample volume (ml)

2.2.4.2 Protein determination with Bradford assay

Protein concentration was determined using Bradford Protein Assay with bovine serum albumin (BSA) as standard (Bradford, 1976).

2.2.4.3 Optimization of expression conditions

Expression conditions were optimised to achieve high level protein production. Therefore, effect of cultivation in different media, growth temperature and also copper concentrations on the expression were tested in this study.

Cells were inoculated in 25 ml of BMGY (buffered glycerol complex medium) or BMG in a 250 ml flask and grown at 28˚C in a shaking incubator (250 rpm) until culture has reached OD_{600} = 6.0. After harvesting cells by centrifugation, pellets were resuspended to an OD_{600} = 1.0 in buffered complex methanol medium (BMMY) or buffered minimal methanol medium (BMM) for expression. The effect of nutrients in different medium compositions, either BMMY or BMM, different methanol concentrations (0.5 %, 0.75 % and 1 %) and also cultivation temperature (23ºC and 28ºC) on the laccase expression were tested for 7 days. The effect of $CuSO_4$ as an inducer was also examined by adding different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mM) of $CuSO_4$ to the growth medium.

2.2.5 Purification of the recombinant laccases

Recombinant laccase purification was carried out with ammonium sulphate precipitation, anion exchange chromatography and also size exclusion chromatography approaches. 200 ml of *Pichia pastoris* culture in 1 l shake flask was sedimented by centrifugation at 10000 xg for 15 minutes and 1 mM phenylmethylsulphonyl fluoride (PMSF) was added to the culture supernatant to inhibit serine proteases.

2.2.5.1 Ammonium sulphate precipitation

Optimisation of the ammonium sulphate concentration to precipitate proteins were carried out by adding ammonium sulphate into the culture supernatant, between 40 % to 90 % concentrations with 10 % increment at 4˚C. Salt was added in small portions to the stirred solution and allowed to completely dissolve before adding the next portion. The mixture was pelleted by centrifugation at 10000 xg for 40 min after standing on ice for 30 min and tested for laccase activity.

Ammonium sulphate precipitate was resuspended in 20 mM sodium phosphate buffer, pH 7.2 and salts were removed by ultrafiltration using centrifugal devices Amicon Ultra-15 centrifugal filter units (Millipore).

2.2.5.2 Anion exchange chromatography

Sample was first applied onto the Q-sepharose resin (GE Healthcare Life Sciences) equilibrated with 20 mM sodium phosphate buffer, pH 7.2 and eluted proteins at a flow rate of 2 ml min^{-1} were detected by the routine laccase activity assay. Pooled active fractions were pooled and concentrated by ultrafiltration.

2.2.5.3 Size exclusion chromatography

Sample was loaded onto the Sephadex G-100 column (GE Healthcare Life Sciences) pre-equilibrated with the 20 mM sodium phosphate buffer, pH 7.2 with 0.05 M NaCl, to seperate proteins depending on their sizes and fractions were obtained with the flow rate of 0.3 ml min^{-1}. Active fractions were pooled and concentrated with Millipore centrifugal devices and stored at 4˚C.

2.2.6 Biochemical characterization of the recombinant laccases

Biochemical characteristics of the purified recombinant laccases were examined by determining the molecular weight, effect of pH, temperature, different substrates and potential inhibitor compounds on the laccase activity. Catalytic properties of the enzymes were also determined..

2.2.6.1 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a 12 % resolving gel according to the protocol of Laemmli (1970) to determine the relative molecular mass of the recombinant laccases and gels were stained with Coomassie Brilliant Blue R-250 after electrophoresis. Molecular weight of the proteins were determined by comparing their profiles with unstained protein molecular weight marker (Fermentas).

2.2.6.2 Activity staining

To determine the laccase activity on the SDS-PAGE, samples were loaded without boiling and after electrophoresis gels were soaked with 100 mM sodium citrate buffer, pH 3.0 for 30 min and incubated in 2.5 mM ABTS, prepared in the assay buffer, at room temperature until green colour has been observed.

2.2.6.3 Effect of temperature on the laccase activity

The effect of temperature on laccase activity was analyzed at different temperatures using 2.5 mM ABTS as substrate. Prior to laccase activity measurement, enzymes were incubated at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C for 2 minutes. Thermal stability of enzyme was also assayed by incubating the enzyme at those temperatures for 7 hours (R-LCC1) and 10 hours (R-LCC2) and measuring the retaining activity at 25°C.

2.2.6.4 Effect of pH on the laccase activity

The optimum pH of the purified recombinant laccases was determined with ABTS (2.5 mM) and DMP (2.5 mM) as the substrates 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).

2.2.6.5 Effect of different substrates and inhibitor compounds on laccase activity

Oxidation of different substrates by recombinant laccases was examined by analyzing laccase activity for 5 mM ABTS, 5 mM DMP, 5 mM guaiacol, 5 mM ferulic acid, 5 mM catechol and 3 mM tyrosine at the specific wavelength of each substrate.

The effect of various potential inhibitors on the recombinant laccase activity was tested using 0,1 mM sodium azide, 1 mM sodium fluoride, 1 mM L-cysteine, 1 mM SDS and 1 mM EDTA after incubation of enzyme at 30°C for 5 min. Laccase activity was measured using ABTS (2.5 mM) as substrate in 100 mM sodium citrate buffer, pH 3.0. Inhibition percentage was calculated by comparing retaining activity with control sample.

2.2.6.6 Catalytic properties of the enzyme

Enzyme activity was measured with different concentrations of ABTS (0.1 – 5 mM) and DMP (0.05 - 10 mM) as substrate. Michaelis-Menten constant Km and Vmax values were calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (Url-5). Catalytic efficiency was explained by turnover number (kcat), calculated by dividing Vmax (moles of substrate sec^{-1} mole

of enzyme site^{-1}) to total enzyme concentration Et (molar enzyme site concentration) used in the assay. Specifity constant was also indicated by kcat/Km value.

2.2.7 Functionality of the recombinant laccase

Subsequently to the characterization of the purified laccase, functionality of the recombinant laccases were demonstrated by biotransformation of 3-hydroxyanthranilic acid into the antibiotic cinnabarin. Textile dye biosynthesis studies were also carried out from different dye precursors and 2 different dyes were produced. Obtained cinnabarinic acid and putative textile dyes were identified with UV visible spectrum analysis characterized with thin layer chromatography approaches.

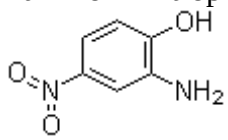
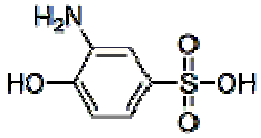
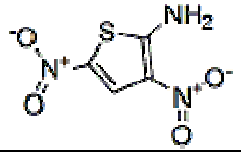
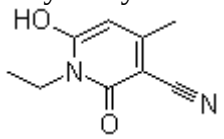
2.2.7.1 Cinnabarinic acid synthesis by oxidation of hydroxyanthranilic acid via laccase

For 3-HAA oxidation studies, 20 µg of recombinant laccase, purified from *P. pastoris* was added to a solution of 1 mM 3-HAA in 1 ml of 50 mM sodium tartrate buffer (pH 4.0) and incubated at 30°C. Oxidation of 3-HAA was monitored spectrophotometrically between 250 and 800 nm with Perkin Elmer .. spectrophotometer at 10, 30, 60 minutes and 6 hours of incubation.

2.2.7.2 Textile dye biosynthesis

Textile dyes were synthesized from different dye precursors (25 mM) by 10 U/ml recombinant laccase in 100 mM tartrate buffer, pH 4.5 with 200 µl of rwaction volume.. Dye precursors are listed in Table 2.7. Parallel reactions using recombinant LCC1 and R-LCC2 from yeast *Pichia pastoris*, commercially available laccase from *Trametes versicolor* and without laccase (blank) were set up in 1.5 ml tubes for 12 hours at 30ºC.

Table 2.7: Different dye precursors used in the dye synthesis reaction.

Dye	Precursor 1	Precursor 2
ITU-11	**S2,** 2-amino 4- nitrophenol 	**S3,** 2-Aminophenol-4-sulfonic acid 
ITU-G	**P6,** 3,5-Dinitro-2-aminothiophene 	**P8,** 3 cyano 4 methyl 6 hydroxy N ethyl 2-Pyridone 

2.2.7.3 Thin layer chromatography

Analysis of 3-HAA and CA by High Performance Thin Layer Chromatography (HPTLC) was performed on silica gel plates (GF grade, Uniplate, Analtech, Newark, DE) using n-butanol, acetic acid, water (4:2:1, vol/vol) as the solvent system. 10 µl of samples were applied onto the plates with automatic applicator (Linomat IV-CAMAG) as bands. After development, plate was removed and dried in the ventilated fume-hood. Visualisation of the plate was performed under UV light at short wave-length (254 nm), long-wave length (366 nm) and also at white light. Chromatogram was scanned between 200 nm and 800 nm wavelengths with CAMAG TLC scanner III and peaks were evaluated depend on the Rf values. Identification of compounds in reaction mixtures was performed by comparing Rf (retntion factor) values of the samples with pure compounds' values.

HPTLC analysis of textile dye precursors and dyes was performed on aluminum plated silica gel plates and dyes were seperated and identified. 10 µl of samples were spotted on the plates and dried. *n*-Butanol, acetone, water, ammonia (5:5:1:2, vol/vol) was used as solvent system and TLC plates was dried for 20 min in a ventilated fume hood. Same visualisation and detection techiques, applied in the identification of CA, was used for characterization of textile dyes.

3. RESULTS AND DISCUSSION

3.1 Screening of Laccase Genes

Laccase isozymes are secreted based on the different growth conditions by almost all fungi examined so far. Laccase screening by conventional methods is performed based on the activity of the enzyme but determination of some laccases including industrially important ones is not possible by using this technique, because of the difference of the enzyme expression type (constitutive or inducible). To overcome this problem, D'Souza et al. (1996) and Hoshida et al. (2001) developed a PCR-based laccase-sequence screening method using degenerate PCR primers specific to conserved sequences around the two pairs of histidines in the N-terminal copper-binding regions of known basidiomycete laccases and rapidly isolated and characterized laccase-specific sequences. Screening of laccase-specific sequences by using this PCR-based method have been efficiently performed to isolate the laccase genes from different fungal sources (Hoshida et al., 2001; Hong et al., 2007; Kim et al., 2001; Cheong et al.. 2006).

In this study, laccase-gene-specific sequences on the genome of *Pycnoporus sanguineus* MUCL 38531 has been screened with PCR method by using the degenerate primers specific to highly conserved copper-binding regions I and IV of known laccases as an initial step. The precise number of laccase genes within the *Pycnoporus sanguineus* MUCL 38531 genome was also identified by this PCR based gene screening method. Sequence of the degenerate primers used in this study is given in the Table 3.1. As expected, the DNA fraqment approximately 1.6 kb in size was succesfully amplified (Figure 3.1) since the lengths of genomic DNA sequences between copper-binding regions I and IV of known fungal laccases ranged from 1.5 and 1.7 kb (Hoshida et al. 2001; Temp et al. 1999).

Table 3.1: Sequence of the degenerate primers.

Primer	Sequence
LAC-N1 (fwd)	5'-CAYTGGCAYGGNTTYTTYCA-3'
LAC-C1 (rev)	5'-TGRAARTCDATRTGRCARTG-3'

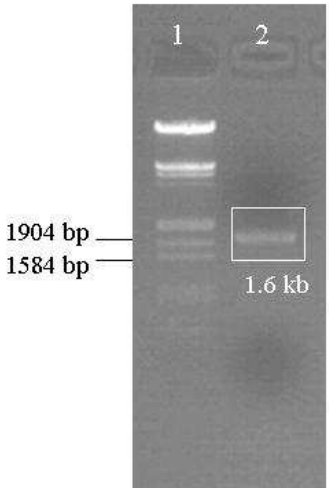


Figure 3.1 : PCR product obtained by using degenerate forward and reverse primers; lane 1. Lambda DNA/EcoRI+HindIII DNA fragments (Marker 10), lane 2. 1.6 kb amplified fragment.

The resultant 1.6 kb of PCR product was eluted from agarose gel and cloned into the pDrive cloning vector. Recombinant vector was used to transform *E.coli* Top10F' electrocompetent cells. As initial screening, plasmid DNAs of 20 *E.coli* Amp^R transformants were isolated (Figure 3.2) and treated with *Eco*RI to verify the cloning of PCR products. Some of the plasmids confirmed with restriction enzyme digestions are given in the Figure 3.3.

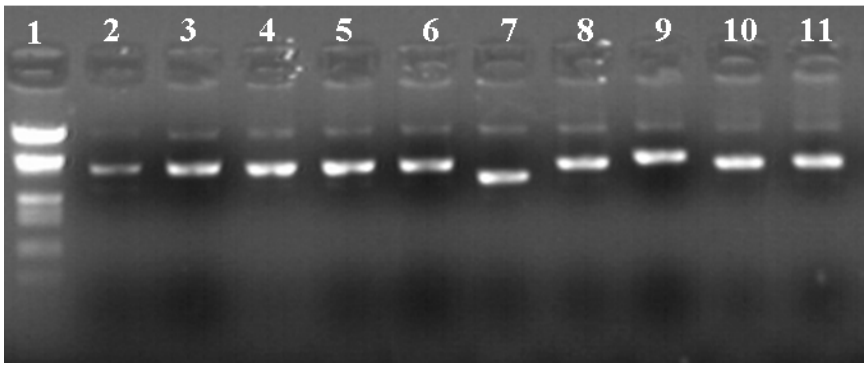


Figure 3.2 : Plasmids DNAs isolated from E.coli Top10 AmpR transformants (lane 2 to lane 11) Lambda DNA/EcoRI+HindIII Marker DNA fragments (lane 1).

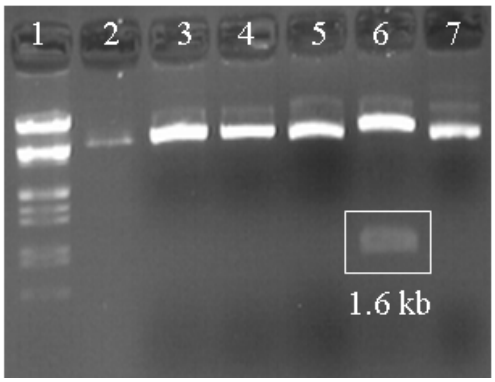


Figure 3.3 : EcoRI digestion of selected plasmid DNAs (lane 2 to lane 11), Lambda DNA/EcoRI+HindIII Marker DNA fragments (lane 1).

Subsequently, nucleotide sequences of 26 selected clones were obtained and compared with previously reported laccase sequences in National Center for Biotechnology Information (NCBI) database by using BLAST search. Two different laccase sequences displaying 62 % similarity to each other were determined on the genome of *Pycnoporus sanguinues* MUCL 38531 and were designated as *lcc1* and *lcc2* (Figure 3.4). Alignment of these two sequences is shown in Figure 3.5.

>lcc1 gDNA partial sequence

5'CACTGGCACGGACTTTTCCAGGAACACACTAACTGGGCTGACGGTCCCGCTTTCGTCAATCAATG
TCCCATTGCTTCTGGACACTCCTTCCTCTACGACTTCCATGTGCCCGATCAAGCCGGTACGTCTCCG
CAGAGCGCACGATGTCTCGGAAACTCAATCTTTCAATCTGGCGTCTAGGCACATACTGGTACCACA
GCCATCTTTCCACGCAGTACTGCGACGGATTGAGAGGGCCGCTTGTCGTGTACGACCCCCACGATC
CTCAGGCGCATCTGTATGATGTTGACAACGATGAGTTCTTTGAATGGCCTGGCGTGCGACACATTT
CTGACGTGCGTGATAGATGACACTGTCATCACTTTGGCGGATTGGTATCATGTCGCGGCCAAGCTA
GGCCCGCAATTCCCGTGCGTCTTCCTCTTCTAATTGTTACGGAGAAGTTCATTCATGTAATGTTGCA
GGAGGGGCGCAAACTCTACGCTCATCAACGGCCTTGGACGAGCGGCGACTGATAGCACTTCCGAT
CTCAGTGTCATTACCGTTGAGCATGGGAAGCGGTAAGCACAGGCAAATTTCGCGTCTTGGAAAGCC
CGCTCATCTTTCGTCATCTTCAGCTATCGTTTCAGGCTTGTATCCATCTCTTGTGATCCGAACCACAC
CTTCAGCATCGATGGCCACAACATGACCATCATCGAAGTCGATGGCGTCAACAGCAAGCCCCTCAC
CGTCGACTCCATCCAGATTTTCGCAGCCCAGCGCTACTCCTTCGTGGTAAGTGAGCTTCACCACGCT
CATGTCGACATAGAACTTATCCGTATCTCCATATAGTTGAATGCTAACCAACCGGTGGACAACTAC
TGGATTCGTGCGAATCCGAGTGGCGGAACCGTGGGTTTCGAGGGCGGCATCAACTCTGCCATTCTC
CGATACAAGGGTGCGCCGGATGCCGAGCCCACGAACACGACCGCGCCGACATCTGTCATTCCTCTG
GTGGAGACGAATTTGCACCCCCTCAAGCCGATGCAAGTGGTACGTGTCATGTGTAGTATTGTCCCT
GCAACGTCCGCTGACCGTACACGAACAGCCCGGCCGGTCTGGTGTCGGTAACGTTGATTATGCGAA
GACACTCAATTTCAACTTCGTGAGTAGCTTGACTCAAGTCATAGACAAATGAACTGAGTGCTGAGA
TATCTTTTATACAATCACCAGAACGGCACCAACTTTTTCATCAACAATGCGACGTTCACCCCGCCCA
CAGTCCCCGTCCTCCTCCAGATCCTGAGCGGAGCGCACAACGCGCAGGACCTCCTCCCCGCCGGGT
CTGTTTACACTCTTCCGCCGCACAGCGCCATCGAGATTACCATGCCGGCTACTACCCTAGCCCCGG
GATCTCCCCACCCCTTCCACTTGCACGGGGTACGCATAAATGCCTCACACTAACTGTCAATCACAC
CTGCTCACTCATCGCCTTCCTGTTCGACAGCACGTCTTCGCTGTCGTACGCAGCGCCGGCAGCACCG
AGTACAACTACCACGACCCCATCTTCCGCGACGTCGTGAGCACCGGCCAGCCCGGCGACAGCGTC
ACGATCCGGTTCATGACGGACAACCCGGGTCCGTGGTTCCTCCATTGCCACATCGACTTCCA-3'

>lcc2 gDNA partial sequence

5'CACTGGCACGGACTATTCCAGAGGTCAACGAACTGGGCGGATGGCCCTGCCTTCGTGAATCAAT
GTCCCATTGCGACTGGGCATTCATTCCTTTATGACTTCCAAGTCCCAGACCAGGCCGGTACGCGCA
TCTCCGAAGCCCGCTAGGATTCTCGCACTAAGTTTGGACTCTGCAGGTACCTTCTGGTATCACAGTC
ATCTGTCCACGCAGTACTGCGATGGGCTGAGGGGCCCATTGGTTGTGTACGATCCCAATGACCCTC
ACGCCAGCCTATACGACGTTGACAACGGTGGGTATCGTCAGTAACTCGCCAGCGACGACATGCTG
ATTGCGTGGCGGTAGATGACACGGTGATCACACTTGCCGACTGGTACCACCTAGCCGCCAAGGTCG
GCCCCAAGTTCCCGTAAGTCTTATCCCAACAGAAAGTTCACGTCGTCGTGGTCATCTCATTTCGTCT
TGATACAGTACACGCTCCGATTCGACGCTGATCAATGGCCGCGGCCGCACGGCTGCAACTATCGCG
GCGGAATTGACGGTCATCAATGTCACTCCGGGAAAGCGGTAGGTTCTACTCGAATTCATGTTCGTA
CAGGCCTCTGATAGACTTCGCGCTGCAGGTATCGTTTCCGTCTTGTGTCAATCTCTTGCGATCCTGC
CTACACTTTCAGCATCGATGGACATGACATGACCGTGATCGAGGCGGATTCAGTCAACACCCAGCC
ACTCGAAGTAGATTCCATTCCCATCTATACCGGGCAACGGTACTCCTTTGTGGTTGAGGCGAACCA
GCCAGTCGACAACTACTGGATTCGCGCAAACCCGATGGCAGGCACGACCGGTTTCGAGGGCGGAA
TCAACTCAGCTATTCTGAGGTACGACGGCGCGCCAGAGCAAGAGCCAACGACGGCCCCGGGCACG
TCCACCAAGCCGTTGAAGGAGACCGATCTCCATCCCCTGGTATCTATGCCTGTGGTAAGCACGCTT
TGATACCAGCATATAGTCAACCACTTATAGTATTATCACACAGCCAGGATCTCCTGTCGCAGGAGG
AGTTGACAAGGCCATCAACTTGGCCTTCCAGTTTGTAAGCGGCATTCTTCGCACAGCTAATGAGCC
AATTGTTGACGGCTAAATCACACCTTTTCTCAGGATGGCACGAACTTCTTCATCAACGGTGCTACCT
TCAAGCCCCCCACTACGCCTGTTCTCCTGCAGATCTTGAGCGGCGCTCAAGCCGCTTCTGACCTCCT
ACCGTCTGGCGATGTCCACGTTTTGCCGTCCAACGCCACGATCGAGCTCTCGTTCCCCGCAACCATC
CAAGCTGGGGCCCCCCACCCCTTCCACTTGCATGGGGTAAGTCTTTGGGTGGTGGAAATCAAGCGT
AGCTTCTCACGTTTCTATTCTTCAGCATACTTTCGCTGTTGTACGCAGCGCAGGCAGTACGGAATAC
AACTACGAGAACCCGATATTCAGAGACGTGGTCAGCACCGGAGTACCTCAGGACAACGACAACGT
GACTATTCGATTCCGGGTGAGTCACCTGTCCGCCCTCGCTTCAAAGATGGCAGATGCTAATCATCG
GCAAGACTGACAACCCCGGCCCGTGGTTCTTGCATTGCCATATCGACTTCCA-3'

Figure 3.4 : Conserved region sequences of *lcc1* and *lcc2* gDNAs from *Pycnoporus sanguineus.*

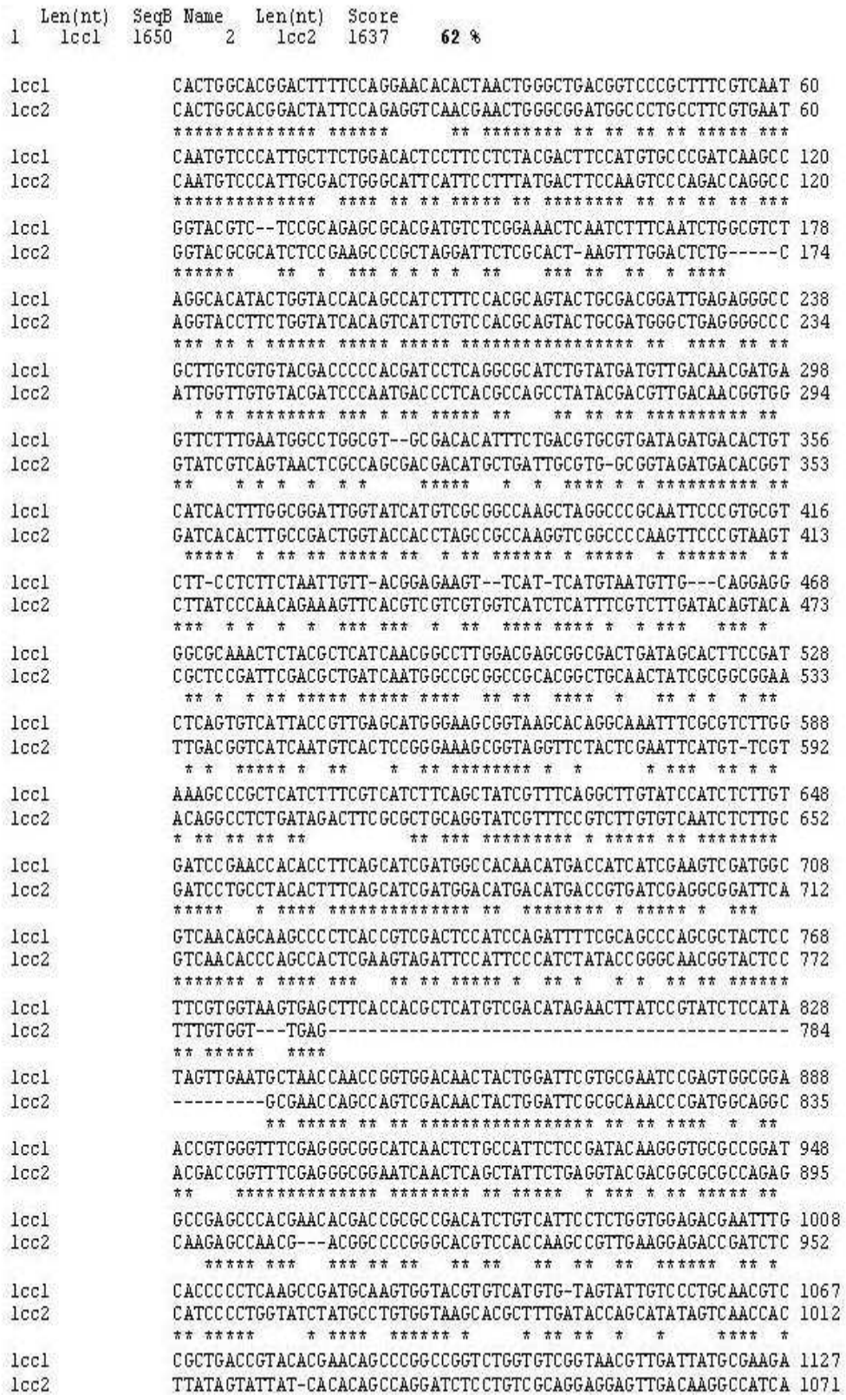


Figure 3.5 : Alignment of the partial *lcc1* and *lcc2* gDNA sequences

```
lcc1        GAGATATCTTTTATACAATCAC-CAGAACGGCACCAACTTTTTCATCAACAATGCGACGT 1246
lcc2        GACGGCTAAATCACACCTTTTCTCAGGATGGCACGAACTTCTTCATCAACGGTGCTACCT 1187
            **    *   * * **  *  * *** * ***** ***** *********  *** ** *
lcc1        TCACCCCGCCCACAGTCCCCGTCCTCCTCCAGATCCTGAGCGGAGCGCACAACGCGCAGG 1306
lcc2        TCAAGCCCCCCACTACGCCTGTTCTCCTGCAGATCTTGAGCGGCGCTCAAGCCGCTTCTG 1247
            ***  ** *****    ** ** ***** ****** ******* ** **   ***    *
lcc1        ACCTCCTCCCCGCCGGGTCTGTTTACACTCTTCCGCCGCACAGCGCCATCGAGATTACCA 1366
lcc2        ACCTCCTACCGTCTGGCGATGTCCACGTTTTGCCGTCCAACGCCACGATCGAGCTCTCGT 1307
            ******* **  * **   ***  **  * * *** *  **  * * ****** *  *
lcc1        TGCCGGCTACTACCCTAGCCCCGGGATCTCCCCACCCCTTCCACTTGCACGGGGTACGCA 1426
lcc2        TCCCCGCAACCATCCAAGCT---GGGGCCCCCCACCCCTTCCACTTGCATGGGGTAAGTC 1364
            * ** ** ** * ** ***    **  * ******************** ****** *
lcc1        TAAATGCCTCACACTAACTGTCAATCACACCTGCTCACTCATCGCCTTCCTGTTCGACAG 1486
lcc2        TTTGGGT---------GGTGGAAATCAAGCGTA---GCTTCTCACGTTTCTATTCTTCAG 1412
            *    *           **  *****  * *     **  ** * ** ** ***  ***
lcc1        CACGTCTTCGCTGTCGTACGCAGCGCCGGCAGCACCGAGTACAACTACCACGACCCCATC 1546
lcc2        CATACTTTCGCTGTTGTACGCAGCGCAGGCAGTACGGAATACAACTACGAGAACCCGATA 1472
            **    ******** *********** ***** ** ** ********* *  **** **
lcc1        TTCCGCGACGTCGTGAGCACCGG------CCAGCCCGGCGACAGCGTCACGATCCGGTTC 1600
lcc2        TTCAGAGACGTGGTCAGCACCGGAGTACCTCAGGACAACGACAACGTGACTATTCGATTC 1532
            *** * ***** ** ********      ***  *  ***** *** ** ** ** ***
lcc1        ATGACGGACAACCCGG--GTCCGTGGTTCCTCCATTGCC-ACATCGACTTCCA------- 1650
lcc2        CGGGTGAGTCACCTGTCCGCCCTCGCTTCAAAGATGGCAGATGCTAATCATCGGCAAGAC 1592
              *  *    *** *   * **  * ***    ** **  *    *    *
lcc1        ------------------------------------------
lcc2        TGACAACCCCGGCCCGTGGTTCTTGCATTGCCATATCGACTTCCA 1637
```

Figure 3.5 (continued): Alignment of the partial *lcc1* and *lcc2* gDNA sequences from *P. sanguineus.*

Highly similar laccases was searched with megablast option of the NCBI alignment tool (Zheng Zhang et al. 2000). Although *lcc1* gDNA gave significant similarity with the previously submitted laccases (Table 3.2), *lcc2* gDNA have no significant similarity with megablast tool and "blastn" option was used to find out somewhat similar sequences (Table 3.3). Partial gDNA sequence of *lcc1* exhibited 88 % identity with *lcc3-2* gene of *Pycnoporus cinnabarinus* (Temp et al. 1999) and 87 % with laccase gene from another *Pycnoporus sanguineus* strain (acc.no. AY510604). Sequence similarity search of *lcc2* gDNA with different laccase genes revealed that, *lcc2* shares 68 % similarity to basidiomycete CECT 20197 pox 3 gene (Mansur et al. 1998) , 70 % to lacC gene from *Trametes sp.* AH 28-2 (Xiao et al. 2004). Not only differences of similarity degrees of *lcc1* and *lcc2* gDNAs to published laccase sequences, but also alignment of these two sequences indicated that *Pycnoporus sanguineus* MUCL 38531 genome contains two different laccase sequences.

Table 3.2: Sequences producing significant alignments with partial sequence of *lcc1* gDNA.

Accession number	Description	Score	Query coverage	Maximum identity
AF123571.1	*Pycnoporus cinnabarinus* laccase (lcc3-2) gene, complete cds	1975	100%	88%
AY510604.1	*Pycnoporus sanguineus* laccase gene, complete cds	1914	100%	87%
AY454306.1	*Favolus alveolaris* laccase gene, partial cds	1312	73%	86%
AY331189.1	*Pycnoporus sanguineus* lacasse gene, partial cds	1277	73%	85%
AY333125.1	*Daedalea quercina* laccase gene, partial cds	1254	73%	85%
AY458017.1	*Pycnoporus sanguineus* laccase mRNA, complete cds	1509	59%	98%
FJ513077.1	*Pycnoporus sanguineus* laccase mRNA, complete cds	1520	63%	96%
AJ626679.1	Uncultured basidiomycete partial lac gene for laccase, exons 1-2, clone S17-Seq1	263	11%	90%
FJ425896.1	*Pycnoporus puniceus* strain MUCL 47087 laccase gene, partial cds	196	10%	88%
FJ425895.1	*Pycnoporus puniceus* strain MUCL 47083 laccase gene, partial cds	193	10%	87%
EU714501.1	*Pycnoporus coccineus* strain MUCL 38525 laccase gene, partial cds	193	10%	87%
EU683254.1	*Pycnoporus coccineus* strain MUCL 38523 laccase gene, partial cds	193	10%	87%
AY147188.1	*Pycnoporus cinnabarinus* laccase (Lcc1) gene, complete cds	193	10%	87%
AF025481.1	*Pycnoporus cinnabarinus* laccase (lcc3-1) gene, complete cds	193	10%	87%
EU684160.1	*Pycnoporus cinnabarinus* strain MUCL 38420 laccase gene, partial cds	187	10%	86%
EU678772.1	*Pycnoporus sanguineus* strain CIRM-BRFM 901 laccase gene, partial cds	176	10%	85%
AF185275.2	*Ganoderma lucidum* strain 7071-9 laccase gene, complete cds	174	26%	75%
FJ473385.2	*Ganoderma lucidum* strain TR6 laccase gene, complete cds	169	26%	74%
EU714499.1	*Pycnoporus sanguineus* strain IMB W3008 laccase gene, partial cds	245	24%	84%
EU683257.1	*Pycnoporus sanguineus* strain IMB G66 laccase gene, partial cds	245	24%	84%
EU678786.1	*Pycnoporus sanguineus* strain CIRM-BRFM 542 laccase gene, partial cds	268	25%	84%
D13372.1	*Coriolus versicolor* CVL3 gene for laccase, complete cds	156	10%	83%

Table 3.3: Similarity of laccase sequences in the NCBI database with partial *lcc2* gDNA sequence.

Accession number	Laccase	Score	Query coverage	Maximum identity
U65401.1	Basidiomycete CECT 20197 phenoloxidase (pox3) gene, complete cds	563	100%	68%
AY839937.1	*Trametes sp.* AH28-2 laccase C (lacC) gene, partial cds	610	89%	70%
M60561.1	*C.hirsutus* ligninolytic phenoloxidase gene, complete cds	275	93%	64%
EU678775.1	*Pycnoporus sanguineus* strain BRFM 118 laccase gene, partial cds	469	94%	87%
EU684155.1	*Pycnoporus sanguineus* strain CIRM-BRFM 881 laccase gene, partial cds	461	94%	87%
EU678772.1	*Pycnoporus sanguineus* strain CIRM-BRFM 901 laccase gene, partial cds	469	94%	89%
M60560.1	*C.hirsutus* ligninolytic phenoloxidase gene, complete cds	257	93%	64%
AY839936.1	*Trametes sp.* AH28-2 laccase A (lacA) gene, complete cds	444	93%	68%
AF388910.1	White-rot fungus AH28-2 laccase gene, partial sequence	444	93%	68%
EU678773.1	*Pycnoporus sanguineus* strain CIRM-BRFM 902 laccase gene, partial cds	449	96%	85%
EU678779.1	*Pycnoporus sanguineus* strain CIRM-BRFM 893 laccase gene, partial cds	443	96%	85%
EU678776.1	*Pycnoporus sanguineus* strain CIRM-BRFM 906 laccase gene, partial cds	443	96%	85%
EU678768.1	*Pycnoporus sanguineus* strain CIRM-BRFM 896 laccase gene, partial cds	442	94%	85%
EU684154.1	*Pycnoporus cinnabarinus* strain BRFM 231 laccase gene, partial cds	436	70%	81%
EU684156.1	*Pycnoporus cinnabarinus* strain BRFM 44 laccase gene, partial cds	445	67%	85%
EU714500.1	*Pycnoporus cinnabarinus* strain CIRM-BRFM 945 laccase gene, partial cds	445	70%	83%
EU683258.1	*Pycnoporus cinnabarinus* strain MUCL 30555 laccase gene, partial cds	445	67%	85%
EU678780.1	*Pycnoporus sanguineus* strain CIRM-BRFM 897 laccase gene, partial cds	415	96%	83%
EU678767.1	*Pycnoporus sanguineus* strain CIRM-BRFM 895 laccase gene, partial cds	409	96%	83%
EU684159.1	*Pycnoporus cinnabarinus* strain CIRM-BRFM 237 laccase gene, partial cds	434	70%	81%
EU684157.1	*Pycnoporus cinnabarinus* strain BRFM 247 laccase gene, partial cds	434	70%	81%
EU678782.1	*Pycnoporus sanguineus* strain CIRM-BRFM 905 laccase gene, partial cds	396	94%	83%
EU678781.1	*Pycnoporus sanguineus* strain BRFM 898 laccase gene, partial cds	406	94%	81%

3.2 cDNA Cloning of the Laccase Gene from *Pycnoporus sanguineus* MUCL 38531

Laccase-specific sequences were screened at the cDNA level by PCR using degenerate primers, designed based on the conserved copper binding regions I and IV of known fungal laccases, and total RNA (Figure 3.6) as template. 1.2 kb of cDNA was amplified (Figure 3.7), purified and ligated into pDrive TA cloning vector (Qiagen). As initial screening, plasmid DNAs of *E.coli* Amp^R transformants were isolated and based on the plasmid profiles, 27 of them were selected and treated with *Eco*RI restriction enzyme to verify the cloning of PCR product (Figure 3.8, Figure 3.9). Subsequently, nucleotide sequences of clones were obtained and compared with previously reported laccase sequences in National Center for Biotechnology Information (NCBI) database by using BLAST search to determine the homology of these sequences to all known laccase genes. Two different partial laccase cDNA sequences from *Pycnoporus sanguineus*, exhibiting 70 % identity to each other were firstly identified by this study (Figure 3.10, Figure 3.11). Determined cDNA sequences gave high homology to all known laccase open reading frames and designated as *lcc1* and *lcc2* in *Pycnoporus sanguineus* MUCL 38531. *lcc1* cDNA was detected dominantly, pointing out that the *lcc1* gene has the highest transcriptional activity in the constructed cDNA library. The partial *lcc1* cDNA reveals 91 % similarity with *Pycnoporus sanguineus* laccase mRNA (Dantan-Gonzalez et al., 2008), 75 % with lac 2 mRNA from *Trametes versicolor* (Fujihiro et al., 2009) and 73 % with *Panus tigrinus* partial mRNA sequence (Quaratino et al., 2008). Maximum identity rates of the partial *lcc1* cDNA with previously submitted laccases are given in Table 3.4.

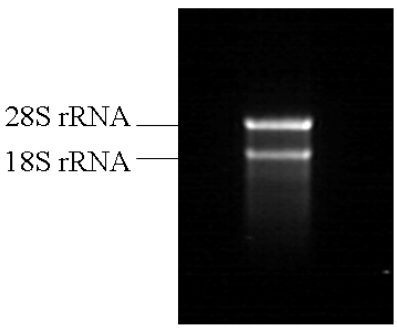


Figure 3.6 : Total RNA from *Pycnoporus sanguineus* grown in maltose medium for 4 days.

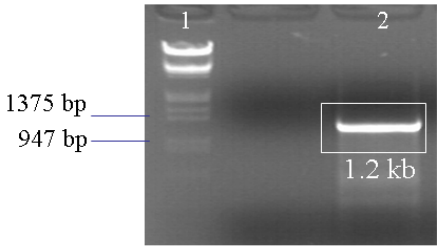


Figure 3.7 : Agarose gel analysis of cDNA fragment amplified with LAC-N1 and LAC-C1 primers. Lambda DNA/EcoRI+HindIII DNA fragments as Marker and 1.2 kb PCR-amplified products.

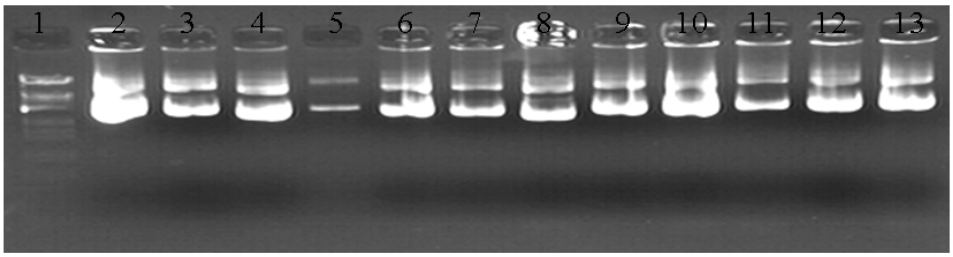


Figure 3.8 : Plasmid DNAs isolated from E.coli Top10 Amp^R transformants containing partial cDNA fragment (1.2 kb) amplified by degenerate primers.

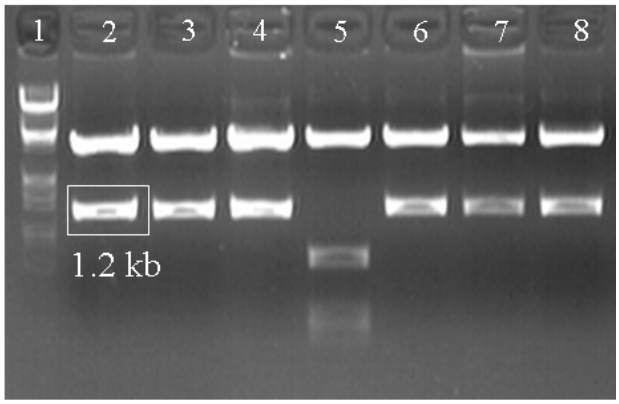


Figure 3.9 : *Eco*RI digestion of selected plasmid DNAs, amplified partial laccase cDNA (1.2 kb) is shown in the box.

Festa *et al.* (2008) reported that, small number of amino acid differences in the active sites of laccases cause changes on the biochemical characteristics of the enzyme, such as pH and temperature optima, by affecting the stability of the enzyme. If similarity between laccase sequences are lower than 97-95 %, those laccase genes are accepted as different. Soden et al. (2002) reported isolation of four different laccase encoding genes, *lac1, lac2, lac3* and *lac4* from and *lac1* has 93% identity with pox1 gene from *Pleurotus ostreatus*, whereas similarity between *lac 4* and *pox2* from *P.ostreatus* was 97%. Consistently, *Coriolus hirsutus po1* gene has 91% similarity to

the *lcc1* from *Trametes villosa* (Eggert et al., 1998). Furthermore, a study on the combined sequence and structural analysis of the more than 100 fungal laccases accepted 95% similarity as a threshold level for identification of the structurally different laccases (Suresh-Kumar et al., 2003). Based on these facts about the different laccases, *lcc1* and *lcc2* from *Pycnoporus sanguineus* MUCL 38531 are certainly different genes with 70% similarity to each other. Besides, *lcc2* cDNA must be considered as a unique gene, because of its maximal identity, which is much more lower than the similarity of *lcc1* to the other laccases. Maximum identity percentages of the *lcc2* partial cDNA with other laccases are given in the Table 3.5 and it gives maximum similarity (75 %) with *pox 3* mRNA from *Trametes sp*. I-62 laccase (Gonzalez et al., 2003), 72 % with *laccase B* precursor mRNA of *Trametes versicolor* (Jolivalt et al., 2005) and 71 % with *lac 1* mRNA from *Trametes versicolor* (O'Callaghan et al., 2002).

> ***lcc1*** **cDNA partial sequence**

5’CACTGGCACGGACTTTTCCAGGAACACACTAACTGGGCTGACGGTCCCGCTTTCGT
CAATCAATGTCCCATTGCTTCTGGACACTCCTTCCTCTACGACTTCCATGTGCCCGAT
CAAGCCGGCACATACTGGTACCACAGCCATCTTTCCACGCAGTACTGCGACGGATTG
AGAGGGCCGCTTGTCGTGTACGACCCCCACGATCCTCAGGCGCATCTGTATGATGTT
GACAACGATGACACTGTCATCACTTTGGCGGATTGGTATCATGTCGCGGCCAAGCTA
GGCCCGCAATTCCCGAGGGGCGCAAACTCTACGCTCATCAACGGCCTTGGACGAGC
GGCGACTGATAGCACTTCCGATCTCAGTGTCATTACCGTTGAGCATGGGAAGCGCTA
TCGTTTCAGGCTTGTATCCATCTCTTGTGATCCGAACCACACCTTCAGCATCGATGGC
CACAACATGACCATCATCGAAGTCGATGGCGTCAACAGCAAGCCCCTCACCGTCGA
CTCCATCCAGATTTTCGCAGCCCAGCGCTACTCCTTCGTGTTGAATGCTAACCAACC
GGTGGACAACTACTGGATTCGTGCGAATCCGAGTGGCGGAACCGTGGGTTTCGAGG
GCGGCATCAACTCTGCCATTCTCCGATACAAGGGTGCGCCGGATGCCGAGCCCACG
AACACGACCGCGCCGACATCTGTCATTCCTCTGGTGGAGACGAATTTGCACCCCCTC
AAGCCGATGCAAGTGCCCGGCCGGTCTGGTGTCGGTAACGTTGATTATGCGAAGAC
ACTCAATTTCAACTTCAACGGCACCAACTTTTTCATCAACAATGCGACGTTCACCCC
GCCCACAGTCCCCGTCCTCCTCCAGATCCTGAGCGGAGCGCACAACGCGCAGGACC
TCCTCCCCGCCGGGTCTGTTTACACTCTTCCGCCGCACAGCGCCATCGAGATTACCA
TGCCGGCTACTACCCTAGCCCCGGGATCTCCCCACCCCTTCCACTTGCACGGGCACG
TCTTCGCTGTCGTACGCAGCGCCGGCAGCACCGAGTACAACTACCACGACCCCATCT
TCCGCGACGTCGTGAGCACCGGCCAGCCCGGCGACAGCGTCACGATCCGGTTCATG
ACGGACAACCCGGGTCCGTGGTTCCTCCATTGCCACATCGACTTCCA-3’

> ***lcc2*** **cDNA partial sequence**

5’CACTGGCACGGACTATTCCAGAGGTCAACGAACTGGGCGGATGGCCCTGCCTTCG
TGAATCAATGTCCCATTGCGACTGGGCATTCATTCCTTTATGACTTCCAAGTCCCAG
ACCAGGCCGGTACCTTCTGGTATCACAGTCATCTGTCCACGCAGTACTGCGATGGGC
TGAGGGGCCCATTGGTTGTGTACGATCCCAATGACCCTCACGCCAGCCTATACGACG
TTGACAACGATGACACGGTGATCACACTTGCCGACTGGTACCACCTAGCCGCCAAG
GTCGGCCCCAAGTTCCCTACACGCTCCGATTCGACGCTGATCAATGGCCGCGGCCGC
ACGGCTGCAACTATCGCGGCGGAATTGACGGTCATCAATGTCACTCCGGGAAAGCG
GTATCGTTTCCGTCTTGTGTCAATCTCTTGCGATCCTGCCTACACTTTCAGCATCGAT
GGACATGACATGACCGTGATCGAGGCGGATTCAGTCAACACCCAGCCACTCGAAGT
AGATTCCATTCCCATCTATACCGGGCAACGGTACTCCTTTGTGGTTGAGGCGAACCA
GCCAGTCGACAACTACTGGATTCGCGCAAACCCGATGGCAGGCACGACCGGTTTCG
AGGGCGGAATCAACTCAGCTATTCTGAGGTACGACGGCGCGCCAGAGCAAGAGCCA
ACGACGGCCCCGGGCACGTCCACCAAGCCGTTGAAGGAGACCGATCTCCATCCCCT
GGTATCTATGCCTGTGCCAGGATCTCCTGTCGCAGGAGGAGTTGACAAGGCCATCAA
CTTGGCCTTCCAGTTTGATGGCACGAACTTCTTCATCAACGGTGCTACCTTCAAGCCC
CCCACTACGCCTGTTCTCCTGCAGATCTTGAGCGGCGCTCAAGCCGCTTCTGACCTC
CTACCGTCTGGCGATGTCCACGTTTTGCCGTCCAACGCCACGATCGAGCTCTCGTTC
CCCGCAACCATCCAAGCTGGGGCCCCCCACCCCTTCCACTTGCATGGGCATACTTTC
GCTGTTGTACGCAGCGCAGGCAGTACGGAATACAACTACGAGAACCCGATATTCAG
AGACGTGGTCAGCACCGGAGTACCTCAGGACAACGACAACGTGACTATTCGATTCC
GGACTGACAACCCCGGCCCGTGGTTCTTGCATTGCCATATCGACTTCCA-3’

Figure 3.10 : Partial sequences of *lcc1* and *lcc2* cDNAs amplified with degenerate primers.

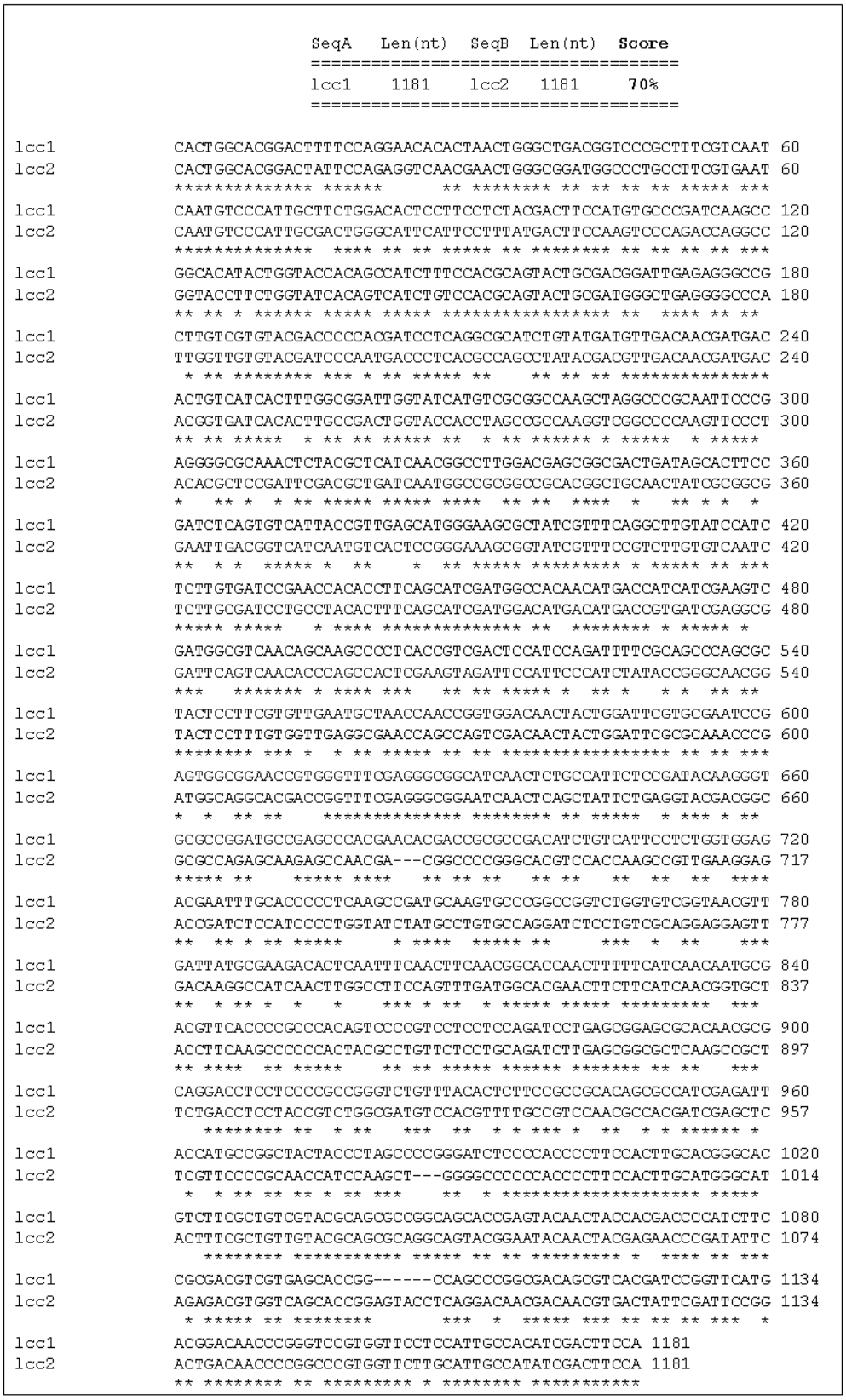


Figure 3.11 : Alignment of the partial sequences of *lcc1* and *lcc2* cDNAs.

Table 3.4: Multiple alignment results of the conserved region sequence of *lcc1* cDNA with previously reported laccases in the NCBI database.

Accession number	Laccase	Score	Maximum identity
AY458017.1	*Pycnoporus sanguineus* laccase mRNA, complete cds	1797	93%
FJ513077.1	*Pycnoporus sanguineus* laccase mRNA, complete cds	1683	91%
U44430.1	*Trametes versicolor* laccase I (lccI) mRNA, complete cds	877	76%
AY049725.1	*Trametes versicolor* laccase 1 (lac1) mRNA, complete cds	874	76%
AY693776.1	*Trametes versicolor* laccase 1 (lcc1) mRNA, complete cds	868	76%
FJ469151.1	*Trametes versicolor* laccase protein mRNA, complete cds	850	76%
AB212732.1	*Trametes versicolor* lac2 mRNA for laccase2, complete cds	836	75%
AF548032.1	*Trametes sp.* I-62 laccase (pox1) mRNA, complete cds	827	75%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	821	75%
AM419158.1	*Panus tigrinus* partial mRNA for laccase (lac1 gene)	706	73%
Y18012.1	*Trametes versicolor* mRNA for laccase	682	73%
AF414109.1	*Trametes versicolor* laccase B precursor (lac1) mRNA, complete cds	661	72%
AF548034.1	*Trametes* sp. I-62 laccase (pox2) mRNA, complete cds	650	72%
AF152170.1	*Pycnoporus cinnabarinus* laccase (lac1) mRNA, complete cds	650	72%
AB072703.1	*Pycnoporus coccineus* lcc1 mRNA for laccase, complete cds	609	71%
FJ688172.1	*Pycnoporus sanguineus* strain H275 multicopper redoxase (lcc1) mRNA, complete cds	607	71%
EF362634.1	*Polyporus brumalis* LAC1 mRNA, complete cds	598	71%
AY081775.2	*Coriolus hirsutus* laccase (072-1) mRNA, complete cds	598	71%
AF176230.1	*Polyporus ciliatus* laccase (lcc3-1) mRNA, complete cds	596	71%
AF491759.1	*Trametes sp.* C30 laccase 1 (lac1) mRNA, complete cds	589	71%
FJ817448.1	*Trametes sp.* C30 laccase 5 (lac5) mRNA, complete cds	569	71%
DQ431716.1	*Coriolopsis gallica* laccase (lac1) mRNA, complete cds	562	71%
FJ598130.1	*Coriolopsis gallica* laccase mRNA, complete cds	553	71%
AY875867.1	*Coriolopsis gallica* laccase (lacA) mRNA, partial cds	553	71%
DQ914876.1	*Ganoderma fornicatum* strain 0814 laccase (lac1) mRNA, complete cds	551	71%
DQ914872.1	*Ganoderma lucidum* strain RZ laccase (lac4) mRNA, complete cds	538	70%
AB212731.1	*Trametes versicolor* lac1 mRNA for laccase1, complete cds	524	70%
FJ656307.1	*Ganoderma lucidum* strain 7071-9 laccase mRNA, complete cds	518	70%
AB212731.1	*Trametes versicolor* lac1 mRNA for laccase1, complete cds	524	70%
FJ656307.1	*Ganoderma lucidum* strain 7071-9 laccase mRNA, complete cds	518	70%
AF176232.1	*Polyporus ciliatus* laccase (lcc3-3) mRNA, partial cds	517	70%
AF548035.1	*Trametes sp*. I-62 laccase (pox3) mRNA, complete cds	511	70%
AM422387.1	*Trametes versicolor* mRNA for multicopper oxidase (klc2 gene)	473	69%
DQ914874.1	*Ganoderma tsugae* strain 1109 laccase (lac1) mRNA, complete cds	471	69%
AB212733.1	*Trametes versicolor* lac3 mRNA for laccase3, complete cds	470	69%
AM419159.1	*Panus tigrinus* partial mRNA for laccase (lac2 gene)	466	69%
AB212734.1	*Trametes versicolor* lac4 mRNA for laccase4, complete cds	464	69%
DQ914868.1	*Ganoderma lucidum* strain RZ laccase (lac1) mRNA, partial cds	457	69%
AJ420900.1	*Pycnoporus cinnabarinus* partial mRNA for laccase (lcc3-1 gene)	455	71%
AF491761.1	Basidiomycete C30 laccase 2 (lac2) mRNA, complete cds	455	68%
AF176231.1	*Polyporus ciliatus* laccase (lcc3-2) mRNA, complete cds	453	69%
AY485829.1	*Ganoderma lucidum* laccase mRNA, complete cds	435	68%

Table 3.5: Multiple alignment results of the partial lcc2 cDNA sequence with previously reported laccases.

Accession number	Laccase	Score	Maximum identity
AF548035.1	*Trametes sp.* I-62 laccase (pox3) mRNA, complete cds	803	75%
Y18012.1	*Trametes versicolor* mRNA for laccase	704	73%
AF414109.1	*Trametes versicolor* laccase B precursor (lac1) mRNA, complete cds	666	72%
FJ688172.1	*Pycnoporus sanguineus* strain H275 multicopper redoxase (lcc1) mRNA, complete cds	637	72%
AF548034.1	*Trametes sp*. I-62 laccase (pox2) mRNA, complete cds	618	71%
AB072703.1	*Pycnoporus coccineus* lcc1 mRNA for laccase, complete cds	596	71%
AM419158.1	*Panus tigrinus* partial mRNA for laccase (lac1 gene)	590	71%
AY081775.2	*Coriolus hirsutus* laccase (072-1) mRNA, complete cds	590	71%
AY049725.1	*Trametes versicolor* laccase 1 (lac1) mRNA, complete cds	572	71%
AY693776.1	*Trametes versicolor* laccase 1 (lcc1) mRNA, complete cds	572	71%
AB212731.1	*Trametes versicolor* lac1 mRNA for laccase1, complete cds	569	71%
U44430.1	*Trametes versicolor* laccase I (lccI) mRNA, complete cds	569	71%
EF362634.1	P*olyporus brumalis* LAC1 mRNA, complete cds	563	71%
AB212732.1	*Trametes versicolor* lac2 mRNA for laccase2, complete cds	558	70%
AF152170.1	*Pycnoporus cinnabarinus* laccase (lac1) mRNA, complete cds	558	70%
FJ469151.1	*Trametes versicolor* laccase protein mRNA, complete cds	554	70%
AF176230.1	*Polyporus ciliatus* laccase (lcc3-1) mRNA, complete cds	547	70%
DQ914872.1	*Ganoderma lucidum* strain RZ laccase (lac4) mRNA, complete cds	526	70%
AF548033.1	*Trametes sp.* I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	524	70%
AF548032.1	*Trametes sp.* I-62 laccase (pox1) mRNA, complete cds	524	70%
AY458017.1	*Pycnoporus sanguineus* laccase mRNA, complete cds	511	70%
FJ513077.1	*Pycnoporus sanguineus* laccase mRNA, complete cds	502	69%
AJ420900.1	*Pycnoporus cinnabarinus* partial mRNA for laccase (lcc3-1 gene)	502	72%
FJ656307.1	*Ganoderma lucidum* strain 7071-9 laccase mRNA, complete cds	499	69%
FJ598130.1	*Coriolopsis gallica* laccase mRNA, complete cds	480	69%
DQ431716.1	*Coriolopsis gallica* laccase (lac1) mRNA, complete cds	480	69%
AF491759.1	*Trametes sp.* C30 laccase 1 (lac1) mRNA, complete cds	475	69%
DQ914874.1	*Ganoderma tsugae strain 1109 laccase (lac1) mRNA, complete cds*	473	69%
AB006824.1	*Ganoderma tsunodae mRNA for bilirubin oxidase, complete cds*	470	69%
AY875867.1	*Coriolopsis gallica* laccase (lacA) mRNA, partial cds	468	69%
FJ817448.1	*Trametes sp.* C30 laccase 5 (lac5) mRNA, complete cds	450	68%
AY485829.1	*Ganoderma lucidum* laccase mRNA, complete cds	434	69%
AF176232.1	*Polyporus ciliatus* laccase (lcc3-3) mRNA, partial cds	432	68%
DQ914876.1	*Ganoderma fornicatum* strain 0814 laccase (lac1) mRNA, complete cds	426	68%
AM419159.1	*Panus tigrinus* partial mRNA for laccase (lac2 gene)	417	68%
AF176231.1	*Polyporus ciliatus* laccase (lcc3-2) mRNA, complete cds	414	68%
DQ914868.1	*Ganoderma lucidum* strain RZ laccase (lac1) mRNA, partial cds	407	68%
AM422387.1	*Trametes versicolor* mRNA for multicopper oxidase (klc2 gene)	407	68%
EF362635.1	*Polyporus brumalis* LAC2 mRNA, complete cds	398	67%
AB212733.1	*Trametes versicolor* lac3 mRNA for laccase3, complete cds	385	67%
AY450406.1	*Flammulina velutipes* laccase mRNA, complete cds	352	67%
AB212734.1	*Trametes versicolor* lac4 mRNA for laccase4, complete cds	352	66%
U44431.1	*Trametes versicolor* laccase IV (lccIV) mRNA, complete cds	329	66%

3.2.1 5’-and 3’-Rapid amplification of cDNA ends (RACE)

Following determination of the partial sequence, gene specific primers used in the 5’- and 3’-RACE protocols were designed to obtain both 5’ and 3’ ends of the laccase cDNA (Table 3.6). Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and either the 3´ or the 5´ end of the mRNA. This simple, rapid and efficient cDNA cloning strategy is developed based on the PCR and overcomes difficulties in obtaining full-length cDNA clones of low-abundance mRNAs. Although PCR requires two sequence-specific primers flanking the sequence to be amplified, amplification of unknown sequences causes a limitation for PCR and those limitations can be overcome by using 3’- and 5’-RACE procedures. RACE has been used for amplification and cloning of rare mRNAs , and also applied to existing cDNA libraries (Frohman et al. 1988, Frohman 1993). Additionally, RACE products can be used to prepare probes (Harvey and Darlison, 1991-) and in conjunction with exon-trapping methods amplification of unknown coding sequences can be performed with the RACE procedures (Adams, and Blakesley, 1991). In order to isolate the laccase cDNAs, utilization of RACE technique has been reported in different studies (Hoshida et al., 2001, Kiiskinen and Saloheimo, 2004, Liu et al., 2003). General strategy applied for cloning of laccase genes is summarized in the Figure 3.12.

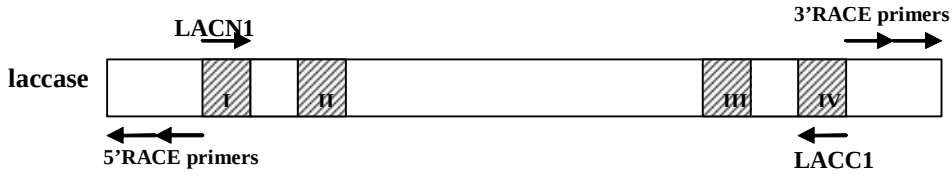


Figure 3.12 : Schematic representation of designing degenerate PCR primers specific to conserved copper binding regions I and IV and primers to be used in 5’ and 3’RACE protocols for laccase amplification.

Table 3.6: Primers used in the RACE experiments.

Primer	Sequence
lcc1-3'RACE-forward	5'-TCCGTGGTTCCTCCATTGCCA -3'
lcc1-3'RACE-nested forward	5'-CCATTGTCACATCGTCTTCCA -3'
lcc1-5'RACE-reverse	5'-ACATTGATTGACGAAAGCGGG -3'
lcc1-5'RACE-nested reverse	5'-GGACATTGATTCACGAAGGCA -3'
lcc2-3'RACE-forward	5'-GAGTACCTCAGGACAACGACAACGTGAC-3'
lcc2-5'RACE-reverse-I	5'-AATGAATGCCCAGTCGCAATG -3'
lcc2-5'RACE-reverse-II	5'- ACGGAGATCGCGAGGGATACCGTC -3'
Lcc1-forward*	5'CCGGAATTCAGCATGGGCTCCGGTCT-3'
Lcc1-reverse*	5'-AACGCGGCCGCCCTAATGGTCGGACT-3'
Lcc2-forward*	5'-CCGGAATTCATGGAGGGATCGAGACCAACT-3'
Lcc2-reverse*	5'-AACGAATTCTCAGACCCCAATAACTA-3'

3.2.1.1 Determination of 5' End of the *lcc1* cDNA

5' RACE system was used to determine the 5'end of the laccase *lcc1* cDNA from *Pycnoporus sanguineus* MUCL 38531. A 600 bp fragment was obtained after PCR reaction using *lcc1*-5'RACE-nested reverse primer and AUAP (Figure 3.13). This fragment was cloned into pDrive TA cloning vector and used to transform electrocompetent *E.coli* Top10F' cells. After plasmid isolation and restriction analysis, fragment was sequenced, compared with previously reported laccase sequences in the databases and significant similarity with other laccases was not observed.

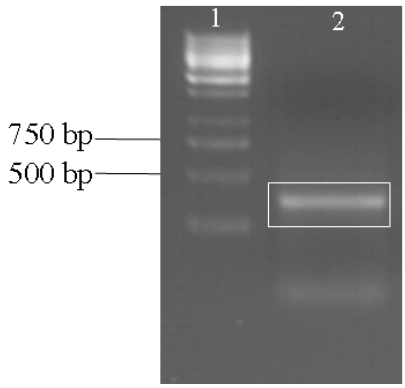


Figure 3.13 : Agarose gel analysis of 5'RACE product using lcc1-5'RACE-nested reverse primer and AUAP. 1. Gene Ruler™ 1kb DNA Ladder as Marker and 2. PCR-amplified product.

To evaluate the specificity of the amplification reaction from the oligo-dC tail, another control reaction omitting TdT was set up in the TdT tailing experiment of cDNA. However, PCR reaction using *lcc1*-5'RACE-reverse and *lcc1*-5'RACE-nested reverse primers and AUAP showed no difference between two PCR products and concluded that a specific 5'-RACE fragment could not be amplified. Although it has not been obtained 5'-RACE product, we decided to use the 5'-end sequence of the another *Pycnoporus sanguineus* laccase (AY458017.1) with % 93 similarity to *lcc1* cDNA of *Pycnoporus sanguineus* MUCL 38531 to amplify full-length *lcc1* cDNA.

3.2.1.2 Determination of 3' end of the *lcc1* cDNA

The 3'-RACE system was used to determine the nucleotide sequence of 3' end of the *Pycnoporus sanguineus MUCL 38531* laccase *lcc1* cDNA. This procedure uses poly(A) tail found in mRNA as a generic priming site for PCR. 3 μg of total RNA was converted into the first strand cDNA by using 10μM oligo (dT)-anchor primer (Adapter primer) and Superscript II reverse transcriptase (Invitrogen 3'-RACE System). "*lcc1*-3'RACE-forward" and "*lcc1*-3'RACE-nested forward" primers, listed in Table 3.4 has annealed to a region of exon sequence and the adapter primer has targeted the poly(A) tail region. By using this way, 3'-mRNA sequences, which lie between the exon and the poly(A) tail, had been captured. The first PCR reaction set up with forward primer and AUAP resulted in the product ca. 500 bp (Figure 3.14-A) and this product was used as a template for the second PCR using nested-forward primer and AUAP. More distint band ca. 500 bp was amplified (Figure 3.14-

B) and cloned into pDrive vector. Isolated plasmids were cut with *Eco*RI (Figure 3.15) and positive clones were sequenced.

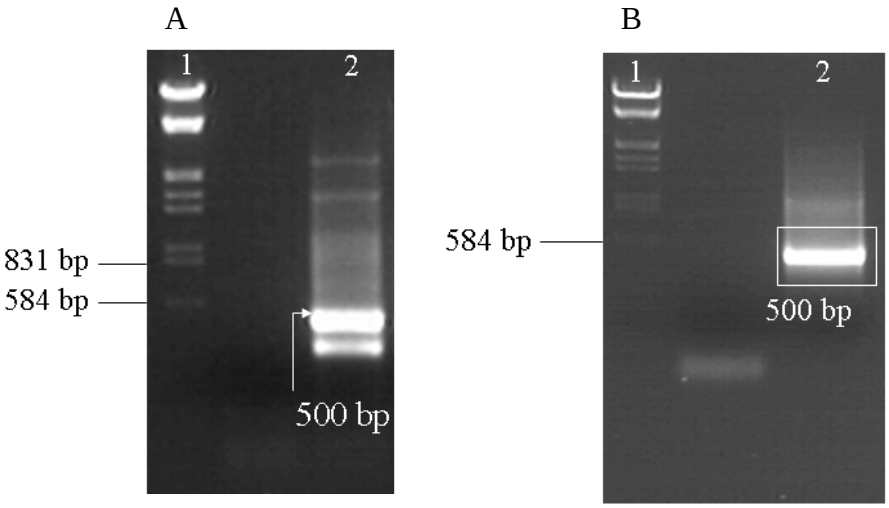


Figure 3.14 : Agarose gel analysis of 3'RACE-products. A. PCR using *Lcc1*-3'RACE- forward primer and AUAP. B. PCR using *Lcc1*-3'RACE-nested forward primer and AUAP. lane 1. Lambda DNA/EcoRI+HindIII DNA fragments as Marker and lane 2. PCR-amplified product.

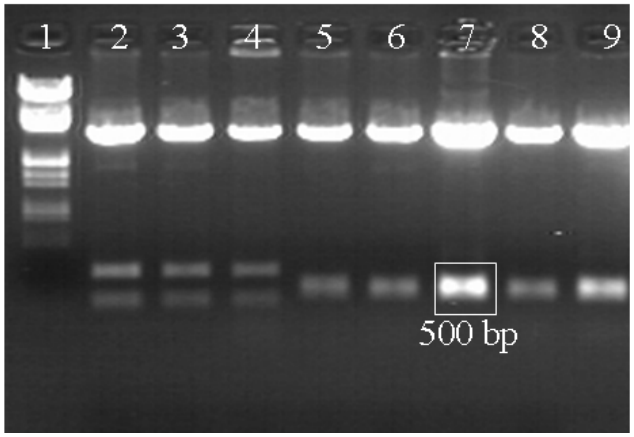


Figure 3.15 : *Eco*RI digestion of selected plasmid DNAs (lane 2 to lane 9), the fragment shown corresponds to the amplified partial laccase cDNA, obtained by 3'-RACE system, Lambda DNA/EcoRI+HindIII Marker DNA fragments (lane 1).

Sequence of 3'-end of *lcc1* cDNA is given in Figure 3.16. In this figure, just a small portion of the conserved copper binding region, previously shown in the Figure 3.12 is displayed. "*lcc1*-3' RACE-forward" primer sequence is underlined and stop codon "TAG" was found at the upstream of the polyadenylation site. Stop codon was defined by translating nucleotide sequences into the deduced amino acids and this

codon was the first codon that has not been translate into the any amino acids. Results of the alignment of the sequence with known laccases in the database is listed in Table 3.7. 3'-end sequence of *lcc1* showed 93 % and 83 % similarity to laccases isolated from different *Pycnoporus sanguineus* strains (Dantan-Gonzalez et al. 2008, Fujihiro et al. 2009). Furthermore, 84 % similarity to *lcc1* mRNA of *Trametes versicolor* (Necochea et al., 2005), 81 % to *Trametes pubescens lap2* gene (Galhaup et al., 2002) and 80 % to LAC1 phenoloxidase from *Basidiomycete C30* has been observed.

5'…..**AGCCCGGCGACAGCGTCACGATCCGGTTCATGACGGACAAC**
CCGGGTCCGTGGTTCCTCCATTGCCACATCGACTTCCATCTCGAG
GCCGGCTTCGCCATCGTGTTTGCCGAGGACGTGAACGATATCAA
GTATGCGAACCCGGTCCCGCCGTCGTGGTCGGAGCTTTGCCCCA
TCTACGACAAGCTCCCGGAGTCCGACCAT*TAG*GTTTGCGCGGGGT
GCGGAGGGGCGGACTCACTTGGGACTATCATTCCTTCGTTGAATTCC
GGATTTCTAGTATCAGAGACATGGAGGCTCTAGGTCTGGTTAATACC
GCGCGGACATTTGGGTAATATTCTCGTAGACCTTGTAGCTCCGAGTA
CTGTATGGGGTTGCTCGCAGTCTACCGGCGCACACTCTTTGGTAGTCC
TGCTTCGGGTCACTATGTATAGTACATTACAGTTTATACCAATGTGAA
TTTGGATTAGAGGTATGATGACTGACAAAAAAAAAAAAAAAAAAAA
GTACTAGTCGACGCGTGGCCAATCTGAATTCGTCGACAAGCTTCTCG
AGCCTAGGCTAGCTCTAGACCACACGTGTGGGGGCCCGAGCTCGCGG
CCGCTGTATTCTATAGTGTCACCTAAATGGCCGCACAATTCACTGGCC
3'

Figure 3.16 : 3'end sequence of *lcc1* cDNA. Partial sequence of conserved copper-binding region is bold, gene-specific forward primer is underlined and stop codon TAG is in italics and bold. Polyadenylation site is also underlined.

3.2.1.3 Determination of 5' end of the *lcc2* cDNA

In order to obtain 5'-end of *lcc2* cDNA, the first strand cDNA was synthesized with "Smart II A Oligonucleotide" and used as a template for 5'-RACE-PCR. Subsequently, second strand cDNA was amplified with the gene specific reverse primer "*lcc2*-5'RACE-reverse-I" and the universal primer (UPM),which recognizes the smart II A oligonucleotide and a fragment of approximately 550 bp was obtained (Figure 3.17-A). The fragment was cloned into the pDrive cloning vector and isolated plasmids were digested with *Eco*RI (Figure 3.17-B). Determination of the 5'-end of *lcc2* cDNA was also confirmed by another RACE-PCR set up with the

gene specific reverse primer "*lcc2*-5'RACE-reverse-II" and the fragment with equal size was obtained.

Table 3.7: Multiple alignment results of the 3'-end sequence of lcc1 cDNA.

Accession number	Laccase	Score	Maximum identity
AY458017.1	Pycnoporus sanguineus laccase mRNA, complete cds	423	82%
AF123571.1	Pycnoporus cinnabarinus laccase (lcc3-2) gene, complete cds	392	80%
AY510604.1	Pycnoporus sanguineus laccase gene, complete cds	327	95%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	311	93%
AB212732.1	Trametes versicolor lac2 mRNA for laccase2, complete cds	188	83%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	185	83%
U44851.1	Trametes versicolor laccase I (lccI) gene, complete cds	185	83%
L49377.1	Trametes villosa (clone LCC2) laccase gene, exons 1-11, complete cds	185	83%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	197	82%
AY693776.1	Trametes versicolor laccase 1 (lcc1) mRNA, complete cds	197	84%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	194	81%
AF414807.1	Trametes pubescens laccase 2 (lap2) gene, complete cds	176	81%
AF162785.1	Basidiomycete C30 polyphenoloxidase (LAC1) gene, complete cds	176	80%
AF491759.1	Trametes sp. C30 laccase 1 (lac1) mRNA, complete cds	176	80%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	174	80%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	174	80%
U65399.1	Basidiomycete CECT 20197 phenoloxidase (pox1) gene, complete cds	174	80%
FJ598130.1	Coriolopsis gallica laccase mRNA, complete cds	172	80%
DQ431716.1	Coriolopsis gallica laccase (lac1) mRNA, complete cds	172	80%
DQ431715.1	Coriolopsis gallica laccase (lac1) gene, complete cds	172	80%
AY081188.1	Trametes versicolor laccase III gene, complete cds	172	80%
AY875867.1	Coriolopsis gallica laccase (lacA) mRNA, partial cds	172	80%
EF362634.1	Polyporus brumalis LAC1 mRNA, complete cds	170	81%
AJ294820.1	Trametes trogii lcc1 gene for laccase, exons 1-11	170	78%
Y18012.1	Trametes versicolor mRNA for laccase	170	81%

Plasmid DNAs containing the inserts were sequenced. The start codon"ATG" has been found as a part of Kozak consensus sequence and followed by another guanine. Kozak consensus sequence, gccRccAUGG (R=A,G) occurs on eukaryotic mRNA at three base upstream of the start codon and plays an important role in the translation initiation with recognition by ribosome. Kozak sequence has variations and strong consensus is explained as occurrence of G at the position +4 and A or G at the position -3 (Kozak, 1986). The *lcc2* cDNA sequence has displayed strong consensus as the start codon is followed by a guanine and the nucleotide at -3 position is an adenine (ACAATGG). In filamentous fungi, the -3 position is 90 % an adenine

(Yaver et al. 1996). The partial sequence of *lcc2* cDNA, containing the 5'-end, is demonstrated in the Figure 3.18. Maximum identity of *lcc2* laccase with other laccases are given in Table 3.8 and have 77 % nucleotide similarity with *Trametes sp.* I-62 *pox 3* mRNA (Gonzalez et al. 2003), 76 % with *T. versicolor lcc1* mRNA (Ong et al. 1997), 75 % with *Trametes sp.* I- 62 *pox 1* mRNA (Gonzalez et al. 2003) and 81 % with *lac 1* mRNA from *Polyporus brumalis* (Ryu et al. 2008).

Partial sequence of the *lcc2* promoter region were also analyzed for putative consensus promoter elements known to be employed in the transcriptional regulation of fungal genes. Approximately 155 bp upstream of the start codon ATG has been sequenced and analyzed. Putative transcription factor binding TATA and CAAT motifs have been identified at -48 and -116 within the promoter of *lcc2* and they are underlined in the Figure 3.18. Meanwhile, it is known that TATA and CAAT motifs are not strictly conserved in the fungal gene promoters (Yaver et al. 1996, Smith et al. 1998). Strong fungal promoters contain a typical long pyrimidine rich region between the TATA box and the translation start site (Galhaup et al. 2002). Here a long pyrimidine rich region has been observed at the defined location. Moreover, a putative metal response element (MRE), adhering to the consensus sequence TGCRCNC (R=A/G, N=any nucleotide) at nucleotide position – 98 and underlined in the Figure 3.18.

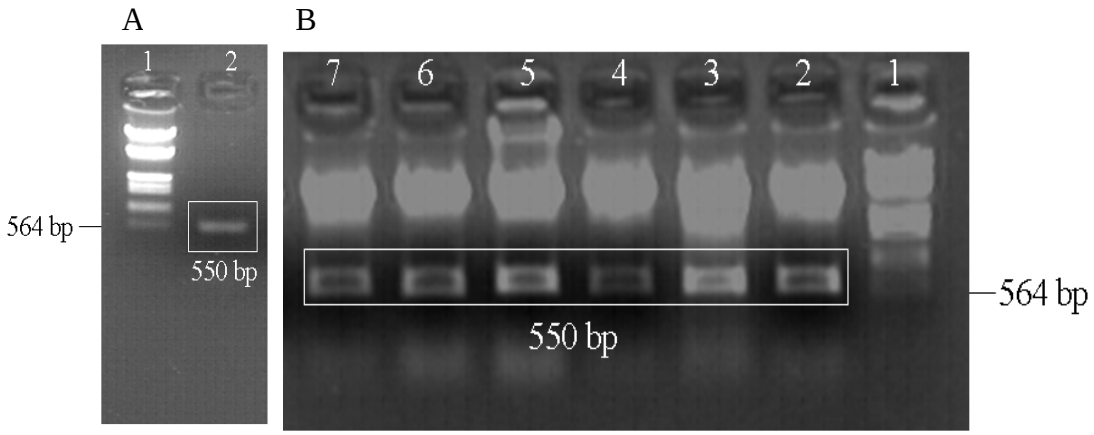


Figure 3.17 : A. 5'-RACE-PCR product amplified by "*lcc2*-5'-RACE-reverse primer" and UPM, B.Restriction enzyme digestion of plasmids having PCR product. lane 1. Lambda DNA/*Eco*RI+*Hind*III DNA fragments as Marker, lane 2-9: cut plasmids, PCR product is shown in the box.

5’*CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT*ACG
CGGGGATCGCTTCCGAGTCCTCTCGTGGCTCATTTTTCCTCTTACTG
CTTCCCTTTTCTATTCTAGCCCCCTCTCTTTCTCGGCCTTAGTTCATT
TTATTTATCCTCTTTGGCCGTTCTGTTTTGATTATTGGGGTTTACGGA
CTTGACA***ATG***GAGGGATCGAGACCAACTCTTCTTCACATGTTCTCGT
CCTTGGTTGTGACGGTATCCCTCGCGATCTCCGTGTTAGCTGGAATA
GGTCCCGTGACGGACTTGACGATCAGCAACGCCGACGTCTCTCCAG
ATGGCTACCAGCGAGCAGCGGTAGTGGCAAACGGTGTCATGCCCGC
ACCACTCATTACGGGCACGAAGGGCGACGAGTTCAAGATCAATGTC
ATCAACAACCTGACGAATCATACCATGCTCAAGTCTACTAGTATT**C**
ACTGGCACGGACTATTCCAGAGGTCAACGAACTGGGCGGATGG
CCCTGCCTTCGTGAATCAATGTCCCATTGCGACTGGGCATTCAT
TCCTTTATGACTTCCAAGTCCCAGACCAGGCCGGTACCTTCTGG
TATCACAGTCATCTGTCCACGCAGTACTGCGATGGGCTGAGGG
GCCCATTGGTTGTGTACGATCCCAATGACCCTCACGCCAGCCTA
TACGACGTTGACAACGATGACACGGTGATCACACTTGCCG…..-3’

Figure 3.18 : 5’-end sequence of the lcc2 cDNA. Partial sequence of the conserved copper binding region is displayed with bold characters. Regions used in designing gene-specific reverse and nested-reverse primers are underlined and the sequence of universal primer is shown in italics. Start codon “ATG” is showed in bold and italics. Lcc2-forward primer sequence used in the full-length cDNA synthesis is highlighted with gray and TATA box in the promoter region is also underlined.

3.2.1.4 Determination of 3’ end of the *lcc2* cDNA

The 3’-end of the *lcc2* cDNA has been determined by 3’-RACE-PCR set up with universal primer mix and gene specific “*Lcc2*-3’RACE-forward” primer, listed in Table 3.6. A fragment ca. 550 bp has been amplified (Figure 3.19) and cloned into the pDrive cloning vector. Plasmid DNAs were isolated from the transformants, cut with *Eco*RI (Figure. 3.20) and the positive recombinant plasmids were sequenced. Partial sequence of *lcc2* cDNA displaying the 3’-end is shown in Figure 3.21 The stop codon “TGA” has shown in italics and a potential polyadenylation signal sequence, which is an eukaryotic consensus sequence, AATTAA, located in the 31 nucleotide downstream of the stop codon is underlined. Nucleotide identity ratios of the *lcc2* cDNA with other laccases in the NCBI database are given in Table 3.9 and the amplified fragment has 77 % similarity with *pox 3* mRNA of *Trametes sp.* (Gonzalez et al. 2003), 77 % with *pox 2* gene of basidiomycete CECT 20197 (Mansur et al. 1997) and 76 % with white-rot fungus AH28-2 laccase gene partial sequence (Xiao et al. 2003).

Table 3.8: Multiple alignment results of 5'-end sequence of the lcc2 cDNA

Accession number	Laccase	Score	Maximum identity
AF548035.1	Trametes sp. I-62 laccase (pox3) mRNA, complete cds	394	77%
DQ914872.1	Ganoderma lucidum strain RZ laccase (lac4) mRNA, complete cds	324	75%
AF152170.1	Pycnoporus cinnabarinus laccase (lac1) mRNA, complete cds	306	75%
AY081775.2	Coriolus hirsutus laccase (072-1) mRNA, complete cds	295	77%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	295	76%
AY693776.1	Trametes versicolor laccase 1 (*lcc1*) mRNA, complete cds	295	76%
AB212732.1	Trametes versicolor lac2 mRNA for laccase2, complete cds	291	76%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	291	76%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	289	75%
AF176230.1	Polyporus ciliatus laccase (lcc3-1) mRNA, complete cds	288	74%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-*lcc1*A allele, complete cds	286	75%
Y18012.1	Trametes versicolor mRNA for laccase	286	74%
DQ914874.1	Ganoderma tsugae strain 1109 laccase (lac1) mRNA, complete cds	284	76%
AY485829.1	Ganoderma lucidum laccase mRNA, complete cds	284	76%
FJ688172.1	Pycnoporus sanguineus strain H275 multicopper redoxase (*lcc1*) mRNA, complete cds	282	73%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	282	75%
DQ431716.1	Coriolopsis gallica laccase (lac1) mRNA, complete cds	280	74%
AB212731.1	Trametes versicolor lac1 mRNA for laccase1, complete cds	277	73%
AF414109.1	Trametes versicolor laccase B precursor (lac1) mRNA, complete cds	275	73%
AY458017.1	Pycnoporus sanguineus laccase mRNA, complete cds	273	75%
AY439331.1	Panus rudis laccase mRNA, partial cds	268	73%
AB072703.1	Pycnoporus coccineus *lcc1* mRNA for laccase, complete cds	266	73%
AY875867.1	Coriolopsis gallica laccase (lacA) mRNA, partial cds	264	76%
AF548034.1	Trametes sp. I-62 laccase (pox2) mRNA, complete cds	255	76%
AM422387.1	Trametes versicolor mRNA for multicopper oxidase (klc2 gene)	244	72%
AF491759.1	Trametes sp. C30 laccase 1 (lac1) mRNA, complete cds	241	72%
AB212734.1	Trametes versicolor lac4 mRNA for laccase4, complete cds	232	72%

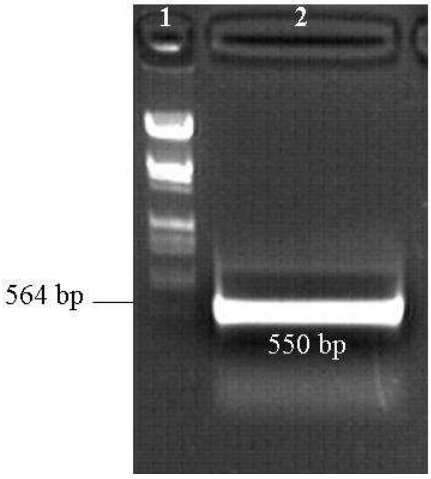


Figure 3.19 : PCR product obtained by lcc2-3'RACE-forward primer and UPM.

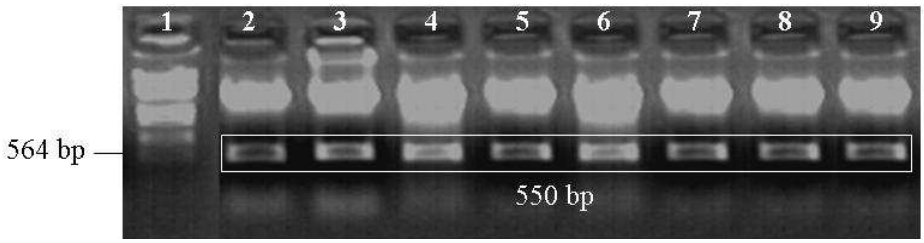


Figure 3.20 : Plasmids containing the 3'-end of the lcc1 cDNA, clones were verified by EcoRI digestion.

5'……TGTCCACGTTTTGCCGTCCAACGCCACGATCGAGCTCTCGTTCCC
CGCAACCATCCAAGCTGGGGCCCCCCACCCCTTCCACTTGCATGGGCA
TACTTTCGCTGTTGTACGCAGCGCAGGCAGTACGGAATACAACTACGA
GAACCCGATATTCAGAGACGTGGTCAGCACCG<u>GAGTACCTCAGGACAA</u>
<u>CGACAACGTGAC</u>TATTCGATTCCGGACTGACAACCCCGGCCCGTGGTT
CTTGCATTGCCATATCGACTTCCACCTCGAGGCCGGCTTCGCCGTCATCAT
GGCCGAAGACACGCCCGAGACCAAGTCGACAACCCCGTGCCTCAATCTTGG
ACGGACCTTTGCCCTATCTACGACGCGCTGGACCCTAGTGATCTGTGAACGC
GGGGTCATCATTGAAGTCGCCTCGCTACACCTTCGCCTTGTTGGACATTCTC
CCATCTTTCTTGCATCGCTATTTGGACATTGGTCACTTGTTAATAAGCCAGTC
ATTTCGTGGATAGTTATTGGGGTC***TGA***GGACCTTGGGATGGACTTAACTTTG
CTTTAC<u>AATTAA</u>CCGGACTAGTACTGTAGATAGCGTATACATTGCATAGTCG
TTACAGACAGAAAAGCCACTCTGTAATGTTCCTCATNCCTGCCATANGGATA
TCTCCCGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAG*TACTCTGCGTTGA*
TACCACTGCTTGCCCTATAGTGAGTCGTATTAG-3'

Figure 3.21 : Sequence of the 3'-end of the lcc2 cDNA. Partial sequence of the conserved copper binding region is displayed with bold characters. Gene-specific forward primer and polyadenylation signal are underlined and universal primer is shown in italics. Stop codon "TGA" is shown in bold and italics. Lcc2-reverse primer used is highlighted.

Table 3.9: Multiple alignment results of the 3'-end sequence of lcc2 cDNA with other laccases.

Accession number	Laccase	Score	Maximum identity
AF548035.1	Trametes sp. I-62 laccase (pox3) mRNA, complete cds	288	77%
AY081188.1	Trametes versicolor laccase III gene, complete cds	286	77%
D13372.1	Coriolus versicolor CVL3 gene for laccase, complete cds	282	76%
L49376.1	Trametes villosa (clone *LCC1*) laccase gene, exons 1-9, complete cds	277	76%
AF548034.1	Trametes sp. I-62 laccase (pox2) mRNA, complete cds	271	75%
AF414807.1	Trametes pubescens laccase 2 (lap2) gene, complete cds	268	76%
Y18012.1	Trametes versicolor mRNA for laccase	268	76%
AF414109.1	Trametes versicolor laccase B precursor (lac1) mRNA, complete cds	264	76%
AB212731.1	Trametes versicolor lac1 mRNA for laccase1, complete cds	260	76%
FJ688172.1	Pycnoporus sanguineus strain H275 multicopper redoxase (*lcc1*) mRNA, complete cds	259	75%
AB072703.1	Pycnoporus coccineus *lcc1* mRNA for laccase, complete cds	259	76%
U65400.1	Basidiomycete CECT 20197 phenoloxidase (pox2) gene, complete cds	282	77%
EF362634.1	Polyporus brumalis LAC1 mRNA, complete cds	237	74%
AY081775.2	Coriolus hirsutus laccase (072-1) mRNA, complete cds	233	74%
AB072704.1	Pycnoporus coccineus *lcc1* gene for laccase, complete cds	233	77%
AF152170.1	Pycnoporus cinnabarinus laccase (lac1) mRNA, complete cds	226	75%
DQ914872.1	Ganoderma lucidum strain RZ laccase (lac4) mRNA, complete cds	221	74%
AF176230.1	Polyporus ciliatus laccase (lcc3-1) mRNA, complete cds	221	72%
DQ431716.1	Coriolopsis gallica laccase (lac1) mRNA, complete cds	219	76%
AY875867.1	Coriolopsis gallica laccase (lacA) mRNA, partial cds	219	76%
AF491759.1	Trametes sp. C30 laccase 1 (lac1) mRNA, complete cds	210	75%
AY147188.1	Pycnoporus cinnabarinus laccase (*Lcc1*) gene, complete cds	258	75%
AF025481.1	Pycnoporus cinnabarinus laccase (lcc3-1) gene, complete cds	258	75%
AJ294820.1	Trametes trogii *lcc1* gene for laccase, exons 1-11	205	77%
AF170093.1	Pycnoporus cinnabarinus laccase (Lac1) gene, complete cds	203	75%
AY693776.1	Trametes versicolor laccase 1 (*lcc1*) mRNA, complete cds	203	72%
FJ656307.1	Ganoderma lucidum strain 7071-9 laccase mRNA, complete cds	201	73%
EU492907.1	Trametes hirsuta laccase (Lac) gene, complete cds	201	76%

3.2.2 Full-length laccase cDNA synthesis

Following to the determination of the both 5'- and 3'-end of the *lcc1* and *lcc2* cDNAs, forward and reverse primers involving the initiation and termination codons, listed in Table 3.6, were designed.

3.2.2.1 Amplification of the full-length *lcc1* cDNA

The full-length *lcc1* cDNA synthesis has been carried out via PCR by using the first strand cDNA, Expand high fidelity polymerase, "*lcc1*-forward" and "*lcc1*-reverse" primers. A fragment ca. 1.6 kb has been amplified (Figure 3.22), cloned into the pDrive cloning vector. Plasmid DNA were isolated from the transformants (Figure 3.23) and positive clones were selected by digestion with *Eco*RI (Figure 3.24) and sequenced. BLAST homology search results of full-length *lcc1* cDNA with laccase sequences in the NCBI database showed 94 % identity with *Pycnoporus cinnabarinus lcc3-2* (Temp et al. 1999), 93 % with *Pycnoporus sanguineus* (AY458017.1) and 91 % with *Pycnoporus sanguineus* (Vite-Vallejo et al. 2009). Homology search results are listed in Table 3.10. Isolated *lcc1* cDNA was submitted to the NCBI database.

Complete mRNA sequence of *lcc1* from ATG start codon to TAG stop codon is 1557 bp and this open reading frame encodes deduced protein with 518 amino acids (Figure 3.25). A signal peptide of 20 residues, responsible for the secretion of the laccase was observed in the sequence.

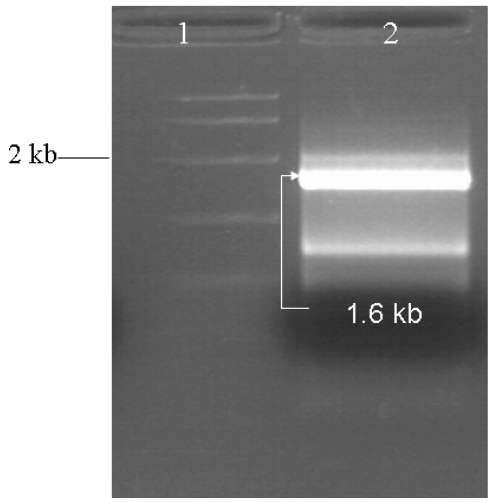


Figure 3.22 : Full-length lcc1 cDNA amplified with lcc1-forward and lcc1-reverse primers.

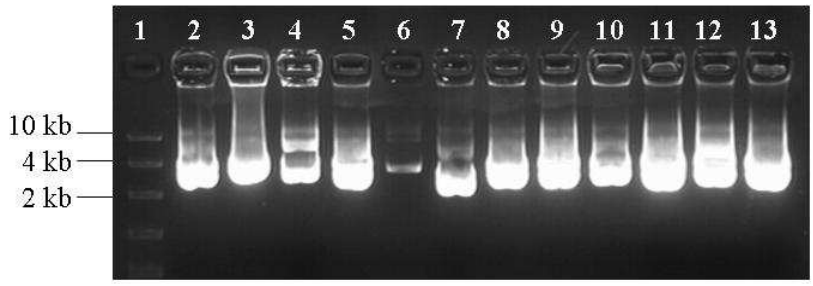


Figure 3.23 : Lcc1/pDrive plasmids (lane 2 to lane 13), Fastruler high range DNA ladder (lane 1).

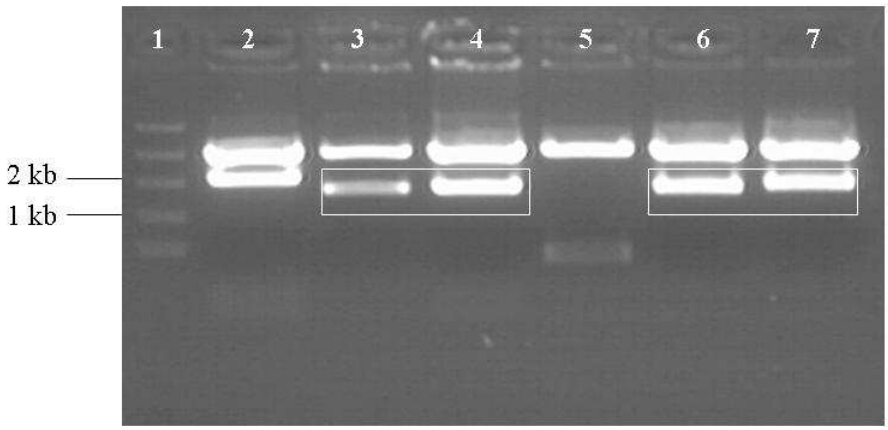


Figure 3.24 : Restriction enzyme digestion results of lcc1/pDrive plasmids (lane 2 to lane 7), Fastruler high range DNA ladder (lane 1).

Lcc1 cDNA encodes deduced laccase protein with 518 amino acids and a typical laccase secretion signal sequence of 20 residues exists in the N-terminus of the protein. As observed in other laccases this peptide has a positively charged amino terminus, a hydrophobic core and small amino acid residues (Liu et al. 2003). Typical copper ligands composed of 10 His and 1 Cys residue, required for coordinating copper atoms on the active site of the enzyme are in the conserved positions of fungal laccases (Figure 3.27). In addition to the six potential N-glycosylation sites, five Cystein residues are available in the deduced amino acid sequence and Cys 472 is the conserved laccase signature acting as a ligand to T1 copper domain. Crystal structures of reported basidiomycete laccases are composed of 3 domains and two disulfide bridges, between domain 1 and 3 and between domain 1 and 2, stabilize the enzyme (Giardina et al. 2010 , Liu et al. 2003).

Table 3.10: Similarity search results of full-length lcc1 with different laccases.

Accession number	Laccase	Score	Maximum identity
AY458017.1	Pycnoporus sanguineus laccase mRNA, complete cds	2316	93%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	2148	91%
AF548034.1	Trametes sp. I-62 laccase (pox2) mRNA, complete cds	483	74%
AY510604.1	Pycnoporus sanguineus laccase gene, complete cds	2055	97%
AF123571.1	Pycnoporus cinnabarinus laccase (lcc3-2) gene, complete cds	1815	96%
AB072703.1	Pycnoporus coccineus lcc1 mRNA for laccase, complete cds	425	73%
FJ688172.1	Pycnoporus sanguineus strain H275 multicopper redoxase (lcc1) mRNA, complete cds	414	73%
Y18012.1	Trametes versicolor mRNA for laccase	715	80%
AF414807.1	Trametes pubescens laccase 2 (lap2) gene, complete cds	339	80%
L49376.1	Trametes villosa (clone LCC1) laccase gene, exons 1-9, complete cds	339	79%
D13372.1	Coriolus versicolor CVL3 gene for laccase, complete cds	320	79%
AY454306.1	Favolus alveolaris laccase gene, partial cds	975	96%
AY333125.1	Daedalea quercina laccase gene, partial cds	935	93%
AY331189.1	Pycnoporus sanguineus lacasse gene, partial cds	953	96%
FJ656307.1	Ganoderma lucidum strain 7071-9 laccase mRNA, complete cds	267	78%
AY147188.1	Pycnoporus cinnabarinus laccase (Lcc1) gene, complete cds	207	81%
AF025481.1	Pycnoporus cinnabarinus laccase (lcc3-1) gene, complete cds	207	81%
FJ425896.1	Pycnoporus puniceus strain MUCL 47087 laccase gene, partial cds	196	87%
FJ425895.1	Pycnoporus puniceus strain MUCL 47083 laccase gene, partial cds	196	87%
EU714501.1	Pycnoporus coccineus strain MUCL 38525 laccase gene, partial cds	196	88%
U65400.1	Basidiomycete CECT 20197 phenoloxidase (pox2) gene, complete cds	195	80%
EU683254.1	Pycnoporus coccineus strain MUCL 38523 laccase gene, partial cds	191	87%
EU684160.1	Pycnoporus cinnabarinus strain MUCL 38420 laccase gene, partial cds	185	87%
AJ626679.1	Uncultured basidiomycete partial lac gene for laccase, exons 1-2, clone S17-Seq1	182	94%
EU678772.1	Pycnoporus sanguineus strain CIRM-BRFM 901 laccase gene, partial cds	174	86%
AF185275.2	Ganoderma lucidum strain 7071-9 laccase gene, complete cds	161	81%
EU714499.1	Pycnoporus sanguineus strain IMB W3008 laccase gene, partial cds	247	85%
EU683257.1	Pycnoporus sanguineus strain IMB G66 laccase gene, partial cds	247	85%
EU678786.1	Pycnoporus sanguineus strain CIRM-BRFM 542 laccase gene, partial cds	253	87%
FJ473385.2	Ganoderma lucidum strain TR6 laccase gene, complete cds	156	81%
AY081188.1	Trametes versicolor laccase III gene, complete cds	122	81%
EU683256.1	Pycnoporus sanguineus strain IMB H2180 laccase gene, partial cds	95.3	87%
EU683255.1	Pycnoporus sanguineus strain IMB G53 laccase gene, partial cds	95.3	87%
EU683253.1	Pycnoporus coccineus strain MUCL 38527 laccase gene, partial cds	95.3	87%
EU678784.1	Pycnoporus sanguineus strain IMB W006-2 laccase gene, partial cds	95.3	87%
AB072704.1	Pycnoporus coccineus lcc1 gene for laccase, complete cds	95.3	87%

Based on the reported laccase structures, it can be supposed that two disulfide bridges is formed between Cys105 and Cys507 and between Cys137 and Cys225 in *Pycnoporus sanguineus* MUCL38531 lcc1 laccase. Phenyl-alanine, leucine or methionine, involved in the coordination of the type I copper atom, can be found ten amino acids downstream of the conserved cysteine. Phe 482 is located at the position mentioned in deduced amino acid sequence of *lcc1* and is assumed to be essential for the high redox potential of the laccases (Temp et al. 1999). Multiple sequence alignment of deduced amino acid sequence of *Lcc1* with other basidiomycete laccase sequences revealed that *Pcynoporus sanguineus* MUCL 38531 shares 98 % and 95 % of identity with laccases from *Pycnoporus sanguineus* reported by Zhao (Genebank accession number AAR20864.1) and Vite-Vallejo et al. (2009) and 97 % with *Pycnoporus cinnabarinus lcc 3-2* (AAD49218.1) (Fig 3.28). It also has 78% identity with *Trametes versicolor laccase I* (AAC49828.1) and *Trametes sp.* I-62 laccase (AAQ12268.1), 73 % identity with *Pycnoporus cinnabarinus lcc1* (AAN71597.1) and *Pycnoporus cinnabarinus lcc3-1 (*AAC39469.1), 72% with *Pycnoporus cinnabarinus lac1* (AAF13052.1) (Figure 3.26). Conserved copper binding regions are shaded in gray and possible N-glycosylation sites are underlined.

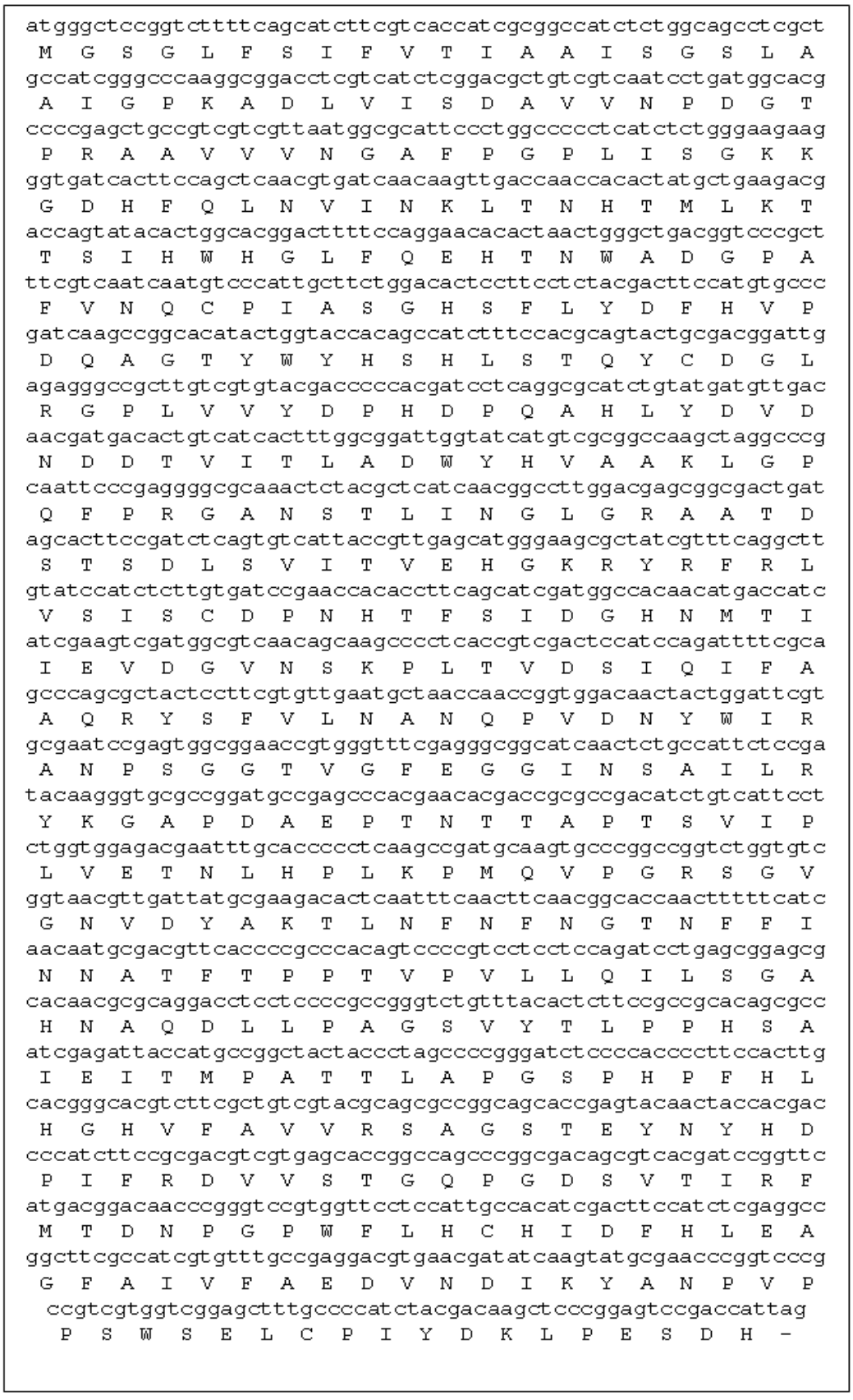


Figure 3.25 : *lcc1* open reading frame consists of 1557 bp and encodes 518 deduced amino acids.

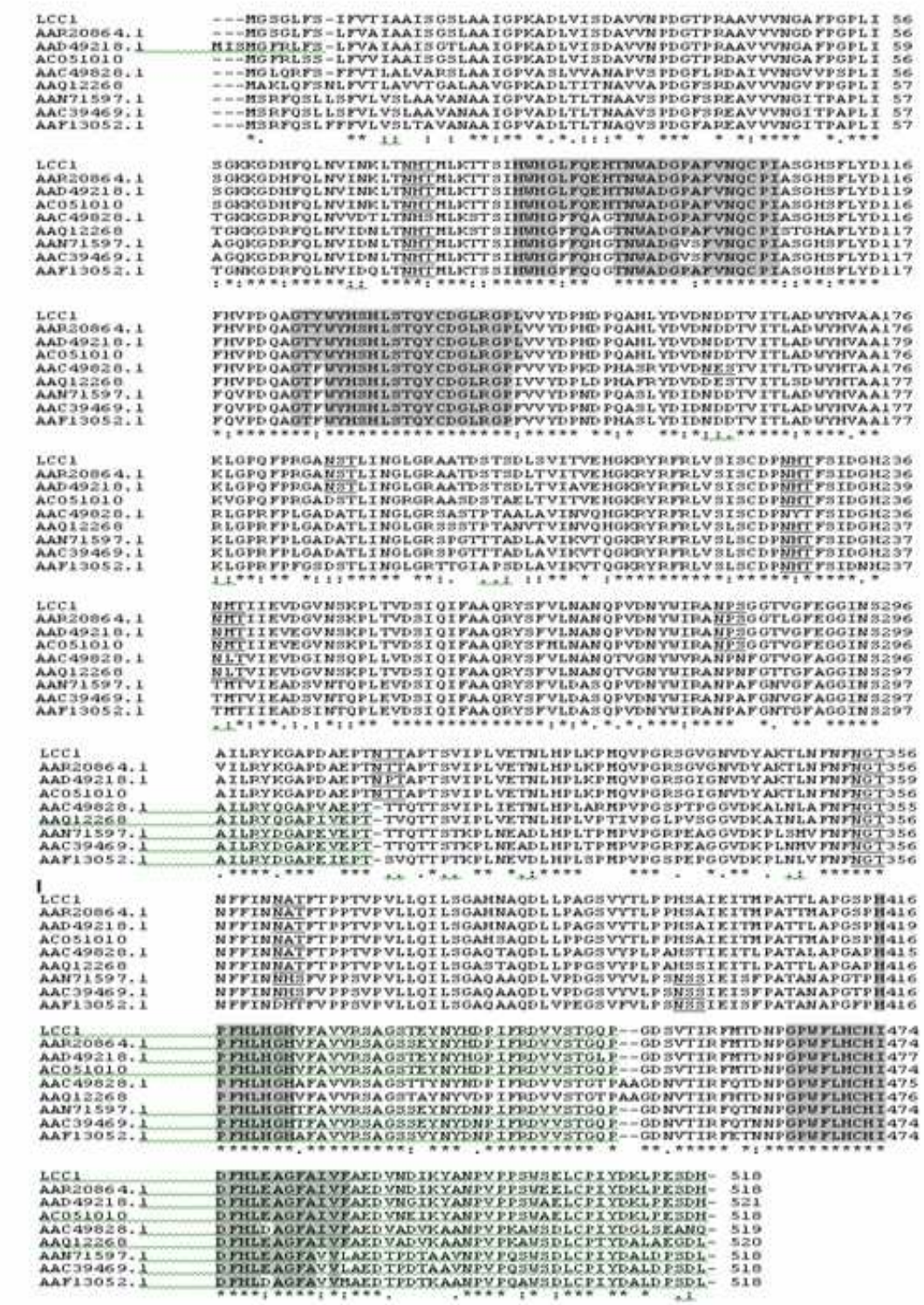


Figure 3.26 : Alignment of putative amino acid sequence of lcc1 with different basidiomycete laccases using CLUSTAL W algorithm, Pycnoporus sanguineus (AAR20864.1), Pycnoporus cinnabarinus lcc3-2 (AAD49218.1), Pycnoporus sanguineus (AC0510109), Trametes versicolor (AAC49828.1), Trametes sp. I-62 (AAQ12268.1), Pycnoporus cinnabarinus lcc1 (AAN71597.1), Pycnoporus cinnabarinus lcc3-1 (AAC39469.1), Pycnoporus cinnabarinus lac1 (AAF13052.1).

3.2.2.2 Amplification of the full-length *lcc2* cDNA

The full length *lcc2* cDNA was synthesized by PCR using "*lcc2*-forward" and "*lcc2*-reverse" primers listed in Table 3.6. The amplified fragment was ca. 1.7 kb (Figure 3.27) and was cloned into the pDrive vector. Plasmids were isolated (Figure 3.28) and sequenced. Similarity of *lcc2* cDNA with different laccase sequences is given in Table 3.11. Maximum identity of *lcc2* cDNA was 75 % with pox 3 laccase mRNA of *Trametes sp.* (Gonzalez et al. 2003), 71 % with *Pycnoporus sanguineus* strain H275 multicopper redoxase mRNA (Lu et al. 2010) and 71 % with *lcc1* mRNA of *Pycnoporus coccineus* (Hoshida et al. 2001). Isolated *lcc2* cDNA was submitted to the NCBI database.

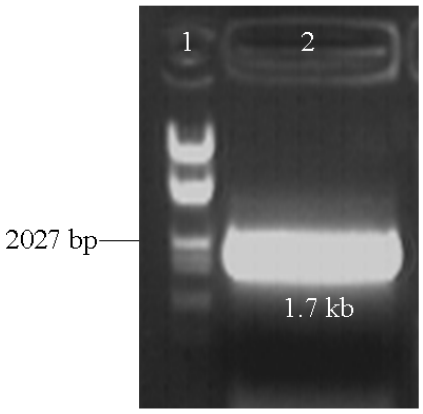


Figure 3.27 : Full-length *lcc2* cDNA amplified with *lcc2*-forward and lcc2-reverse primers.

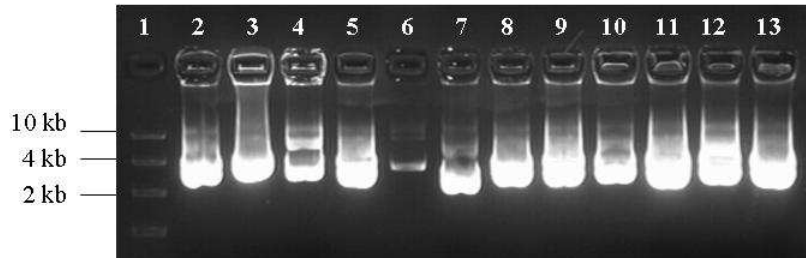


Figure 3.28 : *Lcc2*/pDrive plasmids (lane 2 to lane 13), Fastruler high range DNA ladder (lane 1).

Table 3.11: Alignment results of full-length lcc2 cDNA with previously reported laccases.

Accession number	Laccase	Score	Maximum identity
AF548035.1	Trametes sp. I-62 laccase (pox3) mRNA, complete cds	1050	75%
Y18012.1	Trametes versicolor mRNA for laccase	843	72%
AF414109.1	Trametes versicolor laccase B precursor (lac1) mRNA, complete cds	812	72%
FJ688172.1	Pycnoporus sanguineus strain H275 multicopper redoxase (lcc1) mRNA, complete cds	800	71%
AF548034.1	Trametes sp. I-62 laccase (pox2) mRNA, complete cds	773	72%
AB072703.1	Pycnoporus coccineus lcc1 mRNA for laccase, complete cds	758	71%
AF152170.1	Pycnoporus cinnabarinus laccase (lac1) mRNA, complete cds	744	71%
AY081775.2	Coriolus hirsutus laccase (072-1) mRNA, complete cds	742	71%
AB212731.1	Trametes versicolor lac1 mRNA for laccase1, complete cds	711	71%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	699	71%
AY693776.1	Trametes versicolor laccase 1 (lcc1) mRNA, complete cds	695	71%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	691	70%
AF176230.1	Polyporus ciliatus laccase (lcc3-1) mRNA, complete cds	688	70%
EF362634.1	Polyporus brumalis LAC1 mRNA, complete cds	686	70%
DQ914872.1	Ganoderma lucidum strain RZ laccase (lac4) mRNA, complete cds	684	70%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	672	70%
AB212732.1	Trametes versicolor lac2 mRNA for laccase2, complete cds	672	70%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	661	70%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	657	70%
AB006824.1	Ganoderma tsunodae mRNA for bilirubin oxidase, complete cds	634	70%
DQ431716.1	Coriolopsis gallica laccase (lac1) mRNA, complete cds	630	69%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	627	70%
FJ656307.1	Ganoderma lucidum strain 7071-9 laccase mRNA, complete cds	627	69%
AY458017.1	Pycnoporus sanguineus laccase mRNA, complete cds	625	70%
FJ598130.1	Coriolopsis gallica laccase mRNA, complete cds	621	69%
AY875867.1	Coriolopsis gallica laccase (lacA) mRNA, partial cds	610	70%
DQ914874.1	Ganoderma tsugae strain 1109 laccase (lac1) mRNA, complete cds	603	70%
AF491759.1	Trametes sp. C30 laccase 1 (lac1) mRNA, complete cds	601	69%
AM419158.1	Panus tigrinus partial mRNA for laccase (lac1 gene)	590	71%
FJ817448.1	Trametes sp. C30 laccase 5 (lac5) mRNA, complete cds	565	68%
AY485829.1	Ganoderma lucidum laccase mRNA, complete cds	563	69%
AF176231.1	Polyporus ciliatus laccase (lcc3-2) mRNA, complete cds	529	68%
EF362635.1	Polyporus brumalis LAC2 mRNA, complete cds	524	68%
DQ914876.1	Ganoderma fornicatum strain 0814 laccase (lac1) mRNA, complete cds	522	68%
AF176232.1	Polyporus ciliatus laccase (lcc3-3) mRNA, partial cds	520	69%
AM422387.1	Trametes versicolor mRNA for multicopper oxidase (klc2 gene)	509	68%
AJ420900.1	Pycnoporus cinnabarinus partial mRNA for laccase (lcc3-1 gene)	502	72%
AB212733.1	Trametes versicolor lac3 mRNA for laccase3, complete cds	500	68%
AB212734.1	Trametes versicolor lac4 mRNA for laccase4, complete cds	462	67%
U44431.1	Trametes versicolor laccase IV (lccIV) mRNA, complete cds	461	67%

Complete mRNA sequence of *lcc2* from ATG start codon to TGA stop codon is 1713 bp and this open reading frame encodes deduced protein with 570 amino acids. A

signal peptide of 27 residues, responsible for the secretion of the laccase was observed in the sequence (Figure 3.29). Conserved 10 histidine (H91, H93, H136, H138, H421, H424, H426, H478, H480, H484) and one cysteine (C 479). Phe 489 exists 10 amino acids downstream of the conserved cysteine residue and is known to be involved in the coordination of the type 1 copper atom in the laccase (Temp et al. 1999). Furthermore, it is suggested that the phenyl alanine residue at this position is essential for the high redox potential of laccases as observed in the *lcc1*.

Secreted laccases are glycosylated up to 25% with 3-10 glycosylation sites found at Asn-X-Thr/Ser sites and those functional groups are suggested to be involved in stabilization of copper centres, protein secretion direction, protection against proteolysis and also enhancing thermostability (Rodgers et al. 2009). In the deduced amino acid sequence of *lcc2*, three possible N-glycosylation sites were found at N78, N404 and N462 using N-glycosylation prediction tool (Url-6).

Signal peptide is the polypeptide domain that are involved in the protein targeting of secretory proteins and those peptides are segments of 15-50 amino acids. Signal peptide sequence is composed of hydrophobic central core containing leucine, methionine and phenylalanine and this core is flanked by the positively charged N-terminus and the hydrophilic C-terminus. The secretion signal sequence was determined in the deduced amino acid sequence of *lcc2* and the position was found between 1 and 27 amino acids, whereas the most likely cleavage site was identified between pos. 27 and 28: VLA-GI (Url-7). The similarity percentages of the signal peptide were between 22-48 % with other laccases, aligned previously, and the highest similarity was observed for the laccase C from *Trametes sp.* AH 28-2 (Xiao et al. 2006).

Multiple sequence alignment results of deduced amino acid sequence of *Lcc2* with different basidiomycete laccase sequences indicated that *Pycnoporus sanguineus* MUCL 38531 shares 80 % of identity with *laccase C* from *Trametes sp.* AH28-2 (Xiao et al. 2006) and 76 % with *Trametes sp.* phenoloxidase (Mansur et al. 1997) (Fig 3.30). It also has 72 % identity with *Trametes pubescens laccase 2* (Galhaup et al. 2002), *Pycnoporus sanguineus* strain BRFM 66 laccase gene (Uzan et al. 2010), *Pycnoporus cinnabarinus lac1* (Otterbein et al. 2000), *Pycnoporus sanguineus* multicopper redoxase (Lu et al. 2010) and *Pycnoporus coccineus* laccase (Hoshida et al. 2001) (Figure 3.30).

```
atggagggatcgagaccaactcttcttcacatgttctcgtccttggttgtgacggtatcc
 M  E  G  S  R  P  T  L  L  H  M  F  S  S  L  V  V  T  V  S
ctcgcgatctccgtgttagctggaataggtcccgtgacggacttgacgatcagcaacgcc
 L  A  I  S  V  L  A  G  I  G  P  V  T  D  L  T  I  S  N  A
gacgtctctccagatggctaccagcgagcagcggtagtggcaaacggtgtcatgcccgca
 D  V  S  P  D  G  Y  Q  R  A  A  V  V  A  N  G  V  M  P  A
ccactcattacgggcacgaagggcgacgagttcaagatcaatgtcatcaacaacctgacg
 P  L  I  T  G  T  K  G  D  E  F  K  I  N  V  I  N  N  L  T
aatcataccatgctcaagtctactagtattcactggcacggactattccagaggtcaacg
 N  H  T  M  L  K  S  T  S  I  H  W  H  G  L  F  Q  R  S  T
aactgggcggatggccctgccttcgtgaatcaatgtcccattgcgactgggcattcattc
 N  W  A  D  G  P  A  F  V  N  Q  C  P  I  A  T  G  H  S  F
ctttatgacttccaagtcccagaccaggccggtaccttctggtatcacagtcatctgtcc
 L  Y  D  F  Q  V  P  D  Q  A  G  T  F  W  Y  H  S  H  L  S
acgcagtactgcgatgggctgaggggcccattggttgtgtacgatcccaatgaccctcac
 T  Q  Y  C  D  G  L  R  G  P  L  V  V  Y  D  P  N  D  P  H
gccagcctatacgacgttgacaacgatgacacggtgatcacacttgccgactggtaccac
 A  S  L  Y  D  V  D  N  D  D  T  V  I  T  L  A  D  W  Y  H
ctagccgccaaggtcggccccaagttccctacacgctccgattcgacgctgatcaatggc
 L  A  A  K  V  G  P  K  F  P  T  R  S  D  S  T  L  I  N  G
cgcggccgcacggctgcaactatcgcggcggaattgacggtcatcaatgtcactccggga
 R  G  R  T  A  A  T  I  A  A  E  L  T  V  I  N  V  T  P  G
aagcggtatcgtttccgtcttgtgtcaatctcttgcgatcctgcctacactttcagcatc
 K  R  Y  R  F  R  L  V  S  I  S  C  D  P  A  Y  T  F  S  I
gatggacatgacatgaccgtgatcgaggcggattcagtcaacacccagccactcgaagta
 D  G  H  D  M  T  V  I  E  A  D  S  V  N  T  Q  P  L  E  V
gattccattcccatctataccgggcaacggtactcctttgtggttgaggcgaaccagcca
 D  S  I  P  I  Y  T  G  Q  R  Y  S  F  V  V  E  A  N  Q  P
gtcgacaactactggattcgcgcaaacccgatggcaggcacgaccggtttcgagggcgga
 V  D  N  Y  W  I  R  A  N  P  M  A  G  T  T  G  F  E  G  G
atcaactcagctattctgaggtacgacggcgcgccagagcaagagccaacgacggccccg
 I  N  S  A  I  L  R  Y  D  G  A  P  E  Q  E  P  T  T  A  P
ggcacgtccaccaagccgttgaaggagaccgatctccatcccctggtatctatgcctgtg
 G  T  S  T  K  P  L  K  E  T  D  L  H  P  L  V  S  M  P  V
ccaggatctcctgtcgcaggaggagttgacaaggccatcaacttggccttccagtttgat
 P  G  S  P  V  A  G  G  V  D  K  A  I  N  L  A  F  Q  F  D
ggcacgaacttcttcatcaacggtgctaccttcaagccccccactacgcctgttctcctg
 G  T  N  F  F  I  N  G  A  T  F  K  P  P  T  T  P  V  L  L
cagatcttgagcggcgctcaagccgcttctgacctcctaccgtctggcgatgtccacgtt
 Q  I  L  S  G  A  Q  A  A  S  D  L  L  P  S  G  D  V  H  V
ttgccgtccaacgccacgatcgagctctcgttccccgcaaccatccaagctggggccccc
 L  P  S  N  A  T  I  E  L  S  F  P  A  T  I  Q  A  G  A  P
cacccctccacttgcatgggcatactttcgctgttgtacgcagcgcaggcagtacggaa
 H  P  F  H  L  H  G  H  T  F  A  V  V  R  S  A  G  S  T  E
tacaactacgagaacccgatattcagagacgtggtcagcaccggagtacctcaggacaac
 Y  N  Y  E  N  P  I  F  R  D  V  V  S  T  G  V  P  Q  D  N
gacaacgtgactattcgattccggactgacaaccccggcccgtggttcttgcattgccat
 D  N  V  T  I  R  F  R  T  D  N  P  G  P  W  F  L  H  C  H
atcgacttccacctcgaggccggcttcgccgtcatcatggccgaagacacgcccgagacc
 I  D  F  H  L  E  A  G  F  A  V  I  M  A  E  D  T  P  E  T
aagtcgacaaccccgtgcctcaatcttggacggacctttgccctatctacgacgcgctgg
 K  S  T  T  P  C  L  N  L  G  R  T  F  A  L  S  T  T  R  W
accctagtgatctgtgaacgcggcgtcatcattgaagtcgcctcgctacaccttcgcctt
 T  L  V  I  C  E  R  G  V  I  I  E  V  A  S  L  H  L  R  L
gttggacattctcccatctttcttgcatcgctatttggacattggtcacttgttaataag
 V  G  H  S  P  I  F  L  A  S  L  F  G  H  W  S  L  V  N  K
ccagtcatttcgtggatagttattggggtctga
 P  V  I  S  W  I  V  I  G  V  -
```

Figure 3.29 : *Lcc2* open reading frame isolated from *Pycnoporus sanguineus* MUCL 38531, consists of 1713 bp and encodes 570 deduced amino acids.

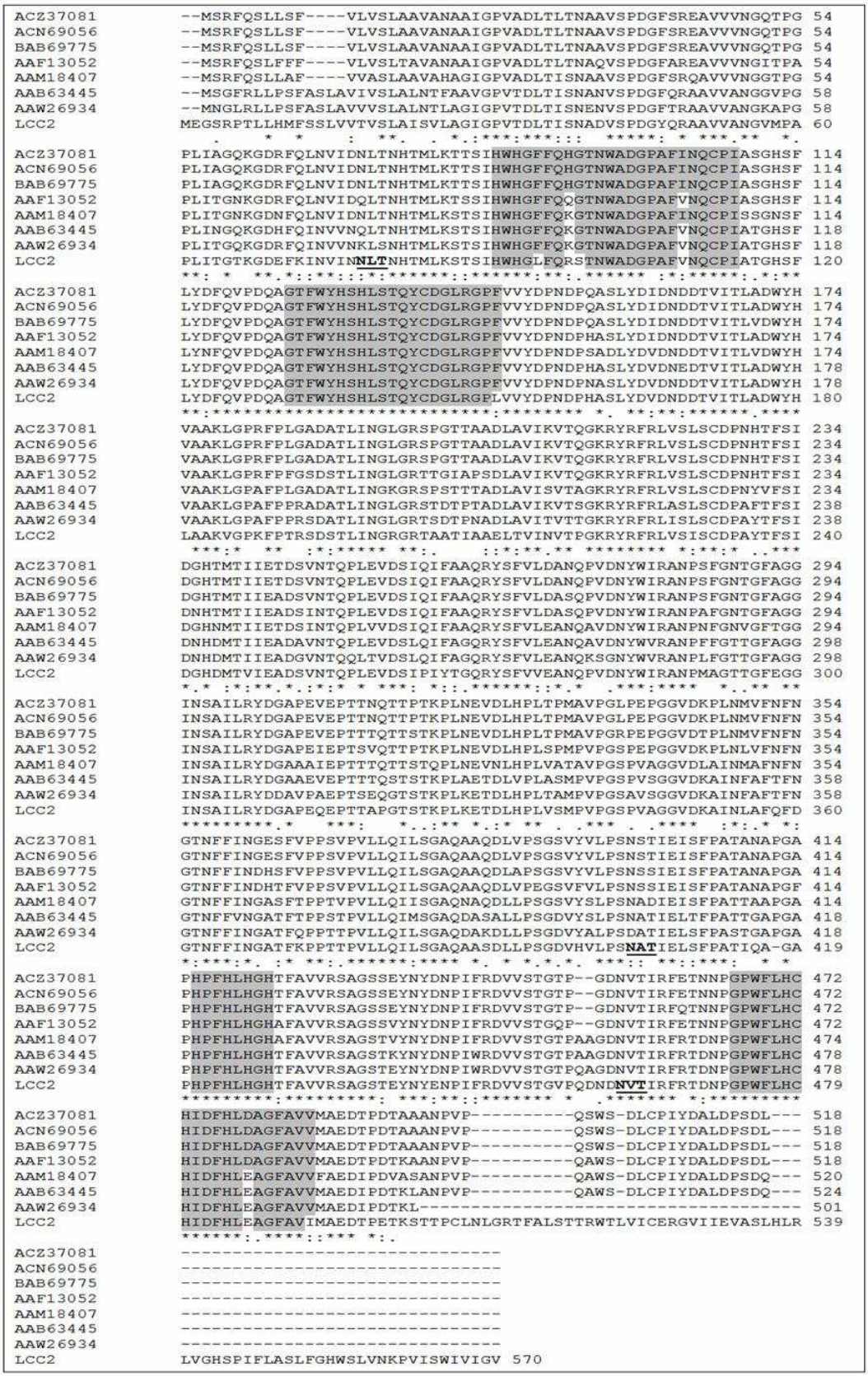


Figure 3.30 : Alignment of putative amino acid sequence of *lcc2* with different basidiomycete laccases using CLUSTAL W algorithm.

Trametes sp. phenoloxidase (AAB63445.1), *Trametes sp.* AH28-2 laccase C (AAW26934.1), *Trametes pubescens* laccase 2 (AAM18407.1), *Pycnoporus sanguineus* strain BRFM 66 laccase gene (ACZ37081), Pycnoporus cinnabarinus lac1 (AAF13052.1), *Pycnoporus sanguineus* multicopper redoxase (ACN69056), *Pycnoporus coccineus* laccase (BAB69775.1).Conserved copper binding regions are shaded in gray and possible N-glycosylation sites are underlined (Figure 3.30).

3.3 Characterization of Laccase Genes

Following to the synthesis of the full-length cDNAs, *lcc1* and *lcc2* genes were isolated and characterized by determining the intron-exon organization. To our knowledge, only 14 complete laccase sequences from *Pycnoporus* genus among 1440 of basidiomycetes laccase genes are available in the NCBI database and this number of sequences are very limited for extensively studied and biotechnologically important fungal laccases. Gene cloning strategies gives the opportunity to understand the structure-function relationships as a primary step for heterologous expression of laccases in different organisms, which are easily cultivated and producing sufficient amount of the protein. The present study constitutes the first report on the cloning of laccase genes from white-rot fungi *Pycnoporus sanguineus* MUCL 38531 and cloned genes were submitted to the NCBI database.

3.3.1 Sequence of *lcc1* gene of *Pycnoporus sanguineus* MUCL 38531

Characterization of *lcc1* gene has been performed following to amplification by PCR using "*lcc1*-forward" and "*lcc1*-reverse"primers and genomic DNA of *Pycnoporus sanguineus* MUCL 38531 as template. The fragment ca. 2 kb has been amplified, cloned into the pDrive cloning vector. Subsequently, positive clones were sequenced and aligned with the sequences of the cDNAs to determine intron-exon organization of laccase genes. Similarity of *lcc1* gene to other laccases in the NCBI database was determined and *lcc1* shared 88 % similarity with *Pycnoporus cinnabarinus lcc3-2* (Temp et al. 1999), 87 % with laccase of another *Pycnoporus sanguineus* strain (AY510604), and 86% with *Favolus alveolaris* laccase gene (AY454306) (Table 3.12).

lcc1 gene was 2132 bp and has an open reading frame of 1557 bp with a 58% GC content (Url-8). The coding region was interrupted by 10 introns of 52-68 bp in length, consistent with the introns of other fungal genes in the literature (Figure 3.31). The coding regions of fungal laccases are generally interrupted by 8-13 introns of about 50-90 bp in length and splicing junctions usually adhere to the 5'-GT….AG-3' rule (Galhaup et al 2002). Several fungal introns that contain consensus splicing sites as observed in the *lcc1* at 5' and 3' intron boundaries can be examplified. The *lap2* gene of *Trametes pubescens* and *lcc1* gene from *Trametes villosa* contain 8 introns (Galhaup et al 2002, Yaver et al. 1996), *lcc3-2* of *Pycnoporus cinnabarinus* and *lcc2* of *Trametes villosa* have 10 introns (Temp et al. 1999, Yaver et al. 1996), *CVLG1* of *Coriolus versicolor* and *Cs-Lcs1* of *Ceriporiopsis subvermispora* possess 11 introns (Mikuni and Morohoshi, 1997, Karahanian et al. 1998).

5'ATGGGCTCCGGTCTTTTCAGCATCTTCGTCACCATCGCGGCCATCTCTGGCAG
CCTCGCTGCCATCGGGCCCAAGGCGGACCTCGTCATCTCGGACGCTGTCGTCA
ATCCTGATGGCACGCCCCGAGCTGCCGTCGTCGTTAATGGCGCATTCCCTGGCC
CCCTCATCTCTGGGAAGAAG*gtaaggggcatgttctctgtatggggtataccacggctaatcgtgctggtgta*
*g*GGTGATCACTTCCAGCTCAACGTGATCAACAAGTTGACCAACCACACTATGCT
GAAGACGACCAGTATA*gtatgccggtctgttctcttggggtagctgcggtactcacgcagccctgaag*CACT
GGCACGGACTTTTCCAGGAACACACTAACTGGGCTGACGGTCCCGCTTTCGTC
AATCAATGTCCCATTGCTTCTGGACACTCCTTCCTCTACGACTTCCATGTGCCC
GATCAAGCCG*gtacgtctccgcagagcgcacgatgtctcggaaactcaatctttcaatctggcgtctag*GCACAT
ACTGGTACCACAGCCATCTTTCCACGCAGTACTGCGACGGATTGAGAGGGCCG
CTTGTCGTGTACGACCCCCACGATCCTCAGGCGCATCTGTATGATGTTGACAAC
GATGA*gttctttgaatggcctggcgtgcgacacatttctgacgtgcgtgatagatga*CACTGTCATCACTTTG
GCGGATTGGTATCATGTCGCGGCCAAGCTAGGCCCGCAATTCCC*gtgcgtcttcctcttct*
*aattgttacggagaagttcattcatgtaatgttgcag*GAGGGGCGCAAACTCTACGCTCATCAACGG
CCTTGGACGAGCGGCGACTGATAGCACTTCCGATCTCAGTGTCATTACCGTTG
AGCATGGGAAGCG*gtaagcacaggcaaatttcgcgtcttggaaagcccgctcatctttcgtcatcttcag*CTATC
GTTTCAGGCTTGTATCCATCTCTTGTGATCCGAACCACACCTTCAGCATCGATG
GCCACAACATGACCATCATCGAAGTCGATGGCGTCAACAGCAAGCCCCTCACC
GTCGACTCCATCCAGATTTTCGCAGCCCAGCGCTACTCCTTCGTG*gtaagtgagcttca*
*ccacgctcatgtcgacatagaacttatccgtatctccatatag*TTGAATGCTAACCAACCGGTGGACAA
CTACTGGATTCGTGCGAATCCGAGTGGCGGAACCGTGGGTTTCGAGGGCGGCA
TCAACTCTGCCATTCTCCGATACAAGGGTGCGCCGGATGCCGAGCCCACGAAC
ACGACCGCGCCGACATCTGTCATTCCTCTGGTGGAGACGAATTTGCACCCCCTC
AAGCCGATGCAAGTG*gtacgtgtcatgtgtagtattgtccctgcaacgtccgctgaccgtacacgaacag*CCC
GGCCGGTCTGGTGTCGGTAACGTTGATTATGCGAAGACACTCAATTTCAACTTC
*gtgagtagcttgactcaagtcatagacaaatgaactgagtgctgagatatcttttatacaatcaccag*AACGGCACCA
ACTTTTTCATCAACAATGCGACGTTCACCCCGCCCACAGTCCCCGTCCTCCTCC
AGATCCTGAGCGGAGCGCACAACGCGCAGGACCTCCTCCCCGCCGGGTCTGTT
TACACTCTTCCGCCGCACAGCGCCATCGAGATTACCATGCCGGCTACTACCCTA
GCCCCGGGATCTCCCCACCCCTTCCACTTGCACGGG*gtacgcataaatgcctcacactaactgt*
*caatcacacctgctcactcatcgccttcctgttcgacag*CACGTCTTCGCTGTCGTACGCAGCGCCGG
CAGCACCGAGTACAACTACCACGACCCCATCTTCCGCGACGTCGTGAGCACCG
GCCAGCCCGGCGACAGCGTCACGATCCGGTTCATGACGGACAACCCGGGTCCG
TGGTTCCTCCATTGCCACATCGACTTCCATCTCGAGGCCGGCTTCGCCATCGTG
TTTGCCGAGGACGTGAACGATATCAAGTATGCGAACCCGGTCCCGCCGTCGTG
GTCGGAGCTTTGCCCCATCTACGACAAGCTCCCGGAGTCCGACCAT**TAG-3'**

Figure 3.31 : Sequence of *lcc1* gene from *Pycnoporus sanguineus* MUCL 38531, open reading frame is interrupted by ten introns.

Table 3.12: Alignment of *lcc1* gene with previously submitted laccase sequences.

Accession number	Laccase	Score	Maximum identity
AF123571.1	Pycnoporus cinnabarinus laccase (lcc3-2) gene, complete cds	2521	88%
AY510604.1	Pycnoporus sanguineus laccase gene, complete cds	2484	87%
AY454306.1	Favolus alveolaris laccase gene, partial cds	1312	86%
AY331189.1	Pycnoporus sanguineus lacasse gene, partial cds	1277	85%
AY333125.1	Daedalea quercina laccase gene, partial cds	1255	85%
AY458017.1	Pycnoporus sanguineus laccase mRNA, complete cds	2062	97%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	1938	95%
AJ626679.1	Uncultured basidiomycete partial lac gene for laccase, exons 1-2, clone S17-Seq1	263	90%
AF185275.2	Ganoderma lucidum strain 7071-9 laccase gene, complete cds	220	76%
U65400.1	Basidiomycete CECT 20197 phenoloxidase (pox2) gene, complete cds	217	75%
AY147188.1	Pycnoporus cinnabarinus laccase (Lcc1) gene, complete cds	211	81%
AF025481.1	Pycnoporus cinnabarinus laccase (lcc3-1) gene, complete cds	211	81%
FJ473385.2	Ganoderma lucidum strain TR6 laccase gene, complete cds	209	75%
FJ425895.1	Pycnoporus puniceus strain MUCL 47083 laccase gene, partial cds	200	87%
EU714501.1	Pycnoporus coccineus strain MUCL 38525 laccase gene, partial cds	200	87%
D13372.1	Coriolus versicolor CVL3 gene for laccase, complete cds	200	80%
FJ425896.1	Pycnoporus puniceus strain MUCL 47087 laccase gene, partial cds	198	87%
EU683254.1	Pycnoporus coccineus strain MUCL 38523 laccase gene, partial cds	195	87%
EU684160.1	Pycnoporus cinnabarinus strain MUCL 38420 laccase gene, partial cds	189	86%
AF548034.1	Trametes sp. I-62 laccase (pox2) mRNA, complete cds	189	80%
EU678772.1	Pycnoporus sanguineus strain CIRM-BRFM 901 laccase gene, partial cds	178	85%
FJ656307.1	Ganoderma lucidum strain 7071-9 laccase mRNA, complete cds	161	81%
EU714499.1	Pycnoporus sanguineus strain IMB W3008 laccase gene, partial cds	247	84%
EU683257.1	Pycnoporus sanguineus strain IMB G66 laccase gene, partial cds	247	84%
EU678786.1	Pycnoporus sanguineus strain CIRM-BRFM 542 laccase gene, partial cds	270	84%
AY081188.1	Trametes versicolor laccase III gene, complete cds	134	82%
Y18012.1	Trametes versicolor mRNA for laccase	122	81%
AB072704.1	Pycnoporus coccineus lcc1 gene for laccase, complete cds	121	75%
EU683256.1	Pycnoporus sanguineus strain IMB H2180 laccase gene, partial cds	117	75%
EU683255.1	Pycnoporus sanguineus strain IMB G53 laccase gene, partial cds	108	75%
EU683253.1	Pycnoporus coccineus strain MUCL 38527 laccase gene, partial cds	108	75%
EU678784.1	Pycnoporus sanguineus strain IMB W006-2 laccase gene, partial cds	106	74%
AB072703.1	Pycnoporus coccineus lcc1 mRNA for laccase, complete cds	95.3	87%
EU678783.1	Pycnoporus sanguineus strain IMB G05.10 laccase gene, partial cds	89.8	74%
AJ420899.1	Ganoderma lucidum partial lac2 gene for laccase, exons 1-7	82.4	80%

3.3.2 Sequence of *lcc2* gene of *Pycnoporus sanguineus* MUCL 38531

Isolation of the *lcc2* gene from the genome of *Pycnoporus sanguineus* MUCL 38531 has been achieved by PCR, set up with "*lcc2*-forward" and "*lcc2*-reverse" primers. The fragment ca. 2.2 kb was cloned into the pDrive cloning vector, positive plasmids were selected and sequenced. Identity of *Lcc2* gene to other fungal laccases was remarkably lower compared to the identitiy of *lcc1* and *lcc2* gene exhibited 68% identity with the complete sequence of *pox 3* gene from the basidiomycete CECT 20197 (Mansur et al. 1997), 78% with *Trametes sp.* AH28-2 laccase C (*lacC*) gene, partial cds (Xiao et al. 2006), 67% with *Trametes sp.* AH28-2 laccase A (*lacA*) gene, complete cds (Xiao et al. 2006) (Table 3.13). Moreover, *lcc2* gene revealed 61% identity with recently reported *Pycnoporus coccineus* strain BRFM 938 laccase gene and 56% with laccase gene from *Pycnoporus sanguineus* strain BRFM 66 (Uzan et al. 2010). Isolated *lcc2* gene has been submitted to the NCBI database.

The *lcc2* gene is 2296 bp and includes an open reading frame of 1713 bp with 56% GC content. The open reading frame is interrupted by 10 introns as observed in the *lcc1* and the length of introns is between 53 and 67 base pairs and consistent with the most fungal introns (Figure 3.32). Furthermore, all introns have the consensus sequences conserved in the eukaryotes at the 5'-splicing site as GTRNGY (Y=C, T; R=A, G; N=A,G, C, T) and at the 3'-splicing site as YAG (Giardina et al. 1999).

3.4 Expression of laccase cDNAs in Methylotrophic Yeast *Pichia pastoris*

Present study is the first report on the heterologous expression of both laccases of *Pycnoporus sanguineus* MUCL 38531. In this study, isolated *lcc1* and *lcc2* cDNAs of *Pycnoporus sanguineus* MUCL 38531 were succesfully expressed in yeast *Pichia pastoris*. Cloning of cDNAs into the expression vector, transformation of the yeast and the studies performed to optimize the expression conditions are explained below.

Figure 3.32 : Sequence of *lcc2* gene from *Pycnoporus sanguineus* MUCL 38531.

Table 3.13: Similarity of *lcc2* gene to other known laccases.

Accession number	Laccase	Score	Maximum identity
U65401.1	Basidiomycete CECT 20197 phenoloxidase (pox3) gene, complete cds	744	68%
AY839937.1	Trametes sp. AH28-2 laccase C (lacC) gene, partial cds	781	78%
M60561.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	288	64%
EU678775.1	Pycnoporus sanguineus strain BRFM 118 laccase gene, partial cds	476	88%
AY839936.1	Trametes sp. AH28-2 laccase A (lacA) gene, complete cds	466	67%
AF388910.1	White-rot fungus AH28-2 laccase gene, partial sequence	466	67%
EU684155.1	Pycnoporus sanguineus strain CIRM-BRFM 881 laccase gene, partial cds	469	88%
EU678772.1	Pycnoporus sanguineus strain CIRM-BRFM 901 laccase gene, partial cds	476	90%
M60560.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	269	64%
AY147188.1	Pycnoporus cinnabarinus laccase (Lcc1) gene, complete cds	540	81%
EU678773.1	Pycnoporus sanguineus strain CIRM-BRFM 902 laccase gene, partial cds	456	86%
AF025481.1	Pycnoporus cinnabarinus laccase (lcc3-1) gene, complete cds	540	80%
AY510604.1	Pycnoporus sanguineus laccase gene, complete cds	504	88%
EU678779.1	Pycnoporus sanguineus strain CIRM-BRFM 893 laccase gene, partial cds	447	84%
EU678776.1	Pycnoporus sanguineus strain CIRM-BRFM 906 laccase gene, partial cds	451	86%
EU678768.1	Pycnoporus sanguineus strain CIRM-BRFM 896 laccase gene, partial cds	449	86%
AF123571.1	Pycnoporus cinnabarinus laccase (lcc3-2) gene, complete cds	490	82%
EU684154.1	Pycnoporus cinnabarinus strain BRFM 231 laccase gene, partial cds	443	83%
EU684156.1	Pycnoporus cinnabarinus strain BRFM 44 laccase gene, partial cds	452	86%
EU714500.1	Pycnoporus cinnabarinus strain CIRM-BRFM 945 laccase gene, partial cds	452	84%
EU683258.1	Pycnoporus cinnabarinus strain MUCL 30555 laccase gene, partial cds	452	86%
AJ420900.1	Pycnoporus cinnabarinus partial mRNA for laccase (lcc3-1 gene)	429	81%
L49377.1	Trametes villosa (clone LCC2) laccase gene, exons 1-11, complete cds	406	88%
EU678780.1	Pycnoporus sanguineus strain CIRM-BRFM 897 laccase gene, partial cds	422	84%
EU678767.1	Pycnoporus sanguineus strain CIRM-BRFM 895 laccase gene, partial cds	416	84%
EU684159.1	Pycnoporus cinnabarinus strain CIRM-BRFM 237 laccase gene, partial cds	441	83%
EU684157.1	Pycnoporus cinnabarinus strain BRFM 247 laccase gene, partial cds	441	83%
EU678782.1	Pycnoporus sanguineus strain CIRM-BRFM 905 laccase gene, partial cds	398	83%
EU678781.1	Pycnoporus sanguineus strain BRFM 898 laccase gene, partial cds	413	83%
EU678771.1	Pycnoporus sanguineus strain CIRM-BRFM 900 laccase gene, partial cds	407	83%
EU678770.1	Pycnoporus sanguineus strain CIRM-BRFM 899 laccase gene, partial cds	418	84%
EU678769.1	Pycnoporus sanguineus strain BRFM 894 laccase gene, partial cds	409	81%

3.4.1 Cloning of the full-length laccase cDNA into the expression vector

Laccase cDNAs were cloned into yeast shuttle expression vector pPICZB with its own secretion signal sequence. Signal peptides are necessary to direct proteins into the secretory pathway and the use of the signal sequence of recombinant proteins's itself is generally succesful for set up a secretory expression system, especially in *Pichia pastoris* (Daly and Hearn, 2005). The pDrive cloning vector having the PCR product and the pPICZB expression vector were digested with *Eco*RI and *Not*I. Resulting fragments were recovered from the gel and ligated with pPICZB using T4 DNA Ligase (Roche). Following to transformation of *E.coli* Top10 F' with *lcc1*/pPICZB and *lcc2*/pPICZB constructs, sequencing was carried out to confirm that the laccase cDNA was in frame with the C-terminal peptide before yeast transformation.

3.4.2 Transformation of yeast competent cells

Selected recombinant vectors were linearized within the 5'*AOX1* region with *Sac*I to integrate into the 5' *AOX1* region of host's genome via homologous recombination . (Daly and Hearn, 2005). Recombinant pPICZB transferred into chemically competent *Pichia pastoris* strain X-33 cells and laccase-producing transformants were selected on minimal methanol plates supplemented with 0.2mM ABTS after incubation for 3 days at 30˚C. Laccase secreting transformants oxidized the ABTS and were identified by the green zone around the *Pichia* colonies (Figure 3.33).

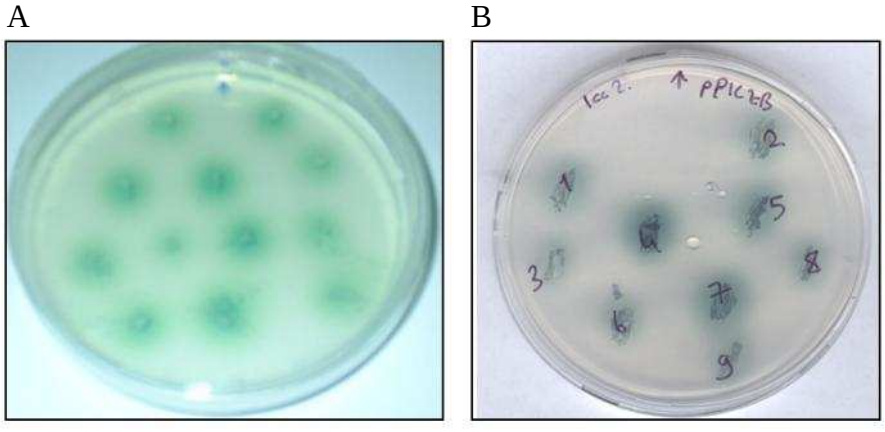


Figure 3.33 : The agar plate assay to select Pichia pastoris recombinants containing laccase cDNA on minimal methanol media supplemented with 0.2mM ABTS, A. lcc1 transformants, B. lcc2 transformants.

3.4.3 Laccase production

Positive transformants were assayed for extracelluar laccase production by growing in liquid buffered minimal methanol medium. The amount of extracellular laccase activity ranged from 10 Ul^{-1} to 55 Ul^{-1} among 27 LCC1-producing clones and clone 3 was selected as the best LCC1 producer (Figure 3.34-A). However, the recombinant LCC2 activity were between 2.5 Ul^{-1} and 35 Ul^{-1} and the maximum activity was observed for clone 8 (Figure 3.34-B). The best laccase producer clones were, then, selected and used to study the time course activity of recombinant enzyme and to characterize the recombinant enzyme.

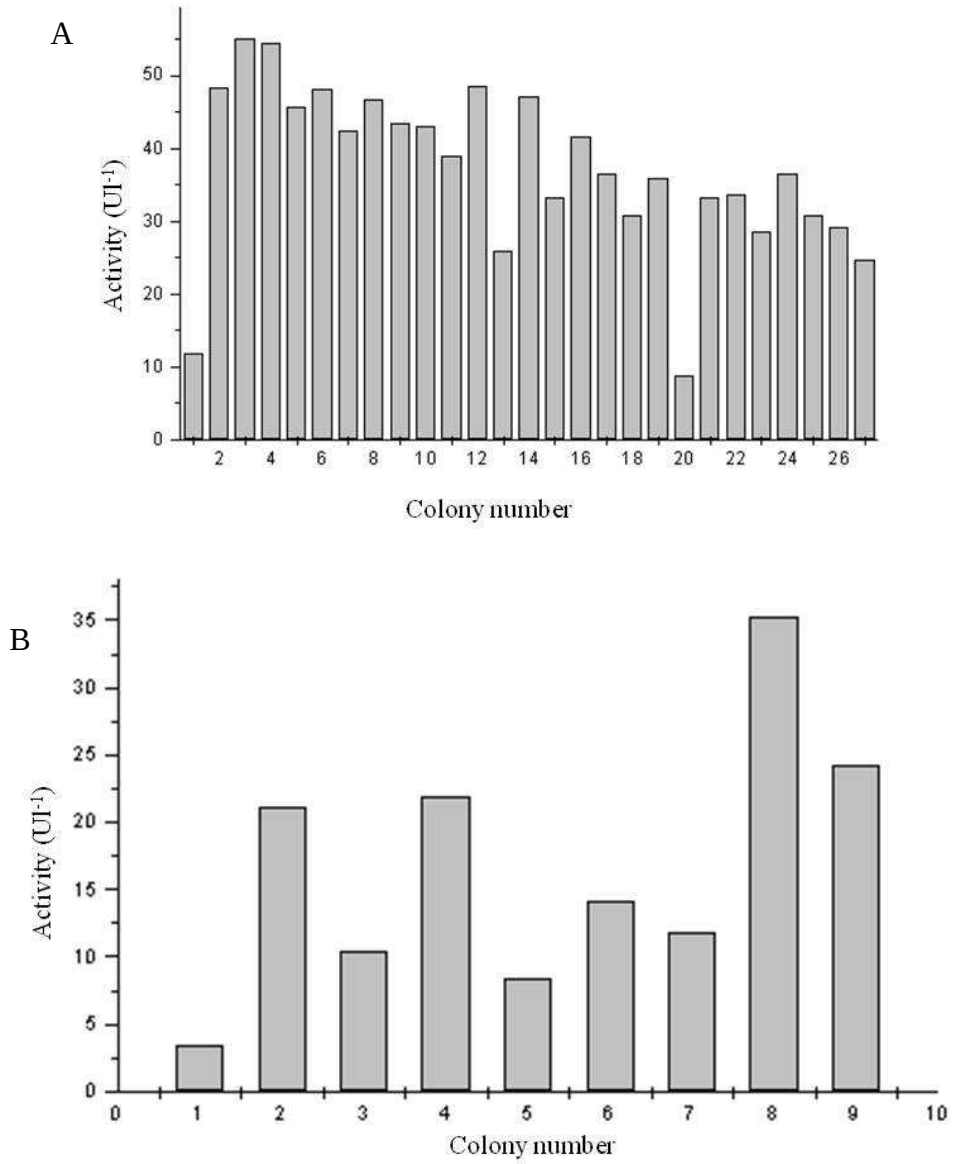


Figure 3.34 : Selection of the transformant with highest laccase activity in buffered minimal methanol medium, A. Lcc1, B. Lcc2 transformants.

3.4.4 Optimisation of expression conditions

High level protein production for any heterologous protein is achieved by optimization of expression conditions. Earlier studies reported the importance of pH and copper availability on the activity of laccase enzyme (O'Callaghan et al. 2002, Liu et al. 2003), so laccase activity was compared in four different media as a preliminary study for expression optimisation. The best laccase producer clones were incubated in different media including buffered minimal methanol medium (BMM), buffered minimal glycerol medium (BMG), 0.2 mM $CuSO_4$ added BMM and 0.2 mM $CuSO_4$ - 0.3 % Ala containing BMM. Protein production of *Pichia pastoris* in the low pH media (pH 3.0) is known to be possible and sometimes it is preferable (Yaver et al. 1996). But laccase activity results presented here revealed that, pH of the buffered minimal methanol medium (pH 6.5) was decreased to acidic region in the enzyme production phase because of the release of ligninolytic enzymes and other excreted acidic factors (Majeau et al. 2010). Low pH in the buffered minimal methanol medium and buffered minimal methanol medium with $CuSO_4$ had decreased the laccase activity compared to 0.2mM $CuSO_4$ and 0,3 % Ala containing buffered minimal methanol medium (pH 6.5) and it may be due to the susceptibility to acidic proteases (Figure 3.35). O'Callaghan et al. (2002) explained the effect of alanine by supposing the metabolism of alanine released ammonia that neutralised acidic end products of methanol metabolism and increased laccase activity was observed in the alanine-added *Trametes versicolor* laccase producing *Pichia pastoris* cultures. pH-controlled culture by alanine increased the laccase activity because of the increased enzyme stability and the activity was reached a maximum of 276 units after 7 days in that medium. Oxygen availability has also an important impact on heterologous production of laccase in *Pichia pastoris* and cultivation have been carried out with 20% total volume of the shaking flasks. Subsequently optimal production of recombinant laccase was carried out in terms of exploring the effect of nutrients and methanol concentration, cultivation temperature on laccase activity. Optimum copper concentration, as an inducer, has also been found for the laccase activity.

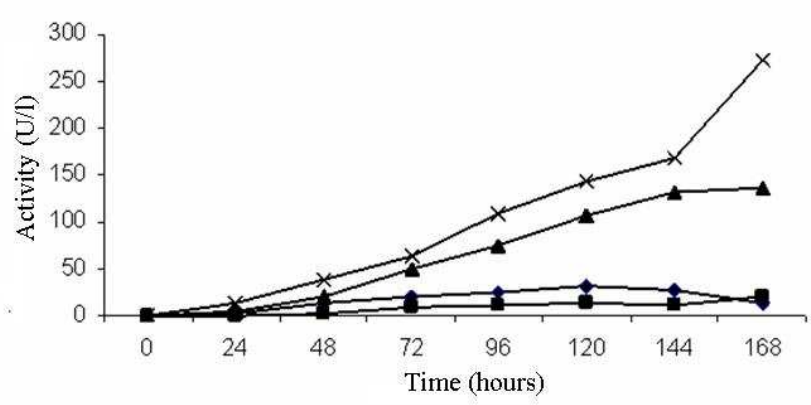


Figure 3.35 : Laccase production of Pichia pastoris transformants in methanol containing buffered minimal medium (♦), methanol free buffered minimal glycerol medium (■), methanol containing buffered minimal medium with 0.2mM CuSO4(▲),methanol containing buffered minimal medium with 0.2mM CuSO4 and 0.8% D-alanine (×).

3.4.4.1 Effect of cultivation temperature

Heterologous protein expression is affected by several important environmental factors, such as pH, osmolarity, nutrients and also growth temperature. Protein folding/aggregation, secretion and stress responses are common responses to those factors (Gasser et al., 2007). Optimum growth temperature for *P. pastoris* is usually defined as 28–30ºC and the previous studies have revealed that a reduction of the growth temperature for example 25ºC could improve the recombinant protein production without preventing the growth (Gasser et al. 2007). Decreasing the cultivation temperature was found to be beneficial for the laccase expression as indicated in the study of Cassland and Jönsson (1999). Changing the growth temperature affects many cellular processes, including the central carbon metabolism, stress response and protein folding. Growth temperature may have a strong impact on specific productivity by affecting regulation of specific genes. Molecular links between protein folding and temperature was explored by different works and specific productivity was revealed at the transcriptional level. Host cells react to recombinant protein production by various metabolic and intrinsic stresses, such as the unfolded protein response (UPR) pathway and induction of oxidative and osmotic stress response genes are overlapped by UPR. It has been explained that a downregulation of environmental stress response genes occurs, while upregulation of ribosome biogenesis genes and enhanced transcription of the secretory pathway components have been observed at lower temperature. Consequently, the higher rate

of correctly folded recombinant protein secretion has been accomplished (Gasser et al. 2007, Graf et al. 2009, Dragosits et al. 2008). In order to observe the effect of the temperature on the laccase activity, cells were grown in buffered minimal methanol medium at 23ºC and 28ºC. Cultures were assayed for the laccase activity for 7 days and the highest values were compared. Laccase activities of the lcc1 culture for seven days is given in the Figure 3.36. Based on these results, both LCC1 and LCC2 cultures were cultivated at 23ºC in this study.

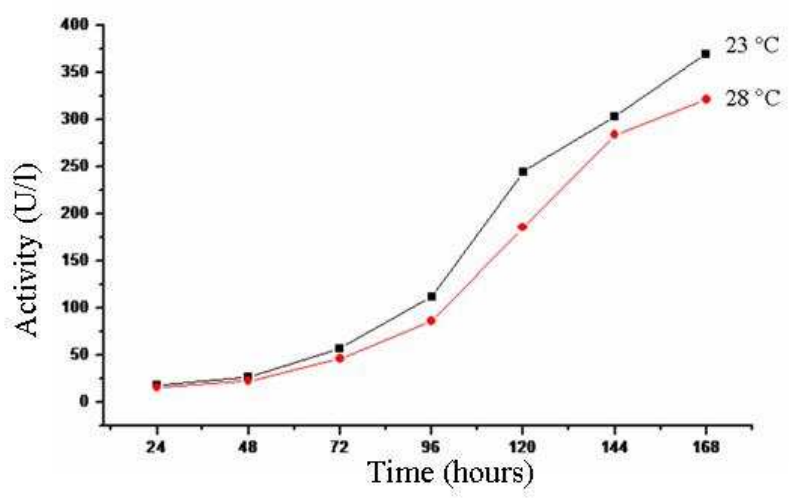


Figure 3.36 : Determination of the optimum cultivation temperature of P.pastoris transformant expressing lcc1 cDNA in buffered minimal methanol medium containing 0.2mM CuSO4 and 0.3% alanine.

3.4.4.2 Effect of Methanol Concentration and Nutrients

The effect of the different media on the laccase activity was tested with buffered complex methanol medium (BMMY) and buffered minimal methanol medium (BMM), containing 0.3 % alanine to eliminate pH effect. *Pichia pastoris* use methanol as a sole carbon source and methanol was added daily to induce *Aox1* promoter to induce laccase expression. Effect of different methanol concentrations (0.5 %, 0.75 % and 1 %) were also tested in the BMMY and BMM medium at 23ºC for 7 days.

Expression of laccase depends on the growth conditions, medium compositions and strain. Tryptone and peptone are the favourite nitrogen sources. Although nitrogen limitation is defined as the major factor for enhancing ligninolytic enzyme production in some species, fungal laccases that are not affected by nitrogen limitation should be preferred for large-scale production as the increasing growth rate

of biomass causes improved laccase activity (Majeau et al. 2010). Collins and Dobson (1997) reported the regulation of laccase expression in *Trametes versicolor* at the transcriptional level by nitrogen, as the increased nutrient nitrogen concentration caused the increased levels of laccase mRNA. Consistently, laccase activity was about 7 fold higher in the BMMY, containing yeast extract and peptone, by preventing the proteolysis of secreted proteins in this study.

Pichia pastoris is a methylotrophic yeast and uses methanol as a sole carbon source. Heterologous expression of the proteins is performed by cloning cDNAs into the expression vector under the control of the promoter *Aox1* of alcohol oxidase gene which is induced by methanol. This strong promoter is controlled at the gene transcription level and is used to drive the expression of high level of recombinant proteins even with the single integrated copy of the gene of interest (Cereghino and Cregg, 2000; Daly and Hearn, 2005). Therefore methanol concentration added to the growth medium should be optimized to induce laccase activity without inhibiting the growth of cells. In this study, 1 % MeOH was the best concentration to induce expression (Figure 3.37), as observed in the recombinant laccases of *Pycnoporus cinnabarinus* and *Trametes versicolor* expressed in *P. pastoris* (Otterbein et al. 2000; O'Callaghan et al. 2002). Consistently, Guo et al. (2006) reported the enhanced activity of recombinant *T. versicolor* laccase expressed in *Pichia methanolica* with increasing methanol concentration and 0.8% methanol has been explained as the optimum concentration. However, Lu et al.(2009) reported the 0.5% methanol as optimum concentration to induce heterologous laccase expression in *Pichia pastoris*. Based on these results, recombinant LCC2 production has been performed in the same medium and cultivation conditions.

In this study, different concentrations of copper sulfate was added into the BMMY medium and the highest level of activity was 120 U l^{-1} in the culture grown in 0.6 mM $CuSO_4$ containing buffered complex methanol medium after 7 days of growth in shake flask and measured laccase activity was about 3 fold higher than the control sample grown in the BMMY without copper sulfate. All measurements were triplicated and standard devitiations have not generally exceeded 10 % of the mean values. It has been observed that the level of laccase activity in the control culture was much more lower than the other cultures supplemented with copper sulfate (Figure 3.39-B).

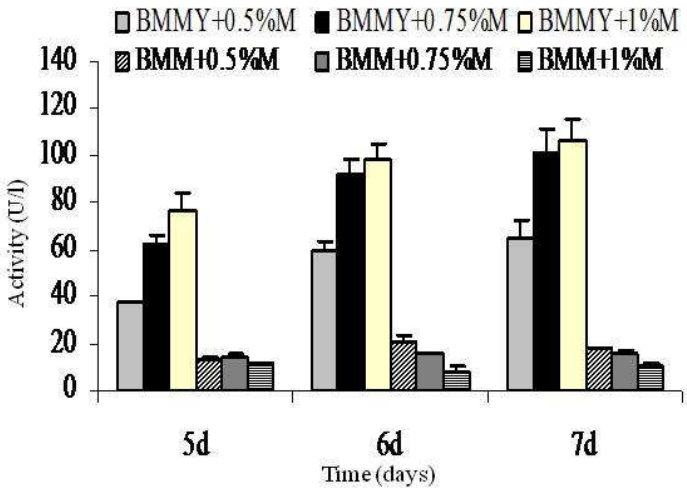


Figure 3.37 : Effect of different media and methanol concentration on the laccase activity.

Total protein concentration reached 143 mg l^{-1} after 8 days of growth and the increasing copper concentration did not affect protein synthesis degree, whereas laccase activity was decreased due to the copper requirement of the enzyme (O'Callaghan et al. 2002). Furthermore biomass of the culture, containing 0.6 mM $CuSO_4$, was lower than the other concentrations and even the control sample, indicating that the increased laccase activity was not due to increased biomass and increasing copper concentrations have not any toxic efffect on the biomass of the cultures (Figure 3.38-A). Collins and Dobson (1997) reported the effect of copper concentrations on the laccase gene transcription level of *Trametes versicolor* and tested different concentrations of copper sulfate. The amount of the highest laccase transcript and activity was observed at 0.4 mM $CuSO_4$, whereas low level of laccase transcripts detected in the absence of copper as observed in this study, suggesting that the existence of a correlation between copper concentration and the of the transcription *lcc1*. The effect of different concentrations of copper sulfate on the LCC2 activity was also detected by adding copper sulfate into the BMMY medium and the highest enzyme activity was 148 U l^{-1} in the culture grown in 2 mM $CuSO_4$ after 6 days of growth and measured laccase activity was about 3 fold higher than the control sample grown in the BMMY without copper sulfate (Figure 3.38-B).

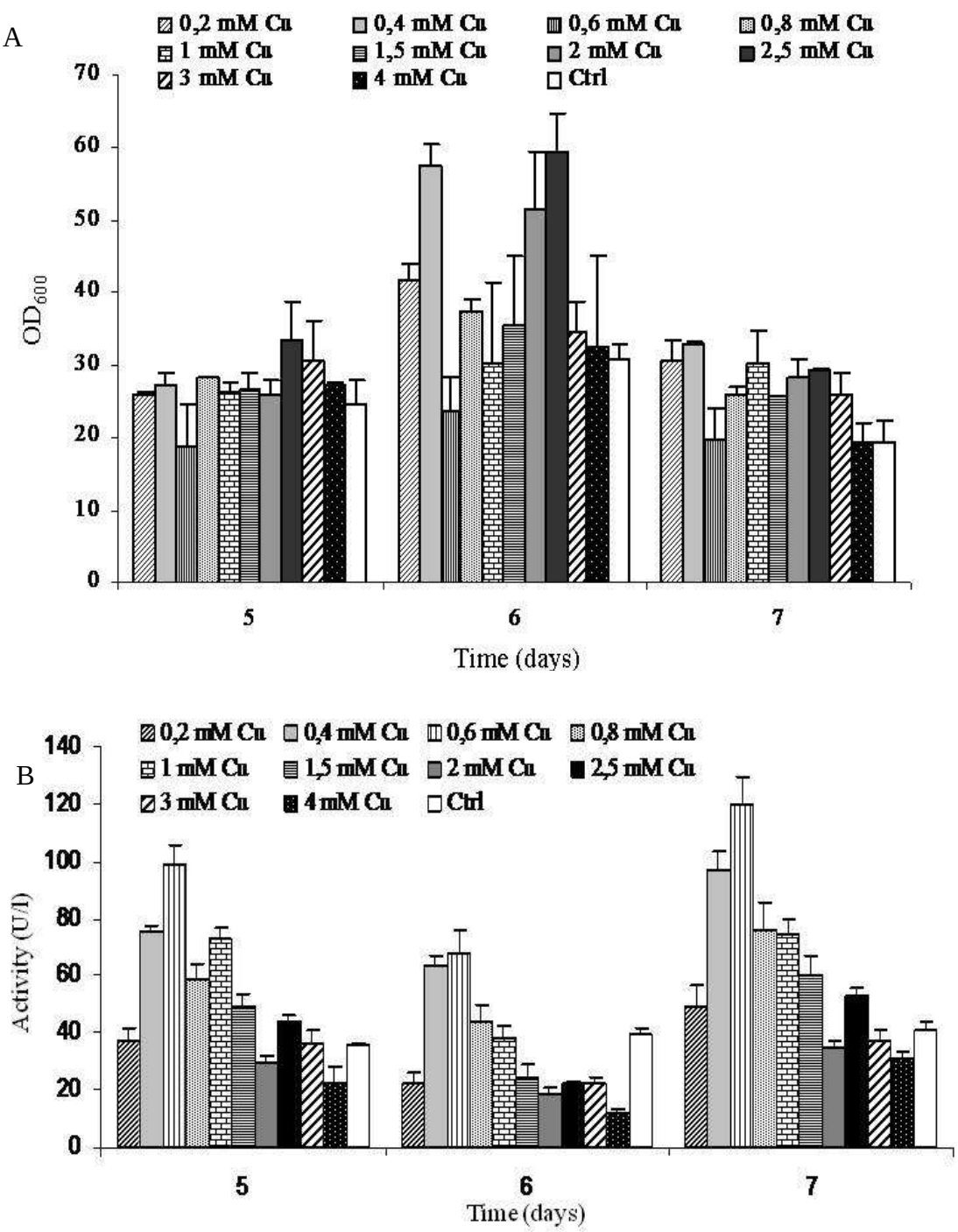


Figure 3.38 : Effect of the different copper concentrations on the LCC1 activity of P.pastoris cultivated in BMMY at 23ºC. A. Growth profile, B.Laccase activity of the culture.

Maximum total protein yield was 104 mg l^{-1} on the 8th day of the growth. The growth of cultures have not been affected with increasing concentration of copper, whereas the OD600 values have decreased in the presence of 3 mM and more copper concentrations (Figure 3.39-A, Figure 3.39-B). Furthermore, laccase activites of the all cultures containing copper sulfate were higher than the control culture without

copper. Fonseca et al. (2010) have reported that, copper produced an increase of constitutive laccases in fungi *G. applanatum*, *Peniophora sp.*, *C. versicolor* and *P. sanguineus*, although biomass of *Pycnoporus sanguineus* underwent a growth inhibition with 1 mM copper.

Expression levels obtained in this study for both LCC1 and LCC2 was remarkably higher than the recombinant laccase of *Pycnoporus cinnabarinus* expressed in *Pichia pastoris* with 8 mg l^{-1} (Otterbein et al. 2000) and recombinant lac4 laccase of *Pleurotus sajor-caju* with 4.85 mgl^{-1} yield (Soden et al. 2002). Acquired concentration of the recombinant protein was also higher than lac1 of *Pycnoporus cinnabarinus* heterologously expressed in *Aspergillus oryzae* (80 mg l^{-1}) and *Aspergillus niger* (70 mg l^{-1}) (Sigoillot et al. 2004). Recombinant laccase activity and protein production can be further increased with fermentor-based production as reported by Colao *et al.* (2006). *Trametes trogii* LCC1 laccase in fermentor culture of *Pichia pastoris* was expressed with 14 fold higher activity than shake flasks culture.. Furthermore LCC2 was expressed by using fermentor with 286 mg l^{-1} total soluble protein concentration on the 6th day of the growth (Colao et al. 2009).

3.5 Purification of the Recombinant Laccases from *Pichia pastoris*

Pichia pastoris culture of LCC1 and LCC2 laccases were grown in 1 l shake flasks containing BMMY supplemented with 1 % MeOH, 0.3 % Ala and optimized concentration of copper sulphate for 8 days. 1 mM phenylmethylsulphonyl fluoride (PMSF) was added to the culture supernatant to inhibit serine proteases.

Following to ammonium sulphate precipitation, proteins were purifed by using anion exchange chromatography and size exclusion chromatography. Purified proteins were visualized on SDS-PAGE and laccase activity was observed by zymogram analysis on the gel. The general purification techniques used for laccases involve ultrafiltration, ammonium sulphate precipitation, ion exchange, gel filtration and hydrophobic interaction chromatographies (Madhavi and Lele, 2009).

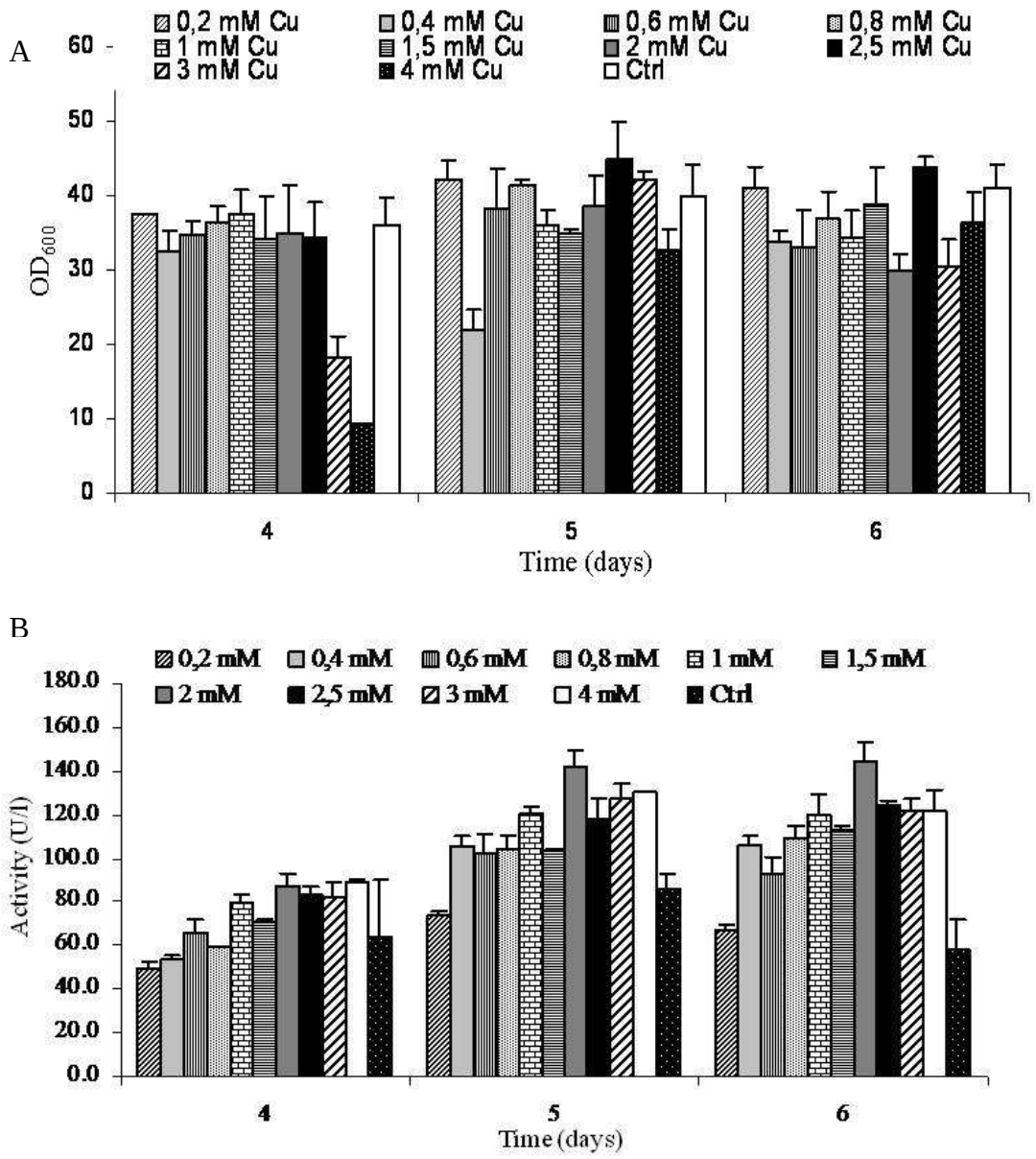


Figure 3.39 : Effect of different copper concentrations on the growth (A) and laccase activity (B) of LCC2 producing P. pastoris culture

3.5.1 Purification of R-LCC1

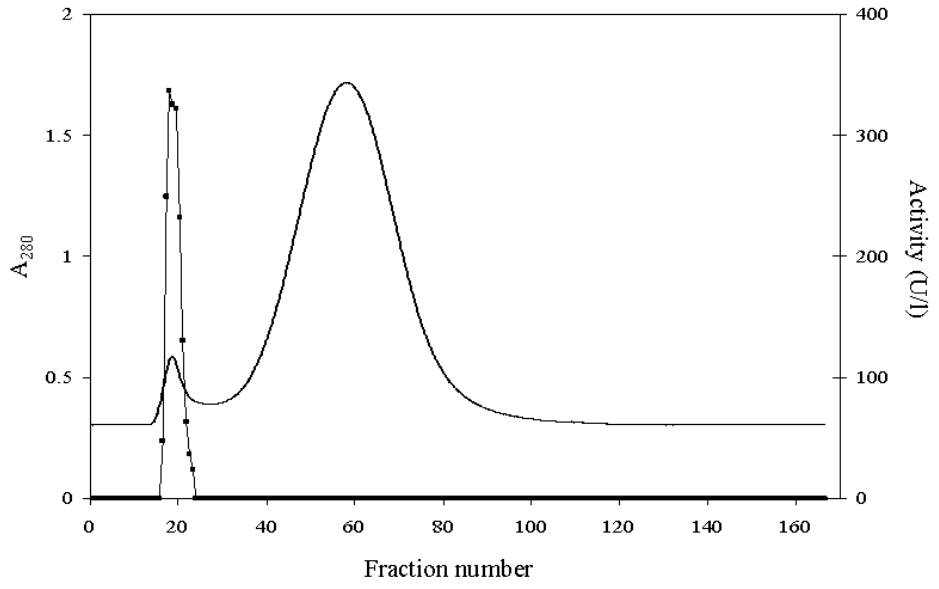
Protein precipitation was carried out by adding $(NH_4)_2SO_4$ to the culture supernatant and optimization of the ammonium sulphate concentration was carried out by adding the salt between 40 % to 90 % concentrations with 10 % increment at 4˚C. Laccase activity of the pellets have been tested and saturation with 90 % salt was found as the ideal amount for laccase precipitation. Following to precipitation, the pellet was resuspended in 20 mM sodium phosphate buffer, pH 7.2 and salts were removed by ultrafiltration using Amicon Ultra-15 centrifugal filter units. Sample was first applied onto the Q-sepharose resin equilibrated with 20 mM sodium phosphate buffer, pH

7.2 and eluted proteins at a flow rate of 2 ml min^{-1} were detected by the routine laccase activity assay. Recombinant laccase was successfully separated from dark brown colour present in the supernatant with the anion exchange chromatography and this procedure on Q-sepharose resin provided 22 fold purification with a final enzyme yield of 83.7 %. Activity of the fractions were assayed by routine measurement procedure. The resultant chromatogram and also active fractions are shown in Figure 3.40-A. Pooled active fractions were concentrated by ultrafiltration and the sample was loaded onto the Sephadex G-100 column pre-equilibrated with the same buffer to seperate proteins depending on their sizes and fractions were obtained with the flow rate of 0.3 ml min^{-1}. Active fractions were collected from the first peak region in Figure 3.40-B , concentrated and resulted in 39.4 fold purification with 44.5 % yield. The laccase activity, specific activity yield percentage and fold purification values at all purification steps are summarized in Table 3.14. Recombinant LCC1 was purified from *Pichia pastoris* culture with 82.7 U mg^{-1} specific activity and 44.5 % yield was more than the yield obtained by hydrophobic interaction chromatography in *Pycnoporus sanguineus* CCT-4518 (Garcia et al. 2007), and it was very close to the *P. sanguineus* CelBMD001 (Vite-Vallejo et al. 2009). Purified recombinant laccase was run on 12% SDS-polyacrylamide gel and purified laccase was monomeric and single band with ca. 60 kDa was observed on the SDS-PAGE. (Figure 3.41-A).

Table 3.14: Recombinant LCC1 laccase purification steps from *Pichia pastoris* culture

Purification step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification Fold
$(NH_4)_2SO_4$ precipitation	46122	22	2096	100	1
Anion exchange	38595	0.835	46221	83.7	22
Size exclusion	20548	0.248	82842	44.5	39.4

A



B

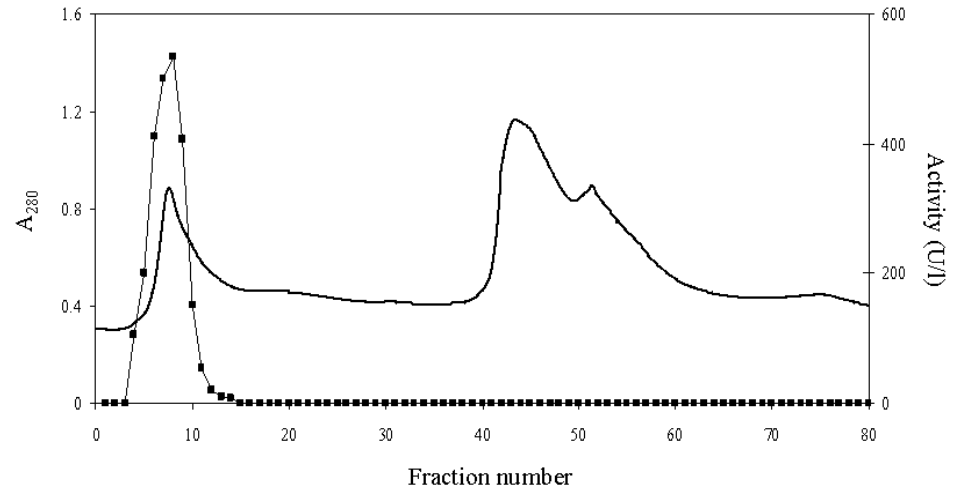


Figure 3.40 : Elution profile of recombinant LCC1 laccase applied on A. size-exclusion and B. anion-exchange chromatography columns.

Laccase activity on the SDS-PAGE was observed by treating gel with ABTS and green colour occured indicating the substrate oxidation after several minutes of incubation (Figure 3.41-B).

Fungal laccases are copper-containing glycoproteins, molecular weight is between 55 and 85 kDa with 15–25 % carbohydrate and generally observed as monomeric proteins (Arora and Sharma 2010, Rodgers et al. 2009). Molecular weight of the recombinant laccase was similar to other fungal laccases and was not hyperglycosylated in contrast to the recombinant Lac protein of *Pycnoporus cinnabarinus* expressed in *Pichia pastoris* (Otterbein et al. 2000). The molecular

weight of the purified laccase was also consistent with the molecular weights of other *Pycnoporus sanguienus* strains reported by Pointing and Vrijmoed (2000) and Garcia et al. (2006). Furthermore, the feature of reacting with ABTS on the SDS-polyacrylamide gel indicates the strength of the enzyme and its ability to use in the applications in the presence of detergents, such as decolorization of textile dyes (Dantan-Gonzalez et al. 2008).

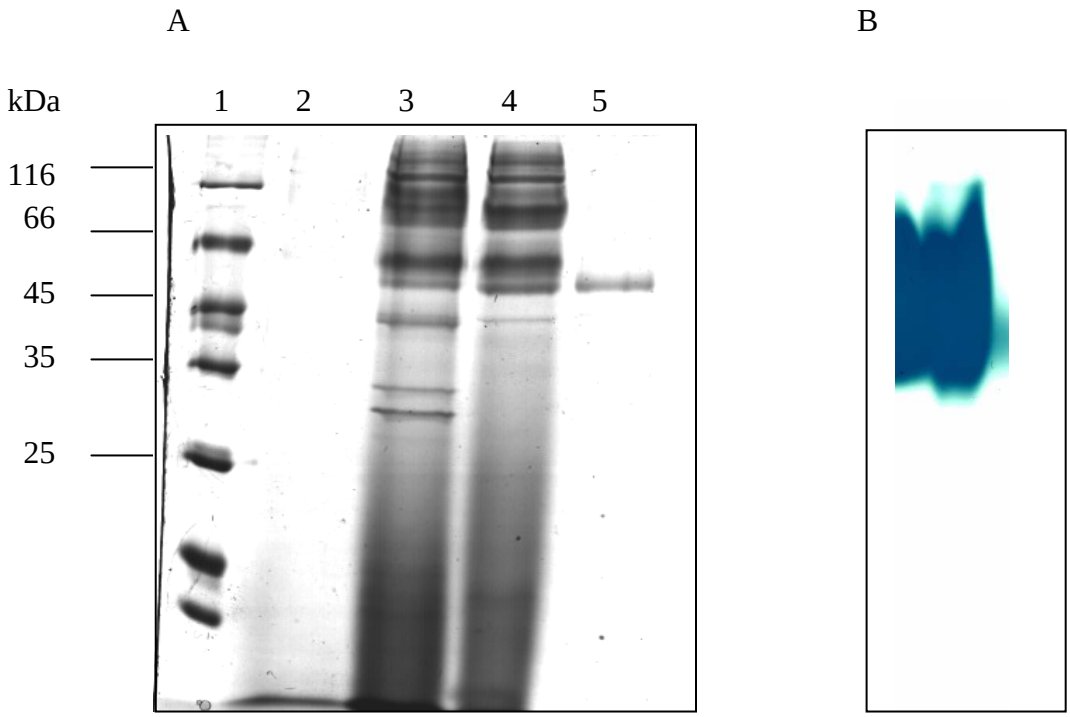


Figure 3.41 : Coomassie brilliant blue stained SDS-PAGE analysis of purified laccase from Pichia pastoris carrying lcc1 cDNA, lane 1: Protein molecular weight marker (Fermentas); lane 2: crude extract of P.pastoris; lane 3: laccase from anion exchange chromatography; lane 4: purified laccase by gel filtration chromatography. B Zymogram analysis on the SDS-PAGE stained with ABTS.

3.5.2 Purification of R-LCC2

Same purification procedures applied for LCC1 was also performed for purification of recombinant LCC2 from *Pichia pastoris* culture. Proteins were separated from dark brown colour present in the supernatant with the anion exchange chromatography and this procedure on Q-sepharose resin provided 26.7 fold purification with a final enzyme yield of 85 %. Elution profile of the anion Exchange chromatography is as in the Figure 3.42-A. Active fractions were pooled, applied onto the Sephadex G100 column and proteins are seperated by size exclusion chromatography. Active fractions collected from the first peak region in Figure 3.42-

B were concentrated and resulted in 17.4 fold purification with 26.6 % yield. The laccase activity, specific activity yield percentage and fold purification values at all purification steps are summarized in Table 3.15.

Table 3.15: Summary of recombinant LCC2 purification from *Pichia pastoris* culture

Purification step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification Fold
Crude	62349	23.2	2687	100	1
$(NH_4)_2SO_4$ precipitate	52937	16.2	3267.7	85	1.22
Anion exchange	53735	0.75	71646.9	86.2	26.7
Size exclusion	16597	0.354	46936.2	26.62	17.47

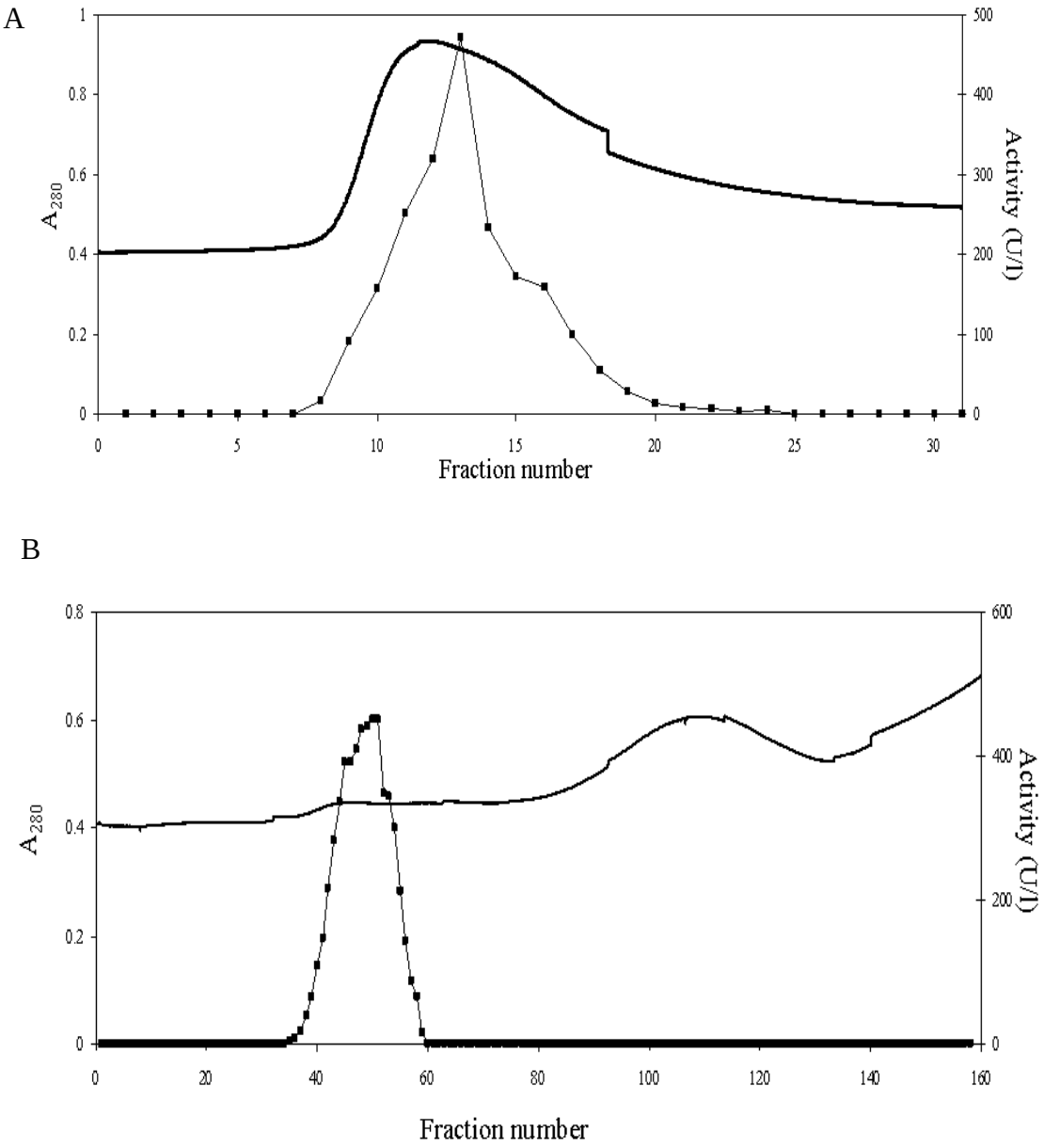


Figure 3.42 : Elution profile of recombinant LCC2 laccase applied on A. anion-exchange chromatography and B. size-exclusion chromatography columns.

Purified recombinant laccase was run on 12% SDS-polyacrylamide gel and the molecular mass was approximately 62 kDa (Figure 3.43-A). Laccase activity on the SDS-PAGE was observed by treating gel with ABTS and green colour occured indicating the substrate oxidation after several minutes of incubation (Figure 3.43-B).

Recently Koschorreck et al. (2008) have been cloned four laccase isoenzymes from *Trametes versicolor* and expressed them in *Pichia pastoris*. Purified laccases were migrated on the SDS-polyacrylamide gel and proteins were about 72 kDa and they supposed that the higher molecular weight compared to the native enzyme was the

result of the hyperglycosylation in *P. pastoris*. Besides, Lu et al. (2010) reported the recombinant laccase from *Pycnoporus sanguineus* H275, expressed in *P. pastoris*, with higher molecular weight than the native one. On the contrary to these results, neither R-LCC1 nor R-LCC2 was hyperglycosylated as the molecular weight of the two laccases on the gel was close to the theoretical molecular weight of the deduced amino acid sequences (Url-9).

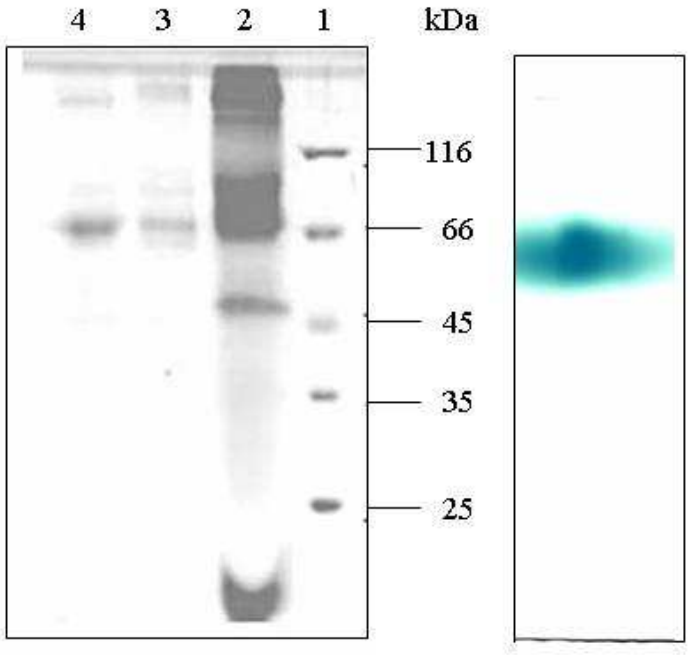


Figure 3.43 : SDS-PAGE analysis of purified laccase from LCC2 producer Pichia pastoris culture, A. Purified laccase, lane 1. Protein molecular weight marker (Fermentas), lane 2. crude extract of P.pastoris, lane 3.pooled laccase fractions from anion exchange chromatography, lane 4. purified laccase, B. Activity assay on SDS-PAGE gel

3.6 Biochemical Characterization of Recombinant Laccases

Biochemical characterization is very important to enlight the properties of enzyme at the molecular and kinetic levels being a prerequisite for usage of those enzymes in biotechnological and industrial applications. Biochemical characteristics of the laccases has been reported extensively from basidiomycetes and ascomycetes group of fungi. In this study, purified recombinant laccases of *Pycnoporus sanguienus* MUCL 38531 were characterized in terms of determining enzyme activities at different pH and temperatures and assaying effect of several inhibitors on laccase activity for the first time in the literature. Spectral characteristics and catalytic properties were also examined.

3.6.1 Determination of Optimum pH

The optimum pH of the recombinant laccases have been determined by measuring activity at broad pH range from pH 2.0 to 7.0 for 2.5 mM ABTS and 2.5 mM DMP respectively. pH optima may vary with different substrates or with laccase isozymes of the same organism and each substrate of laccase has its own specific pH optima for its oxidation (Jordaan et al 2004; Arora and Sharma 2010). The optimum pH of the most fungal laccases are between 3.0 and 7.0 for phenolic substrates, whereas optimum pH of plant laccases can range up to 9.0 for plant laccases. Furthermore, the optimum pH for fungal laccases usually lies in the acidic range (between pH 3 and pH 5) when ABTS used as substrate and increasing pH values causes loss of enzyme activity. Optimum pH of the laccases from white-rot fungi is generally lower than the laccases of brown rot fungi and this may be related to the involvement of the white-rot laccases to the lignin breakdown, whereas the latter act as detoxification agents (Madhavi and Lele, 2009; Sinsabaugh 2010).

pH optima of both recombinant laccases characterized in this study was pH 3.0 for ABTS, whereas pH 4.0 for DMP with a bell-shaped profile (Figure 3.44) as proposed by Xu et al. (2007). Those values were identical with native laccase from *Pycnoporus sanguineus* SCC 108 (Litthauer et al. 2007) and also consistent with the other laccases, POXC from *Pleurotus ostreatus* (Palmieri et al., 1997) and *Trametes pubescens* (Galhaup et al., 2002). Furthermore, theoretical pI values of LCC1 and LCC2 were predicted as 5.7 and 5.5, respectively (Url-10).

Optimum pH profiles of laccases towards phenolic substrates are generally bell-shaped as observed in this study and varies among different laccases, because of the varied contribution of laccase, phenolic substrate and oxygen to the pH activity profile. Activity profile of a laccase for a phenolic substrate is affected by both the hydroxide anion binding to the T2/T3 coppers, causing to an inhibitory effect and also the redox potential difference betweeen the T1 copper site and reducing substrate at higher pH (Xu 1997; Madhavi and Lele, 2009; Sinsabaugh, 2010).

The structure and conserved residues of laccases have the important roles on the pH profile. The architecture of the reducing substrate binding cavity of the laccases has two conserved residues histidine and aspartic acid, which are located in close proximity to one other. Both residues are responsible for the initiation of catalytic cycle and the existence of these two dissociable polar residues associated with the

optimal acidic pH for the substrate oxidation. Substitution of this residue with an uncharged residue such as asparagine or alanine was resulted in the decreasing laccase activity at low pHs (Colao et al., 2009). Furthermore, replacement of the aspartic acid residue with less polar or apolar residues (Asn or Ala) shifted the optimal pH to higher pH values for phenolic substrates. Madzak et al. (2006) reported a shift in the optimal pH for DMP towards higher pH with Asp→Ala and Asp→Asn substitutions. As the pH optima of recombinant laccases of *Pycnopurus sanguineus* are acidic, it may be suggested that there is no conserved amino acid substitutions in the substrate binding site.

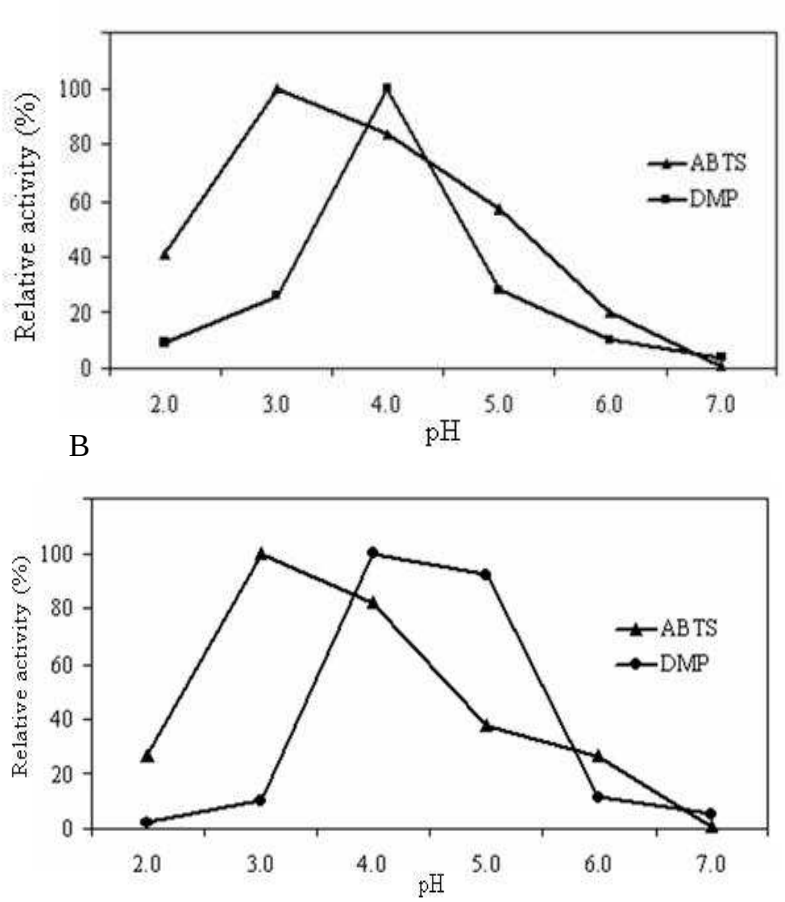


Figure 3.44 : pH optima of LCC1 (A) and LCC2 (B) laccases for ABTS and DMP substrates

3.6.2 Determination of Optimum Temperature and Thermal Stability

The optimum temperature of laccases may vary between the strains of an organism. Here, the effect of temperature on LCC1 activity was analyzed at different temperatures using ABTS as substrate. Thermal stability of enzyme was also assayed by incubating enzyme at those temperatures for 7 hours and measuring the retaining

laccase activity at 25°C. Optimal temperature is about 60 °C using ABTS as substrate and the activity has decreased with increasing temperature (Figure 3.45-A). The enzyme was stable at 30°C more than a week and $t_{1/2}$ were higher than 7 hours for both 30°C and 40°C. Half-lives of the enzyme were 80 min and 60 min for, 50°C and 60°C respectively. Activity rapidly decreased above 70°C and % 50 of activity was retained after 30 minutes of incubation at 80°C (Figure 3.45-B).

Optimum temperature for laccase activity is between 20-37 °C and laccases of most white-rot fungi is stable below 50 °C (Arora and Sharma 2010). Maximum laccase activity was observed at 60 °C and this value was higher than the optimum temperature of different *Pycnoporus sanguineus* strains (Garcia et al. 2006, Trovaslet et al. 2007). Half-life of the R-LCC1 was 80 min, 60 min and 30 min for 50°C, 60°C, 70°C and 80°C respectively. Thermal stability of the enzyme was higher than the recombinant laccase of *Pleurotus eryngii* expressed in *Aspergillus oryzae*, which lost its activity by incubation at 65°C for 30 min (Rodriguez et al. 2008).

A

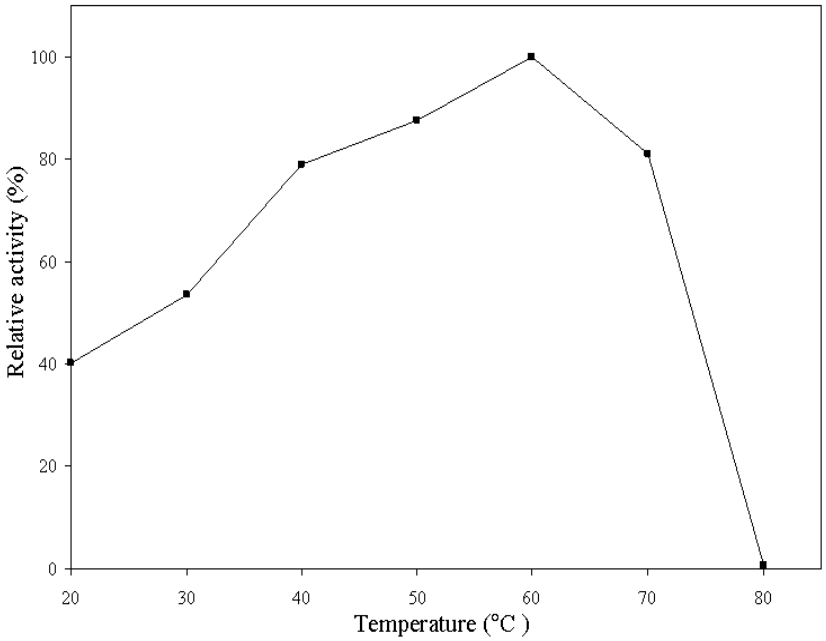


Figure 3.45 : Optimum temperature (A) and thermal stability (B) of recombinant LCC1

B

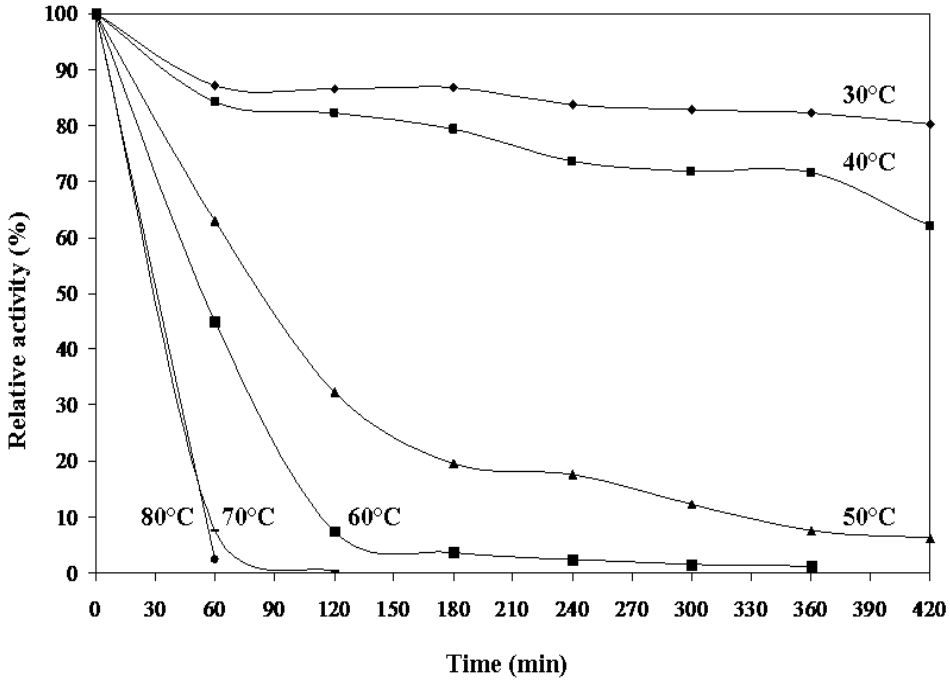


Figure 3.45 (continued): Optimum temperature (A) and thermal stability (B) of recombinant LCC1

The recombinant LCC2 laccase has also been tested for its thermal properties by determining its thermotolerance and thermostability. To determine the optimum temperature activity was measured at the concerned temperatures without pre-incubation. Optimal temperature is about 30 °C using ABTS as substrate and the activity has decreased with increasing temperature (Figure 3.46-A). The half-life ($t_{1/2}$) of the enzyme at 30°C and 40°C. were about 250 min and 45 min. Half-lives of the enzyme were 30 min for 50°C and rapidly decreased above 50°C and % 50 of activity was retained after 10 minutes of incubation at 70°C and 80°C (Figure 3.46-B).

Optimum temperature of LCC2 was lower than LCC1 and also LCC2 was less stable because of the lower half-life at 30°C. Activity was decreased more quickly in increasing temperatures compared to LCC1. Although half-live of the lcc2 seems very low at higher temperatures, optimum temperature of another white laccase from *Pleurotus ostreatus*, POXA2 was about 25-35 °C and $t_{½}$ was 12 min at 60 °C (Palmieri et al. 1997). Similar results were reported in the study performed with another strain of *Pycnoporus sanguineus*. Two laccase isoenzymes, namely Lac I and LacII were purified and molecular weight of LacI was higher than LacII as observed for R-LCC2. Optimum temperatures were 30°C and 50°C respectively.

Thermostability of LacI was less than LacII and those results were consistent with our data (Garcia et al. 2006).

Thermal stability of laccases correlates with the growth temperature of the source organism significantly. Thermostable laccases have generally been isolated from bacteria since fungal laccases quickly losses their activity at temperature over 60°C, but *Coriolopsis* and *Trametes* species are higher stability than other groups of basidiomycete. Although they have higher stability, thermal properties of different isozymes and isolates may differ (Hilden et al. 2009). Consistently, thermal characteristics of LCC1 and LCC2 laccases are different both in thermostability and thermotolerance. Thermotolerant response and thermostability of laccases result from several factors including 3D protein fold and packing, presence of metal ions, such as existence of four copper ions, and internal protein features, like salt bridges (ionic bonds) and dense hydrogen bonding. For example depletion of copper ions both inactivate laccase and uncouple the protein domains and changes in protein conformation causes to loss of activity at elevated temperatures (Bonomo et al. 2001, Hilden et al. 2009).

Laccases from *Pycnoporus* genus also have similar optimum temperature and stability characteristics with *Coriolopsis*. Although LCC1 stability was higher than LCC2 and some laccases from different *Pycnoporus sanguineus* strains, $t_{1/2}$ of laccase from SCC108 was 170 min at 75°C for the oxidation of DMP (Litthauer et al. 2007). This could be due to the lack of hyperglycosylation, providing stability to the enzyme by stabilizing the conformation and preventing the heat denaturation. Furthermore glycosylation type and its position also affects the thermal behaviour more than the attached amount of glycosylation to the protein. The carbohydrate composition of laccases are between 10 and 25 % and glycosylation is suggested to have roles in the stabilization of the conformation, the secretion of proteins and sensitivity to proteases (Hilden et al. 2009, Enguita et al. 2003, Uzan et al. 2010). Proline content has also known as its property of increasing the stability by causing tight packing. Proline content of LCC1 (8.3 %) was more than the proline contentof LCC2 (7.5 %), assuming the reason of the thermal stability of LCC1 compared to LCC2.

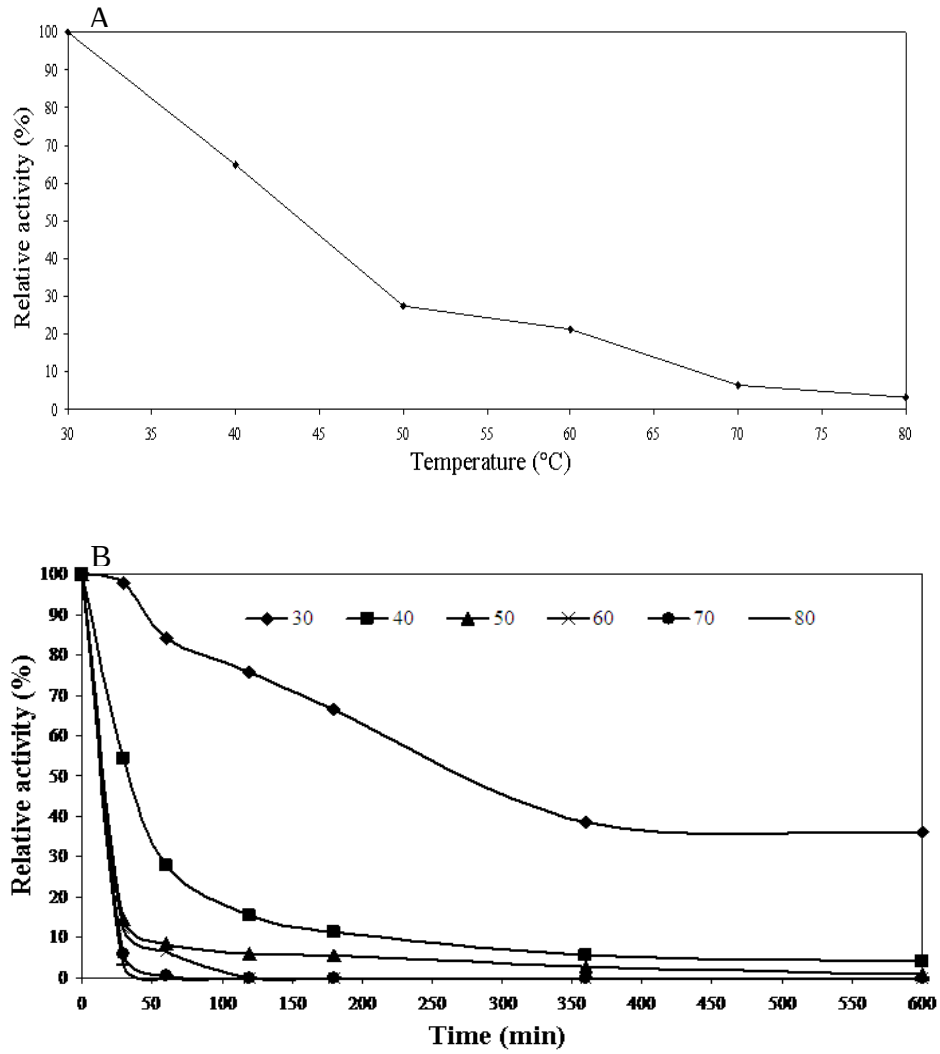


Figure 3.46 : Temperature optima (A) and thermal stability (B) of recombinant LCC2

3.6.3 Substrate specifity

The substrate specifity of the laccase was examined for different compounds as in the order of ABTS, DMP, guaiacol, ferulic acid, catechol and tyrosine by analyzing laccase activity. Extinction coefficient and wavelengths used in the activity calculation is listed in Table 3.16. As observed in the other laccases purified from different Pycnoporus sanguineus strains, the recombinant *P. sanguineus* laccases LCC1 and LCC2 oxidized a range of substrates, including nonphenolic heterocyclic compound ABTS and monoaromatic phenolic substrate DMP (Lu et al. 2007, Garcia et al. 2007, Uzan et al. 2010). Laccase activity of both LCC1 and LCC2 for ABTS was higher than the other substrates. Laccase activity for ABTS and DMP were also higher than guaiacol, ferulic acid and catechol. Recombinant LCC1and LCC2 laccases did not oxidize tyrosine as a typical laccase.

Laccases are classified as low-, medium- and high-reduction potentials for the copper sites and fungal laccases have higher reduction potentials than other organism's laccases (Rodgers et al. 2009). Phenols are typical substrates of laccases and have lower reduction potentials allowing electron abstraction by type 1 copper. DMP, guiacol and syringaldazine are commonly used phenolic substrates. Laccases are also able to oxidize other electron donating substrates, for example the most commonly used substrate ABTS which is not pH dependent and does not form quinones (Giardina et al. 2010; Majeau et al. 2010).

Table 3.16: Laccase activity of LCC1 and LCC2 for different substrates

Substrate	Concentration	εmax (M^{-1} cm^{-1})	Wavelength (nm)	Activity (U ml^{-})	
				LCC1	LCC2
ABTS	5.0	36000	420	13,7	1,52
DMP	5.0	35645	470	1,2	0,25
Guaiacol	5.0	6400	436	0,175	ND*
Ferulic acid	5.0	12483	287	0,033	0,215
Catechol	5.0	2211	450	0,028	0,02
Tyrosine	3.0		280	0	0

*ND: Not determined

3.6.4 Effect of inhibitor compounds on laccase activity

Inhibitory effects of known laccase inhibitors, sodium azide, sodium fluoride, L-cysteine, SDS and metal chelator EDTA on the laccase activity were analyzed after the incubation of enzyme with those compounds at 30°C for 5 min. Inhibition percentage was calculated by comparing retaining activity with control sample.

The general inhibitors of metal-containing oxidases are sodium azide or fluoride, several sulfhydryl organic compounds, such as dithiothreitol, thioglycolic acid, L-cysteine, metal ions (e.g. Mg^{2+}, Ca^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}), fatty acids, EDTA and quarternary ammonium reagents (Baldrian, 2006; Johannes and Majcherczyk, 2000, Arora and Sharma, 2010). In this study, LCC1 laccase was strongly inhibited by typical inhibitors of Pycnoporus genus, sodium azide and

sodium fluoride with 99.97 % and 99.0 %, respectively. 1.0 mM L-cysteine had also caused 96 % inhibition, whereas the most effective inhibition was observed with 100 % at 1.0 mM L-cysteine for LCC2 (Table 3.17). Furthermore, a respectable inhibitory effect of SDS has been observed on both LCC1 and LCC2. Laccase purified from *Pycnoporus sanguineus* CCT-4518 was also strongly inhibited by sodium azide, sodium fluoride and L-cysteine (Garcia et al. 2007). Chelating agents, such as potassium cyanide, cysteine, sodium azide and a EDTA are present in some wastewaters and are the potential laccase inhibitors. Ionic strength of the solution affects enzymatic performance as well as the solubility of reaction products and high ionic strength can totally inhibit laccase activity (Majeau et al. 2010). Smaller and more electronegative halides inhibit laccase activity than other halides in the periodic table (fluoride>chloride>bromide). Small anion inhibitors such as halides bind to the type 2 and type 3 copper and cause interruption of the internal electron transfer and activity inhibition by blocking Access to the trinuclear copper cluster. Furthermore, X-ray structures revealed that an azide sits among the type 3 coppers in one bacterial laccase structure and the most effective inhibitors were declared as the azide and fluoride in terms of their capacity of lowering activity by 50% at micromolar concentrations (Rodgers et al. 2009, Colao et al. 2009). The inhibitory effect of reducing agents L-cysteine and B-mercaptoethanol has been suggested due to breaking disulfide bonds between sulfur containing amino acids in the enzyme (Garcia et al. 2007). Metal chelator EDTA was not an efficient inhibitor, especially for LCC2, consistent with the other reported laccases (Jordaan et al. 2004).

Table 3.17: Inhibitory effect of different compounds on laccase activity

Compound	Concentration (mM)	Inhibition (%)	
		LCC1	LCC2
Sodium azide	0.01	96.75	97.36
	0.1	99.97	99.96
Sodium fluoride	0.1	90.2	ND
	1	99.0	ND
L-cysteine	0.1	40.5	99.14
	1	96.0	100
SDS	0.1	90.93	91.2
	1	91.73	98.89

3.6.5 Catalytic properties

Catalytic performance of the recombinant enzyme were measured by determining Michaelis constant (Km), turnover number (kcat), and specifity constant (kcat/Km).

Enzyme activity was measured with different concentrations of ABTS (0.1 – 5 mM) and DMP (0.05 - 10 mM) as substrate. Michaelis-Menten constant Km and Vmax values were calculated using GraphPad Prism program. Km values of R-LCC1 were 1.355 mM and 0.442 mM for ABTS and DMP respectively, whereas R-LCC2 have 0.277 mM and 8.39 mM towards ABTS and DMP . Both turnover number and specifity constant values were higher than the kcat and kcat/Km ratio for DMP (Table 3.18).

Kinetic parameters of R-LCC1 and R-LCC2 were determined by using ABTS and DMP as substrates. Although recombinant LCC1 seems have lower affinity for ABTS than DMP with its higher Km value, turnover number for ABTS was 39.5 fold higher than DMP proving that ABTS was oxidized faster than DMP and ABTS seems a better substrate for R-LCC1 in terms of specifity constant. However, affinity of R-LCC2 for ABTS was remarkably higher than the DMP, since Km value of R-LCC2 for ABTS was lower than the DMP. Moreover, turnover number for ABTS has been 5.4 fold higher than the turnover number of DMP and it bares the faster oxidation of ABTS than DMP as observed in the R-LCC1. The turnover number can be diverse and varies with each laccase from different organisms or strains. The Km value of R-LCC1 for ABTS was 1.85 fold lower and kcat was 1.3 fold higher than the Lac4 laccase from *Pleurotus sajor-caju* expressed in *Pichia pastoris* while Km for DMP of R-LAC4 was 3.7 fold and kcat was 24 fold higher than R-LCC1 (Soden et al. 2002). Although affinity of R-LCC1 for both ABTS and DMP were lower than native laccases from different reported *Pycnoporus sanguineus* strains (Trovaslet et al. 2007, Litthauer et al. 2007, Vite-Vallejo et al. 2009, Lu et al. 2007), catalytic efficiency (kcat) values indicating that the rate of conversion for the substrate of the recombinant enzyme remarkably was higher than native ones. Specifity constants of the reported native laccases are generally closed to each other and were two fold lower than the recombinant laccase for ABTS. Furthermore, native laccase reported by Lu et al. (2007) had 79.6 fold lower constant than the recombinant LCC1. Catalytic properties of the several native or recombinant fungal laccases are given in the Table 3.19.

Table 3.18: Kinetic parameters of recombinant LCC1 and LCC2 laccases of *P. sanguienus* MUCL 38531

Laccase	Substrate	Vmax ($\mu molmin^{-1}l^{-1}$)	Km (mM)	kcat (min^{-1})	kcat /Km ($mM^{-1}min^{-1}$)
LCC1	ABTS	11538	1,355	$9,65.10^4$	$7,12.10^4$
	DMP	292,15	0,4415	$2,44.10^3$	$5,52.10^4$
LCC2	ABTS	1907,7	0,2768	$1,7.10^4$	$6,14.10^4$
	DMP	350,9	8,389	$3,14.10^3$	$3,74.10^2$

Table 3.19: Catalytic properties of previously reported fungal laccases

Laccase	Substrate	Km (mM)	kcat (min^{-1})	kcat/Km ($mM^{-1}min^{-1}$)	Reference
P. sanguineus CELBMD 001	ABTS	0,106	$3,56.10^3$	$3,36.10^4$	Vite-Vallejo et al. 2009
P. sanguineus China	ABTS	0,077	68,87	$8,94.10^2$	Lu et al. 2007
P. sanguineus SCC108	ABTS	0,13	$2,88.10^3$	$2,2.10^4$	Litthauer et al. 2007
P. sanguineus SCC108	DMP	0,052	$1,26.10^3$	$2,4.10^4$	Litthauer et al. 2007
P.sanguineus MUCL 41582	ABTS	0,018	570	$3,16.10^4$	Trovaslet et al. 2007
P. sanguineus BRFM 902	ABTS	0,032	$1,42.10^4$	$4,4.10^5$	Uzan et al. 2010
P. sanguineus BRFM 66	ABTS	0,033	$1,28.10^4$	$3,9.10^5$	Uzan et al. 2010
Pycnoporus coccineus BRFM 938	ABTS	0,026	$1,3.10^4$	5.10^5	Uzan et al. 2010
P. sanguineus H275 expressed in P.pastoris	ABTS	0,0026	$6,33.10^2$	$2,4.10^5$	Lu et al. 2010
Wt-MtL_fungus	ABTS	290	$7,9.10^2$	2,7	Bulter et al. 2003
Wt-MtL_yeast	ABTS	270	80	0,3	Bulter et al. 2003
Pleurotus sajor-caju Lac 4	ABTS	2,5	$7,4.10^4$	$2,96.10^4$	Soden et al. 2002

Recently Uzan et al. (2010) reported two laccases from *Pycnoporus sanguienus* BRFM 66 and BRFM 902, which Km values have been lower than the values of other described *Pycnoporus sanguineus* laccases and proved that the catalytic properties of the laccases from the different strains of the same species could be varied.

Difference between catalytic properties of the recombinant laccase isoenzymes may have been resulted from the difference of the structures of both laccases. Colao et al. (2009) observed a difference between the molecular architecture of two recombinant laccase of *Trametes trogii* with regard to two spatially closed residues surrounding the substrate cavity, Thr164 and Ser264 of Lcc1 were replaced by Phe 163 and Ile 265 in Lcc2 by affecting the capacity of enzymes to interact with polar groups on the ligands.

3.7 Demonstration of the Functionality of Recombinant Laccases

Following to characterization of the purified recombinant laccase LCC1 And LCC2, functionality studies of the recombinant laccase were performed. In vitro synthesis of antibiotic cinnabarin and textile dye biosynthesis were performed by using the recombinant laccases.

3.7.1 Synthesis of Cinnabarinic Acid by in-vitro oxidation of 3-HAA

In this part of the study, the formation of the phenoxazinone derivative, cinnabarinic acid synthesis by recombinant laccases LCC1 and LCC2 purified from *P. pastoris* has been aimed. The o-aminophenol 3-hydroxyanthranilic acid (3-HAA) is one of the tryptophan metabolites along kynurenine pathway and is the precursor of the phenoxazinone derivative, cinnabarinic acid (CA). 3-HAA is converted into CA through the oxidation by horseradish peroxidase, myeloperoxidase, catalase, tyrosinase, glucose oxidase and also laccase. The formation of phenoxazinone ring from anthranilate precursors starts with 6 electron oxidation of precursor 3-HAA and is followed by any of several reaction sequences all of which proceed reactive intermediates such as quinone imines and results in the phenoxazinone ring of cinnabarinic acid. In this study, 20 µg of recombinant laccases LCC1 and LCC2 was added to a solution of 1 mM 3-HAA in dissolved 1 ml of 50 mM sodium tartrate buffer (pH 4.0) and prepared mixture was incubated at 30°C. Oxidation of 3-HAA

was monitored spectrophotometrically at 10, 30 and 60 minutes and 6 hours of incubation. For all time intervals, oxidation of 3-HAA was also monitored as increasing absorbance at the 325 nm wavelength. Lyophilized form of cinnabarin pigment, chemically synthesized in our laboratory before, has been also prepared in the 50 mM sodium tartrate buffer (pH 4.0) and used as positive control in all of the HAA oxidation studies. Both laccase with HAA reaction and lyophilized form of cinnabarin was scanned between 250 nm and 500 nm wavelengths with UV visible spectrophotometer. An increment in the absorbance at 450 nm and red pigment formation were observed with time, revealing that CA was formed by oxidation of 3-HAA (Figure 3.47 and Figure 3.48). As consistent with our data. in the literature it was reported that cinnabarinic acid (CA) had formed after oxidation of the precursor 3-hydroxyanthranilic acid (3-HAA), by laccases (Eggert et al. 1995, Bruyneel et al. 2008).

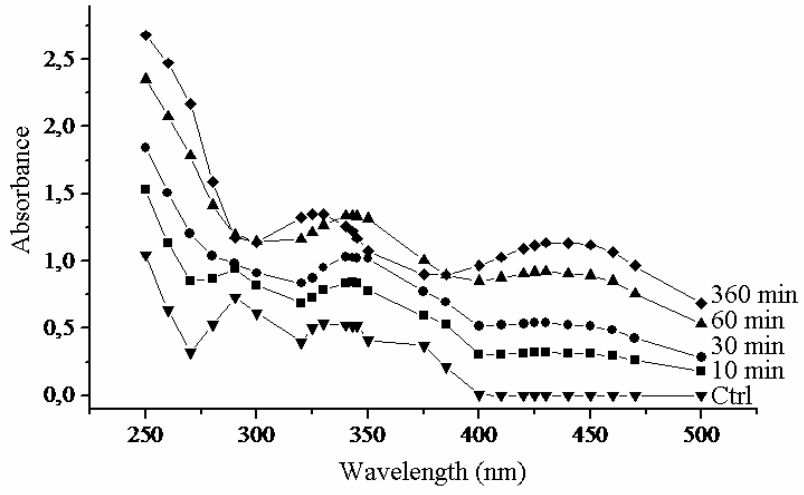


Figure 3.47 : Changes in the UV-visible spectrum during the oxidation of 3-hydroxyanthranilic acid by P cinnabarinus laccase. Conditions: 50 mM sodium tartrate buffer (pH 4.0), 1 mM substrate, and 20 ,ug enzyme at 30°C in a 1-ml reaction volume. Time intervals are given in

minutes

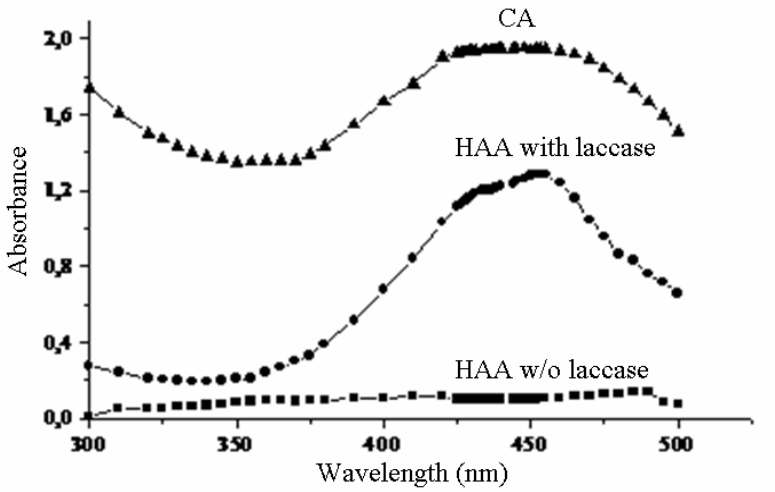


Figure 3.48 : Comparison of the UV-visible spectrums of chemically synthesized cinnabarinic acid, HAA with lcc1 laccase and HAA without laccase samples

In order to also characterize the observed red pigment, HPTLC analysis of 3-HAA, recombinant laccase-catalyzed reaction of 3-HAA, which was synthesized at 6 hours of incubation and also CA, was performed on silica gel plates using butanol/acetic acid/water (4:2:1) as the mobile phase. After development, the plates were dried and scanned (Camag TLC scanner 3 under software control of WinCats v. 1.3.2) at 325 and 450 nm wavelengths. The images of the HPTLC plate of the samples, visualized at white light and UV light at 366 nm and 254 nm wavelength is diplayed in Figure 3.49. Blue fluorescence has been observed for the spots of 3-HAA, whereas CA has observed as orange-red colour at 366 nm wavelength.

Following to the scanning of plate at 325 nm and 450 nm wavelength, major significant spots were appeared at about Rf 0.78 for 3-HAA, at Rf 0.58 for CA and Rf 0.59 and 0.78 for 3-HAA oxidation samples catalyzed with LCC1 and LCC2. 3D visualization of HPTLC chromatogram is given in Figure 3.50. The Rf values for 3-HAA and CA was closed to the reported Rf values 0.82 and 0.51 respectively (Eggert et al. 1995).

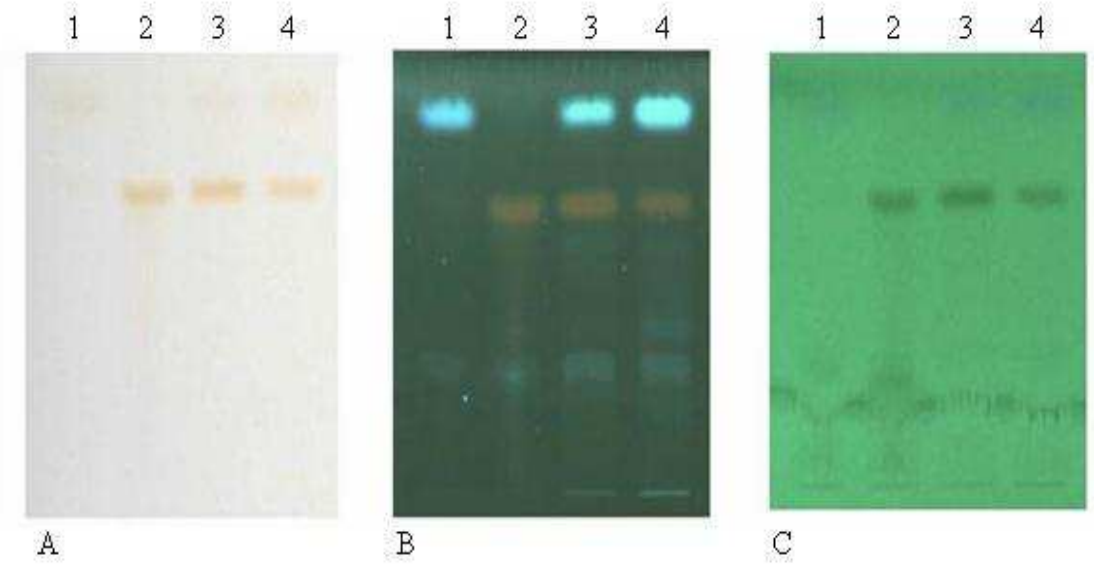


Figure 3.49 : Separation of laccase catalyzed oxidation of 3-hydroxyanthranilic acid products by high performance thin layer chromatography (HPTLC), Plate was displayed at white light (A), 366 nm (B) and 254 nm(C); lane 1. Negative control, containing 1 mM Hydroxy-anthranilic acid without enzyme, lane 2. Positive control, cinnabarinic acid without enzyme, lane 3. 1mM hydroxyanthranilic acid with LCC1, lane 4. 1mM hydroxyanthranilic acid with LCC2

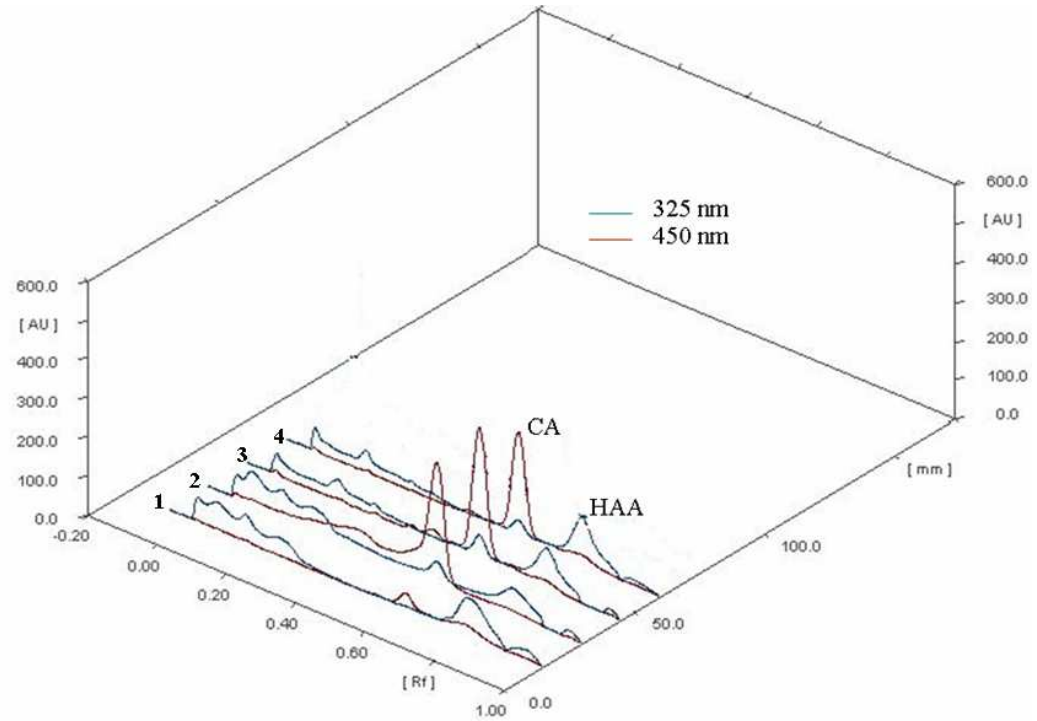


Figure 3.50 : Three dimensional analysis visualization of seperated samples on TLC plate at 325 nm and 450 nm wavelengths, sample 1. Negative control, containing 1 mM Hydroxy-anthranilic acid without enzyme, sample 2. Positive control, cinnabarinic acid without enzyme, sample 3. 1mM hydroxyanthranilic acid with LCC1, sample 4. 1mM hydroxyanthranilic acid with LCC2

Absorbance values were consistent with the wavelength maxima of HAA and CA and CA spots had higher values than HAA spots at 450 nm, whereas 3-HAA spots absorbance of were higher at 325 nm (Table 3.20, Table 3.21). All of the obtained results by both UV-visible spectrophotometry and HPTLC indicated that, the red pigment synthesized by recombinant laccase catalyzed oxidation of 3-HAA was the phenoxazinone derivative cinnabarin.

Table 3.20: Rf values of significant peaks and absorbances of the samples on the TLC plate scanned at 450 nm wavelength

Sample / 450 nm	Peak number	Rf value	Absorbance (AU)
Negative control	1	0,60	39,4
	2	0,78	24,1
Positive control	1	0	12,0
	2	0,35	49,7
	3	**0,58**	304,5
HAA with LCC1	1	0	12,2
	2	0,4	16,9
	3	0,46	27,5
	4	**0,59**	336,7
	5	0,77	19,1
HAA with LCC2	1	**0,59**	265,6
	2	0,78	23,0

Table 3.21: Rf values of significant peaks and absorbances of the samples on the TLC plate scanned at 325 nm wavelength

Sample / 325 nm	Peak number	Rf value	Absorbance (AU)
Negative control (3-HAA)	1	0,01	61,4
	2	0,06	70,7
	3	0,14	69,0
	4	0,24	46,1
	5	0,78	95,3
	6	0,95	24,5

Table 3.21 (continued): Rf values of significant peaks and absorbances of the samples on the TLC plate scanned at 325 nm wavelength

Sample	Peak number	Rf value	Absorbance (AU)
325 nm			
Positive control (CA)	1	0,01	61,6
	2	0,05	84,3
	3	0,14	70,1
	4	0,22	58,1
	5	**0,58**	52,8
	6	0,80	65,8
	7	0,97	19,5
HAA with LCC1	1	0,01	55,6
	2	0,18	53,9
	3	0,29	32,2
	4	**0,59**	64,1
	5	0,78	101,3
	6	0,97	16,2
HAA with LCC2	1	0	59,7
	2	0,15	57,8
	3	0,28	27,9
	4	0,34	18,1
	5	**0,59**	41,2
	6	0,78	119
	7	0,92	14,4

3.7.2 Textile Dye Biosynthesis by Recombinant Laccases

Natural dye biosynthesis for textile coloration as an alternative to synthetic dyes because of being friender to the environment and also strict environmental standards accepted by many countries has been growing recently. Several novel textile dyes had been obtained by screening of fifteen precursors and their coupling reactions by our group previously (Kahraman, 2008). In this study, synthesis of an azo dye named as ITU-G, which can be used in textile coloration, via recombinant laccase-mediated coupling of dye precursors 3,5-Dinitro-2-aminothiophene (P6) and 3 cyano 4 methyl 6 hydroxy N ethyl 2-Pyridone (P8) and ITU-11 from 2-amino 4-

nitrophenol (S2) and 2-Aminophenol-4-sulfonic acid (S3) have been aimed. Dyes were synthesized succesfully from different dye precursors (25 mM) by incubating with 10 U/ml recombinant laccase in 100 mM tartrate buffer, pH 4.5.

Parallel reactions were set up with individual precursors without laccase (blank), two precursors without enzyme, individual precursors with recombinant LCC1 or LCC2 enzyme and two precursors with enzyme in 1.5 ml tubes and incubated for 12 hours at 30ºC. The reaction of S2 and S3 precursors with recombinant laccases resulted in orange-red colored ITU-11, while green color obtained from laccase catalyzed oxidation of P6 and P8 precursors. Synthesized dyes were shown in Figure 3.51 as in the order of ITU-11 (A) and ITU-G (B). As shown in Figure 3.51 there was no color change in the reactions containing precursors without laccase as opposed to the reactions containing laccase.

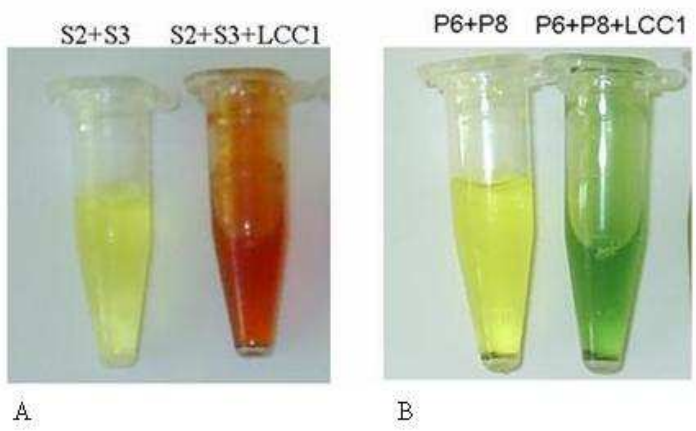


Figure 3.51 : ITU-11 (A) and ITU-G (B) dyes synthesized from relevant precursors by laccase catalyzed reaction

To characterize the reactions mentioned above, high performance thin layer chromatography was carried out and dye mixtures were seperated into the individual components. The analysis was performed on aluminum plated silica gel plates and 10 µl samples were spotted on the plates and *n*-Butanol, acetone, water, ammonia (5:5:1:2, vol/vol) was used as mobile phase. The movement of the spots on the plate was observed at white light, 366 nm and 254 nm wavelengths of UV light. HPTLC plate images irradiated at 366 nm wavelength are shown in Figure 3.52 for ITU-G and ITU-11 respectively.

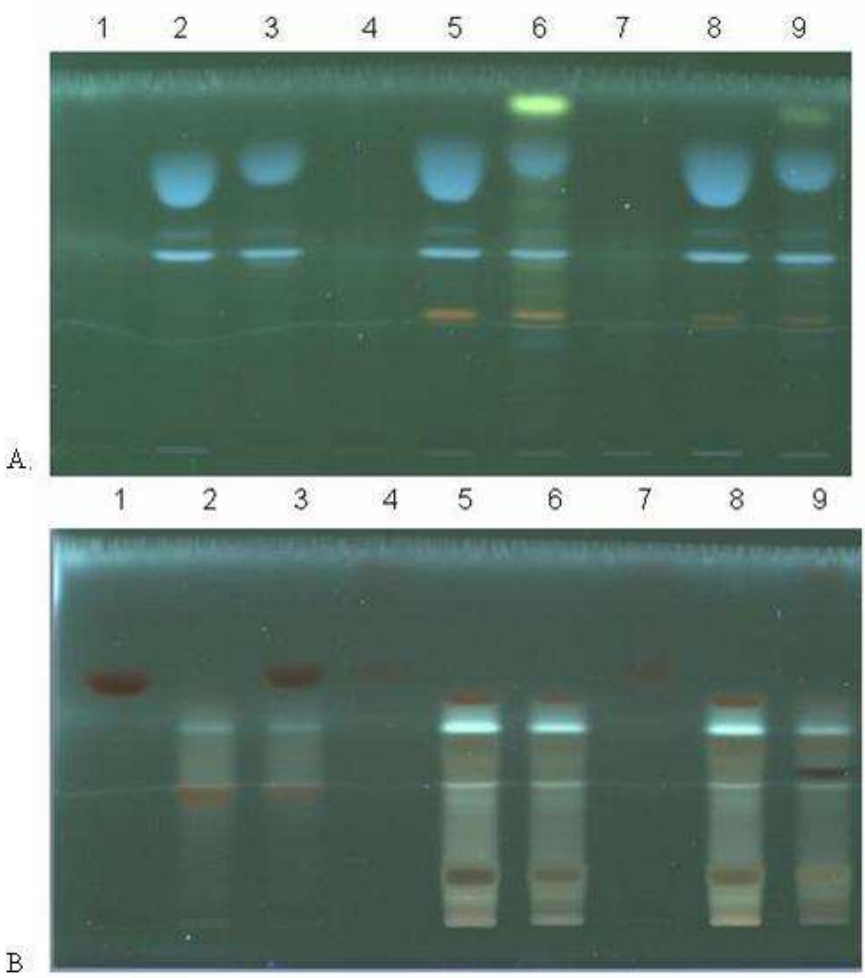


Figure 3.52 : High performance thin layer chromatographic seperation of laccase catalyzed textile dyes, (A) ITU-G; sample 1. P6, sample 2. P8, sample 3. P6+P8, sample 4. P6+LCC1, sample 5. P8+LCC1, sample 6. P6+P8+LCC1, sample 7. P6+LCC2, sample 8. P8+LCC2, sample 9. P6+P8+LCC2, (B) ITU-11; lane 1. S2, lane 2. S3, lane 3. S2+S3, lane 4. S2+LCC1, lane 5. S3+LCC1, lane 6. S2+S3+LCC1, lane 7. S2+LCC2, lane 8. S3+LCC2, lane 9. S2+S3+LCC2

Negative control reactions containing dye precursors without enzyme and positive control reactions of individual precursors with enzymes were set up to compare the profiles of the spots on the TLC plate. Plates were scanned between 200 nm and 800 nm wavelengths and separated components were analyzed by comparing the rates of migration and also absorption values of the samples. Based on the multiwavelength profiles of the plates 400 nm was chosen for evaluation of the Rf values.

The 3D-HPTLC chromatogram at 400 nm in Fig 3.53 show the characteristic traces of ITU-G with their peaks. Specific and significant Rf values of seperated ITU-G

samples are shown in Table 3.22. Characterization of the synthesized dyes were carried out by comparing the retention factors. According to the compared Rf values of significant spots, P6+P8+LCC1 sample gave the major spot at about Rf 0.90 with green color, while LCC2 gave this spot at Rf 0.87 with the same color. Because of the difference of those Rf values from the other samples and observed green spots could be the result of laccase-catalyzed oxidative coupling of P6 and P8 precursors.

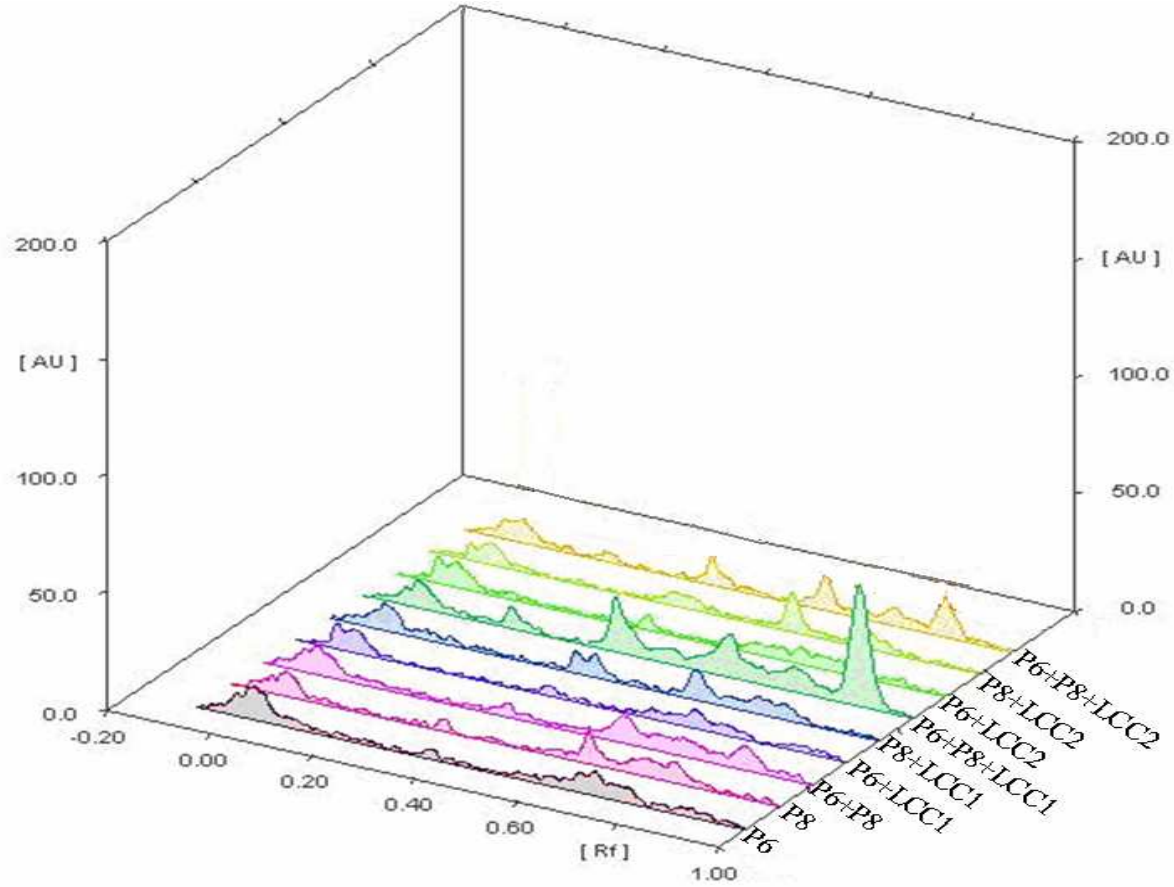


Figure 3.53 : 3D visualization of seperated samples on HPTLC plate at 400 nm wavelength

The 3D visualized HPTLC chromatogram of ITU-11 at 400 nm is given in Figure 3.54 and significant Rf values of the characteristic traces with their peaks are demonstrated in Table 3.23. The compared Rf values of significant spots revealed that, all S2+S3, S3+laccase and S2+S3+laccase have significant spots, appearing at Rf 0.07, Rf 0.5, Rf 0.69 and Rf 0.77, whereas S3+laccase and S2+S3+laccase reactions gave one more spot at Rf 0.81. Besides, absorbance of the mentioned two precursors with enzyme was about 1.61 fold more than individual S3 with laccase.

Considering these results, it can be concluded that ITU11 had resulted from laccase-catalyzed oxidation of S3 precursor, not coupling between S2 and S3.

Table 3.22: Rf of significant peaks detected in ITU-G synthesis reactions by HPTLC

ITU-G (400 nm)	Peak number	Rf value	Absorbance (AU)
P6	1	0,04	14,1
P8	1	0,63	14
P6+P8	1	0,02	12,4
P6+LCC1	1	0	10,8
P8+LCC1	1	0,64	12,8
P6+P8+LCC1	1	0,42	23,8
	2	0,64	18,6
	3	**0,90**	**51,3**
P6+LCC2	0	0	0
P8+LCC2	1	0,63	17,2
P6+P8+LCC2	1	0,41	12,5
	2	0,64	14,9
	3	**0,87**	**16,7**

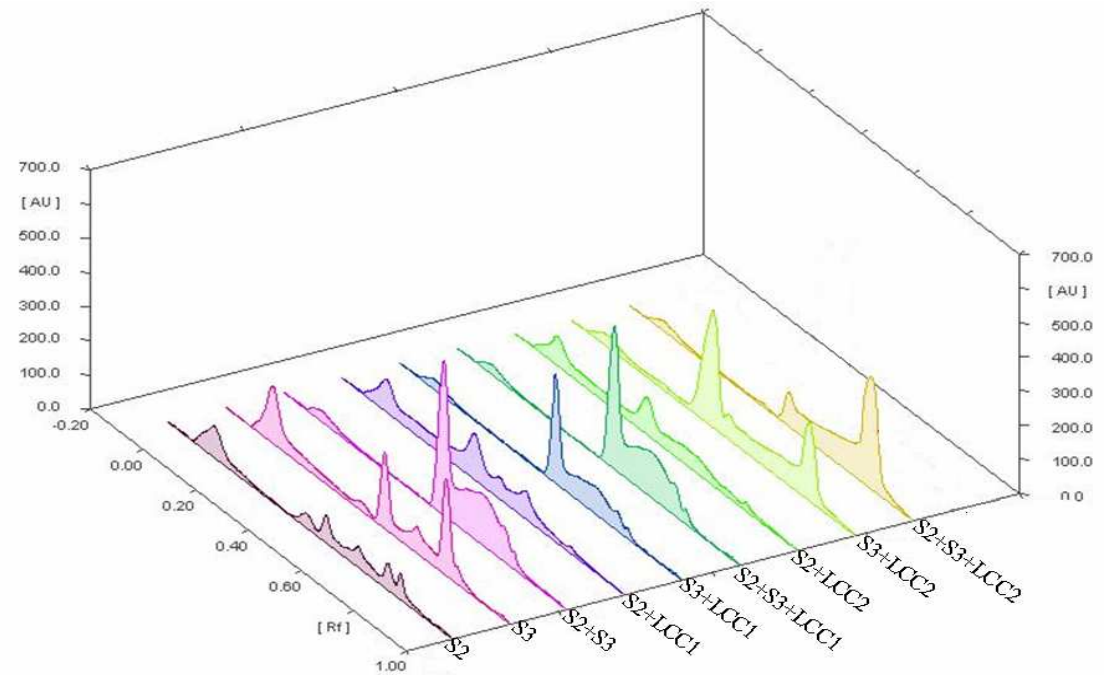


Figure 3.54 : 3D visualization of ITU-11 samples on HPTLC plate at 400 nm wavelength

Table 3.23: Rf values of significant peaks detected in ITU-11 synthesis reactions by HPTLC

ITU-11 (400 nm)	Peak number	Rf value	Absorbance (AU)
S2	1	0,07	120
	2	0,45	70
	3	0,53	105
	4	0,65	81
	5	0,75	102
S3	1	0,1	205
	2	0,45	70
	3	0,52	260
	4	0,65	125
	5	0,75	325
S2+S3	1	0,07	51
	2	0,5	500
	3	0,69	190
	4	0,77	150
S2+LCC1	1	0,1	125
	2	0,42	158
	3	0,52	82
	4	0,63	100
	5	0,7	25
S3+LCC1	1	0,07	60
	2	0,5	350
	3	0,69	120
	4	0,77	80
	5	**0,81**	**60**
S2+S3+LCC1	1	0,07	75
	2	0,5	470
	3	0,69	180
	4	0,77	125
	5	**0,81**	**97**
S2+LCC2	1	0,1	108
	2	0,43	130
	3	0,62	75
	4	0,72	42
S3+LCC2	1	0,07	68
	2	0,46	380
	3	0,51	115
	4	0,73	102
	5	**0,81**	**263**
S2+S3+LCC2	1	0,07	70
	2	0,51	138
	3	**0,81**	**360**

Although continuous search of several industries to find out new harmless ways for dye synthesis, there are only limited number of studies exist in the literature about laccase-catalyzed natural dye biosynthesis. Biosynthesis of yellow colored food colorants by laccase-catalyzed oxidation of ferulic acid in a biphasic hydro-organic medium had been reported recently. By achieving improved control of synthesis, color stability in the food coloring had been obtained and these compounds were suggested as food colorant for the replacement of synthetic colorants like tartrazine (Mustafa et al. 2005). Enaud et al. (2010) reported the biosynthesis of the first sulfonic azoanthraquinone dye, named Laccase Acid Red 1, through enzymatic coupling of aromatic amine monomers. The commercial laccase from *Trametes versicolor* was used as biocatalyst in the study and the obtained dye was not cytotoxic, showed no mutagenic effect and the dyeing properties showed its industrial potential. By means of the synthesis of this ecofriendly compound, safe and environmentally friendly routes to azo sye biosynthesis had been opened. Similarly, following the characterization of the synthesized colored solutions ITU-G and ITU-11, dyeing capability experiments should be performed and it should also be tested in terms of its antimicrobial activity, toxicity for mammalian cell lines, mutagenicity and also for ecotoxicity in future works.

4. CONCLUSION AND RECOMMENDATIONS

The purpose of this research was to achieve isolation of laccase encoding genes from white-rot fungi *Pycnoporus sanguineus* MUCL 38531, the heterologous expression of those laccase cDNAs and also purification and biochemical characterization of the recombinant laccases. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multifunctional enzymes as oxidizing diverse list of aromatic compounds containing hydroxy or aromatic group and these features make them good candidates for applications in many industrial, agricultural and medicinal applications. Those applications have been carried out either in laboratory or pilot plant scale (Gİardina et al. 2010, Arora and Sharma, 2010, Aro et al. 2005). Large-scale applications need sufficient enzyme stocks and production of this enzyme in the fungal sources need expensive redox mediators and inducers. Cloning of laccase genes from the fungal sources and overexpression of the functional enzymes with sufficient yields in yeasts overcome these problems. Thus, our study was firstly focused on the determination and isolation of the laccase-encoding cDNAs from white-rot fungi *Pycnoporus sanguineus* MUCL 38531, then laccase cDNAs were heterologously expressed in yeast *Pichia pastoris* and recombinant laccases were biochemically characterized. The laccase-specific sequences of *Pycnoporus sanguineus* MUCL 38531 has been screened and two different laccase-specific sequences, displaying similarity to previously reported laccases, were identified both on the cDNA and gDNA levels and denominated as *lcc1* and *lcc2*. *lcc1* cDNA was detected dominantly in the constructed cDNA library, pointing out the highest transcriptional activity of *lcc1* laccase gene. 5'- and 3'-ends of the laccase cDNAs were determined and isolation of the full-length cDNAs have been carried out. The corresponding open reading frame for full length *lcc1* cDNA is 1557 bp, coding for 518 amino acids with a putative 20-residue signal sequence. Furthermore *lcc2* gene contains an open reading frame 1713 bp in length, coding for 570 deduced amino acids and the genomic DNA sequence of both *lcc1* and *lcc2* genes revealed the 10 introns. Typical copper signatures composed of 10 His and 1 Cys residue, required for coordinating copper atoms on the active site of the enzyme, are in the conserved positions of fungal laccases in

both *lcc1* and *lcc2* genes. The present work constitutes the first report on the molecular cloning of laccase genes from *Pycnoporus sanguineus* MUCL 38531 and the sequences of *lcc1* and *lcc2* gDNAs and mRNAs were submitted to the GenBank genetic sequence database the NCBI with the accession numbers HM106995 and HM106997.

The heterologous production of laccases have been reported as the most suitable way to obtain higher yields of functionally active laccase in the shorter period of time than the fungi itself. Yeasts can serve as ideal host for the overexpression of the eucaryotic proteins, requiring post-translational modifications. Ligninolytic enzymes are generally difficult to overexpress in heterologous organisms in their active form, however the expression of active recombinant laccases has been reported in the yeasts *Saccharomyces cerevisiae, Kluyveromyces lactis* (Piscitelli et al. 2005), *Yarrowia lipolytica* (Madzak et al. 2005), *Pichia pastoris* (Soden et al. 2002) so far. Both laccase encoding cDNAs of *P. sanguineus* MUCL 38531 were successfully expressed in yeast *Pichia pastoris* in this study. High level protein production for any heterologous protein is achieved by optimization of expression conditions. Results of our study revealed that the alanine should be added to neutralize the pH of the culture medium and it prevents the negative effect of the acidic proteases on the laccase activity. Decreasing cultivation temperature was also found beneficial to obtain increased laccase activity, thus *P. pastoris* cultures were cultivated at 23°C. The effect of nutrients on the laccase activity were tested by growing yeast on either minimal or complex media and appropriate methanol concentration to induce laccase expression was also determined. Laccase activity was higher in the complex medium, containing yeast extract and peptone by preventing proteolysis of secreted protein, and 1% methanol was the best concentration to induce *Aox1* promoter. Laccases are copper-containing enzymes and requirement of laccase for copper ions and the effect of copper concentrations on laccase activity were indicated in previous studies (O'Callaghan et al. 2002, Liu et al. 2003). Copper atoms have a role in the catalytic core of the laccase as a cofactor and a minimum concentration of copper in milimolar range is necessary for laccase activity. Optimum copper concentration is specific to strain of a single species or to different species. Different concentrations of copper sulfate was added into the complex medium and the highest level of activity was observed 0.6 mM and 2.0 mM CuSO4 for LCC1 and LCC2 respectively. Laccase

activites of the all cultures containing copper sulfate were higher than the control culture without copper. Expression level obtained in this study was remarkably higher than the most of the fungal recombinant laccases that are produced in *P. pastoris* or other heterologus host organisms. The biomass of the R-LCC1 producing culture, containing 0.6 mM $CuSO_4$, was lower than the other concentrations and even than the control sample, indicating that the increased laccase activity was not due to increased biomass and increasing copper concentrations have not any toxic efffect on the biomass of the cultures. The growth of R-LCC2 cultures have not been affected with increasing concentration of copper, whereas the OD_{600} values have decreased in the presence of 3 mM and more copper concentrations.

Recombinant laccases were purified from *Pichia pastoris* culture and both laccases were monomeric and single band with 56 kDa and 62 kDa was observed on the SDS-PAGE for LCC1 and LCC2, respectively. Molecular weight of the recombinant laccases was similar to other fungal laccases and was not hyperglycosylated. Biochemical characterization is very important to enlight the properties of enzyme at the molecular and kinetic levels being a prerequisite for usage of those enzymes in biotechnological and industrial applications. pH optima may vary with different substrates or with laccase isozymes of the same organism and the optimum pH for fungal laccases usually lies in the acidic range and each substrate for its oxidation has its own specific pH optima (Jordan et al. 2004, Arora and Sharma 2009). The pH optima for ABTS was pH 3.0 in this study, whereas pH 4.0 for DMP with a bell-shaped profile. Optimum temperature for laccase activity is generally between 20-37 °C and laccases of most white-rot fungi is stable below 50 °C (Arora and Sharma 2010). Maximum laccase activity was observed at 60 °C and 30 °C for lcc1 and lcc2 and this values were similar to the the optimum temperatures of different *Pycnoporus sanguineus* strains (Garcia et al. 2006, Trovaslet et al. 2007). Laccases generally have four copper atoms in the active site and T1 site, containing mononuclear copper ion, is the primary electron acceptor and is responsible for the typical blue colour of the enzyme, characterized absorption peak at 600 nm of UV-visible spectrum. No peak was observed at 600 nm in the UV-visible spectrum of the purified recombinant LCC1 and LCC2, while there was a peak at 410 nm similar with the other reported white laccases. Inhibitory effects of different compounds on the recombinant laccase was also assayed and both laccases were strongly inhibited by common laccase

inhibitors, sodium azide, sodium fluoride, L-cysteine, SDS and metal chelator EDTA. Kinetic parameters of recombinant LCC1 and LCC2 were determined with ABTS and DMP substrates. Although recombinant LCC1 seems have lower affinity for ABTS than DMP with its higher Km value, turnover number for ABTS was higher than DMP proving that ABTS was oxidized faster than DMP and ABTS seems a better substrate for recombinant LCC1 in terms of specifity constant. The affinity of R-LCC2 for ABTS was remarkably higher than the DMP, since Km value of R-LCC2 for ABTS was lower than the DMP. Moreover, turnover number for ABTS was higher than the turnover number of DMP and it bares the faster oxidation of ABTS than DMP as observed in the R-LCC1. Results presented here, proved the existence of various laccases having different optimum temperatures and also catalytic efficiency parameters. It is also evident that, the molecular basis of these differences in terms of the determination of the number of laccase encoding genes has been investigated by this dissertation.

Following to characterization of the recombinant laccases LCC1 and LCC2, functionality studies of the recombinant laccases have been performed. In vitro synthesis of orange-red compound cinnabarin after oxidation of the precursor 3-hydroxyanthranilic acid with recombinant laccases has been carried out. Although continuous search of several industries to find out new harmless ways for dye synthesis, there are only limited number of studies exist in the literature about laccase-catalyzed natural dye biosynthesis. Thus, synthesis of two dyes named as ITU-G and ITU-11, which can be used in textile coloration, have been occured via recombinant laccase-mediated coupling of dye precursors in this study. These functionality studies were firstly reported for recombinant laccases in the literature and demonstrates the synthesis of textile dyes or pigments, which will be used possibly as colorants for different purposes, with eco-friendly enzymatical methods as an alternative to conventional chemical synthesis.

As a consequence, constructed vectors containing *lcc1* and *lcc2* cDNAs and heterologously expressed laccases of *P. sanguineus* serve as a template for further studies on the determination of structure/function relationships or as a green tool in many biotechnological processes by the help of their optimized expression conditions, purification procedures and determined biochemical characteristics. It has

also been shown that, recombinant laccases have similar biochemical and molecular characteristics to the native enzymes and may provide cheaper alternatives.

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APPENDICES

APPENDIX A.1 : Compositions and Preparation of Culture Media

APPENDIX A.2 : Compositions of Buffers and Solutions

APPENDIX A.3 : Chemicals and Enzymes

APPENDIX A.4 : Laboratory Equipment

APPENDIX A.1 : Compositions and Preparation of Culture Media

Maltose medium

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) were dissolved in 1000 ml distilled water and sterilized by autoclaving for 15 minutes on liquid cycle.

Nutrient agar medium (1000ml)

Potato dextrose agar 39 g
Distilled H_2O was added up to 1000ml and then autoclaved for 15 minutes.

Luria Bertani (LB) Medium (1000ml)

Tryptone 10 g
Yeast Extract 5 g
NaCl 5 g
Distilled H_2O was added up to 1000ml and 15 g of agar was added for solid medium then autoclaved for 15 minutes.

2xYT Medium (1000ml)

Tryptone 16 g
Yeast Extract 10 g
NaCl 5 g
Distilled H_2O was added up to 1000ml and 15 g of agar was added for solid medium then autoclaved for 15 minutes.

Yeast Extract Peptone Dextrose Medium (1000 ml)

Yeast Extract	1 %
Mycological peptone	2 %
Dextrose (D-glucose)	2 %

10 g of yeast extract and 20 g of mycological peptone were dissolved in 900 ml of water and sterilized for 15 minutes on liquid cycle. 100 ml of filter-sterilized 20 % dextrose was added.

Yeast Extract Peptone Dextrose Sorbitol Medium with Zeocin™ (1000 ml)

Yeast Extract	1 %
Mycological peptone	2 %
Dextrose (D-glucose)	2 %
Sorbitol	1 M
Zeocin™	100 µg/ml

10 g of yeast extract and 20 g of mycological peptone and 182.2 g of sorbitol were dissolved in 900 ml of water, 20 g of agar was added and sterilized for 15 minutes on liquid cycle. 100 ml of filter-sterilized 20 % dextrose was added. Medium was cooled to ~60°C and poured after adding 1.0 ml of 100 mg/ml Zeocin™ .

Minimal Dextrose Medium (1000 ml)

YNB	1.34 %
Biotin	4×10^{-5} %
Dextrose	2 %

13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 900 ml of water, 15 g of agar was added and sterilized for 15 minutes on liquid cycle. 100 ml of filter-sterilized 20 % dextrose and 2 ml of filter-sterilized 500x biotin were added. Medium was cooled to ~60°C and poured.

Minimal Methanol Medium (1000 ml)

YNB	1.34 %
Biotin	4×10^{-5} %
Methanol	0.5 %

13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 993 ml of water, 15 g of agar was added and sterilized for 15 minutes on liquid cycle. 5 ml of methanol and 2 ml of filter-sterilized 500x biotin were added. Medium was cooled to ~60°C and poured.

Buffered Minimal Glycerol and Buffered Minimal Methanol Media, BMG and BMM (1000 ml)

100 mM potassium phosphate buffer, pH 6.0

YNB	1.34 %
Biotin	4×10^{-5} %
Methanol	0.5 % or
Glycerol	1 %

13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 890 ml of distilled water and sterilized for 15 minutes on liquid cycle.

Following to cooling the medium to ~60°C, 100 ml of pre-sterilized 1 M potassium phosphate buffer, pH 6.0, 2 ml of filter-sterilized 500x biotin and 10 ml of glycerol was added. 5 ml of methanol was added for BMM instead of glycerol.

Buffered Glycerol-complex Medium, BMGY and BMMY (1000 ml)

YNB	1.34 %
Yeast Extract	1 %
Mycological peptone	2 %
100 mM potassium phosphate buffer, pH 6.0	
Biotin	4×10^{-5} %
Methanol	0.5 % or
Glycerol	1 %

10 g of yeast extract, 20 g of mycological peptone and 13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 890 ml of distilled water and sterilized for 15 minutes on liquid cycle. Following to cooling the medium to ~60°C, 100 ml of pre-sterilized 1 M potassium phosphate buffer, pH 6.0, 2 ml of filter-sterilized 500x biotin and 10 ml of glycerol was added. 5 ml of methanol was added for BMMY instead of glycerol.

APPENDIX A.2 : Compositions of Buffers and Solutions

TAE Buffer (50X): 242 g Tris Base, 57.1 ml Glacial acetic acid, 100 ml EDTA (0.5M, pH 8.0) mixed, distilled water was added up to 1 L and pH was adjusted to 8.0 by adding HCl.

TBE Buffer (10x): 108 g **T**ris base, 55 g Boric acid, 40 ml EDTA(0.5M, pH 8.0) mixed, distilled water was added up to 1 L and the solution was autoclaved for 20 min.

Agarose Gel (1%): 0.5 g Agarose was melted in 50 ml TAE Buffer (1X), following to cooling down the gel, 0.5 µg/ml EtBr was added and gel was poured into the tray.

FA Gel (1.2 %): 1.2 g agarose, 10 ml 10x FA gel buffer, RNase-free water was added up to 100 ml and the mixture was heated to melt the agarose. Following to cooling down the gel to 65°C in a water bath, 1.8 ml of 37% (12.3 M) formaldehyde and gel was poured into the tray (size 10 x 14 x 0.7 cm).

10x FA gel buffer: 200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH to 7.0 with NaOH.

1x FA gel running buffer: 100 ml 10x FA gel buffer, 20 ml 37% (12.3 M) formaldehyde, 880 ml RNase-free water.

P1 Buffer : 6.06 g Tris-base, 3.72 g EDTA.$2H_2O$ were mxed pH adjusted to 8.0 with HCl after dissolving components and distilled water was added up to 1L. 100 mg RNase A was added for 1 L buffer.

P2 Buffer: 8 g NaOH, 50 ml SDS (20% w/v), NaOH was dissolved in 950 ml distilled water and 50 ml of SDS was added.

P3 Buffer: 294.5 g potassium acetate dissolved in 500 ml distilled water adn pH was adjusted to 5.5 by adding acetic acid.

1 M potassium phosphate buffer, pH 6.0: 132 ml 1 M K_2HPO_4, 868 ml 1 M KH_2PO_4 mixed and confirm the pH = 6.0 ± 0.1 and sterilized by autoclaving.

100 mM sodium-citrate buffer, pH 3.0 (1000 ml): 820 ml 0.1M-citric acid and 180 ml 0.1M-trisodium citrate were mixed.

100 mM Tartrate Buffer, pH 4.5 (1000 ml): 15.08 g tartaric acid was dissolved in 1000 ml distilled water and pH was adjusted to 4.5 with NaOH.

20 mM Sodium Phosphate Buffer, pH 7.2 (1000 ml)

360 ml 0.2M-Na_2HPO_4 and 140 ml 0.2M-NaH_2PO_4 were mixed and diluted to 1000 ml with H_2O.

30 % Acrylamide solution: 58.4 g acrylamide and 1.6 g bisacrylamide were dissolved in distilled water and stored at 4C in the dark.

4X Separating gel buffer: 1.5 M Tris-HCl (pH 8.8) was dissolved in 200 ml of distilled water.

4X Stacking gel buffer for SDS-PAGE: 0.5 M Tris-HCl (pH 6.8) was dissolved in 50 ml of distilled water.

2X sample buffer for SDS-PAGE: 2.5 ml 4x stacking buffer, 4 ml 10 % SDS, 2 ml glycerol, 1 ml β-Mercaptoethanol, 0.05 % (w/v) Bromophenol blue were dissolved in 10 ml of distilled water.

SDS-PAGE Running Buffer: 3 g Tris, 14.4 g Glycine, 10 ml SDS (0.1%) were dissolved in 1 l of distilled water.

SDS-PAGE Staining Buffer: 0.1 g Coomassie Brilliant Blue R-250, 50 ml methanol, 10 ml acetic acid dissolved in 100 ml of distilled water.

SDS-PAGE Destaining Solution: 5 ml Methanol, 10 ml Acetic acid dissolved in 100 ml of distilled water.

Ammonium persulphate (APS): 10% (w/v).

Sodium dodecyl sulphate (SDS): 10% (w/v).

APPENDIX A.3 : Chemicals and Enzymes

Chemicals	Supplier
ABTS	Sigma-Aldrich (Germany)
2,6-dimethoxyphenol (DMP)	Fluka (Switzerland)
Acetic acid	Merck (Germany)
Acrylamide	Sigma –Aldrich
Agar	Sigma
Agarose	Sigma
Ammonium persulphate	Merck
Ammonium sulfate	Riedel-deHaen (Germany)
Bisacrylamide	Sigma-Aldrich
Boric acid	Merck
Bovine serum albumine	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
Calcium chlorid ($CaCl_2$)	Merck
Catechol	Sigma-Aldrich
Coomassie Brilliant Blue	Bio-Rad (USA).
Copper sulfate pentahydrate	Merck
D-biotin	Fluka
Dextrose	Riedel-de Haën
di Potassium hydrogen phosphate (K_2HPO_4)	Riedel-de Haën
Diammonium tartarate	Sigma-Aldrich
Di-potassium hydrogen phosphate	Merck
Disodium hydrogen phosphate	Riedel-de Haen
Dithiothreitol	Merck
Ferulic acid	Sigma-Aldrich
Glycerol	Fluka
Glycine	Merck
Guaiacol	Merck
Hydrochloric acid	Merck
Hydroquinone	Sigma-Aldrich
L- alanine	Merck
L-cysteine	Merck
Methanol	Riedel-deHaen
Mycological peptone	Bio-Chemika
Natruim hydroxid (NaOH)	Riedel-de Haën
Potassium di hydrogen phosphate (KH_2PO_4)	Riedel-de Haën
Potassium dihydrogen phosphate	Fluka
Sodium acetate was obtained from	J.T.Baker (USA).
Sodium azide	Merck
Sodium chloride (NaCl)	Riedel-de Haën
Sodium dihydrogen phosphate	Riedel-de Haen

Sodium dodecyl sulphate	Sigma-Aldrich
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
Sodium hydroxide	Riedel-de Haen
Sorbitol	Sigma
Tris (hydrocymethyl) aminomethane	Merck
Triton-X100	Sigma
Tryptone	Sigma
Yeast Extract	Sigma
Yeast nitrogen base with ammonium sulfate and w/o amino acids	Sigma
Zeocin	Invitrogen
B-Mercaptoethanol	Merck

Enzymes	**Supplier**
*Eco*RI	Fermentas
*Sac*I	Fermentas
*Not*I	Fermentas
Taq polymerase	Fermantas
Long DNA Polymerase Mix	Fermantas
Expand High Fidelity Polymerase	Roche
T4 DNA Ligase	Fermentas

APPENDIX A.4 : Laboratory Equipment

Autoclave: Nüve OT 4060 Steam Sterilizer (Turkey), Tuttnauer 2540 ML, (Switzerland), Tuttnauer 3870 ELVC (Switzerland)

Balances: Precisa XB620C SCS (Germany), Precisa 125 A SCS (Germany)

Centrifuge rotor: F241.5P, JA-30.50 Ti Beckman Coulter (Germany)

Centrifuges: Beckman Coulter, Microfuge 18 (Germany), Avanti J-30I Beckman Coulter (Germany), Allegra™ 25R Centrifuge, Beckman Coulter (Germany)

Deep freezes and refrigerators: -80°C Heto Ultrafreeze 4410, - 80 ºC New Brunswick Scientific U410 Premium (England), Heto Polar Bear 4410 ultra freezer, JOUAN, 2021 D refrigerator, Arcelik, -20°C Arçelik 209lt,+4°C Arçelik

Electrophoresis equipments: E – C mini cell primo EC320, The Mini Protean III System Bio-Rad (USA).

FPLC: BioLogic Duo-Flow, Bio-Rad (USA)

Fraction collector: Model 2128, Bio-Rad (USA)

Gel documentation system: UVI PHoto MW Version 99.05 for Windows

Ice machine: AF 10, Scotsman (UK)

Incubators: Nüve EN400, Nüve EN500

Laminar flow cabinet: Biolab Faster BH-EN2003 (Italy)

Magnetic stirrer : AGE 10.0164, VELP Scientifica srl.

Micropipettes: Gilson pipetteman 10 µl, 20 µl, 200 µl, 1000 µl, Volumate Mettler Toledo 10 µl, 20 µl, 200 µl, 1000 µl, Eppendorf research 10 µl, 20 µl, 200 µl, 1000 µl

Micro-plate reader : Bio-rad Benchmark Microplate Reader

Orbital shaker incubators: Shell lab 1575R-2E (USA), Certomat S II, Product# 886 252 4, B. Braun Biotech Internationnal GmbH, Thermo Electron Corporation 430 (USA)

pH meter: MP 220, Metter Toledo International Inc.Wissenchaftlich-Technische Werstätten

Pure water systems: USF Elga UHQ-PS-MK3, Elga Labwater

UV-Visible Spectrophotometers: Perkin Elmer Inst. Lambda 25 (USA), Shimadzu UV-Pharmaspec 1700 (Japan)

Thermocycler: Techne FTGENE 5D

Thermomixer: Eppendorf thermomixer comfort (1.5ml)

Transillumunator: Biorad UV transilluminator 2000

Vortex machine: Heidolph Raax top (Germany)

Waterbaths: Memmert wb-22

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