

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

CLONING OF A cDNA ENCODING LACCASE FROM
Coriolopsis polyzona* MUCL 38443 AND EXPRESSION IN *Pichia pastoris

Ph.D. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Ayten KARATAŞ

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

***Coriolopsis polyzona* MUCL 38443'TEN LAKKAZ KODLAYAN BİR cDNA'NIN
KLONLANMASI VE *Pichia pastoris*'TE İFADE EDİLMESİ**

DOKTORA TEZİ

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To my parents and wife,



FOREWORD

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ABBREVIATIONS

3-HAA	: 3-hydroxyanthranilic acid
1-HBT	: 1-hydrobenzotriazole
ABTS	: 2-2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)
Amp	: Ampicillin
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
cDNA	: Complementary Deoxyribonucleic acid
dATP	: Deoxyadenosine triphosphate
DMP	: 2,6-dimethoxyphenol
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleoside triphosphate
EB	: Elution buffer
EDTA	: Ethylene diamine tetra-acetic acid
FA	: Formaldehyde
FPLC	: Fast protein liquid chromatography
gDNA	: Genomic Deoxyribonucleic acid
kb	: Kilobase
k_{cat}	: Turnover number
kDa	: Kilodalton
K_m	: Michaelis-Menten constant
LB	: Luria-Bertani
M	: Molar
min	: Minute
ml	: Milliliter
mM	: Millimolar
mRNA	: Messenger RNA
MUCL	: Mycotheque de l'Universite catholique de Louvain
Native-PAGE	: Native-Polyacrylamide gel electrophoresis
NCBI	: National Center for Biotechnology Information Database
NUP	: Nested Universal Primer
PCoA	: Principal Coordinates Analysis
PCR	: Polymerase chain reaction
PDA	: Potato Dextrose Agar
RACE	: Rapid amplification of cDNA ends
RNA	: Ribonucleic acid
rpm	: Rounds per minute
rRNA	: Ribosomal RNA
RT-PCR	: Reverse transcriptase-polymerase chain reaction
SDS-PAGE	: Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
sec	: Second
TAE	: Tris-Acetic acid-EDTA
T_m	: Melting temperature
TNT	: 2,4,6-trinitrotoluene

U	: Units
UPM	: Universal Primer Mix
UV	: Ultraviolet
μg	: Microgram
μl	: Microliter



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**CLONING OF A cDNA ENCODING LACCASE FROM
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SUMMARY

Laccases (EC 1.10.3.2) are members of the protein superfamily of multi-copper oxidases isolated from various fungi, plants, bacteria and insects that are able to catalyze the oxidation of non-phenolic and phenolic lignin related compounds and environmental pollutants. Up to date, over 120 laccases have been isolated from fungal cultures. Their biochemical and catalytic properties have been identified. They are important for several biotechnological processes such as the medical diagnosis, bioremediation (to clean up pesticides, herbicides etc.), the detoxification of industrial effluents (from paper, pulp, textile and petrochemical industries), the processing of the beverages (wine, fruit juice and beer), the gelation of the sugar beet pectin, the determination of the ascorbic acid, baking, detection processes as biosensors and being as ingredients in cosmetics and pharmaceutical area.

Lignocellulose degradation is a critical process for carbon recycling of earth's ecosystems and particularly dead wood that represents globally 5 % of organic carbon in the forest ecosystems. White-rot fungi are the only microorganisms able to degrade lignocellulosic complex by a multi-enzyme system which plays very important role in the global carbon cycle. Especially, the white rot Basidiomycetes are able to decay all wood lignocellulose components, both in softwood and hardwood, as they produce unique extracellular oxidative enzymes such as laccases. The white-rot Basidiomycetes have been a focus for lignin degradation when compared to Ascomycetes and Deuteromycetes. *Collybia velutipes*, *Fomes annosus*, *Fomes fomentarius*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Pholiota mutabilis*, *Pleurotus ostreatus*, *Poria subacida*, *Sporotrichum pulverulentum*, *Trametes sanguinea* and *Trametes versicolor* are Basidiomycetes known as best laccase producer and lignin degraders. Basidiomycete *Coriolopsis polyzona* is a white rot fungus belonging to the family of *Coriolaceae* and a promising high-yield ligninolytic enzyme producer.

In the present research, a novel laccase gene, named as *Cplcc1*, and its corresponding cDNA were isolated from the *Coriolopsis polyzona* MUCL 38443 strain, heterologously expressed and characterized. Fundamentally, a partial laccase cDNA library of *C. polyzona* MUCL 38443 was constructed. Three distinct partial laccase cDNA sequences were identified and described as *Cplcc1*, *Cplcc2* and *Cplcc3*. These laccase specific cDNA sequences showed 80 % similarity with previously submitted laccase encoding sequences in National Center for Biotechnology Information (NCBI) database. In the partial cDNA library, the dominant cDNA sequences were belonging to *Cplcc1*, which appeared to be the major expressed laccase gene in *Coriolopsis polyzona* MUCL 38443.

The *Cplcc1* gene consists of a 1563 bp open reading frame encoding a 520 amino acids protein with a 20-residue putative signal peptide. The molecular weight of the Cplcc1 laccase protein was determined as 54 kDa with theoretical MW (Compute pI/MW, ExPasy) calculator using full-length deduced amino acid sequence. While the

complete cDNA sequence of *Cplcc1* indicates 82 % similarity with *Trametes hirsuta* laccase A mRNA, the deduced amino acid sequence of *Cplcc1* also revealed 88 % identity with *Trametes hirsuta* laccase A (AIZ72721.1).

In addition, the *Cplcc1* gene was also sequenced and characterized. The size of the *Cplcc1* gene is 2106 bp with 57 % GC content and interrupted with ten introns ranging from 51-58 bp and contains five potential N-glycosylation sites. Thus, *Cplcc1* gene has 76 % identity with *Trametes hirsuta* laccase (Lac) gene (EU492907.1). The nucleotide sequences of cDNA and genomic DNA sequences of *Cplcc1* have been deposited in the GenBank database under accession number KT802746 and KT802747, respectively.

Furthermore, the full-length *Cplcc1* cDNA was successfully cloned into the yeast shuttle expression vector pPICZC with their own secretion signal sequence and expressed in methylotrophic yeast *Pichia pastoris*. To optimize the recombinant laccase production, optimum cultivation conditions including the effect of methanol concentration, copper concentration, L-Alanine concentration and growth temperature were investigated. As a result of optimization study, BMGY/BMMY medium supplemented with 1.5 % methanol, 0.8 mM CuSO₄ and 0.6 % L-Alanine with 250 rpm and 28°C were obtained as optimum conditions. The maximum recombinant CpLcc1 laccase activity was found as 800 U l⁻¹. As a result of optimization studies, laccase activity was increased approximately 9.8 fold.

Then, recombinant laccase was partially purified by ammonium sulfate precipitation, Q-sepharose anion exchange and Superdex-75 gel filtration chromatography. Based on the SDS-PAGE, the relative molecular mass of the partially purified recombinant laccase CpLcc1 was estimated as 54 kDa with the SDS-PAGE analysis which is consistent with theoretical MW and in the range reported for most of the fungal laccases. The maximum recombinant laccase activity was observed for 2,2'-azinobis-(3-ethylbenzthiaoline-6-sulfonic acid) (ABTS) as the substrate with optimum temperature as 70°C and pH value as 3.0. On the other hand, recombinant CpLcc1 was stable at 30°C more than a week and t_{1/2} were higher than 8 hours. While half-life of CpLcc1 at 40°C for 8 hours and at 50°C for 30 minutes, activity decreased dramatically in few minutes at 60°C, 70°C and 80°C. K_m, V_{max}, k_{cat} and k_{cat} K_m⁻¹ values of the recombinant laccase were identified as 0.137 mM, 288.6 μmol min⁻¹ l⁻¹, 5.73×10⁵ min⁻¹ and 4.18×10⁶ min⁻¹ mM⁻¹, respectively. Sodium azide, L-cysteine and SDS were found as usual inhibitors.

Present study is the first report on the cloning and the characterization of a laccase gene and its encoding laccase from the white rot fungus strain *Coriolopsis polyzona* MUCL 38443.

***Coriopsis polyzona* MUCL 38443'TEN LAKKAZ KODLAYAN BİR cDNA'NIN KLONLANMASI VE *Pichia pastoris*'TE İFADE EDİLMESİ**

ÖZET

Lakkazlar (EC 1.10.3.2), birçok farklı mantar, bitki, bakteri ve böcek türlerinden elde edilebilen, fenolik olan ve fenolik olmayan lignin içerikli bileşiklerin ve çevre kirleticilerin oksidasyonunu katalize edebilen, çoklu bakır oksidazların protein süper ailesinin bir üyesidir.

Bugüne kadar 120'den fazla lakkaz enzimi fungal kültürlerden izole edilerek, biyokimyasal ve katalitik özellikleri aydınlatılmıştır. Bu noktada, tıbbi tanı kitleri, biyoremediasyon (pestisit ve herbisit gibi maddelerin temizlenmesi), endüstriyel atıkların detoksifiye edilmesi (kâğıt, tekstil ve petrokimya endüstrilerinden kaynaklı), içeceklerin işlenmesi (şarap, meyve suyu ve bira), şeker pancarı pektininden jel oluşturulması, askorbik asit belirlenmesi, biyosensör yapısında belirleme için kullanılması ile kozmetik ve ilaç malzemesi olarak kullanılması gibi farklı biyoteknolojik prosesler açısından önem taşımaktadırlar.

Lignoselülozların parçalanması dünyanın ekosisteminde, özellikle orman ekosistemlerinde yer alan organik karbonların yüzde beşini oluşturan ölü odunsu yapılardan sağlanan karbon geri kazanımının gerçekleşmesi için kritik bir öneme sahiptir.

Küresel karbon döngüsü için çok önemli bir rol oynayan lignoselülozik kompleksleri degrade edebilme özelliğine sahip çoklu enzim sistemine sahip tek mikroorganizma tipi beyaz çürükçül mantarlardır. Özellikle beyaz çürükçül Basidiomisetler, tüm odunsu lignoselüloz bileşiklerinin yumuşak ve sert odunsu kısımlarının çürütülmesini sağlayan hücre dışına salgılanan lakkaz benzeri özel oksidatif enzimler üretirler. Askomiset ve Döteromisetler ile karşılaştırıldığında, beyaz çürükçül Basidiomisetler ligninin parçalanması konusunda ön plana çıkmaktadır. *Collybia velutipes*, *Fomes annosus*, *Fomes fomentarius*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Pholiota mutabilis*, *Pleurotus ostreatus*, *Poria subacida*, *Sporotrichum pulverulentum*, *Trametes sanguinea* ve *Trametes versicolor* türleri bilinen en iyi lakkaz üreticileri ve lignin parçalayıcılarını oluşturmaktadır. Basidiomycete *Coriopsis polyzona*, *Coriolaceae* ailesine ait beyaz bir çürük mantar olup, yüksek verimli bir ligninolitik enzim üreticisidir.

Heterolok ekspresyon sistemlerinin kurulması, enzim özelliklerinin iyileştirilmesini sağlayacak, protein mühendisliği çalışmalarının önünü açacak önemli bir basamaktır. Ayrıca, farklı kaynaklardan yeni lakkazların elde edilmesini kapsayan çalışmalar, endüstride yeni kullanım alanına sahip ve/veya düşük maliyetli yeni lakkazların yakalanmasını sağlaması açısından, önemli bulunmaktadır.

Bu araştırmada, *Coriopsis polyzona* MUCL 38443 suşundan izole edilen ve *Cplcc1* olarak adlandırılan yeni bir lakkaz genine ait cDNA, *Pichia pastoris*'de heterolok olarak eksprese edilmiş ve karakterizasyonu gerçekleştirilmiştir. Öncelikle, *Coriopsis polyzona* MUCL 38443 ait, kısmi lakkaz cDNA kütüphanesi kurulmuştur. Bu kütüphaneden yaklaşık 52 klona ait parça lakkaz nükleotit dizileri elde edilmiş, birbirleri ile benzerlikleri ClustalW2 ile "Principal Coordinates Analysis (PCoA)

pogramları ile karşılaştırılmıştır. Sonuç olarak kurulan kısmi lakkaz cDNA kütüphanesinde, sadece 3 farklı kısmi lakkaz cDNA dizisinin bulunduğu belirlenmiş ve *Cplcc1*, *Cplcc2* ve *Cplcc3* olarak isimlendirilmişlerdir. Bulunan bu kısmi lakkaz cDNA dizileri, Ulusal Biyoteknoloji Bilgi Merkezi (NCBI) veri tabanına önceden yüklenmiş olan ve lakkaz kodlayan diziler ile karşılaştırıldığında, yaklaşık % 80 benzerlik gösterdiği belirlenmiştir. Oluşturulmuş olan kısmi cDNA kütüphanesinde, belirlenen bu kısmi üç cDNA dizisi arasında da, en sık *Cplcc1* cDNA dizisine rastlanmıştır. Bu sonuca dayanarak, *Cplcc1* lakkaz geninin, *Coriolopsis polyzona* MUCL 38443’de en baskın ifade edilen lakkaz geni olduğu anlaşılmıştır.

Cplcc1 geninin 1563 bç uzunluğuna sahip ve 520 amino asit kodlayan bir açık okuma çerçevesi ile 20 amino asitlik sinyal peptidini içerdiği belirlenmiştir. *Cplcc1* cDNA dizisi “*Trametes hirsuta* laccase A mRNA” dizisi ile % 82 benzerlik gösterirken, *Cplcc1* genine ait çıkarılmış amino asit dizisi ise benzer şekilde “*Trametes hirsuta* laccase A” (AIZ72721.1) ile % 88 benzerlik göstermiştir. *CpLcc1* lakkazına ait moleküler ağırlık değeri, teorik moleküler ağırlık hesaplama aracı ile yaklaşık 54 kDa olarak (Compute pI/MW, ExPasy) hesaplanmıştır.

Buna ek olarak, *Cplcc1* geninin boyutu 2106 bç uzunluğunda ve % 57 GC içeriğine sahip olduğu, 51-58 bç uzunluğunda on adet intron ve beş adet potansiyel N-glikosilasyon bölgesini içerdiği tespit edilmiştir. Bununla birlikte, *Cplcc1* geni *Trametes hirsuta* lakkaz geni (Lac) (EU492907.1) ile % 76 benzerlik göstermiştir. *Cplcc1* cDNA ve genomik DNA’sına ait nükleotit sekansları GenBank veri tabanına sırasıyla KT802746 ve KT802747 erişim numarası ile kaydedilmiştir.

Ayrıca, tam boyuttaki *Cplcc1* cDNA’sı maya mekik ifade vektörü olan pPICZC’ye kendi sinyal dizisi ile birlikte başarılı bir şekilde klonlanmış ve metilotrofik bir maya olan *Pichia pastoris*’te eksprese edilmiştir.

En iyi kültür koşullarını belirlemek amacıyla, metanol konsantrasyonu, bakır konsantrasyonu, L-Alanin konsantrasyonu ve büyüme sıcaklığının etkisi araştırılmıştır. Optimizasyon çalışmaları sonucunda, optimum koşullar, BMGY/BMMY besiyeri içerisinde % 1,5 metanol, 0.8mM CuSO₄ ve % 0,6 L-Alanin eklenerek, 250 rpm ve 28°C olarak belirlenmiştir. Elde edilen en yüksek rekombinant *CpLcc1* lakkaz aktivitesi 800 U l⁻¹ olarak bulunmuş ve optimizasyon çalışmaları boyunca lakkaz aktivitesi 9,8 kat arttırılabilmektedir.

Optimizasyon çalışmaları sonrasında, rekombinant lakkazın kısmi olarak saflaştırılması, amonyum sülfat çöktürmesi, Q-sepharose anyon değişim ve Superdex-75 boyut kromatografisi adımları ile gerçekleştirilmiştir.

Kısmi saflaştırılmış rekombinant *CpLcc1* lakkazına ait rölatif moleküler ağırlık değeri SDS-PAGE analizi ile, teorik moleküler ağırlık değeri ile benzer biçimde 54kDa olarak ayrıca bulunmuştur. Bu değer önceden raporlanmış çalışmalarla uygunluk göstermektedir.

Rekombinant lakkaz aktivitesine ait maksimum değer 2,2’-azino-bis (3-etilbenzotiazolin-6-sulfonik asit) (ABTS) substratı için optimum sıcaklık olan 70°C ve optimum pH 3.0’de gözlemlenmiştir. Diğer yandan rekombinant *CpLcc1* 30°C’de bir haftadan fazla stabil kalabilirken, yarılanma ömrü ise 8 saatten yüksektir. Ayrıca 40°C ve 50°C’deki yarılanma süresi sırasıyla 8 saat ve 30 dakika iken, enzim aktivitesi 60°C, 70°C ve 80°C’lerde ise dakikalar içerisinde ani düşüş göstermektedir.

K_m , V_{max} , k_{cat} ve k_{cat}/K_m değerleri sırasıyla 0,137 mM, 288,6 $\mu\text{mol d}^{-1} \text{l}^{-1}$, $5.73 \times 10^5 \text{ d}^{-1}$ and $4.18 \times 10^6 \text{ d}^{-1} \text{ mM}^{-1}$, olarak hesaplanmıştır. Sodyum azid, L-sistein ve SDS maddeleri ise olağan inhibitörler olarak belirlenmiştir.

Sonuç olarak, bu çalışma ile ilk kez, *Coriolopsis polyzona* MUCL 38443 suşunda ifade edilen lakkaz genleri belirlenmiş, bunların arasından *CpLcc1* olarak adlandırılan

lakkaz enziminin *P. pastoris* mayasında heterolok üretimi, kısmi saflaştırılması başarı ile gerçekleştirilmiş ve biyokimyasal karakterizasyonu tamamlanmıştır.





1. INTRODUCTION

1.1 The Structure of Wood

Forests comprise 31 % of the world's land and provide the wood which is the largest commercial product (Sedjo and Lyon, 2015).

The wood consumption of the world is 3500 million m³ that increased over 65 % since 1960 especially for pulp and paper industry (Martinez et al, 2005). Lignocelluloses are especially obtained from wood (Figure 1.1), forestry based wastes and other agricultural residues and approximately 140 billion tons of biomass per year comes from plant primary metabolism. On the other hand, cellulose is a linear and mostly consisted of cellobiose which displayed 50 % of wood load and formed with D-glucose subunits linked by β -1,4 glycosidic bonds, which generates long chains called elemental fibrils linked by hydrogen bonds and van der Waals forces (Perez et al, 2002; Bouws et al, 2008; Martinez et al, 2005; Lundell et al, 2010).

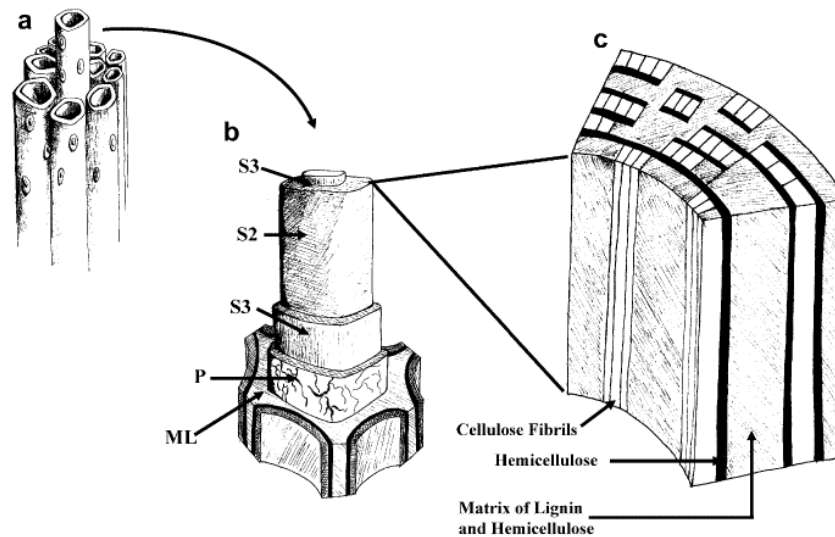


Figure 1.1 : The structure of wood: **a.** adjacent cells, **b.** cell wall layers, **S1**, **S2** and **S3** are secondary cell wall layers, **ML.** the middle lamella, **P.** the primary wall, **c.** lignin, cellulose and hemicelluloses distribution in the secondary cell wall (Perez *et al*, 2002).

Hemicelluloses forming approximately 30 % of wood weight, are composed of pentose and hexose residues as D-xylose, D-glucose, D-galactose, D-mannose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids which are bond with

rarely β -1,3-glycosidic and generally β -1,4-glycosidic bonds. They are often acetylated, and generally form branched chains. In reference to cellulose, hemicellulose is shorter than cellulose including 150-200 sugar residues. Hemicelluloses are consisted of mainly xylans together with variable percentages of galactose, arabinose, rhamnose and methylglucuronic acid units and acetyl groups in hardwood while with glucomannan in softwood (Kirk and Cullen, 1998; Perez et al, 2002; Martinez et al, 2005). The term lignin derives from the Latin word 'lignum' which means wood and is one of the three components of lignocellulosic biomass along with cellulose and hemicellulose. Lignin is the second most abundant natural substance in nature after cellulose. Additionally, lignin typically comprises 20-32 % by weight of the lignocellulosic biomass. Lignin together with hemicelluloses, an amorphous matrix in which the cellulose fibrils are embedded makes wood highly resistant to both chemical and biological degradation and this property provides mechanical resistance to wood. Lignin is a water insoluble, an amorphous and optically inactive cross-linked macromolecular material which is derived from oxidative coupling of monolignols, mainly hydroxycinnamyl alcohols as p-coumaryl, coniferyl and synapyl alcohols (Munk et al, 2015; Pollegioni et al, 2015; Martinez et al, 2005; Perez et al, 2002; Fengel and Wegener, 1984; Palonen, 2004).

The biological degradation of cellulose, hemicellulose and lignin attract the attention of scientists for many years. Fungi are the best-known microorganisms capable of degrading these polymers with extracellular enzymes (Perez et al, 2002).

1.2 White Rot Fungus and *Coriolopsis polyzona*

Lignocellulose degradation is a crucial process for carbon recycling of earth's ecosystems and especially of dead wood that represents globally 5 % of organic carbon in forest ecosystems. On the other hand, this degradation is mostly performed by fungi. Saprophytic asco- and basidiomycetes are essential microorganisms for degradation of lignocelluloses in nature. Due to the high lignin content in wood (20-40 %), this degradation is necessary for the mineralization of dead wood and increases the accessibility of the carbohydrates for other organisms such as non-ligninolytic fungi, bacteria or arthropods. The wood rotting fungi can be classified into three types: soft-rot fungi, brown-rot fungi and white-rot fungi based on their degradation mode on the different wood cell walls and components. While white rot fungi degrade lignin and hemicelluloses, brown rot fungi generally attack cellulose and hemicelluloses with its

inability to decompose lignin. Addition to this information, the ligninolytic system of soft-rot fungi does not have an oxidative potential to attack the recalcitrant guaiacyl lignin but they have the ability to oxidize and mineralize syringyl lignin. These fungi perform degradation of wood by forming microscopic cavities in the secondary cell wall. Therefore, basidiomycetes are the only microorganisms known that can perform lignin degradation with enzymatic process. Basidiomycetes particularly have different ecological functions like being symbionts and endophytes as in lichens, plant root mycorrhizas, leaves and needles; parasites and pathogens for plants and animals; or saprotrophs for conversion lignocelluloses in plant into simple sugars, oligosaccharides, and humic substances in soil. For instance, *Cryptococcus neoformans* laccase converts catecholamine into melanine in host, induces damage to the host and works as a virulence factor (Arnstadt et al, 2016; Bouws et al, 2008, Martinez et al, 2005; Arora and Sharma, 2010; Baldrian, 2006, Lundell et al, 2010; Riva et al, 2006).

The white rot Basidiomycetes are especially able to decay all wood lignocellulose components, both in softwood and hardwood, as they produce unique extracellular oxidative enzymes that degrade lignin which protects cellulose microfibrils of plant cell walls. This production causes a great attention on white rot fungi basidiomycetes. On the other hand, the white rot fungi only organisms known to mineralize lignin to CO₂ and H₂O entirely with a white fibrous material remaining and have the inability to use lignin as a sole carbon and energy source. But they have ability to oxidize side chains and cleave the aromatic component, remaining aliphatic carboxyl-rich fragments in the polymer. (Lundell et al, 2010; Wesenberg et al, 2003; Ward, 2012).

The white-rot fungi display two separated patterns of decay as simultaneous and delignification decay. In simultaneous decay, the cellulose, hemicellulose, and lignin are removed less or more simultaneously. In this type of decay, erosion channels are observed beneath the hyphae. As result, cell wall structure changes and becomes gradually thinner, often in a non-uniform manner, with holes appearing between cells as decay advances. On the other hand, the degree of decay generally varies from cell to cell. In delignification pattern, lignin and hemicelluloses are eliminated before the removal of cellulose but cell walls do not lose their morphology when compared to the simultaneous pattern. They just become unreactive to lignin stains and more reactive to cellulose stains because of the lignin coating removal (Kirk and Cullen, 1998).

Lignin peroxidase, LiP (EC 1.11.1.14), manganese peroxidase, MnP (EC 1.11.1.13) and laccase are the three major oxidative and lignin-modifying enzymes (LME) secreted by white rot fungi (Eriksson et al, 1990; Lundell et al, 2010). While other microorganisms produce only one or two of LME, some white rot fungi produce all three (Hatakka, 1994). Lignin peroxidase (LiP, 1,2-bis (3,4-dimethoxyphenyl) propane-1,3-diol: hydrogen peroxide oxidoreductase) and manganese peroxidase (MnP, Mn(II): hydrogen-peroxide oxidoreductase) were first discovered in 1980s.

LME are essential for lignin degradation, generally combined with additional enzymes. During lignin degradation, various auxiliary enzymes such as superoxide dismutase and glyoxal oxidase, that are unable to degrade lignin by themselves, are responsible for the intracellular production of H_2O_2 as LiP and MnP co-substrate. On the other hand, glucose oxidase, aryl alcohol oxidase and cellobiose dehydrogenase are involved in feedback circuits and linking lignin breakdown with cellulose and hemicellulose degradation (Leonowicz et al, 1999; Wesenberg et al, 2003).

Basidiomycete *Coriolopsis polyzona* (Figure 1.2) is a white rot fungus belonging to the family of *Coriolaceae* and known high-yield ligninolytic enzyme producer (Erikson et al, 1990; Jaouani et al, 2006).



Figure 1.2 : *Coriolopsis polyzona* (Url-1).

1.3 Laccases

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are the multicopper-containing oxidases catalyzing monoelectronic oxidation of a wide variety of organic and inorganic substrates via the reduction of oxygen to water and accompanying the oxidation of substrate molecules to produce radicals which are isolated from various fungi, plants, bacteria and insects (Giardina et al, 2010, Arora and Sharma, 2010; Riva, 2006; Martinez et al, 2005; Rivera-Hoyos et al, 2013). They are generally monomeric extracellular glycoproteins consist of 15-30 % carbohydrate with variable molecular

weight, ranging between 50 to 140 kDa, with a great diversity both in size and glycosylation patterns (Rivera-Hoyos et al, 2013; Chandra and Chowdhary, 2015).

Laccases not only charge in lignin degradation but also have various roles, such as the mediation of interaction between fungal pathogens and host plants, stress defense and morphogenesis (Thurston, 1994).

Laccase was first isolated from Japanese lacquer tree *Rhus vernicifera* by Yoshida in 1883. Therefore, laccase is one of the oldest enzymes that has been discovered and the first ligninolytic enzyme that has been investigated. On the other hand, in 2002, complete molecular structure of laccase was revealed from basidiomycete *Trametes versicolor* and the ascomycete *Melanocarpus albomyces* (Mayer and Staples, 2002; Martinez et al, 2005). After *Rhus vernicifera* laccase, another laccase was discovered in *Acer pseudoplatanus* (sycamore) cells, apricots, mung bean hypocotyls cell walls, stems of *Zinnia elegans*, rosemary cell suspension, mango fruits and in tobacco. On the other hand, phenoloxidases with laccase feature were isolated from *Drosophila melanogaster*, *Lucilia cuprina*, *Manduca sexta*, and *Sarcophaga bulata* and bacteria *Azospirillum lipoferum* and cyanobacterium *Anabaena azollae* (Gianfreda et al, 1999). In the fungi as primary laccase source, the white-rot Basidiomycetes have been a focus for lignin degradation when compared to Ascomycetes and Deuteromycetes. *Monocillium indicum* is the first ascomycete that secretes laccase showing peroxidative activity. *Sinorhizobium meliloti* laccase is the best characterized bacterial laccase as 45 kDa periplasmic protein with isoelectric point at pH 6.2 and able to oxidize syringaldazine. *Collybia velutipes*, *Fomes annosus*, *Fomes fomentarius*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Pholiota mutabilis*, *Pleurotus ostreatus*, *Poria subacida*, *Sporotrichum pulverulentum*, *Trametes sanguinea* and *Trametes versicolor* are Basidiomycetes known as the best laccase producer and lignin degraders (Baldrian, 2006; Rosconi et al, 2005).

Up to this point, over 120 laccases have been isolated from fungal cultures and their biochemical and catalytic properties have been identified (Du et al, 2015). Four ungapped conservative regions, L1-L4, were revealed which are specific for all laccases. One cysteine and ten histidine residues are located within these four 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).

Various mechanism and physicochemical characteristic, such as molecular weight, pH and optimum temperature, isoelectric point, carbohydrate content, and especially kinetic properties are different between laccases (Duran et al, 2002).

1.3.1 Molecular properties of laccase

UV/visible and electroparamagnetic resonance (EPR) spectroscopy can be used for the identification of three copper centers in laccase structure. Laccase active site contain four copper ions, which can be classified into three groups according to their electron paramagnetic resonance (EPR) features: Type 1 (T1) or blue copper, Type 2 (T2) or “normal” copper and Type 3 (T3), an antiferromagnetically coupled binuclear copper pair and the copper ions are coordinated by one cysteine and 10 histidines. Type 1 copper in the T1 site has a strong absorption band at around 600 nm and this property causes the exhibition of blue color. On the other hand, T1-type copper atoms lacking laccases are not blue. These laccases were entitled as “white or yellow” laccases and are unable to show absorption band around 600 nm named as yellow or white laccases such as *Pleurotus ostreatus* laccases (Palmieri et al, 1997; Baldrian, 2006; Gunne et al, 2014; Pollegioni et al, 2015).

While the distance between the T2 and T3 sites of the enzyme is approximately 4 Å, the T1 copper is located at the distance of about 12 Å from T2-T3 trinuclear copper center. The catalytic mechanism (Figure 1.3) is based on one-electron oxidation and four-electron reduction of molecular oxygen to water. After the electrons are taken by Type-1 (T1) copper as substrate oxidation site, these electrons are transferred to one Type-2 (T2) and two Type-3 (T3) coppers where oxygen reduction takes place (Baldrian, 2006; Morozova et al, 2007; Gunne et al, 2014; Munk et al, 2015).

The first step of the laccase reaction mechanism is the substrate oxidation that is carried by a primary electron acceptor, T1 copper. Then, electron is transferred to the T2/T3 trinuclear cluster through His-Cys-His motif which is the rate-limiting step for catalysis. While 4 electron oxidation from different substrate molecules were performed, laccase enzyme turns into fully reduced form. After that, the molecular oxygen binds to the T2/T3 site and reduced to two molecules of water (Figure 1.4) (Mate et al, 2010; Dwivedi et al, 2011; Shleev et al, 2012; Pollegioni et al, 2015).

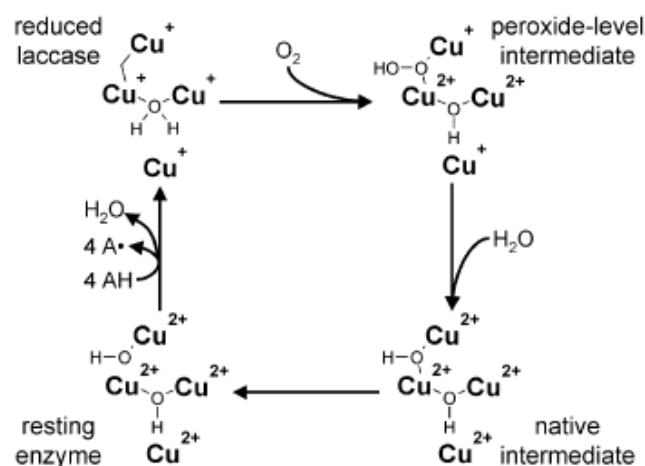


Figure 1.3 : The catalytic cycle of laccase (Wesenberg et al, 2003).

Laccase activity on phenol oxidation is known to be proportional to the redox potential difference between the T1 copper and the substrate (Mate et al, 2010). *Trametes versicolor* laccase as the most widely studied fungal laccase has shown that the catalytically important copper ions are located in the different domains with the T1 copper positioned on the surface of the enzyme, whereas one T2 and two T3 copper atoms, respectively, that form a trinuclear copper cluster (T2/T3), are situated centrally at the interface between two domains in the enzyme that provide the ligand residues for copper atoms coordination (Sitarz et al, 2014). The phenolic substrate is firstly subjected to one-electron oxidation giving rise to an aryloxyradical and subsequently, it 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 in charge with decarboxylation and demethylation (Wesenberg et al, 2003; Giardina et al, 2010; Duran et al, 2002).

1.3.2 Laccase oxidation on different substrates

Laccases can oxidize a variety of aromatic and non-aromatic compounds with a broad substrate range and especially act with low specificity on o-phenols and p-phenols and mostly also on aminophenols and phenylenediamines with four electrons transfer from organic substrate to molecular oxygen. (Baldrian, 2006; Kües, 2015). These enzymes generally use phenolic compounds as hydrogen donors for reduction of O₂ to water and catalyze single electron transfer from different substrates, including mono-, di-

and polyphenols, aminophenols, methoxyphenols (Figure 1.5) (Galhaup et al, 2002; Baldrian, 2006; Yan et al, 2014). DMP, guaiacol and syringaldazine are common phenolic substrates for laccase with the ability to be oxidized to phenoxy radicals (Figure 1.6). In this process, they can polymerase via radical coupling or rearrange themselves leading to quinones through disproportionation, C α oxidation, alkyl-aryl cleavage, cleavage of C α -C β bond or aromatic ring (Giardina et al, 2010).

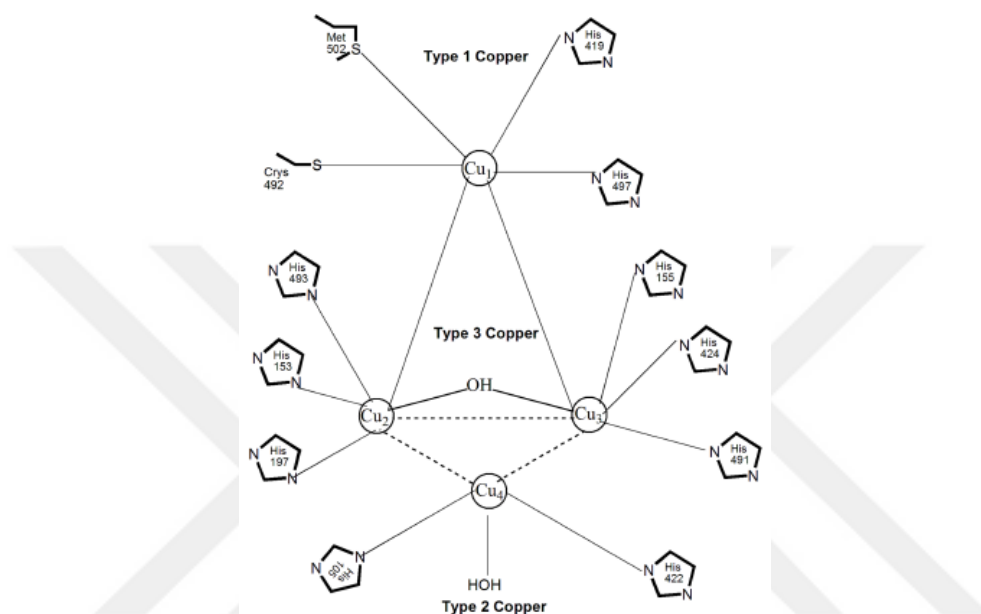


Figure 1.4 : The copper coordination of laccase structure (Christopher et al, 2014).

Higher rate substrate oxidation has two main reasons; high redox potential of laccase or lower oxidation potential of substrate (Madhavi and Lele, 2009).

Oxidation of some substrates could not be performed by laccases. There are two possible reasons for this situation. These substrates can be too large for the penetration to active site of laccase or have higher redox potentials when compared to laccases (ten Have and Teunissen, 2001). In order to overcome with these problems, the laccase mediator system (LMS) was applied and “mediators” that are intermediate substrates for the laccase were used. These chemical mediators can make enzymes able to oxidize non-phenolic compounds (Figure 1.5). During this oxidation, these mediators are also reduced by substrates at the same time (Riva, 2006; Couto and Herrera, 2006a; Christopher et al, 2014).

The laccase mediator system (LMS) was first described in 1990 with the use of ABTS (2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) to overcome problems in wood pulps bio-bleaching (Bourbonnais and Paice, 1990).

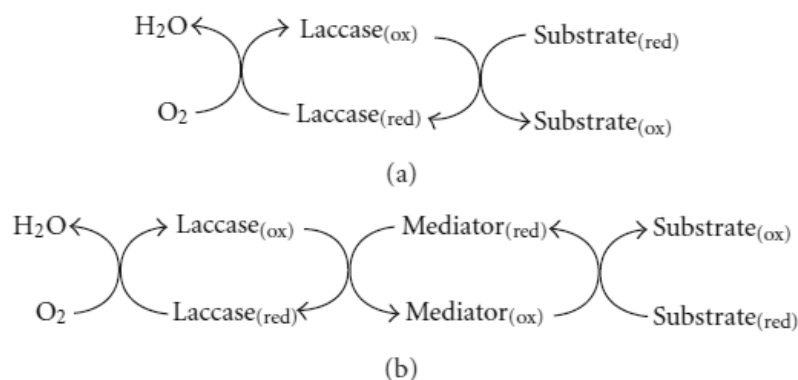


Figure 1.5 : Laccase-catalyzed redox cycles scheme for substrates oxidation in the absence **(a)** or in the presence **(b)** of redox mediators (Riva, 2006).

Several chemical mediators as ABTS, 1-hydrobenzotriazole (1-HBT), violuric acid and various natural mediators as 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol, and 3-hydroxyanthranilate have been discovered (ten Have and Teunissen, 2001).

Laccase oxidation on its substrate in a liquid medium with oxygen supply from air and at atmospheric pressure is dependent on the: (a) laccase structure and redox potential, (b) structure and redox potential of the substrate, (c) Buffer pH, (d) temperature of incubation, (e) solvent concentration, and (f) laccase activity. All these factors are crucial for the comparison of the different laccases with their cleavage and synthesis efficiencies (Hahn et al, 2014).

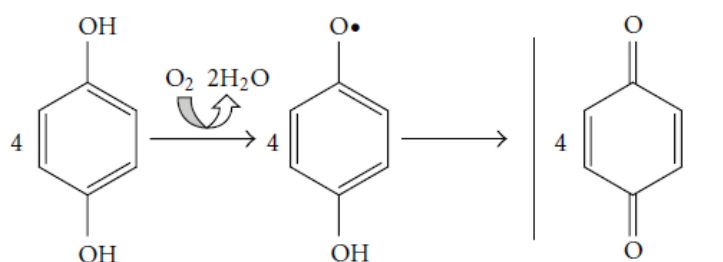


Figure 1.6 : Reactions on phenolic compounds catalyzed by laccases (Osma et al, 2010).

1.3.3 Inhibition of laccase

Various compounds have the ability to inhibit several laccases. While small ions such as halides (excluding iodide), cyanide, azide, and hydroxide could bind to the Type-2 and Type-3 Cu atoms causing an interruption of the internal electron transfer and activity inhibition, fatty acids, sulfhydryl reagents, hydroxylglycine, kojic acid, EDTA and cationic quarternary ammonium detergents may result in amino acid residue

modification, copper chelation, or conformational change. The inhibitory effect of reducing agents L-cysteine and β -mercaptoethanol has been suggested as breaking disulfide bonds between sulfur-containing amino acids in laccases. Dithiothreitol, thioglycolic acid, cysteine and diethyldithiocarbamic acid are previously reported as laccase inhibitors, which have no ability to reduce the oxygen consumption by laccase during the catalysis. The inhibition by heavy metals (e.g. Hg^{2+} , Mg^{2+} , Ca^{2+} , Sn^{2+} , Ba^{2+} , Co^{2+} , Cd^{2+} , Mn^{2+} , Zn^{2+}) must be considered due to the natural occurrence of laccases in soil. Some laccases exhibit sensitivity towards heavy metals. The ionic strength of the solution affects both the solubility of reaction products and enzymatic performance. Therefore, ionic strength can totally inhibit laccase activity. Under alkaline pH conditions, laccase inhibition by OH^- ions can dominate catalysis. On the other hand, various substrates of laccase can competitively inhibit the transformation of other compounds in the complex environment of soil or decaying lignocellulosic material (Madhavi and Lele, 2009; Gianfreda et al, 1999; Garcia et al, 2007; Majeau et al, 2010; Baldrian, 2006).

1.4 Recombinant Expression of Laccases

Large amounts and highly active enzymes are necessary for biotechnological and environmental applications. Otherwise, laccase secreted from plant or fungal organisms may not be proper for these applications because of low yield, economical disadvantage and unwanted preparation procedures (e.g. using toxic inducers). Herein, the cloning of laccase genes and heterologous expression are performed in bacteria, yeasts, filamentous fungi and plants for better laccase production. Recombinant laccase production could be optimized using strong promoters and signal sequences which cause extracellular secretion directly to the medium culture with lower purification costs. Therefore, since the beginning of the nineties, recombinant expression of laccases has been very attractive for many research groups (Strong et al, 2011; You et al, 2014; Viswanath et al, 2014; Antořová and Sychrová, 2016; Piscitelli et al, 2010).

The first laccase sequences were recorded from *N. crassa* (Germann and Lerch, 1986), the ascomycete fungus. In 1990's, *Coriolus hirsutus* (Kojima et al, 1990), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), *Phlebia radiata* (Saloheimo et al, 1991), *Agaricus bisporus* (Perry et al, 1993), *Podospora anserina* (Fernandez Larrea and

Stahl, 1996), *Trametes versicolor* (Jönsson et al, 1997), *Ganoderma lucidum*, *Phlebia brevispora*, *Lentinula edodes*, *Lentinus tigrinus* (D'Souza et al, 1999), *Pycnoporus cinnabarinus* (Eggert et al, 1998), *Coprinus congregatus* (Leem et al, 1999) and *Pleurotus ostreatus* (Giardina et al, 1999) laccase sequences as approximately between 520 and 550 amino acids were published.

Bacteria were used for recombinant laccase production due to easy and cost effective manipulation (Antošová and Sychrová, 2016). In bacteria, extracellular recombinant laccase yields are generally in the range of 2-5600 U l⁻¹ (Durao et al, 2008; Ihssen et al, 2015). *Escherichia coli* was used for the heterologous expression of laccases from several bacterial sources, such as *Bacillus licheniformis* (Koschorreck et al, 2008), *Bacillus clausii* (Brander et al, 2015), *Bacillus subtilis* (Durao et al, 2006; Beneyton et al, 2014), *Bacillus halodurans* (Ruijsenaars et al, 2004) *Klebsiella sp.* (Li et al, 2008), *Marinomonas mediterranea* (Sanchez-Amat et al, 2001), *Aeromonas hydrophila* (Ng et al, 2013), *Streptomyces griseorubens* (Feng et al, 2015) and also for the fungal laccase from *Cyathus bulleri* (Garg et al, 2008) and laccase from white-rot basidiomycete *Rigidoporus lignosus* (Nicolini et al, 2013). On the other hand, the heterologous expression of laccases in *E. coli* generally have low yields because of aggregation of recombinant proteins which cause difficulty for purifications steps (Antošová and Sychrová, 2016). When compared to *E. coli*, *Streptomyces lividans* was a better host for high yielded laccase production and purification (Dube et al, 2008).

Plants were used as hosts for the recombinant expression of several fungal and plant laccases such as LCC1 laccase from *Trametes versicolor* expressed in *Zea mays* (Bailey et al, 2004), LAC1 from *Gossypium arboreum* (Wang et al, 2004) and SofLAC from sugarcane expressed in *Arabidopsis thaliana* (Cesarino et al, 2013).

Filamentous fungi have a natural ability to secrete laccase enzymes, extracellularly. On the other hand, few number of filamentous fungi have been used for heterologous protein production because of genetic manipulation problems. Popular filamentous fungi hosts for recombinant laccase production are the asexually reproducing *Aspergillus niger*, *Aspergillus oryza*, *Trichoderma reesei* and *Aspergillus nidulans* (Antošová and Sychrová, 2016; Piscitelli et al, 2010). For example, Gene IV from *Trametes versicolor* (Baker et al, 2000), LAC1 from *Melanocarpus albomyces* (Hakulinen et al, 2008), *lacA* gene from *Trametes sp* AH28-2 (Zhang et al, 2012) and laccase from *Stachbotrys chartarum* (Mander et al, 2006) were expressed in *Trichoderma reesei*; LCC1 and LCC2 laccases from *Trametes versicolor* (Bohlin et

al, 2006), Gene IV from *Trametes versicolor* (Téllez-Jurado et al, 2006), LAC1 from *Pycnoporus cinnabarinus* (Ravalason et al, 2009) and *pox2* from *Pleurotus ostreatus* (Weenink et al, 2006) were expressed in *Aspergillus niger*; *Lac 1* and *Lac 4* genes from *Trametes versicolor* (Fujihira et al, 2009), LCC1 from *Rhizoctonia solani* (Wahleithner et al, 1996), LCC1 from *Trametes villosa* (López-Cruz et al, 2006), LCC1 from *Lentinula edodes* were expressed in *Aspergillus oryza* (Yano et al, 2009) and LCS-1 from *Ceriporiopsis subvermispora* was expressed in *Aspergillus nidulans* (Larrondo et al, 2003).

Yeast cells take part in the biotechnological production of recombinant proteins of eukaryotic origin with various advantages such as being economical, high yield production, lower time and effort demand. They have eukaryote-specific post-translational modification ability (e.g. proteolytic processing, disulfide bridge formation, glycosylation). Furthermore, yeasts are in charge for developing with desired properties. *Saccharomyces cerevisiae* and *Pichia pastoris* are especially popular yeasts for laccase recombinant expression. In addition, plant laccases were produced in these two yeasts. Since 2005, studies become available for the heterologous expression of fungal laccases in the non-conventional yeasts such as *Pichia methanolica*, *Kluyveromyces lactis* and *Yarrowia lipolytica* (Strong et al, 2011; Antořová and Sychrová, 2016; Piscitelli et al, 2010). Many laccase genes and cDNAs were cloned from different microorganisms and heterologously expressed in yeasts *Saccharomyces cerevisiae* (Cassland and Jönsson, 1999; Necochea et al, 2005; Bleve et al, 2008), *Pichia pastoris* (O'callaghan et al, 2002; Gu et al, 2014; Liu et al, 2015), *Pichia methalonica* (Guo et al. 2006), *Yarrowia lipolytica* (Madzak et al, 2005; Theerachat et al, 2012) and *Kluyveromyces lactis* (Pezzella et al, 2009; Arana-Cuenca et al, 2010).

Laccase heterologous expression systems have also been used to measure the effectiveness of different promoters with other aims such as (i) to examine the effect of constitutive activation of the unfolded-protein response pathway on the heterologous protein production, (ii) to investigate the protein quality control mechanism, (iii) to select higher heterologous proteins producers, (iv) to analyze the physiological state of an organism in biotechnical processes, (v) to reduce the effect of protease activity on recombinant enzyme (Piscitelli et al, 2010).

Generally fungal species have more than one laccase-encoding gene. Although the primary structure of these laccase isoenzymes are highly similar, expression level and

physicochemical characteristics are quite different. Therefore, investigation of each laccase properties of isoenzymes is very crucial for application of suitable laccases in particular industrial fields. High yielded recombinant laccase production with desired properties is essential for industrial applications. Gene cloning strategies give the opportunity to understand the structure-function relationships as a primary step for heterologous expression of laccases in various organisms, which are easily cultivated and producing the desired amount of the protein with low cost (Gu et al, 2014; Piscitelli et al, 2010; Kunamneni et al, 2008a).

To date, laccases have been isolated and characterized from diverse organisms such as fungi, plants, insects, bacteria and archaea. However, fungal laccases are still dominant in the industrial applications. In this regard, efficient recombinant expression of fungal laccases is, often, required due to the low yield of fungal laccases, which makes their application limited for commercial use. Another interesting feature is that their recombinant expression allows directed evolution towards producing improved laccase properties with desirable physicochemical characters with respect to higher redox potential, further extreme optimal pH values and better thermostability compared to the native ones (Piscitelli et al, 2010).

1.5 *Pichia pastoris*, A Work Horse

Yeasts are favorable host organisms for the production of recombinant proteins with eukaryotic origin because of easy genetic manipulation (e.g. site-directed mutagenesis, directed evolution or DNA shuffling), rapid growth at high yield on cost-effective media, extracellular enzyme production and ability to perform complex post-translational modifications. Furthermore, protein expression level in yeasts can be controlled by a variety of strong inducible or constitutive promoters and cultivation conditions, such as composition and pH of growth medium or temperature. Yeast or fungal signal sequences can be used for secretion of the laccases into the expression medium. Methylophilic yeast *Pichia pastoris* which has a total genome size of 9.43 Mbp with four chromosomes is able to metabolize methanol as sole carbon source for energy with a specific metabolic pathway involving various enzymes. *Pichia pastoris* has become a very popular as a host organism for the production of heterologous proteins with ability to increase the bioactivity of various heterologous proteins by reducing proteolytic degradation of the expressed recombinant protein (Cereghino et al, 2002; Antořová and Sychrová, 2016; Macauley-Patrick et al, 2005; Fickers, 2014).

Since 1980s, *P. pastoris* becomes a successful tool for the production of heterologous proteins due to the tightly regulated and strong promoter of the *AOX1* gene (pAOX1). While *AOX1* and *AOX2* encode alcohol oxidase, *Aox1* shows 90 % of the total alcohol oxidase activity (Cos et al, 2006). On the other hand, pAOX1 is repressed in the presence of glucose, glycerol or ethanol and strongly induced in the presence of methanol. Sorbitol has no significant repressive effect on its induction. Several expression vectors for recombinant protein production have been developed for *P. pastoris*. All of these vectors are shuttle vectors that can work both *E. coli* and *P. pastoris*. They have an origin of replication for plasmid DNA maintenance and propagation in *E. coli* and selectable markers for both organisms. Most of these vectors contain a multiple cloning site (MCS) for insertion of a heterologous coding sequence, flanked by promoter and termination sequences most of the time derived from *AOX1*. These vectors contain the sequence for in-frame fusion between recombinant protein and various secretion signals (Ahmad et al, 2014; Fickers, 2014; Inan and Meagher, 2001; Çalık et al, 2010; Niu et al, 2013). *Pichia pastoris* is now a desirable industrial platform for the production of heterologous proteins because of being effective and ideal expression system for production of extracellular proteins with high yield (Liu et al, 2015).

1.6 Industrial Applications of Laccases

The conventional oxidation technologies possess some disadvantages during oxidation reactions in several industrial applications. These disadvantages are generally undesirable, non-specific side reactions and use of chemicals which are hazardous to environment. In order to overcome these drawbacks, new oxidation technologies based on biological systems such as enzymatic oxidation were investigated.

Enzymes are specific and biodegradable catalysts and their reactions are also carried out in mild conditions. Enzymatic oxidation techniques are applied in various industrial fields including the pulp and paper, textile and food industries. Recycling on molecular oxygen as an electron acceptor is an attractive property for enzymes in industrial applications. At this point, laccases are particularly promising enzymes for the development of highly efficient, sustainable, and ecofriendly industrial applications with various properties such as being specific, energy-saving, and biodegradable. Laccases have widespread applications including pulp and paper, textile, bioremediation, food, pharmaceuticals, cosmetics, nanobiotechnology and

analytical applications (Couto and Herrera, 2006a; Madhavi and Lele, 2009; Kunamneni et al, 2008b; Osma et al, 2010; Kudanga and Le Roes-Hill, 2014).

1.6.1 Food and beverage industries

Biocatalysts have been widely applied in food and beverage industries for a long time and involve the application of whole microbial cells or cell extracts, purified enzymes, immobilized cells or immobilized enzymes as catalysts for several processes. Food and beverage enzymes compose the largest part of industrial enzymes. While the market for food industrial enzymes was near \$1.2 billion in 2011 which is also expected to grow to \$1.8 billion by 2016 with annual growth rate of 10.4 %. Laccase application in the food industry is generally based on its ability on polymerization of molecules. Potential applications of laccases exist in various aspects of food industry such as beverage processing and stabilization, ascorbic acid determination, sugar-beet pectin, gelation, baking, etc. Many laccase substrates, mostly phenols, thiol-containing proteins and unsaturated fatty acids are common components of several foods and beverages. The modification of these components, which make food industry benefit from laccase application, may lead to cost reduction, new functions and finally quality improvement. Laccases mainly avoids undesirable changes such as discoloration, clouding, haze, or flavour changes in beer, fruit juices, and wine with improvement of their shelf life by removing phenols (e.g. coumaric acids, flavans, and anthocyanins) (Adrio and Demain, 2014; Pezzella et al, 2015; Arora and Sharma, 2010; Pannu and Kapoor, 2005; Viswanath et al, 2014).

1.6.1.1 Bioremediation of food industry wastewater

The classical methods used in the treatment (refinement) of wastewater (classical precipitation, ion exchange, ozone treatment, coagulation, flocculation, adsorption, etc.) are far from being practical and economical because of their investment and management costs and also reemergence of new pollutants after a certain period. Laccase activity and occurrence of laccases in fungus species were demonstrated in these studies (Kılıç et al., 2016).

Laccase immobilized on polyethersulphone showed chemical and physical properties potentially useful for decreasing phenol concentration in a model wastewater solution (Lante et al, 2000). For instance, some fractions of beer-factory wastewaters represent an important environmental concern due to their high content of polyphenols and dark-

brown color. Yague et al. (2000) reported a white-rot fungus laccase producer *Coriolopsis gallica*, for degradation of this high-tannin-containing wastewater. A *Trametes sp.*, another white-rot fungus laccase producer, was tested in bioremediation of a wastewater which is generated during ethanol production from fermentation of sugar-cane molasses, called vinasses. Vinasses contain a high content of soluble organic matter with a dark-brown color. Maximum decolorization was held with seven days of fungal treatment as 73.3 % and 61.7 %, respectively (Gonzalez et al, 2000). Olive mill wastewaters are a characteristic by-product of olive oil production which is a major environmental problem in the Mediterranean area. Two opposite approaches can be conducted with this effluent are disposal of sewage and spreading on soils. At this point, reported studies indicated that olive oil mill wastewater can also be used in laccase production by fungi (Minussi et al, 2002).

1.6.1.2 Sugar beet pectin gelation

The sugar beet pectin is a functional food ingredient to form thermo-irreversible gels. Therefore, these gels can be heated while maintaining the gel structure. Generally, peroxidases are main agents for this kind of gelation. Nowadays, hydrogen peroxide addition was forbidden to food products in many countries. In some research, it was reported that laccases are more efficient as gelling agents than peroxidases (e.g. luncheon meat and milk). On the other hand, different studies indicated that laccases have the ability to cross-link beet pectin molecules covalently (Osma et al, 2010).

1.6.1.3 Baking

Laccases are important enzymes in baking due to their ability to cross-link biopolymers. With the addition of a laccase enzyme to the dough, laccases can apply an oxidizing effect on the dough constituents and serve to improve the strength of gluten structures. Generally, the use of laccase in baking is reported to result in an increased strength, stability, and reduced stickiness. This situation causes improved machinability of the dough, an improved crumb structure and softer baked product (Minussi et al, 2002; Osma et al, 2010). A *Trametes hirsuta* laccase increased the maximum dough resistance and decreased the extensibility of dough in both flour and gluten dough (Selinheimo et al, 2006).

1.6.1.4 Wine and beer stabilization

The high concentration of phenolics and polyphenols show up during the crushing and pressing steps of wine production. The high concentration of polyphenols from stems, seeds, and skins have negative effects on wine color and astringency. Minussi et al. (2007) suggested a treatment with laccase for the removal of polyphenols selectively. A commercialized laccase (Suberzyme®) was used for preparing cork stoppers for wine bottles (Kunamneni et al, 2008b).

The use of laccase for stabilization is not limited to wine; the beer industry also benefits from the treatment of laccases. Classic haze formation in beer is attributed protein precipitation stimulated by small quantities of proanthocyanidins polyphenols. This complex formed is commonly referred to as chill haze which occurs upon cooling of the beer. The addition of laccase at the end of processing has the effect on the removal of polyphenols and reduced oxygen content results in a longer shelf life of beer (Minussi et al, 2002; Brijwani et al, 2010).

1.6.1.5 Fruit juice clarification

Naturally occurring phenols in fruit juices which interact with proteins cause formation of haze or sediments. In addition, juice browning also imputable with both enzymatic and chemical oxidation phenomena involving phenols turns out into a final product which is more preferable by consumers. Several authors have proposed the use of laccases as stabilizing agents, due to their ability to oxidize most of the phenols in juices. When laccase treatment was followed by a filtration step, juice quality could be highly improved (Minussi et al, 2002; Pezzella et al, 2015). Neifar et al. (2011) used a laccase ultrafiltration process for controlling the haze formation and pomegranate juice browning. Recently, Lattera et al. (2016) used a recombinant POXA1b laccase from *P. ostreatus* expressed in *Pichia pastoris* for a laccase-based fruit juice mixture with 45 % phenol reduction.

1.6.1.6 Detection in food industry

Laccase secreted by *Botrytis cinerea* may cause various forms of rot in grapes. The enzyme is present only in musts and wines made with grapes infected by the fungus. Different methods have been developed to detect laccase activity in must as an indicator of *Botrytis cinerea* infection in grapes (Minussi et al, 2002).

1.6.2 Pulp and paper industry

In paper industry, the separation and degradation of lignin in wood pulp are applied using chlorine- or oxygen-based chemical oxidants. The formation of chlorinated aliphatic and aromatic compounds that could be toxic, mutagenic and carcinogenic. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. At this point, laccases can provide milder and cleaner strategies of delignification with ability to depolymerize lignin and delignify wood pulps, kraft pulp fibers and chlorine-free in the biopolpation process. Laccases are able to delignify pulp when they are used together with mediators. Mediators could be used to oxidize the non-phenolic residues from the oxygen delignification. The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates. Laccases are also suitable to the bioremediation of pulp and paper industry waste by effecting direct dechlorination, the removal of chlorophenols and chlorolignins from bleach effluents. Other uses of laccases for the pulp and paper industry contain reduction of the kappa number of pulp and an improvement in the paper making properties of pulp. In another application, laccases can be even used for deinking and decolorizing a printed paper. Fungal laccases (e.g. laccases from *T. villosa*, *T. versicolor*, *P. sanguineus*, *P. cinnabarinus*, *T. harzianum* WL1 and *P. eryngii*) are generally used for the treatment of effluents from pulp mills or chlorolignins or phenolic effluents from other industries. Furthermore, the application of laccases in pulp-kraft bleaching may result in higher pulp yields and energy savings (Mayer and Staples, 2002; Kunamneni et al, 2008b; Virk et al, 2012; Viswanath et al, 2014; Pannu and Kapoor, 2015).

1.6.3 Textile industry

The chemical reagents used in textile industry have been ranging from inorganic compounds to polymers and organic products with various chemical compositions. The most of the several chemicals are hard to decolorize due to their synthetic origin. Conventional processes for the treatment of dye wastewater are ineffective and not economical. Therefore, the development of laccase based processes seems an attractive solution due to their potential in degrading dyes of diverse chemical structure, including synthetic dyes currently employed in the industry. The use of laccases in

textile industry provides a “green” way at mild process conditions. The application of laccase in the textile industry range from cotton fiber washing, textile dye and bleaching as well as precursor of production to elimination of the former coloring matter in waste-waters. Laccase is used in commercial textile applications to improve the whiteness in conventional cotton bleaching and biostoning. Potential benefits of the application include chemicals, energy, and water saving. Laccases find potential applications for cleansing, such as cloth- and dishwashing. Laccase also could be included in a cleansing formulation in order to eliminate the odor on fabrics, including cloth, sofa surface, and curtain, or in a detergent to eliminate the odor generated during cloth washing (Pannu and Kapoor, 2015; Kunamneni et al, 2008b; Rivera-Hoyos et al, 2013).

A laccase from *M. thermophila* has been used for indigo dye oxidation in denim cloth (Riva, 2006). Lantto et al. (2004) also investigated that wool fibers can be activated with laccase mediator system. Therefore, the use of laccase for anti-shrink treatment of wool seems very attractive. A patent application about the use of LMS to increase the shrink resistance of wool has been already published (Yoon, 1998).

The degradation of textile dyes by laccases of different fungi has been reported. Laccase from *P. 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).

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

1.6.4 Bioremediation

Laccases and laccase mediator systems are applied to remove toxic or recalcitrant pollutants like herbicides, pesticides, dyes, various xenobiotics, polycyclic aromatic hydrocarbons, chlorophenols and other contaminants. Phenolic compounds are present in wastes from several industrial processes, as petroleum refining, coal conversion,

production of organic chemicals and olive oil production among others. Laccases were responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone. Laccase mediator systems have been also used to oxidize alkenes, carbazole, fluorene, N-ethylcarbazole and dibenzothiophene. The most studied mediators for laccases are 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxybenzotriazole (1-HBT), and 3-hydroxyanthranilic acid (3-HAA). Oxidation is different for ABTS when compared to HBT, involving a di-cation and a benzotriazolyl-1-oxide radical, respectively. Oxygen uptake by the laccase is faster with ABTS than with HBT, but the oxidation of non-phenolic substrate is comparable for both the mediators. Isoxaflutole is an herbicide which is activated in soils and plants to its diketonitrile derivative, the active form of the herbicide. At this point, laccases are able to convert the diketonitrile into the acid for bioremediation purpose. Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills. On the other hand, laccases could be used for decolorizing dye house effluents that are difficult to decolorize by conventional sewage treatment plants. In addition to dye house effluents, laccases also can decolorize waste waters from olive oil and pulp mills by removing colored phenolic materials. Thus, laccases are able to mediate the coupling of reduced 2,4,6-trinitrotoluene (TNT) to an organic soil matrix, which resulted in detoxification of the munition residue. Otherwise, laccase may be applied to degrade plastic waste having olefin units (Kunamneni et al, 2008b; Brijwani et al, 2010; Varjani, 2016; Chandra and Chowdhary, 2015; Pannu and Kapoor, 2015; Couto and Herrera, 2006a).

1.6.5 Nanobiotechnology

Bioelectrochemistry has received increased attention during the past two decades. Progress on bioelectrochemistry has been integrated into analytical applications. A biosensor is an integrated biological-component probe with an electronic transducer, thus converting a biochemical signal into electrical response in quantity that detects, transmits and records information regarding a physiological or biochemical change. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, real-time analysis and operation simplicity. Laccases could be applied as biosensors or bioreporters. Furthermore, laccase containing biosensors have been developed to detect various phenolic compounds, oxygen, glucose, aromatic amines or azides, since laccases are able to catalyze electron transfer reactions without

additional cofactors. Laccases are covalently conjugated to a biobinding molecule which can be used as a reporter for immunochemical (e.g. Western blotting, ELISA), histochemical, cytochemical or nucleic acid detection assays. The bioreporter applications are of interest for the high-sensitivity diagnostic field (Couto and Herrera, 2006a; Kunamneni et al, 2008b; Varjani, 2016). Nurmi et al. (2005) applied dimetallic nanoparticles as an effective oxidant instead of granular zero-valency metal in the cleanup of environmental pollutants. In terms of high redox potential laccases, a mutation was recently used as to design a laccase that could function in human blood, so that it could be incorporated into 3D nanobiodevices for biomedical purposes (Mate et al, 2013).

In addition to biosensors, immobilization of laccases on the cathode of biofuel cells is possible for providing power such as for small transmitter systems. Fuel cells are remarkable energy sources, particularly at micro-, mini-, portable-, or mobile-scale, that potentially have higher energy conversion/usage efficiency and lower pollution effect than any of the existing energy sources. Laccase may be applied as a biocatalyst for the electrode reactions. A laccase-based miniature biological fuel cell is of particular interest for many medical applications calling for a power source implanted in a human body (Kunamneni et al, 2008b; Couto and Herrera, 2006a; Chen et al, 2001).

1.6.6 Pharmaceutical and cosmetic industries

Laccases have the ability to catalyze the synthesis of several therapeutics such as antibiotics, antimicrobials and anti-cancer drugs. The first industrial application of laccase with a pharmaceutical approach using laccase enzyme is actinocin synthesis which has been prepared from 4-methyl-3-hydroxyanthranilic acid. This compound provides anticancer capability and works by blocking the transcription of DNA from the tumor cell (Burton, 2003). Recently, the laccase-catalyzed synthesis of anhydrovinblastine, mitomycin and structurally similar anti-cancer compounds has been reported. Laccases from *T. giganteum*, *L. ventriospora*, *L. edodes*, *P. cornucopiae*, *C. comatus*, *L. tigrinus*, *H. coralloides*, *A. placomyces* and *A. biennis* have been reported as inhibitors against HIV-1 reverse transcriptase activity (Pezzella et al, 2015).

Laccase mediator system was also used for the determination of monoclonal antibody of azelaic acid from oleic acid (Kunamneni et al, 2008b).

Another novel application field for laccases is in cosmetics. An increasing interest has been focused on laccase application in the cosmetics as a biocatalyst in the hair dye synthesis and in the formulation of personal hygiene products, including toothpaste, deodorant, mouthwash, detergent and soap (Kunamneni et al, 2008b). Cosmetic and dermatological preparations containing laccases for skin lightening have been developed (Golz-Berner et al, 2004).

Laccase based hair dying is an emerging field of research because traditional chemical based hair coloring products are irritant, difficult to handle and not safe for this purpose, several laccases were proposed instead of H₂O₂ as an oxidizing agent in the dye formulation (Pezzella et al, 2015) such as an alkaline laccase secreted by *Flammulina velutipes* (Saito et al, 2012), a thermoalkali laccase from the actinomycete *Thermobifida fusca* (Chen et al, 2013) and bacterial laccase Lac15D (Fang et al, 2014).

1.7 Aim of the Thesis

The white-rot fungus *Coriolopsis polyzona* MUCL 38443 promising industrial enzyme producer (Jaouani et al, 2006) with several enzymes which could be used in wide range of biotechnological and industrial applications. Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are one of the most important enzyme group among those enzymes. Heterologous expression of the cloned laccase coding genes in suitable hosts and modern recombinant DNA techniques permit to carry out the production of enzymes in large scale with desired modifications, low costs, reduced timing and cultivation disadvantage, when compared to production with fungi natively.

In this thesis, it was aimed to isolate and characterize a full-length cDNA encoding laccase from *Coriolopsis polyzona* MUCL 38443. Additionally, it was also aimed to achieve the heterologous expression of the cDNA encoding laccase in methylotrophic yeast *Pichia pastoris* and the biochemical characterization of the recombinant laccase. This is the first study on the cloning and the characterization of a laccase encoding gene and its corresponding encoding laccase from the white rot fungus strain *Coriolopsis polyzona* MUCL 38443.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains

The white rot fungus *Coriolopsis polyzona* MUCL 38443 was obtained through EU-FP6 SMI-IP (IP: 505899). White rot fungus was firstly grown on Potato Dextrose Agar (PDA) at 28°C for 7 days in order to active cells kept at -80°C. *E. coli* DH5 α bacteria were used for subcloning studies and it was grown in Luria- Bertani (LB) medium at 37°C. The wild-type methylotrophic yeast *Pichia pastoris* X-33 was used as heterologous host with an easy selection of Zeocin™ antibiotic on YPD medium at 28°C in this study.

2.1.2 Plasmid DNAs

pGEM®-T Easy vector for the cloning of PCR products and a shuttle expression vector pPICZC were obtained from Promega and Invitrogen, respectively.

2.1.2.1 pGEM®-T Easy vector

pGEM®-T Easy Vector System (Figure 2.1) is a convenient system for the cloning of PCR products. The vectors are prepared by cutting with *EcoRV* and adding a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site increase the efficiency of ligation of a PCR product into the plasmid DNAs by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often add a single deoxyadenosine (dA), in a template independent manner, to the 3'-ends of the amplified fragments.

The high copy number pGEM®-T Easy Vector contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase. Insertional inactivation of the alpha-peptide allows recombinant clones to be directly identified by blue/white screening on plates.

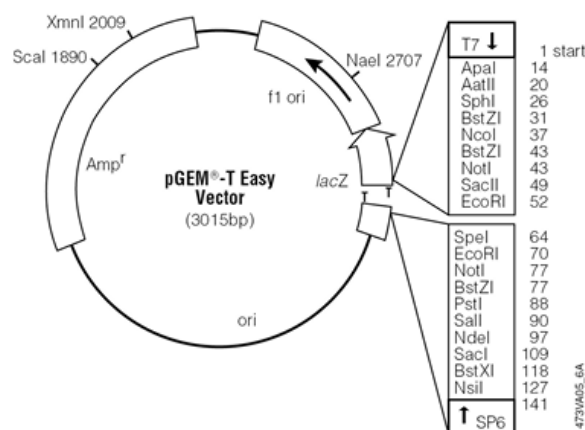


Figure 2.1 : Genomic map of pGEM®-T Easy Vector including restriction map and functional gene, *lacZ*.

2.1.2.2 The shuttle expression vector, pPICZC

pPICZC expression vector (Figure 2.2) is a 3.3 kb expression vector used to express recombinant proteins in *Pichia pastoris*. In this system, recombinant proteins are expressed as fusions to a C-terminal peptide containing the c-myc epitope and a polyhistidine (6xHis) tag (Table 2.1). The vector allows methanol inducible expression of the gene of interest in *Pichia* strain with high-level expression, and can be used in several *Pichia* strains including X-33, GS115, SMD1168H and KM71H. pPICZC has 5' fragment containing the *AOX1* promoter for tightly regulated, methanol induced expression of the gene of interest. On the other hand, the positive selection of transformants in both *E. coli* and *Pichia pastoris* is performed by Zeocin™ resistance gene in the vector.

Zeocin™ ($C_{55}H_{83}N_{19}O_{21}S_2Cu$) is an antibiotic isolated from *Streptomyces*, belongs to a member of the bleomycin/phleomycin family, and highly effective in a variety of organisms, including mammalian, insect cell lines, yeast, bacteria, and plants. As an effect mechanism, Zeocin™ causes the reduction of Cu^{2+} to Cu^{+} and is removed by sulfhydryl compounds in the cell and Zeocin™ becomes activated and causes cell death by intercalating into and cleaving DNA.

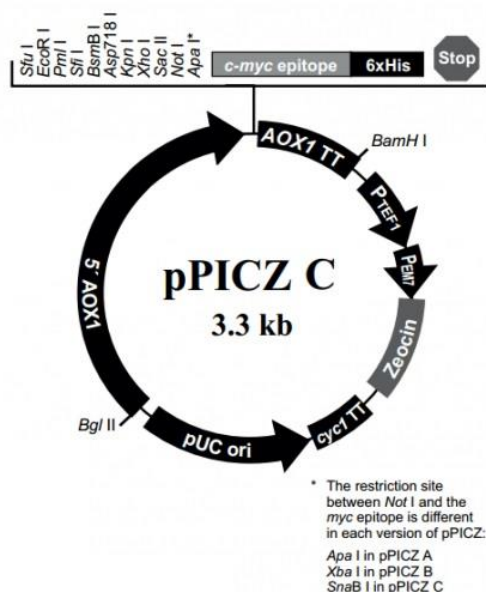


Figure 2.2 : Genomic map of pPICZC Expression Vector including restriction map.

2.2 Methods

2.2.1 Cultivation of bacteria and yeast strains

White-rot fungus *Coriolopsis polyzona* MUCL 38443 was grown on solid Potato dextrose agar (PDA) and cultivated in liquid LSK medium (Hüner, 2007) on a rotary shaker at 150 rpm at 28°C in this study.

E. coli DH5 α strain was grown in Luria-Bertani (LB) liquid medium at 37°C. Storage of the bacteria for longer times was performed by preparation of 10 % glycerol stock and it was kept at -80°C. For cloning with pGEM[®]-T Easy vector, ampicillin (*Amp*) antibiotic (100 μ g/ml) was used as the selective agent for *E. coli* DH5 α strain. On the other hand, Zeocin[™] was used for the selection of recombinant colonies for the expression where pPICZC shuttle vector was used. *Pichia pastoris* wild-type strain X-33 has grown in YPD. The growth temperature of *Pichia pastoris* is 28°C for liquid cultures and solid plates. Glycerol stocks of the *Pichia* cultures were prepared in YPD containing 30 % glycerol and stored at -80°C. The 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 OD₆₀₀ = ~5 x 10⁷ cells/ml for *Pichia pastoris*. Transformant bacteria were selected on the low salt LB plates supplemented with 25 μ g/ml Zeocin, whereas transformant *Pichia* cells were selected in the YPDS plate with 100 μ g/ml Zeocin. Before inoculation of liquid mediums, both *E. coli* and *Pichia* strain were

activated on low salt LB plates and YPD plates from -80°C stocks, respectively. Methanol was added every day to methanol containing growth media, to compensate for methanol loss as a result of evaporation or consumption and also to sustain the induction of expression.

Table 2.1 : Components in the pPICZC expression vector.

Components	Properties
5'-AOX	A fragment (942 bp) containing the <i>AOX1</i> promoter, allows methanol-inducible expression in <i>Pichia</i> and also targets Plasmid DNA integration into the <i>AOX1</i> locus of <i>Pichia</i> genome.
Multiple cloning site with 10 restriction sites	Cloning of interested gene into the expression vector.
C-terminal myc epitope tag	Detection of fusion protein by Anti-myc Antibody or Anti-HRP antibody.
<i>SacI</i> , <i>PmeI</i> , <i>BstXI</i>	Unique restriction sites permitting linearization of vectors at <i>AOX1</i> locus for the integration into the <i>Pichia</i> genome.
C-terminal polyhistidine tag	Purification of recombinant fusion protein on metal chelating Resin.
<i>AOX1</i> Transcription termination	Native transcription termination and polyadenylation signal of <i>AOX1</i> gene (260bp) allows 3' mRNA processing, including poly-A for increased mRNA stability.
<i>TEF1</i> promoter	Transcription elongation factor 1 gene promoter of <i>S. cerevisiae</i> driving <i>Sh ble</i> gene expression in <i>Pichia</i> , conferring Zeocin resistance.
EM7(synthetic prokaryotic promoter)	Synthetic prokaryotic promoter that drives constitutive expression of the Zeocin™ resistance gene in <i>E. coli</i> .
Zeocin resistance	Ble gene (<i>Streptoalloteichus hindustanus ble</i> gene) for selection of <i>E coli</i> and <i>Pichia</i> strains.
CYC1 transcription termination region	3'-end of the <i>S. cerevisiae</i> <i>CYC1</i> gene allowing 3'-mRNA processing of the <i>Sh ble</i> gene for increased stability.
pUC origin	Allows replication and maintenance of plasmid DNA in <i>E.coli</i> .

2.2.2 Culture media composition

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

2.2.3 Buffers and solutions

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

2.2.4 Chemicals and enzymes

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

2.2.5 Laboratory equipment

The laboratory equipment used during this study is listed in Appendix A4.

2.2.6 Nucleic acid manipulation techniques

Nucleic acid manipulation techniques including genomic DNA, and total RNA from *Coriolopsis polyzona* MUCL 38443, 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 are given below.

2.2.6.1 Genomic DNA isolation

Genomic DNA isolation from *Coriolopsis polyzona* MUCL 38443 procedure was carried out with Fast Prep Instrument and FastDNA™ Spin Kit (MPBio). First, frozen 100 mg *Coriolopsis polyzona* pellet was resuspended with 0.8 ml CLS-TC and transferred into Lysing Matrix A. Then, Fastprep Instrument was used at level 4 for 20 seconds for cell disruption. After that, in order to discard pellet debris, centrifugation was applied at 14000 rpm for 10 min. After centrifugation, supernatant (700 µl) was transferred into new clean 1.5 ml tube. In order to bind the DNA into the matrix, 500 µl Binding matrix was added into transferred supernatant and samples were inverted for 5 min at room temperature. After binding, centrifugation was applied at 14000 rpm for 1 min and the supernatant was discarded. 500 µl SEWS-M was added and the pellet was resuspended with pipeting. Then, resuspended Binding Matrix was transferred into SPIN Module (Spin filter and catch tube) and centrifugation was applied at 14000 rpm for 1 min. After that, catch tube contents were discarded, replaced and centrifuged at 14000 rpm for 1 min. In order to get genomic DNA from Spin filter, catch tube was replaced with recovery tube. After adding DES, resuspension with Binding matrix was made above spin filter and incubated for 5 min at 55°C in a heat block. After incubation, centrifugation was applied at 14000 rpm for 1 min to get eluted DNA into Recovery tube and the spin filter was discarded. Isolated genomic DNA was aliquoted and stored at -20°C.

2.2.6.2 Total RNA isolation

Coriolopsis polyzona MUCL 38443 was grown in LSK medium at 28°C for 7 days, in which laccase activity in the culture is in maximum level, were collected and total RNA was isolated with RNeasy Plant Mini Kit (Qiagen). All steps were carried out at room temperature quickly in order to avoid RNA degradation. 10 µl β-mercaptoethanol was added to lysis buffer RLC, containing guanidine hydrochloride, just before use.

Cells were pelleted by centrifugation at 12000 rpm for 30 min. The supernatant was completely removed and biomass was stored at -80°C before isolation. 100 mg *Coriopsis polyzona* pellet was ground to a fine powder with liquid nitrogen using a mortar and pestle. Tissue powder was transferred to an appropriately 2 ml tube and liquid nitrogen have 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 and centrifuged for 2 min at 14000 rpm. The supernatant of the flow-through was then transferred to a new microcentrifuge tube carefully. 0.5 volume of ethanol (96-100 %) was added to the cleared lysate, and mixed by pipetting. The sample was transferred to the RNeasy spin column and centrifuged for 15 sec at 10000 rpm. Flow-through was discarded, 350 µl Buffer RW1 was added to the spin column and centrifuged for 15 sec at 10000 rpm. 10 µl DNase I stock solution was added to 70 µl Buffer RDD and mixed by gently inverting the tube to achieve DNase I incubation mix. Then, the DNase I incubation mix (80 µl) was added directly to the RNeasy spin column membrane, and placed on the benchtop for 15 min. After that, 350 µl Buffer RW1 was added to the spin column and centrifuged for 15 sec at 10000 rpm. 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 sec at 10000 rpm 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 10000 rpm. 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 40 µl RNase-free water directly to the spin column membrane, centrifuged for 1 min at 10000 rpm to elute the RNA.

2.2.6.3 Plasmid DNA isolation

Plasmid DNA isolation from *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 A2.

The bacterial cells were harvested by centrifugation at 13000 rpm for 5 min. 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

min. 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 min on ice and then centrifuged at 13000 rpm for 15 min. 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 min. The pellet was washed with 1 ml of 70 % ethanol by centrifugation at 13000 rpm for 5 min. The supernatant was removed and ethanol was dried out at 37°C for 45 min. The pellet was then dissolved in 15 µl of elution buffer (EB), incubated at 37°C for 30 min and stored at -20°C. The isolated DNA was run on 1 % agarose gel.

2.2.6.4 Polymerase chain reaction (PCR)

Polymerase chain reaction was carried out in different steps of this dissertation. Total RNA screening of *Coriopsis polyzona* MUCL 38443 with degenerate primers, cloning of laccase encoding cDNA in terms of RACE-PCRs and the full-length *Cplcc1* cDNA synthesis were performed with PCR using different primers and conditions. All conditions in terms of PCR were explained in the relevant parts. The annealing temperatures were calculated with T_m values of primers and the final elongation time was used according to the size of the fragment to be amplified as 1 min for 1 kb fragment.

2.2.6.5 Enzymatic digestion

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

2.2.6.6 Agarose gel electrophoresis

Neutral agarose gel system, composed of 1 % agarose gel containing 1X TAE buffer (Appendix A.2) and SYBR Green I Nucleic Acid Gel Stain (Thermo) 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 was adjusted with the purpose of the electrophoresis and the size of the nucleic acid molecule.

2.2.6.7 Gel extraction from agarose gel

Gel extraction protocol was used for extraction and purification of PCR products and linear plasmid DNAs from agarose gel using the “QIAquick Gel Extraction Kit” (Qiagen). The DNA fragment was cut off from the gel, 3 volumes of QG buffer was added according to the weight of the fragment and the solution was incubated for 10 min at 50°C by vortexing every 2-3 min 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 min. The flow through was discarded, 500 µl buffer QG was added to the column and centrifuged at 13000 rpm for 1 min. 750 µl buffer PE was added to wash the column, then stood for 2-5 min and centrifuged at 13000 rpm for 1 min which was followed with an additional 1 min at 13000 rpm. The column was placed into a clean 1.5 ml microfuge tube, approximately 30 µl EB buffer was applied to the center of the QIAquick membrane, stood for 1 min and was centrifuged for 1 min at 13000 rpm. Eluted DNA fragment was run on 1 % agarose gel and stored at -20°C for further analysis.

2.2.6.8 Formaldehyde denaturing gel

Isolated total RNA integrity was controlled 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. The ratio of 28S rRNA to 18S RNA generally should be 2:1. As a result of gel degradation of RNA can be understood if the ribosomal bands are not sharp and appear as a smear with smaller sized RNAs.

2.2.6.9 Screening of laccase-specific sequences at the cDNA level with degenerate primers

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. Transcriptor First Strand Synthesis Kit (Roche) was used for laccase gene-specific cDNA sequences using 6.5 µl total RNA as template, 1 µl oligo (dT) primer and PCR grade dH₂O and completed to 13 µl and the mixture was incubated at 65°C for 15 min in PCR device. 4 µl 5X Reverse transcriptase buffer, 0.5 µl inhibitor, 2 µl dNTP mixture and 0.5 ml Reverse Transcriptase enzyme were added to this reaction to complete the volume up to 20 µl and were incubated at 55°C for 35 min and reverse transcriptase was inactivated at 85°C for 5 min. After cDNA first strand

synthesis, this reaction mix was used as template for PCR reaction using Expand High Fidelity PCR System (Roche). 2 µl template (cDNA), 5 µl 10X Expand High Fidelity buffer with MgCl₂, 1 µl dNTP mix, 2 µl LAC-N1 primer, 2 µl LAC-C1 primer, 0.5 µl Expand High Fidelity enzyme and 37.5 µl PCR grade dH₂O were used for 50 µl final reaction volume. PCR conditions; 94°C for 3 min, 94°C for 1 min, 50°C for 1 min, at 72°C for 2 min and 30 sec (30 cycles) and 72°C for 10 min as a final extension.

2.2.6.10 *Cplcc1* Gene Isolation from *Coriolopsis polyzona* MUCL 38443 genomic DNA

Pfu polymerase enzyme (Fermentas) was used for the amplification of *Cplcc1* gene. In PCR procedure, 0.5 µl *Coriolopsis polyzona* genomic DNA (300 ng) as template, 5 µl 10X *Pfu* Buffer (with MgSO₄), 1 µl dNTP mix (2 mM each), 1 µl *Cplcc1* forward primer (10 mM) and 1 µl *Cplcc1* reverse primer (10 mM), 1 µl *Pfu* DNA polymerase and 36.5 µl PCR grade dH₂O were used for 50 µl final reaction volume. PCR conditions; 95°C for 3 min for initial denaturation, 35 cycles; 95°C for 30 sec, 70°C for 30 sec, 72°C for 4.5 min and finally 72°C for 15 min for the final extension. Therefore, this fragment was polyadenylated for further TA cloning steps using Taq polymerase. In order to apply this procedure, 2 µl 10X PCR buffer (Intron), 5 µl purified *Cplcc1* gene fragment, 0.5 µl i-Taq™ DNA Polymerase (5U/µl) (Intron), 0.5 µl dATP (10 mM) and 12 µl PCR Grade dH₂O were mixed and incubated at 72°C for 30 min.

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

Reverse Transcriptase PCR (RT-PCR) and 5'-3' RACE techniques (Rapid Amplification of cDNA ends) were used for the isolation of the full-length laccase cDNA of *Coriolopsis polyzona* MUCL 38443 from total RNA. Rapid Amplification of cDNA Ends (RACE) is a procedure which is applied 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 (Yaver et al, 1996; Hoshida et al, 2001) (Figure 2.3).

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). 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. (Frohman et al, 1988 and 1993). In this thesis, 5' and 3'- ends of *Cplcc1* cDNA was obtained with BD SMARTer™ RACE cDNA Amplification Kit (Clontech).

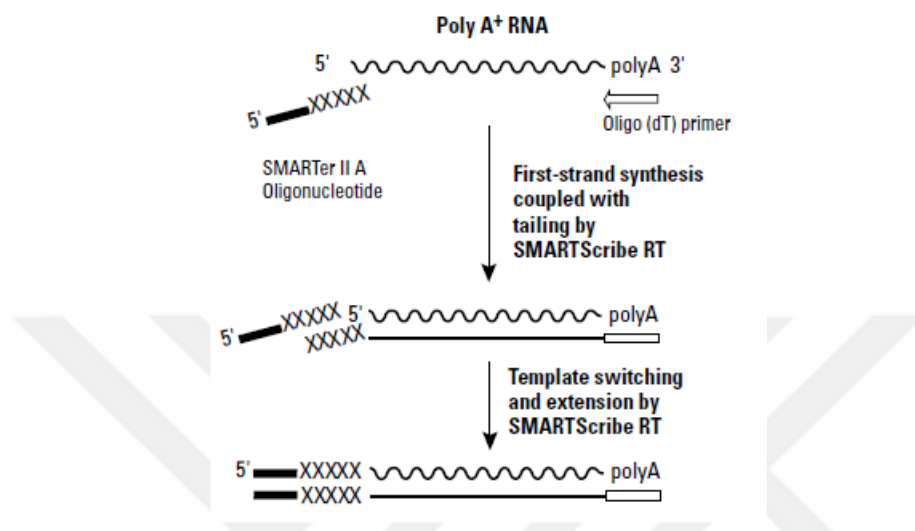


Figure 2.3 : Mechanism of SMARTer™ cDNA synthesis. First-strand synthesis primed with a modified oligo (dT) primer and after SMARTScribe Reverse Transcriptase (RT) reaches the end of the mRNA template, it adds several nontemplate residues.

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. 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 (Figure 2.4). Primers should be 23-28 nt long and should have a GC content of 50-70 % with a T_m of at least 65°C. Primers both designed and commercially used in RACE experiments are designated in Table 2.2.

Determination of 5' end of the *Cplcc1* cDNA

5' RACE ready cDNA synthesis

First, 2 µl of total RNA and 1 µl 5'-CDS primer A were mixed and completed to 3.75 µl with sterile dH₂O. This mixture was incubated at 72°C for 3 min and 42°C for 2 min. Then, 1 µl SMARTer IIA Oligo was added to this mixture. After that, 2 µl 5X First-Strand Buffer, 1 µl DTT (20 mM) and 1 µl dNTP mix (10 mM), 0.25 µl RNase

inhibitor (40 U/ μ l) and 1 μ l SMARTScribe™ Reverse Transcriptase (100 U) were added to this reaction for 10 μ l final volume. This final mixture was incubated at 42°C for 90 min. and then 70°C for 10 min. for cDNA synthesis. Finally, 40 μ l Tricine-EDTA Buffer was added to this mixture and stored at -20°C with aliquots.

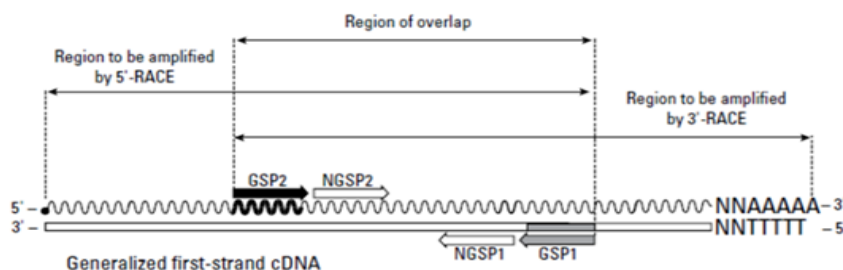


Figure 2.4 : Schematic representation of the 5'- and 3'- RACE PCR for cDNA amplification, the relationship of gene-specific primers to the cDNA template is shown. In terms of laccase specific cDNA, primers were designed based on the sequence of the conserved copper binding region.

5' RACE PCR reaction

PCR amplification of 5' RACE cDNA synthesis was accomplished by using gene specific 5'RACE reverse primer. In PCR procedure; 34.5 μ l PCR grade water, 5 μ l 10X Advantage 2 PCR Buffer, 1 μ l dNTP mix (10 mM), 2.5 μ l 5' RACE cDNA, 5 μ l Universal Primer Mix (10X), 1 μ l 5'RACE reverse primer (10 μ M) and 1 μ l 50X Advantage 2 Polymerase Mixture were mixed for 50 μ l final volume. PCR conditions were held as 94°C for 30 sec, 68°C for 30 sec and 72°C for 2 min (30 cycle). Amplified 5' RACE fragment was cloned into the pGEM®-T Easy Cloning Vector (Promega) and sequenced with ABI PRISM® 3100 Genetic Analyzer.

5' RACE Nested PCR reaction

As PCR mixture, 38.5 μ l PCR grade water, 5 μ l 10X Advantage 2 PCR Buffer, 1 μ l dNTP mix (10 mM), 2.5 μ l 5' RACE product (1/50 dilution), 1 μ l Nested Universal Primer Mix (10X), 1 μ l 5'RACE nested reverse primer (10 μ M) and 1 μ l 50X Advantage 2 Polymerase Mixture for 50 μ l final volume. PCR conditions were held as 94°C for 30 sec, 68°C for 30 sec and 72°C for 2 min (15 cycle). Amplified 5' Nested RACE fragment was cloned into the pGEM®-T Easy Cloning Vector (Promega) and sequenced with ABI PRISM® 3100 Genetic Analyzer.

Table 2.2 : Designed and commercial primers used in the first strand synthesis, RACE Reactions and DNA sequencing.

Primer	Sequence
LAC-N1	5'-CAYTGGCAYGGNTTYTTCYCA-3'
LAC-C1	5'-TGRAARTCDATRTGRCARTG-3'
5'RACE reverse	5'-AGCCTGGTCAGGGACCTGGAAGTCGT-3'
5'RACE nested reverse	5'-ATGAACGCGGGACCATCGGCCAGTT-3'
3'RACE forward	5'-CGACAACCCGATCTTCCGCGACGTCG-3'
3'RACE nested forward	5'-CACGATCCGCTTCCGCACCGACAACC-3'
<i>Cplcc1</i> forward primer	5'-CCGGAATTCAAGCATGTTCGCGTTCAACCAGCTC-3'
<i>Cplcc1</i> reverse primer	5'-AACGCGGCCGCTTACTGGTCGTTGGGGTCGAG-3'
3'-RACE CDS Primer A	5'-AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ V N-3' (N = A, C, G, or T; V = A, G, or C)
5'-RACE CDS Primer A	5'-(T) ₂₅ V N-3' (N = A, C, G, or T; V = A, G, or C)
SMARTer II A Oligonucleotide	5'-AAGCAGTGGTATCAACGCAGAGTACXXXXX-3'
10X Universal Primer A Mix (UPM)	Long (0.4 µM): 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' Short (0.2 µM): 5'-CTAATACGACTCACTATAGGGC-3'
Nested Universal Primer A (NUP)	5'-AAGCAGTGGTATCAACGCAGAGT-3'
M13 reverse	5'-AACAGCTATGACCATG-3'
M13 forward	5'-GTTTTCCCAGTCACGAC-3'

Determination of 3' end of the *Cplcc1* cDNA

3' RACE ready cDNA synthesis

First, 2 µl of total RNA and 1 µl 3'-CDS primer A were mixed and completed to 4.75 µl with sterile dH₂O. This mixture was incubated at 72°C for 3 min and 42°C for 2 min. Then, 2 µl 5X First-Strand Buffer, 1 µl DTT (20 mM), 1 µl dNTP mix (10 mM), 0.25 µl RNase inhibitor (40 U/µl) and 1 µl SMARTScribe™ Reverse Transcriptase (100 U) were added to this reaction for 10 µl final volume. This final mixture was incubated at 42°C for 90 min and then 70°C for 10 min for cDNA synthesis. Finally, 40 µl Tricine-EDTA Buffer was added to this mixture and stored at -20°C with aliquots.

3' RACE PCR reaction

PCR amplification of 3' RACE cDNA synthesis was accomplished by using gene specific 3'RACE forward primer. PCR mixture; 34.5 µl PCR grade water, 5 µl 10X

Advantage 2 PCR Buffer, 1 μ l dNTP mix (10 mM), 2.5 μ l 3' RACE cDNA, 5 μ l Universal Primer Mix (10X), 1 μ l 3' RACE forward primer (10 μ M) and 1 μ l 50X Advantage 2 Polymerase Mixture were mixed for 50 μ l final volume. Touchdown PCR was carried out in this step. Because Touchdown PCR was recommended by SMARTer™ RACE cDNA Amplification Kit Manual if T_m value of gene specific primer is over 70°C. PCR conditions were held 5 cycle; at 94°C for 30 sec and at 72°C for 2 min., 5 cycle; at 94°C for 30 sec, at 70°C for 30 sec and at 72°C for 2 min. 30 cycle; at 94°C for 30 sec, at 68°C for 30 sec and at 72°C for 2 min as final step. Amplified 3' RACE fragment was cloned into the pGEM®-T Easy Cloning Vector (Promega) and sequenced with ABI PRISM® 3100 Genetic Analyzer.

3' RACE Nested PCR reaction

As PCR mixture; 38.5 μ l PCR grade water, 5 μ l 10X Advantage 2 PCR Buffer, 1 μ l dNTP mix (10 mM), 2.5 μ l 3' RACE product (1/50 diluted), 5 μ l Nested Universal Primer Mix (10X), 1 μ l 3' RACE nested forward primer (10 μ M) and 1 μ l 50X Advantage 2 Polymerase Mixture for 50 μ l final volume. PCR conditions; 15 cycle; at 94°C for 30 sec, at 68°C for 30 sec and at 72°C for 2 min. Amplified 3' Nested RACE fragment was cloned into the pGEM®-T Easy Cloning Vector (Promega) and sequenced with ABI PRISM® 3100 Genetic Analyzer.

2.2.6.12 The amplification of full length *Cplcc1* laccase cDNA

Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used for full length *Cplcc1* cDNA synthesis. For this purpose, first step as cDNA synthesis, 2 μ l total RNA, 1 μ l Anchored-oligo(dT)18 Primer (50 mol/ μ l) and 8.4 μ l PCR grade dH₂O were mixed in PCR tube. Then, this reaction was incubated in PCR device at 65°C for 10 min and cooled on ice. 4 μ l Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer (5X), 0.5 μ l Protector RNase Inhibitor (40 U/ μ l), 2 μ l dNTP mixture (10 mM), 1 μ l DTT ve 1.1 μ l Transcriptor High Fidelity Reverse Transcriptase Enzyme were added into this reaction for 20 μ l final volume. After that, this mixture was incubated at 55°C for 10 min, then at 85°C for 5 min for reverse transcriptase inactivation and cooled on ice. 5 μ l of product was used for the PCR step and rest of reaction was aliquoted and kept at -20°C for a short time.

PCR amplification of full-length *Cplcc1* laccase cDNA was held with Expand High Fidelity PCR System (Roche). 5 μ l template cDNA, 5 μ l Expand High Fidelity Buffer

(with 15 mM MgCl₂, 10X), 1 µl dNTP mix (10 mM), 1.5 µl *Cplcc1* Forward primer (10 mM) ve 1.5 µl *Cplcc1* reverse primer (10 mM) (Table 2.2), 0.75 µl Expand High Fidelity Enzyme ve 35.25 µl PCR grade dH₂O were used for 50 µl final reaction volume. PCR conditions were 94°C for 2 min for initial denaturation, 5 cycle; 94°C for 30 sec, 55°C for for 30 sec, 72°C for 2.5 min and 25 cycle; 94°C for 30 sec, 60°C for 30 sec, 72°C for 2.5 min and 72°C for 10 min as final extension.

2.2.6.13 Ligation of the PCR products into the cloning vector

Purified PCR fragments using gel extraction were ligated into pGEM®-T Easy Cloning Vector (Promega) according to the pGEM®-T Easy Vector Systems Protocol. As ligation reaction; 5 µl 2X Rapid ligation buffer, 1 µl pGEM-T Easy vector, 2-3 µl PCR product, 0-1 µl PCR grade dH₂O (depends on PCR product volume) and 1 µl T4 DNA ligase enzyme were mixed. Reaction mixture was briefly mixed and incubated at 25°C for 1 hour then for the maximum number of transformants is required, the reactions was incubated overnight at 4°C. After incubation, the reaction was incubated at 65°C for 10 min for the inhibition of ligase.

2.2.6.14 Cloning into shuttle pPICZC vector for expression

Laccase cDNAs were cloned into yeast shuttle expression vector pPICZC (Invitrogen) with own secretion signal sequence of laccases. Shuttle vectors can propagate in two different host species. Therefore, a DNA in a shuttle vector could be manipulated in two different cell types (Strathern and Higgins, 1991). The pGEM®-T Easy cloning vector including PCR product and the pPICZC expression vector were digested with suitable restriction enzymes, *EcoRI* and *NotI*. After gel extraction, 20 µl ligation reaction was set as 2 µl 10X Ligation buffer (Fermentas), 2 µl pPICZC vector (~100 ng), 2 µl *Cplcc1* laccase cDNA from The pGEM®-T Easy cloning vector, 13 µl PCR grade water and 1 µl T4 DNA ligase enzyme (Fermentas). Then, this reaction was incubated at 22°C for 1 hour, then overnight at 4°C in order to increase colony number. After that, reaction was incubated 65°C for 10 min for the full inactivation of T4 DNA Ligase. The ligation product was transformed into *E. coli* DH5α chemically competent cells, spreaded Zeocin-LB agar plates (25 µg/mL final concentration) and incubated overnight at 37°C.

2.2.7 Transformation of *E. coli* DH5 α chemically competent cells

Obtained recombinant plasmid DNAs were cloned into chemically competent *Escherichia coli* DH5 α cells subsequent to ligation.

2.2.7.1 Preparation of *E. coli* DH5 α chemically competent cells

Chemically competent *E. coli* cells were prepared for this protocol. 500 μ l of overnight LB broth *E. coli* DH5 α culture was transferred into 50 ml LB broth in 500 ml flask and incubated at 37°C and 180 rpm until OD₆₀₀ value of culture reached 0.4-0.5. Cells were separated into precooled sterile tubes at 20 ml volume and incubated on ice for 10 min. Then, these cultures were centrifuged at 4000 rpm, 4°C for 10 min. The supernatant was separated and pellets were resuspended with 1 ml cold 100 mM CaCl₂ and transferred into precooled 1.5 ml tubes at 100 μ l volume. For the storage at -80°C, 11 μ l 100 % glycerol was added to each tube.

2.2.7.2 Transformation of *E. coli* DH5 α with recombinant plasmid DNA

For transformation step, 5-10 μ l of ligation product was added into competent cells tube and incubated on ice for 45 min. Then, tube including competent cells and ligation product, were incubated on waterbath at 37°C for heat shock for 5 min. After that, reaction mixture was added into 1 ml LB broth in 2 ml tube and incubated at 37°C, 180 rpm for 1 hour. Finally, mixture was centrifugated at 5000 rpm for 10 min, pellet was resuspended with 100 μ l saline solution (0.9 % NaCl), spreaded on X-gal/IPTG/Amp LB agar plates for The pGEM[®]-T Easy cloning vector including desired PCR products or Zeocin-LB agar plates for The pPICZC shuttle vector including desired PCR products and incubated overnight at 37°C.

2.2.8 DNA Sequence Analysis

The cloned fragments into cloning and expression vectors were sequenced with sequencing primers M13 reverse primer, M13 forward primer and laccase specific other primers (Table 2.2). 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. Sequence similarities for both cDNA and gDNA sequences were determined by alignment of laccase gene sequences from GenBank-EMBL-DDBJ-PDB databases using the BLAST homology search. Multiple

alignment was achieved using ClustalW2. Putative amino acids sequence encoded by *Cplcc1* was determined with ExPASy-Translate tool. While multiple alignment was achieved using ClustalW2, Molecular Evolutionary Genetics Analysis (MEGA) Version 6 and GenAlEx 6.41 softwares were used for distance-based analysis of laccase specific cDNA sequences in order to investigate different laccase isomers in *Corioloropsis polyzona* MUCL 38443. Phylogenetic analysis was performed using the MEGA version 6.0 software. The phylogenetic tree was constructed using neighbor-joining method that incorporates a Poisson correction model.

2.2.9 Transformation of methylophilic yeast *Pichia pastoris* X-33

2.2.9.1 Preparation of *Pichia pastoris* X-33 chemically competent cells

Pichia EasyComp™ Transformation Kit (Invitrogen) was used for competent cell preparation and transformation of *Pichia pastoris* cells. As chemically competent cell preparation protocol; 10 ml of Yeast Extract Peptone Dextrose (YPD) was inoculated with a single colony of *Pichia pastoris* X-33 and grown overnight at 30°C in a shaking incubator at 250 rpm.

Cells were diluted in 10 ml YPD from the overnight culture to an OD₆₀₀ of 0.1-0.2. Cells were grown at 30°C until the OD₆₀₀ is 0.8 (approximately 4-6 hours). Cells were precipitated by centrifugation at 500 x g for 5 min at room temperature and the supernatant was discarded. Pellet was resuspended 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 min at room temperature, supernatant was 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.

2.2.9.2 Transformation of *Pichia pastoris* with recombinant pPICZC including PCR product

According to *Pichia pastoris* X-33 transformation protocol; Solution II and Solution III were equilibrated in room temperature before transformation. Two tubes of *Pichia pastoris* competent cells for each transformation were thawed at room temperature. *SacI* and *PmeI* restriction enzymes could be used for linearization of recombinant

plasmid DNAs. These enzymes were applied into full-length *Cplcc1* laccase cDNA fragments in order to control that these enzymes were appropriate for this purpose or not. These enzymes must not digest full-length laccase cDNA fragments in the middle which can cause unfunctional/short genes. After this experiment step, *PmeI* was considered as suitable enzyme for linearization. 4 µg linearized (with *PmeI* restriction enzyme) recombinant pPICZC shuttle vectors including desired PCR product were added to each 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 min by vortexing to increase transformation efficiency. This mixture was incubated in 42°C heat block for 10 min to apply heat shock and reaction was split into 2 ml tubes (approximately 525 µl per tube). 1 ml of YPD medium was added to each tube and cells were incubated at 30°C without shaking for 1 hour to allow expression of Zeocin™ resistance. Cells were sedimented centrifugation at 3,000 x g for 5 min at room temperature. Pellets were resuspended in 500 µl of Solution III (Salt solution for washing and plating transformed cells) and cells were collected into one tube. Following to last sedimentation at 3000 x g for 5 min at room temperature, pellet was dissolved in 100 µl of Solution III. Finally, resuspended cells were spreaded on Yeast Extract Peptone Dextrose Sorbitol Agar (YPDS) containing Zeocin (100 µg/ml) and plates were incubated at 30°C for 5 days. Subsequently, 120 transformant were obtained and collected onto YPD plates.

2.2.10 Heterologous expression of the full-length laccase *Cplcc1* cDNA in yeast *Pichia Pastoris*

For heterologous expression of laccase in *Pichia pastoris*, Buffered Glycerol Complex Medium (BMGY) and Buffered Methanol Complex Medium (BMMY) (Recipes are given in Appendix A1) were used. In order to obtain efficient expression, cultures were always adjusted to 10 % of total flask volume and incubation temperature was set to 28°C. 1.0 % methanol added daily because of the loss by evaporation. 0.2-0.5 ml of culture sample was collected every 24 hours.

2.2.10.1 Investigation of *Pichia pastoris* X-33 transformants on solid plates and in liquid mediums for laccase activity

After collection of *Pichia pastoris* transformants on YPD plates, subsequently, all colonies were plated onto minimal methanol plates supplemented with 0.2 mM of substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to detect best laccase-producing transformants according to green zones and plates were incubated for 3 days at 30°C. Positive transformants were assayed for extracellular laccase production by growing in liquid Buffered Minimal Glycerol Medium (BMG) and Buffered Minimal Methanol Medium (BMM) (Recipes are given in Appendix A1) in order to determine the best producer transformant. In order to investigate laccase activity in liquid medium, BMG and BMM were used. For this purpose, first, a single colony was inoculated in 5 ml BMG medium and incubated at 28°C, 250 rpm for 16 h. After incubation, OD₆₀₀ values were measured. These pre-cultures were used to adjust OD₆₀₀ values to 1.0 in 5 ml BMM for expression. For this step, cells were sedimented with centrifuge at 5000 x g for 10 min, supernatants were discarded and cells were resuspended in BMM. Cultures for all transformants were incubated at 28°C for 7 days. Activity assay was applied to supernatant for laccase activity calculation at the end of week.

At last, in order to choose the best laccase producer of these three candidates, laccase activities were also investigated in complex medium. BMGY and BMMY were used for this purpose. At this step, first, a single colony was inoculated in 5 ml BMGY medium and incubated at 28°C, 250 rpm for 16 h. After incubation, OD₆₀₀ values were measured. These pre-cultures were used to adjust OD₆₀₀ values to 1.0 in 10 ml BMMY for expression. For this step, cells were sedimented with centrifuge at 5000 x g for 10 min, supernatants were discarded and cells were resuspended in BMMY. Cultures for all transformants were incubated at 28°C for 8 days. Activity assay was applied to supernatant for laccase activity at 7th and 8th days. As a result of one of three candidates was selected as the best recombinant laccase producer.

2.2.10.2 Laccase assay

Laccase activity of the culture supernatant was determined by measuring the oxidation of 2.5 mM ABTS in 100 mM sodium citrate buffer (pH 3.0) incubated for 3 min at room temperature ($\epsilon_{420}=3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). One unit (U) of laccase activity was defined

as the amount of enzyme, which oxidizes 1 μmol of ABTS per min under the assay conditions. All assays were carried out in triplicate.

$$U\ l^{-1} = [(\Delta A/t) / \epsilon \cdot d] \times (1 \times 10^6 \mu\text{mole/mole}) \times (V/v) \quad (2.1)$$

ΔA = Absorbance change at 420 nm; ϵ = Extinction coefficient of substrate; d = Light path (cm); V = total reaction volume (ml); v = sample volume (ml)

Laccase activities were measured on a Shimadzu UV-Pharmaspec 1700 (Japan) calculated using equation displayed above and explained in unit per liter.

2.2.10.3 Protein determination with Bradford assay

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

2.2.10.4 Optimization of expression conditions

High level protein production for any heterologous protein is achieved by optimization of expression conditions. Therefore, in order to reach maximum laccase activity and production, laccase production capacity of laccase producing transformants could be increased by optimization studies. Oxygen availability has an important impact on heterologous production of laccase in *Pichia pastoris*. Therefore, cultivation have been carried out with 25 ml culture in 250 ml flask (10 % total volume) with 250 rpm. BMGY and BMMY complex mediums were used for further heterologous expression steps. Cells were inoculated in 25 ml of BMGY in a 250 ml flask and grown at 28°C in a shaking incubator (250 rpm) for 18 hours until culture has reached $OD_{600} = 6.0$ – 18.0 . After harvesting cells by centrifugation, pellets were resuspended to an $OD_{600} = 1.0$ in buffered complex methanol medium (BMMY) for heterologous expression. In this part of study, the effect of various methanol concentrations (0 % to 1.5 %), CuSO_4 concentrations (0 mM to 4 mM) and L-Alanine concentration (0 % to 1.0 %) were investigated. The effect of low cultivation temperature (23°C) on the laccase expression was also monitored.

2.2.11 Partial purification of the recombinant laccases

Recombinant laccase purification was carried out with ammonium sulfate precipitation, ultrafiltration, anion exchange chromatography and gel filtration

chromatography. After production, *Pichia pastoris* culture was sedimented by centrifugation at 5000 x g for 20 min in order to obtain the supernatant that includes laccase enzyme.

2.2.11.1 Ammonium sulphate precipitation

Optimization of the ammonium sulfate concentration to precipitate proteins were carried out by adding in small portions to the stirred culture supernatant at 4°C and allowed to completely dissolve before adding the next portion. 90 % of ammonium sulfate salt concentration was obtained as optimum. The mixture was pelleted by centrifugation at 5000 x g for 1 hour.

Ammonium sulfate precipitates was resuspended in 50 mM Sodium phosphate buffer at pH 7.35 according to pI value which is 5.35. After that, ammonium sulfate salt was removed by ultrafiltration using and centrifugal devices with Amicon Ultra-15 centrifugal filter units (Millipore).

2.2.11.2 Anion exchange chromatography

The sample was first applied onto the Q-sepharose resin (GE Healthcare Life Sciences) pre-equilibrated with 50 mM Sodium phosphate buffer, at pH 7.35 and eluted proteins at a flow rate of 2 ml/min. The gradient was formed with 0.5 M NaCl. After fractions (5 ml each) were detected by the laccase activity assay, active fractions were pooled and concentrated and washed with gel filtration buffer (20 mM Sodium acetate buffer with 0.15 M NaCl at pH 5.35) by ultrafiltration again. Protein concentration was determined by Bradford method.

2.2.11.3 Gel filtration chromatography

Sample was loaded onto the Superdex 75 prep gel column (GE Healthcare Life Sciences) pre-equilibrated with the 20 mM Sodium acetate buffer with 0.15 M NaCl at pH 5.35 to separate proteins depending on their sizes and fractions (0.3 ml each) were obtained with the flow rate of 0.3 ml/min. Active fractions were pooled and concentrated with ultrafiltration. Protein concentration was again determined by Bradford method.

2.2.11.4 Polyacrylamide gel electrophoresis (PAGE) and Zymogram

Native-PAGE and SDS-PAGE were performed on 12 % polyacrylamide gels by the method of Laemmli, 1970. In SDS-PAGE, Unstained Protein MW Marker (26610, Pierce™) was used. Constructed SDS-PAGE Molecular Weight Standard Curve: Log MW of used SDS-protein marker versus their R_f values was given as supplementary file (Appendix A.5). PAGE-Gels were stained with Coomassie Brilliant Blue G-250 after electrophoresis. Protein sample used for SDS-PAGE-zymogram was not denatured. There was no sample boiling, nor the addition of reducing agents β -mercaptoethanol. Zymograms were obtained by soaking the gels with 100 mM sodium citrate buffer at pH 3.0 for three times in every 15 min and then incubating in 2.5 mM ABTS in the assay buffer at room temperature until green color has been observed as bands on gel.

2.2.12 Characterization of recombinant laccase CpLcc1

2.2.12.1 Effect of temperature on the laccase activity

The effect of temperature on partially purified laccase activity was analyzed at different temperatures ranging from 20°C to 90°C using 2.5 mM ABTS as substrate. Thermal stability of the enzyme was also assayed by incubating the enzyme at same temperatures up to 8 hours prior to activity assay except for 30°C at which enzyme was incubated for one week.

2.2.12.2 Effect of pH on the laccase activity

The optimum pH of the partially purified recombinant laccases was determined with 2.5 mM ABTS as the substrate in 100 mM HCl/KCl buffer (pH 2.0), 100 mM Citrate buffers (pH 3.0 to 6.0) and 100 mM Sodium phosphate buffers (pH 7.0 to 8.0).

2.2.12.3 Effect of different substrates on laccase activity

Oxidation of different substrates by recombinant laccases was examined by analyzing laccase activity for 5 mM ABTS, 5 mM 2,6-Dimethoxyphenol (DMP), 5 mM guaiacol as substrate.

2.2.12.4 Effect of different inhibitors on laccase activity

The effect of various potential inhibitors on the recombinant laccase activity was tested using 0,1 mM/1mM sodium azide, 0.1 mM/1 mM L-cysteine, 1 mM SDS 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.

2.2.12.5 Catalytic properties of the enzyme

K_m and V_{max} values of the recombinant laccase CpLcc1 were determined by measuring the activity of enzyme with various concentrations (0.1-0.6 mM) of ABTS for 3 min in the presence of 100 mM sodium citrate buffer at pH 3.0 at 25°C. Kinetic constants (K_m and V_{max} values) were calculated by Michaelis-Menten method by using GraphPad Prism 9 version 5.00 program (GraphPad Software, San Diego California USA). Catalytic efficiency was explained by k_{cat} (turnover number) and specificity constant was also indicated by $k_{cat} K_m^{-1}$ value.

3. RESULTS AND DISCUSSION

3.1 Cultivation Conditions for *Coriolopsis polyzona* MUCL 38443

First, *C. polyzona* MUCL 38443 was grown on Potato Dextrose Agar (PDA) (Acumedia) plates for 7 days at 28°C. Then, one slice of *C. polyzona* on PDA agar (Figure 3.1) was used as inoculum for the spore production on long-grain rice at 28°C for 8 days for the spore production step, long-grain rice was washed with deionized water for 1 hour and autoclaved for 15 min in Fernbach Erlenmeyer flasks (150 g of rice per 2 L flask), and inoculated with the mycelial block (Figure 3.2). Humidity kept high (5 ml sterile distilled water was added daily) in order to maintain rice moist for spore formation during incubation.

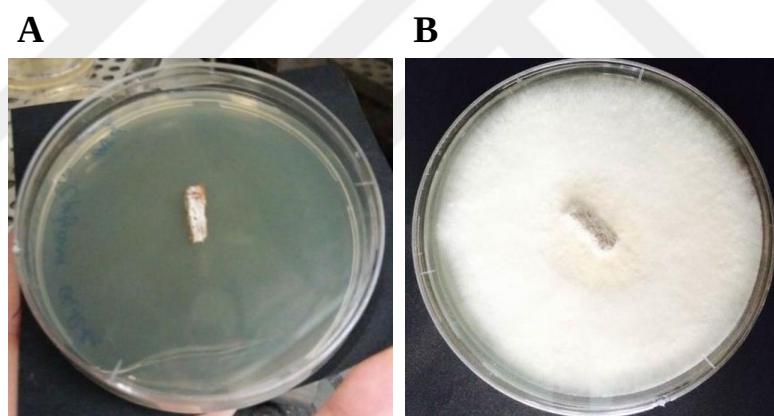


Figure 3.1 : *C. polyzona* growth on PDA agar. **A.** Inoculation day **B.** 7th day.

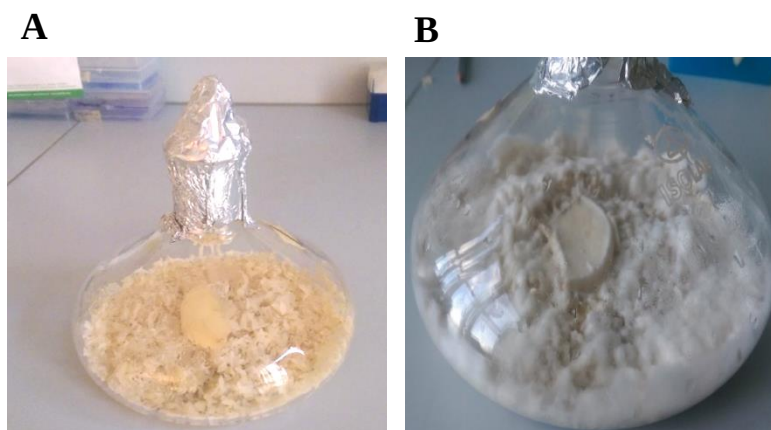


Figure 3.2 : *C. polyzona* growth on long-grain rice. **A.** Inoculation day **B.** 8th day.

After incubation, the fungal spores were harvested aseptically by extraction with 0.9 % NaCl solution and filtered through six layers of cheesecloth and washed with 0.9 % NaCl for 5 days (Figure 3.3).

After extraction of clean aseptic *C. polyzona* spores, the number of spores was determined using Hemocytometer (Neubauer) and the spore suspension was adjusted to a concentration of about 1×10^8 spores/ml stored in 0.9 % NaCl in an aluminium folio covered glass bottle at 4°C.

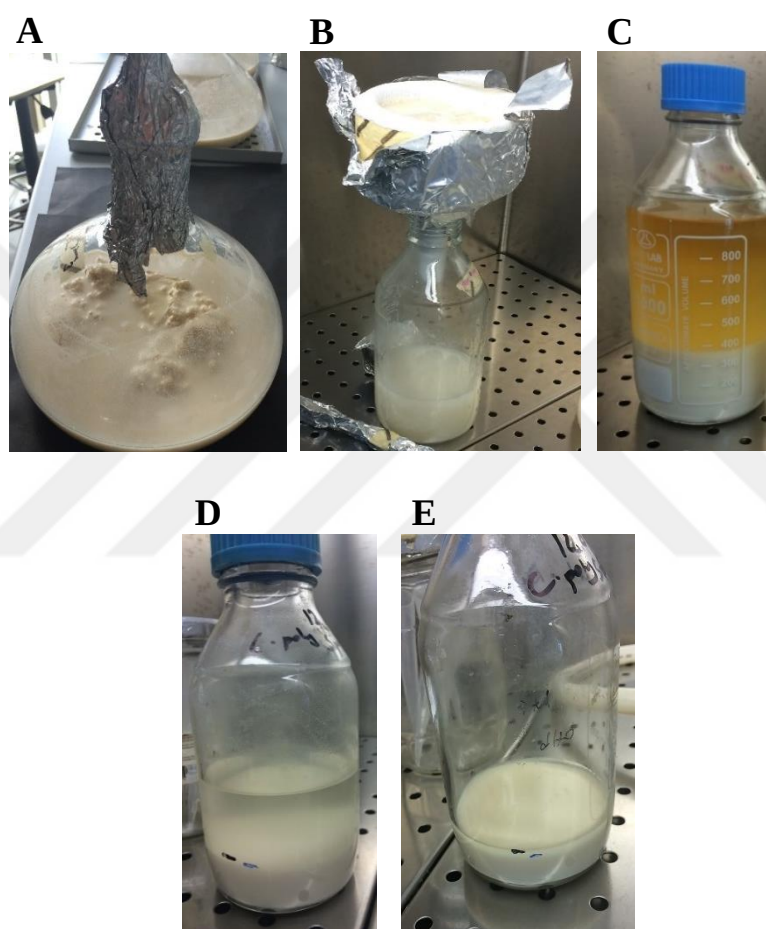


Figure 3.3 : **A.** Separation of *C. polyzona* culture and spores long grain rice using 0.9 % NaCl solution **B.** Filtration through six layers of cheesecloth **C.** Extraction of fungal spores **D.** Washing fungal spores for deposition of dirty supernatant **E.** Final extraction of clean *C. polyzona* spores.

Then, 1.0 ml of the spore suspension was added to 100 ml of LSK medium in 500 ml flasks. In a previous study, LSK medium was obtained as optimum medium for maximum laccase production from *Coriolopsis polyzona* MUCL 38443. The components of LSK medium; 55 g glucose monohydrate, 17 g Bacteriological

peptone, 2.5 g K_2HPO_4 , 1.027 g $MgSO_4 \cdot 7H_2O$, 31.27 mg $CuSO_4 \cdot 5H_2O$, 55.90 mg $MnSO_4 \cdot H_2O$ and 10 mg Thiamine-HCl per liter (Hüner, 2007).

C. polyzona culture was incubated at 28°C for 10 days on a rotary shaker (150 rpm) (Figure 3.4). *C. polyzona* pellets were discarded aseptically with centrifuge and mycelium was removed from the supernatant with filtration. *C. polyzona* pellets was later used for total RNA isolation. Crude laccase activity from supernatant of *C. polyzona* culture was approximately measured as 2900 U l⁻¹.

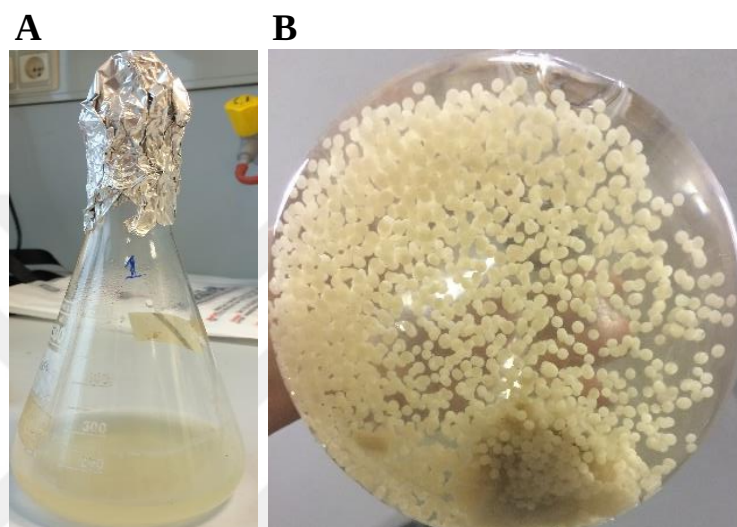


Figure 3.4 : **A.** Inoculation of *C. polyzona* spores in LSK medium at 1st day **B.** *C. polyzona* culture in LSK medium at 10th day.

3.2 Screening of *Cplcc1* Laccase cDNA

Laccase screening in fungal species by conventional methods is generally performed based on the laccase activity. Generally fungal species have more than one laccase-encoding gene. For example, in the *Coprinopsis cinerea* genome, at least 9 of 17 identified non-allelic laccase genes are translated into functional laccase products (Kilaru et al, 2006). Addition to this example, five laccase genes have been found in *Trametes villosa* (Yaver et al, 1996) and four different cDNA sequences were revealed in *Rhizoctonia solani* (Wahleithner et al, 1996). However, primary structures of these laccase isoenzymes were highly similar, expression level and physicochemical characteristics were quite different (Gu et al, 2014). In order to investigate the importance of these laccases as industrial or not, a PCR based laccase-sequence screening method was developed using degenerate PCR primers specific to conserved sequences around the two pairs of histidine in the N-terminal copper binding regions

of known basidiomycete laccases and rapidly isolated and then characterized laccase-specific sequences (D'Souza et al, 1996; Hoshida et al, 2001).

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; Lu et al, 2010; Fan et al, 2011).

A small number of amino acid differences in the active sites of laccases cause changes in the biochemical characteristics of the enzyme like optimum temperature, pH etc. via affecting the stability of the enzyme. If the similarity between laccase sequences is lower than 95 %, those laccase genes have to be accepted as different (Festa et al, 2008).

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). As another example *Coriolus hirsutus po1* gene has 91 % similarity to the *lcc1* from *Trametes villosa* (Eggert et al, 1998). On the other hand, four different laccase encoding genes, *lac1*, *lac2*, *lac3* and *lac4* from and *lac1* has 93 % identity with *Pleurotus ostreatus pox1* gene. At the same time, 97 % similarity between *lac 4* and *pox2* from *P. ostreatus* was revealed (Soden et al, 2002). In this study, laccase specific cDNA sequences on the total RNA of *Corioloopsis polyzona* MUCL 38443 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. Sequences of the degenerate primers used in this study are given in Table 2.2.

Laccase-specific sequences were screened by PCR using degenerate primers designed based on the conserved copper binding regions I and IV of known fungal laccases, and isolated total RNA from *C. polyzona* as template (Figure 3.5).

As a result of PCR using degenerate primers, 1.2 kb of cDNA was successfully amplified (Figure 3.6), cloned into pGEM[®]-T Easy Cloning Vector (Promega) and transferred in to competent *E. coli* cells in order to construct a laccase specific partial cDNA library. Transformants were selected on IPTG X-Gal Ampicillin containing LB agar plates. Plasmid DNAs were isolated from Amp^R white clones (Figure 3.7) and digested with *EcoRI* restriction enzyme to confirm the cloning of 1.2 kb cDNA fragment (Figure 3.8). Nucleotide sequences of selected 52 recombinant clones were then obtained using Universal pUC-M13 Reverse Primer and compared with

previously reported laccase sequences in National Center for Biotechnology Information (NCBI) database, using BLAST search to determine the homology of these sequences to known laccase genes.

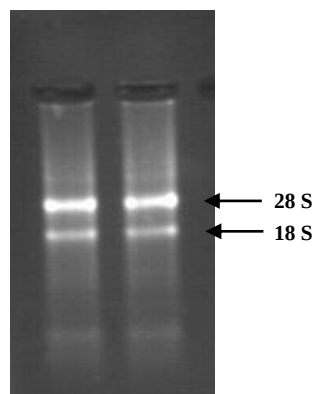


Figure 3.5 : Total RNA isolation from *Coriolopsis polyzona* MUCL 38443 pellets grown in LSK medium for 10 days.

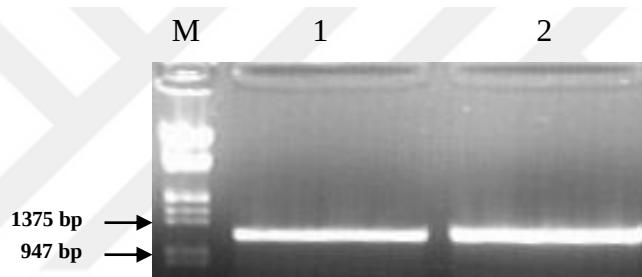


Figure 3.6 : Agarose gel analysis of 1.2 kb cDNA fragment amplified with LAC-N1 and LAC-C1 primers. **Marker (M):** Lambda DNA/*Eco*RI +*Hind*III DNA Marker 3 (Fermentas).

30 clones exhibited homology with known laccases: 2, 2(2), 3, 4, 5(2), 6, 6(2), 6(3), 7(3), 9, 9(2), 10, 10(2), 11, 11(2), 16, 18, 23, 31(2), 35, 35(2), 37, 38 (2), 39, 39(2), 42, 43, 43(2), 46 and 46(2). In order to investigate the differences between laccase positive clones more sensitively, Clone Number 2 was selected as core sequence and also sequenced not only with M13 reverse primer, but also with M13 forward primer and then, complete multiple alignment of these sequences using ClustalW2 was achieved.

As a result of multiple alignment, at least three different partial laccase cDNA sequences were detected as expressed in the white-rot fungus *Coriolopsis polyzona* MUCL 38443 named as *Cplcc1*, *Cplcc2* and *Cplcc3*, respectively (Figure 3.9). These three partial cDNAs showed minimum 79 % homology (Table 3.1) to known laccase cDNAs in National Center for Biotechnology Information (NCBI) database.

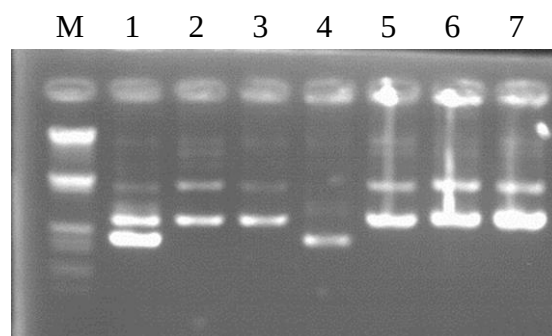


Figure 3.7 : Plasmid DNAs isolated from *E.coli* DH5α Amp^R transformants containing partial cDNA fragment (1.2 kb) amplified by degenerate primers. **Marker (M):** Lambda DNA/*Eco*RI+*Hind*III DNA Marker 3 (Fermentas).

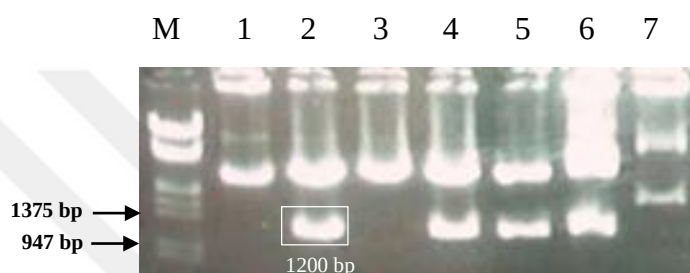


Figure 3.8 : *Eco*RI digestion of plasmid DNAs isolated from *E.coli* DH5α Amp^R transformants. **Marker (M):** Lambda DNA/*Eco*RI+*Hind*III DNA Marker 3 (Fermentas).

For further verification, these sequences were also analyzed with Principal Coordinates Analysis (PCoA). For this purpose, first, pairwise distances were calculated using Molecular Evolutionary Genetics Analysis (MEGA) Version 6 software. Then, a matrix was constructed using distances and a distance-based analysis was achieved with GenAlEx 6.41.

In PCoA (Figure 3.10), 4 different groups were shown. The first area in PCoA consisted of clones 2(2), 6, 6(2), 9(2), 10, 10(2), 16, 18, 23, 35, 37, 38 (2), 39, 43, 43(2), 46 and 46 (2) that have homology on close to 3' prime of clone number 2 cDNA and second area in PCoA consisted of clones 4, 5(2), 6(3), 35(2) and 39(2) that have homology on close to 5' prime of clone number 2 cDNA.

Therefore, the first and the second area that refers to first group of multiple alignments. This proportion seems like different than multiple alignments but in fact, they are very alike. The reason of this situation is because of some sequences which are close to 5' of core sequence clone 2 and while some of them are similar to 3' of core sequence clone 2. The third area in PCoA consisted of clones 3, 7(3) and 9 that refers to second group of multiple alignment. At last, the forth area in PCoA consisted of clones 11,

11(2), 31(2) and 42 that refers to third group of multiple alignment. Finally, Principal Coordinates Analysis (PCoA) confirmed the correction of constructed groups via multiple alignments ClustalW2.

> *Cplcc1* partial cDNA, sequenced with M13 reverse and forward primer

CATTGGCACGGCTTGTTCAGCATGGAACCAACTGGGCGCATGGTCCCAGCTTCATCAACCAGTG
CCCCGTCTCACCGGGTCACGCCTTCCTGTACGACTTCCAGGTCCCTGACCAGGCTGGTACATTCTG
GTACCACAGTCATTTGTCCACGCAGTACTGCGATGGCCTGAGGGGAGCCTTCGTTGTCTACGACCC
CAACGACCCCCACAAGAGCCGCTATGACTTTGACAACGACGACACCGTCATCATTGTGTCGGATTG
GTACCACGTCGCTGCCAAGTTGGGGCCGCGTTTCCCGTTGGGTGCGGACGCTACTTTGATCAATGG
CAAGGGGCGATCGCCCAGCACCGTGTCCGCACCGACCTGGCGGTGGAATTAAATCGTTACCGCTGG
CAAAAACGCTACCGTTTTTCCGTCTAGCCTCTCTGTCCCTGCGAATTCCCAACCCAATGTGTTTACG
ATCGACGGTCACACGATGACGAAATTCATCGAGGCTGACTCGGTCAACCACGCAGCCTTCTTGAGG
TCGACTCCATTCCAGATCTTCGCCGCGGCCAGCGCTACTCCTTTGTGTTGGAGGCTAACCAAGCCA
GTGGACAACACTACTGGATCCGCGCCAACCCGAACCTTCGGGTAACACCGGCTTCGCAGGGTGGCATCA
ACTCGGCCATCTCGCTATGACGGCGCGCCTGCCGTGCGAGCCTACCACTACCCAGACTCGCTCTA
CCAAGCCTCTCAACGAGGTGACCTCCACCCTCTCGAGTCTATGCCGTGGCAAGCCTGTGTCG
CCGGGGTGGTGTGGAACAGGCCATCAACATGGCATTCAACTTCAACGGCACAACTTCTTTATCAA
CGGCGCCCTCCTTCGTGCCCCCACCCTGGCCCGGTGTTTGCTCCAGATCCCTCAGCCGGTGC
GACTGCTCAGGACCGGTGCTCCCGGAAGGGTAGCAATCTACGTGCTCCCCAACCAACGCGGAACAT
CGAGAATTCTCCTTCCCCGCGACTACCGCCCGCCCCGGGGGTGCTCCCCATCCCTTCCCATCTCCA
CGGGTCACACCTTTCGCCGTGTTCCCGCAGCGCAAGGGCAGCACCAGTACAACCTACGACAACCC
GATCTTCCGCGACGTGTCAGCACGGGGACGCTGCGGCCAACGACAACGTACGATCCGCTTCCG
CACCGACAACCCCGGCCCGTGGTTCCTCCACTGCCATATCGTCTTCCA

> *Cplcc2* partial cDNA, sequenced with M13 reverse primer

ACGGGTTTTTCCAGGCAGGCACCAACTGGGCAGACGGCCCCGCGTTTCGTGAATCAGTGTCTATCG
CGTCCGGGCACTCGTTCTTGTACGACTTCCGTGTGCCGGACCAAGCCGGTACCTTCTGGTATCATA
GTCATCTTTTCGACCCAGTACTGCGACGGGTGAGGGGGCCATTTCGTGGTCTATGACCCGAAGGACC
CCCATGCCAGTCGTTACGATGTTGACGACGAGAGCACGGTGATCACCTTGCTGACTGGTACCACA
CCGCGGCTCGCTCGGGCCTAGGTTCCCATTGGGGGCGGACTCCACGCTCATCAATGGTCTCGGT
GATCGTCTCGACCCCTACAGCCCCCTTGGCGGTTCATCAATGTGCAACGTGGAAAACGCTATCGCT
TCCGCTCGTGTGCTCTCATGCGACCCGAACCACGTGTTTACGATCGACGGTCAACATGACCA
TCATCGGGGTGACGCGGTCAACAGCAAGCCTCTCACTGTGGACTCCATCCAAATCTTCGCTGCGC
AACGCTACCTCCTTGTGTTTGAAGCAAATCAACCGGGGGGCAACTACTGGGATCCGTGGCAACCC
CAACTTCGGGACGACCGGGTTCGCGGGCGGGATTAACCTCCCC

> *Cplcc3* partial cDNA, sequenced with M13 reverse primer

CCAGATTTTGGCGGGGACGCGTAATCCTTTGTTTTGAACGCCAAATCGACCTGGGGCCAATAACG
GAAACCGGCAAACCCCCCAATTTCCGCCAGGCCGGTTTCGGGGGCGGGTCAACTCCCGCCTTCCTC
CGCTACAAGGGAGCCCCGGCTATTGACCCGACGACCACTCAAACACCTTCCGTCAACCCACTCGTT
GAGACGAACCTCCACCCCACTCACACGCACGCCGTGCCTGGTCTCCACACCCGGAGGCGTGGA
CAAGGCGATCAACCTCGCTTCAACTTTAATGGGTCCAACCTTCTTCATCAACGACGCGACGTTTAC
ACCACCCAGCGTCCCCGTGCTGCTCCAAATTTCTGAGCGGTGCGCAGACCGCTCAGGACCTCCTGCC
CCCCGGGCTCCGTCTACCTCTCCCGGGCCACTCCTCCATTGAGCTCACGCTGCCGCGACGCGGAT
GGCCCCAGGCGCGCCCCACCCCTTTCACCTGCATGGTCACGTCTTCGCCGTGCTCCGACGCGGG
CAGCACCGAGTACAACCTACAACGACCCGATCTTCCGCGACGTGCTGAACACGGGACGCCCCGCGG
CAACGACAACGTACGATCCGCTTCCGGACGGACAACCCCGGCCCGTGGTTCCTCCACTGCCACAT
AGTCTTCCA

Figure 3.9 : Partial sequences of *Cplcc1*, *Cplcc2* and *Cplcc3* cDNAs.

As a result of screening steps, *Cplcc1* sequence was considered as the most dominant laccase specific cDNA because partial sequences belong to *Cplcc1* were the most frequently detected sequence in cDNA library of *C. polyzona*. Therefore, *Cplcc1* was chosen for further RACE, cloning and heterologous expression experiments.

Table 3.1 : BLAST analysis of the partial *Cplcc1*, *Cplcc2* and *Cplcc3* sequences from *Coriopolis polyzona* MUCL 38443 with previously reported laccases.

<i>Cplcc1</i>			
Accession Number	Description	Score	Homology
XM_008034423.1	<i>Trametes versicolor</i> FP-101664 SS1 laccase B precursor (TvLac3), partial mRNA	778	79 %
KP027478.1	<i>Trametes hirsuta</i> laccase A mRNA, complete cds	756	79 %
Y18012.1	<i>Trametes versicolor</i> mRNA for laccase	756	79 %
<i>Cplcc2</i>			
Accession Number	Description	Score	Homology
JQ828930.1	<i>Trametes versicolor</i> strain NL-1 laccase (lccA) gene, complete cds	544	82 %
XM_008034546.1	<i>Trametes versicolor</i> FP-101664 SS1 laccase I (TvLac1), partial mRNA	527	82 %
AY049725.1	<i>Trametes versicolor</i> laccase 1 (lac1) mRNA, complete cds	521	82 %
<i>Cplcc3</i>			
Accession Number	Description	Score	Homology
AB212732.1	<i>Trametes versicolor</i> lac2 mRNA for laccase2, complete cds	505	83 %
GU738021.1	<i>Trametes velutina</i> strain 5930 laccase (lac5930-1) mRNA, complete cds	503	83 %
JQ828930.1	<i>Trametes versicolor</i> strain NL-1 laccase (lccA) gene, complete cds	544	82 %

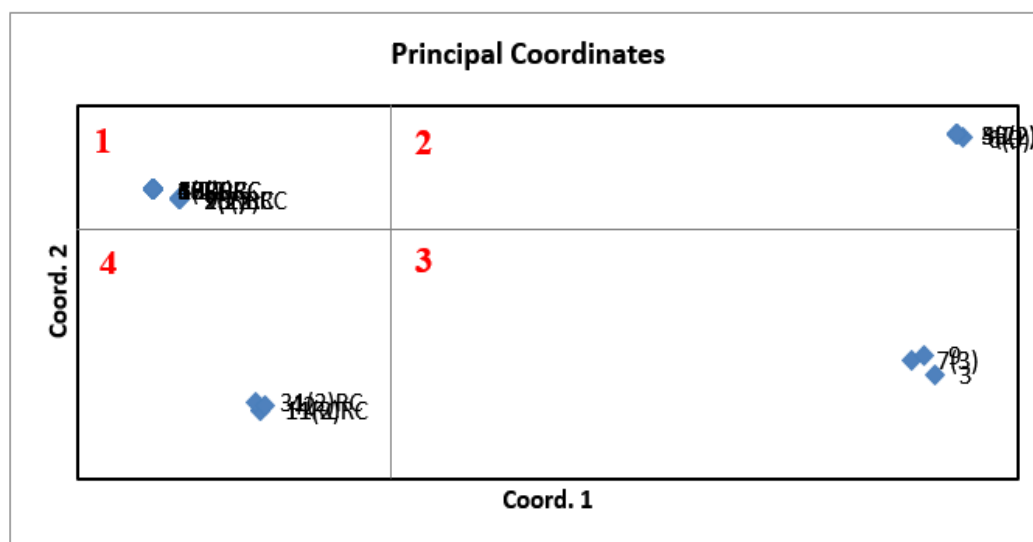


Figure 3.10 : Principal Coordinates Analysis (PCoA) of 30 laccase cDNA from positive clones.

3.3 5'-and 3'-Rapid Amplification of cDNA Ends (RACE)

Following determination of the partial sequence, gene specific primers (Figure 3.11) used in the 5'- and 3'-RACE protocols were designed to obtain both 5' and 3' ends of the partial *Cplcc1* laccase cDNA Table 2.2.

As mentioned previously, 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 mRNA's 3' or the 5' end. 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 and reported in various studies to isolate and identify laccase cDNAs from different microorganisms (Frohman et al, 1988; Hoshida et al, 2001; Kiiskinen and Saloheimo, 2004).

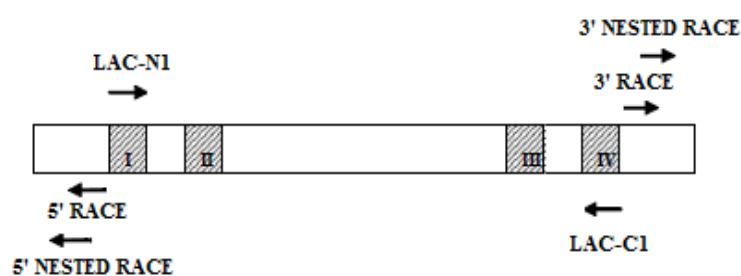


Figure 3.11 : Schematic representation of designing degenerate PCR primers specific to conserved copper binding regions I and IV and primers to be used in 5' and 3' RACE protocols for laccase amplification.

3.3.1 Determination of 5' end of the *Cplcc1* cDNA

3.3.1.1 5' RACE Protocol

5' RACE protocol was applied to determine the 5' end of the *Cplcc1* laccase cDNA from *Coriolopsis polyzona* MUCL 38443. 500 bp fragment was obtained with PCR using Universal Primer Mix and 5' RACE reverse primers and shown on 1% agarose gel (Figure 3.12A). After gel extraction from 1% agarose gel (Figure 3.12B), 5' RACE PCR product was ligated into pGEM[®]-T Easy vector and transferred into *E. coli* DH5 α chemical competent cells. Transformants were selected on IPTG X-Gal Ampicillin containing LB agar plates. After plasmid DNA isolation from Amp^R white clones (Figure 3.13A) *Eco*RI enzyme digestions were performed to detect the plasmid DNA containing 500 bp insert (Figure 3.13B). Finally, appropriate plasmid DNA was

sequenced and compared with previously reported laccase sequences in the databases. However, no significant similarity with other laccases was observed.

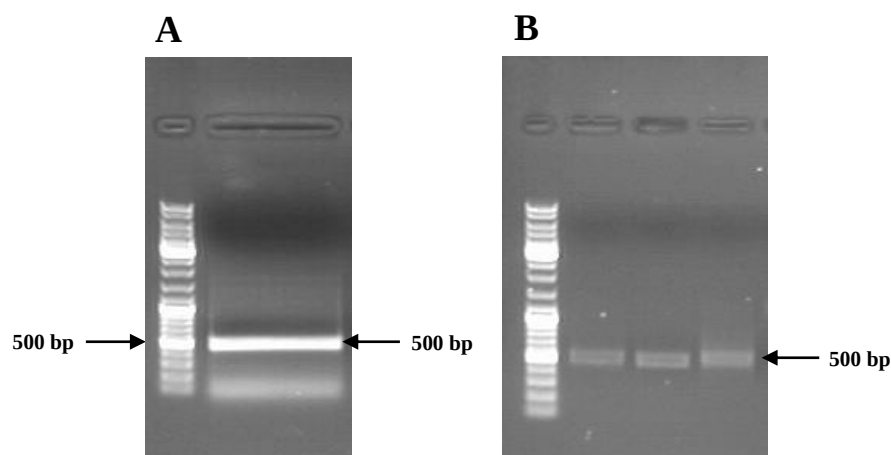


Figure 3.12 : **A.** 5' RACE PCR product before gel extraction, **B.** 5' RACE PCR product after gel extraction. **Marker:** Generuler DNA Ladder Mix (Fermentas).

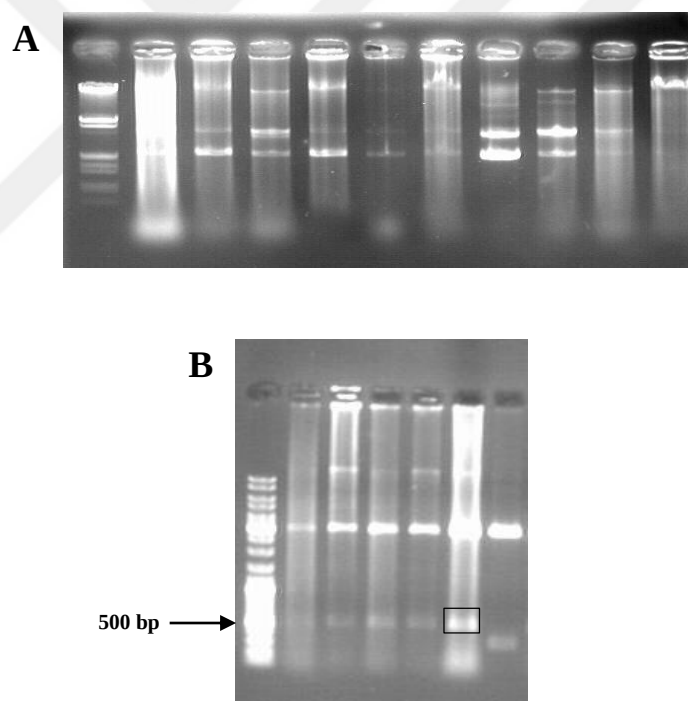


Figure 3.13 : **A.** Plasmid DNAs isolated from *E.coli* DH5 α Amp^R transformants containing 5' RACE fragments. **Marker:** Lambda DNA/*Eco*RI+ *Hind*III (Fermentas), **B.** *Eco*RI digestion analysis of plasmid DNAs isolated from *E.coli* DH5 α Amp^R transformants. **Marker:** Generuler DNA Ladder Mix (Fermentas).

3.3.1.2 5' Nested RACE protocol

5' Nested RACE protocol was applied to determine the 5' end of the *Cplcc1* laccase cDNA from *C. polyzona* MUCL 38443 more accurately. As a result of RACE PCR, 400 bp fragment was obtained using Nested Universal Primer Mix and 5' RACE nested reverse primers with 5' RACE product as template and shown on 1 % agarose gel (Figure 3.14A). After gel extraction from 1 % agarose gel (Figure 3.14B), 5' Nested RACE PCR product was ligated into pGEM[®]-T Easy vector and ligation product was transferred into *E. coli* DH5 α chemical competent cells. After plasmid DNAs isolation (Figure 3.15A), *Eco*RI enzyme digestion were performed to detect the plasmids containing 400 bp 5' Nested RACE fragment (Figure 3.15B). Finally, appropriate plasmids were sequenced and compared with previously reported laccase sequences in the databases.

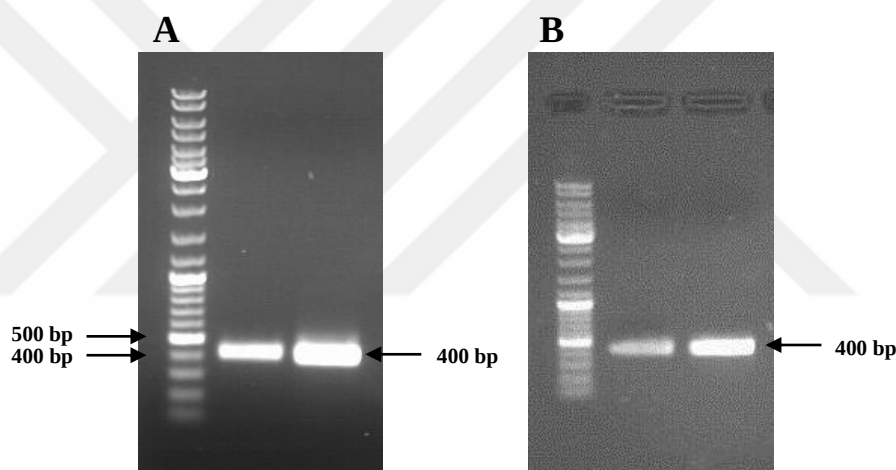


Figure 3.14 : **A.** 5' Nested RACE PCR product before gel extraction, **B.** 5' Nested RACE PCR product after gel extraction, **Marker:** Generuler DNA Ladder Mix (Fermentas).

The sequence of 5' RACE Nested PCR fragment (**Figure 3.16**) was aligned with known laccases and it showed 83 % similarity with *Trametes hirsuta* laccase A mRNA and 83 % similarity with *Coriolus hirsutus* laccase (072-1) mRNA. “ATG” start codon was also detected in the sequence of 5' RACE Nested fragment, indicating that it was reached to the 5' end of *Cplcc1* cDNA.

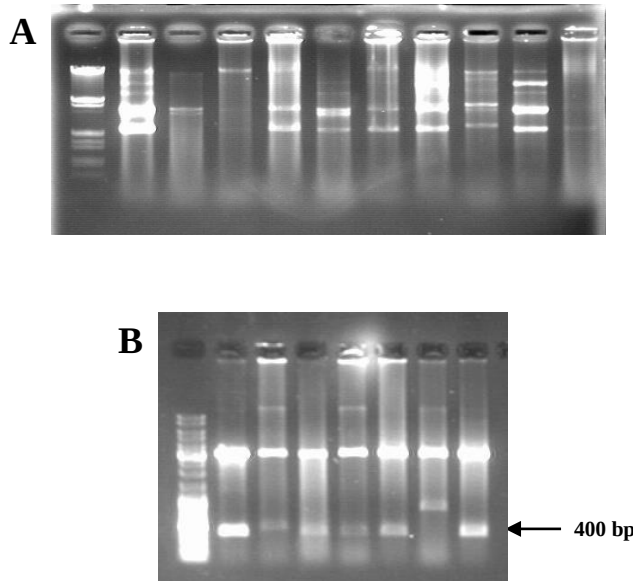


Figure 3.15 : **A.** Plasmid DNAs isolated from *E.coli* DH5 α Amp^R transformants containing 5' Nested RACE fragments. **Marker:** Lambda DNA/*Eco*RI+ *Hind*III (Fermentas) **B.** *Eco*RI digestion analysis of plasmid DNAs isolated from *E.coli* DH5 α Amp^R transformants. **Marker:** Generuler DNA Ladder Mix (Fermentas).

```

5' ...GGCGATTGGGCCCGACGTCGCATGCTCCGCCGCCATGGCGGCCGCGGGAATTCGATTAAG
CAGTGGTATCAACGCAGAGTACATGGGGAACTCGAGTCCCACTTGAGTTACAGCCGGGTCGTC
TCAACCGCCTCATTCTTCCTCAACGATGTCGCGGTTCAACCAGCTCCTCGCTTTCGTCATCGC
TTCCCTTTCGGCAGTGGCCCTTGCTGCCGTGGGCCCTGTGGCCGACCTCACCATCTCCAACGC
TGAGATTAGCCCAGACGGTTTCCCTCGTCAGGCCGTTGTGGTCAACGGTGTCTCTCTCTGGCCC
CATCATCTCGGGTAACAAGGGCGATCGCTTTCAGCTCAACGTGATCGATAACCTGACGAACCA
TACTATGCTCAAGAGTACCAGTATCCACTGGCACGGCTTCTTCCAGCATGGAACCAACTGGGC
CGATGGTCCCGCGTTCATAATCACTAGTGAATTGCGGCCCGCCTGCAGGTCGACCATATGGGA
GAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATCTATAGGCACCTAAATGT...3'

```

Figure 3.16 : 5'-end sequence of *Cplcc1* cDNA. *Cplcc1* Gene-specific forward, 5' RACE Nested and Nested Universal primers are highlighted with yellow, green and dark pink colors, respectively. Start codon (ATG) and Kozak (*italic and underlined*) sequence were also determined. Start codon takes part in Kozak sequence.

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). *Cplcc1* forward primer including strong Kozak sequence ("AGC" codon) and *Eco*RI (5'-GAATTC-3') site was designed for full length *Cplcc1* cDNA amplification (Table 2.2).

3.3.2 Determination of 3' end of the *Cplcc1* cDNA

3.3.2.1 3' RACE protocol

3' RACE protocol was applied to determine the 3' end of the *Cplcc1* laccase cDNA from *C. polyzona* MUCL 38443. 500 bp fragment was obtained with PCR using Universal Primer Mix and 3' RACE reverse primers and shown on 1% agarose gel (Figure 3.17A). After gel extraction from 1 % agarose gel (Figure 3.17B), 3' RACE PCR product was ligated into pGEM[®]-T Easy (Promega) and ligation product was directly transformed into *E. coli* DH5 α chemically competent cells. After plasmid DNA isolation (Figure 3.18) *Eco*RI enzyme digestion was performed to detect the plasmids containing 500 bp 3' RACE fragment. Finally, plasmids from positive clones were sequenced and compared with previously reported laccase sequences in the databases.

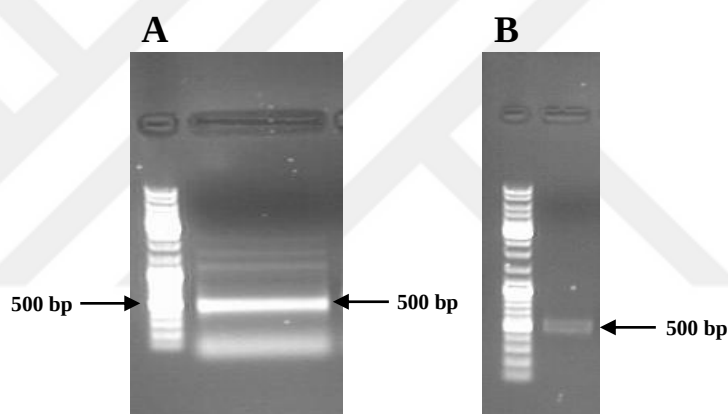


Figure 3.17 : **A.** 3' RACE PCR product before gel extraction, **B.** 3' RACE PCR product after gel extraction. **Marker:** Generuler DNA Ladder Mix (Fermentas).

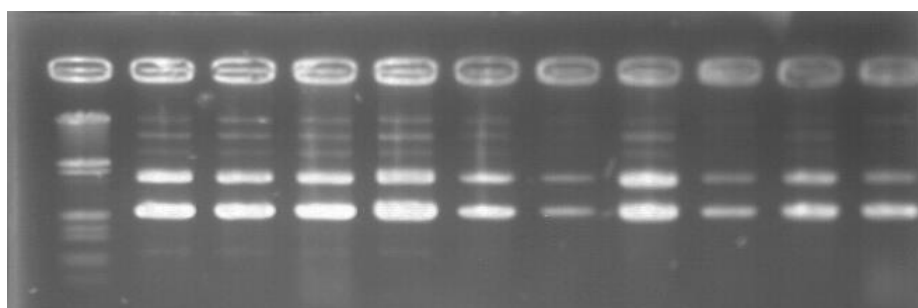


Figure 3.18 : Plasmid DNAs isolated from positive colonies including 3' RACE fragment. **Marker:** Lambda DNA/*Eco*RI+*Hind*III Marker (Fermentas).

3.3.2.2 3' Nested RACE protocol

3' Nested RACE protocol was applied to determine the 3' end of the *Cplcc1* laccase cDNA from *Coriopsis polyzona* MUCL 38443 more sensitively. As a result of RACE PCR, 400 bp fragment was obtained using Nested Universal Primer Mix and 3' RACE nested reverse primers with 3' RACE product as the template and shown on 1 % agarose gel (Figure 3.19A). After gel extraction from 1 % agarose gel (Figure 3.19B), 3' Nested RACE PCR product was ligated into pGEM[®]-T Easy (Promega) and ligation product was transferred into *E. coli* DH5 α chemical competent cells. After plasmid isolation (Figure 3.20), *Eco*RI enzyme digestion were performed to detect the plasmids containing 400 bp 3' Nested RACE fragment. Finally, appropriate plasmids were sequenced and compared with previously reported laccase sequences in the databases.

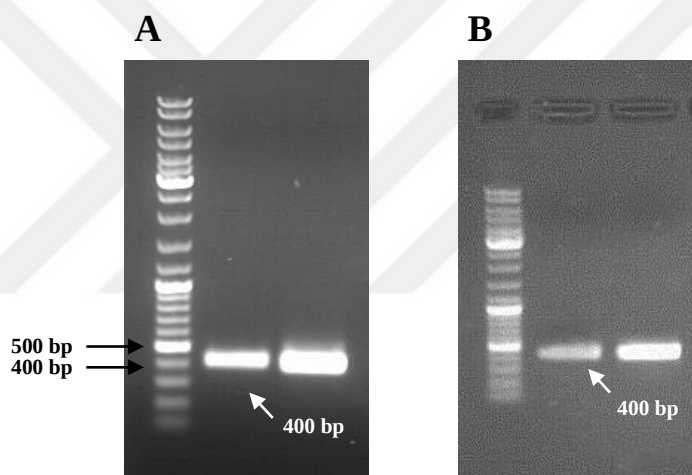


Figure 3.19 : A. 3' Nested RACE PCR product before gel extraction, B. 3' Nested RACE PCR product after gel extraction. **Marker:** Generuler DNA Ladder Mix (Fermentas).

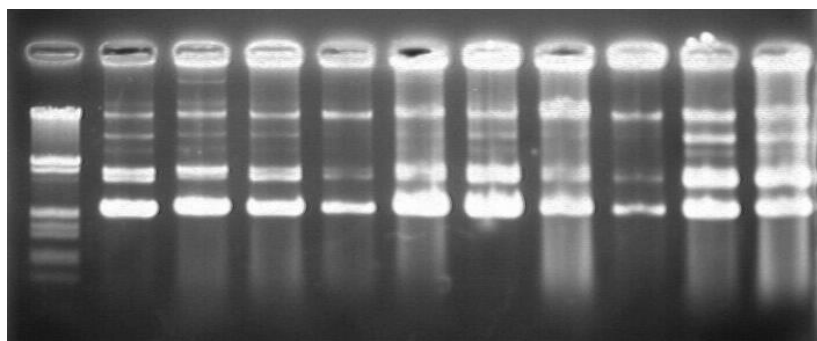


Figure 3.20 : Plasmid DNAs isolated from positive clones including 3' Nested RACE fragment. **Marker:** Lambda DNA/*Eco*RI + *Hind*III Marker (Fermentas).

The sequence of 3' RACE PCR fragment (Figure 3.21) was aligned with known laccases and it showed 90 % similarity with *Trametes hirsuta* laccase A mRNA and 90 % similarity with *Trametes versicolor* FP-101664 SS1 laccase B precursor (TvLac3). "TAA" stop codon was also detected in the sequence of obtained 3' RACE PCR fragment, indicating that we reached to the 3' end of *Cplcc1* cDNA (Figure 3.21). Finally, *Cplcc1* reverse primer was designed for synthesis of full length cDNA with *NotI* (5'- GCGGCCGC-3') site Table 2.2. However, sequence of 3' Nested RACE fragment did not exhibit any similarity with other laccases.

```

5' ...GGGGGGGGTTCATTCCGCCATCCAGGCTGCGCAACTGTTGGGAAAGGGCGATCGGTGCGGG
CCTCTTCGGTTATTACGCCAGCTGGCGAAAAGGGGGATGTGCTGCCAGGGCGATTAAAGTTGGG
TAACGCCAGGGTTTTCCAGTCACGACGTTGTAAAAACGACGCCAGTGAATTGTAATACGAC
TCACTATAGGGCGAATTGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAAT
TCGATTCTGACAAACCGATCTTCCGCGACGTCGTCAGCACGGGGACGCCCTGCGGCCAACGACAA
CGTCACGATCCGCTTCCGCACCGACAACCCCGGCCCGTGGTTCTCCACTGCCACATCGACTT
CCACCTCGAGGGCGGCTTCGCGGTCGTCATGGCTGAGGACACTCCGGACGTGAAGGCCGTCAA
CCCTGTCCCGCAGGCGTGGTCCGACCTCTGCCCTACCTACGACGCGCTCGACCCCAACGACCA
GTAAACGTTTAGTTTCGGTCTGGCATAGACGGACTCTTGGCTACAATCATGCATACGGACTTC
TGTTATGGTAAATCACTAGCTACTGTTAGGTGGCAGTGATGAGAACATGGGCTTTCGGCAGGA
ATGGTTGTACAATACGATAATGGGATGAGAAATGATGTCTATTGCATTTCATGACAAAAA
AAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTTGCCCTATAGTGAGTC...3'

```

Figure 3.21 : 3'-end sequence of *Cplcc1* cDNA. 3' RACE forward primer and *Cplcc1* reverse primer are highlighted with green and yellow colors, respectively. Stop codon (TAA) (*italic and underlined*) was also determined.

3.4 Full-length laccase *Cplcc1* cDNA synthesis

Following to the determination of the both 5'- and 3'-end of the *Cplcc1* cDNA (Figure 3.22), *Cplcc1* forward primer and *Cplcc1* reverse primer involving the initiation and termination codons were designed for the amplification of the full-length *Cplcc1* cDNA (Table 2.2).

3.4.1 Amplification of the full-length *Cplcc1* cDNA

The full-length *Cplcc1* cDNA synthesis has been carried out via RT-PCR technique by using total RNA Transcriptor First Strand Synthesis Kit (Roche), Expand High Fidelity PCR System (Roche), *Cplcc1* forward primer and *Cplcc1* reverse primer. A fragment approximately 1600 bp has been amplified (Figure 3.23A). After gel extraction, full-length *Cplcc1* cDNA (Figure 3.23B) ligated into pGEM-T Easy (Promega) cloning vector and ligation product was transferred into *E. coli* DH5 α chemically competent cells. Transformants were selected on X-gal/IPTG/Amp

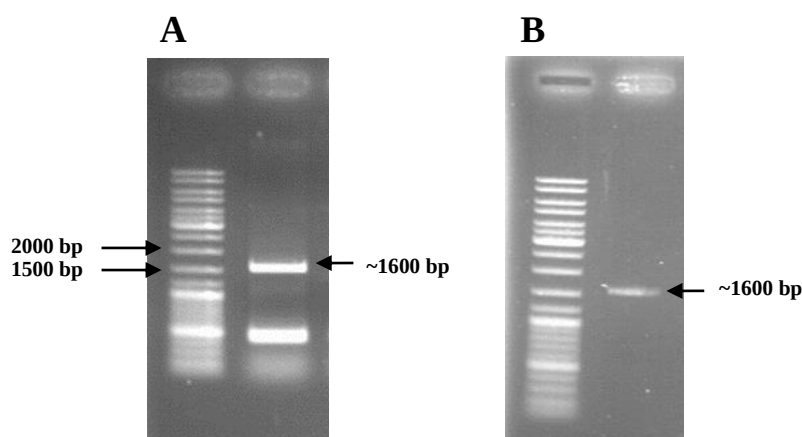


Figure 3.23 : **A.** Full-length *Cplcc1* cDNA amplified with *Cplcc1* forward primer and *Cplcc1* reverse primer before gel extraction. **B.** Full-length *Cplcc1* cDNA amplified with *Cplcc1* forward primer and *Cplcc1* reverse primer. **Marker:** Generuler DNA Ladder Mix (Fermentas).

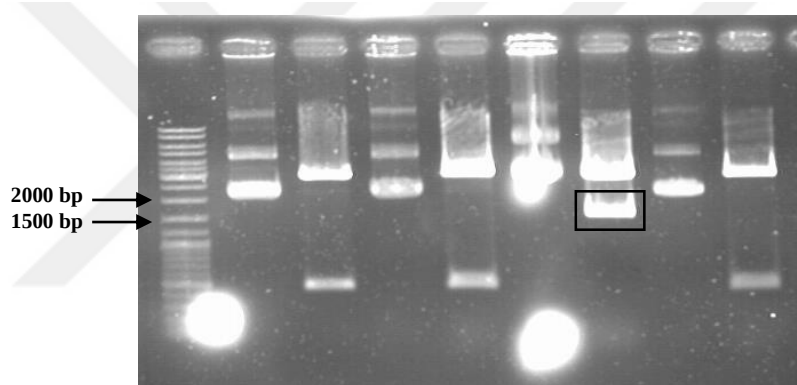


Figure 3.24 : *EcoRI* digestion analysis of *Cplcc1*/pGEM®-T plasmid DNAs. **Marker:** GeneRuler DNA Ladder Mix.

3.5 Isolation and Characterization of *Cplcc1* gene

Following to the synthesis of the full-length cDNA, *Cplcc1* gene was also isolated and characterized by determining the intron-exon organization.

3.5.1 *Cplcc1* gene sequence of *Coriolopsis polyzona* MUCL 38443

Isolation of the *Cplcc1* gene from the genome of *Coriolopsis polyzona* MUCL 38443 has been achieved by PCR using “*Cplcc1* forward” and “*Cplcc1* reverse” primers (Table 2.2) and genomic DNA of *Coriolopsis polyzona* MUCL 38443 as template (Figure 3.26). The fragment approximately 2.2 kb was amplified (Figure 3.27) and cloned into the pGEM®-T Easy cloning vector (Figure 3.28) and then sequenced. The

nucleotide sequences of genomic DNA of *Cplcc1* has been deposited in the GenBank database under accession number KT802747.

```
5' ...ATGTCGCGGTTCAACCAGCTCCTCGCTTTTCGTCATCGCTTCCCTTTCGGCAGTGGGCCCTTGCT
GCCGTGGGCCATGTGGCCGACCTCACCATCTCCAACGCTGAGATTAGCCCAGACGGTTTCCCTCGT
CAGGCCGTTGTGGTCAACGGTGTCTCTCCTGGCCCCATCATCTCGGGTAACAAGGTAGATCGCTTT
CAGCTCAACGTGATCGATAACCTGACGAACCATACTATGCTCAAGAGTACCAGTATCCACTGGCAC
GGCTTCTTCCAGCATGGAACCAACTGGGCCGATGGTCCCGGTTTCATCAACCAGTGCCCCGTCTCA
CCGGGTACGCCTTCTGTACGACTTCCAGGTCCCTGACCAGGCTGGTACATTCTGGTACCACAGT
CATTTGTCCACGCAGTACTGCGATGGCCTGAGGGGAGCCTTCGTTGTCTACGACCCCAACGACCCC
CACAAGAGCCGCTATGACTTTGACAACGACGACACCGTCATCACTTTGTGCGATTGGTACCACGTC
GCTGCCAAGTTGGGGCCGCGTTTCCCGTTGGGTGCGGACGCTACTTTGATCAATGGCAAGGGGCGA
TCGCCCAGCACCGTGTCCGCAGACCTGGCGGTGATTAACGTTACCGCTGGCAAACGCTACCGTTTC
CGTCTAGCCTCTCTGTCTGCGATCCCAACCATGTGTTTCAGCATCGACGGTCACACGATGACGATC
ATCGAGGCTGACTCGGTCAACACGCAGCCTCTTGAGGTGCGACTCCATCCAGATCTTCGCCGCGCAG
CGCTACTCCTTTGTGTTGGAGGCTAACAGCCAGTGGACAACACTACTGGATCCGCGCCAACCCGAAC
TTCGGTAACACCGGCTTCGCAGGTGGCATCAACTCGGCCATCCTGCGCTATGACGGCGCGCCTGCC
GTCGAGCCTACCACTACCCAGACTGCGTCTACCAAGCCTCTCAACGAGGTCGACCTCCACCCCTCTC
GAGTCTATGCCTGTGCCTGGCAAGCCTGTGCGGGGTGGTGTGACAAGGCCATCAACATGGCATTC
AACTTCAACGGCACAACCTTCTTTATCAACGGCGCCTCCTTCGTGCCCCCACCCTGCCGGTGTG
CTCCAGATCCTCAGCGGTGCGCAGACTGCTCAGGACCTGCTCCCGGAGGGTAGCATCTACGTGCTC
CCCAACAACGCGGACATCGAGATCTCCTTCCCCGCGACTACCGCCGCCCCGGGTGCTCCCCATCCC
TTCCATCTCCACGGTCACACCTTCGCCGTGCTCCGCAGCGCAGGCAGCACCCGAGTACAACCTACGAC
AACCCGATCTTCCGCGACGTCGTCAGCACGGGGACGCTGCGGCCAACGACAACGTCACGATCCGC
TTCCGCACCGACAACCCCGGCCCGTGGTTCTTCCACTGCCACATCGACTTCCACCTCGAGGGCGGC
TTCGCGGTGTCATGGCTGAGGACACTCCGGACGTGAAGGCCGTCAACCTGTCCCGCAGGCGTGG
TCCGACCTCTGCCCTACCTACGACGCGCTCGACCCCAACGACCAGTAA...3'
```

Figure 3.25 : *Cplcc1* open reading frame isolated from *Coriopsis polyzona* MUCL 38443, consists of 1563 bp.

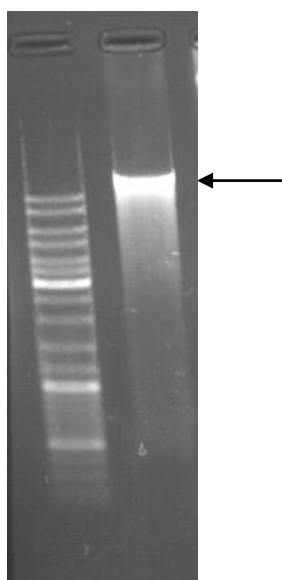


Figure 3.26 : Genomic DNA isolated from *Coriopsis polyzona* MUCL 38443.

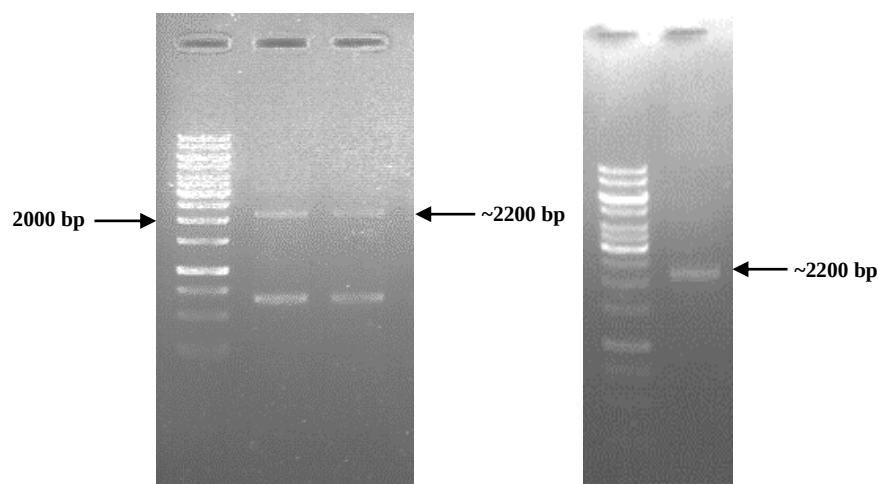


Figure 3.27 : A. Full-length *Cplcc1* gene amplified with *Cplcc1* forward primer and *Cplcc1* reverse primer from genome of *Coriolopsis polyzona* MUCL 38443 before gel extraction. **B.** Full-length *Cplcc1* gene amplified with *Cplcc1* forward primer and *Cplcc1* reverse primer from genome of *Coriolopsis polyzona* MUCL 38443 after gel extraction, **Marker:** Generuler DNA Ladder Mix (Fermentas).

The full length *Cplcc1* gene is 2106 bp and includes an open reading frame of 1563 bp with 57 % GC content (Figure 3.29). Several fungal introns contain consensus splicing sites. 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). In literature, *lap2* gene of *Trametes pubescens* and *lcc1* gene from *Trametes villosa* contain 8 introns (Galhaup et al, 2002; Yaver et al, 1996), *lcc2* of *Trametes villosa* has 10 introns (Yaver et al, 1996), CVLG1 of *Coriolus versicolor*, Cs-Lcs1 of *Ceriporiopsis subvermispora* have 11 introns (Mikuni and Morohoshi, 1997; Karahanian et al, 1998), *lac1* and *lac2* genes from *P. rivulosus* are interrupted by 11 and 12 introns, respectively (Hildén et al, 2013) and the eleven identified laccase genes from the white rot basidiomycete *Flammulina velutipes* contain 6–17 introns (Wang et al, 2015). Consistently, sequence comparison of the genomic and cDNA sequences of *Cplcc1* revealed 10 introns with sizes ranging from 51 to 58 base pairs (Figure 3.29). *Cplcc1* gene has maximum 76 % similarity with *Trametes hirsuta* laccase (Lac) gene (EU492907.1).

Cplcc1 open reading frame encodes deduced protein with 520 amino acids (Figure 3.29) and the deduced amino acid sequence of *Cplcc1* was compared with the sequence of other laccases reported in GenBank, revealing that CpLcc1 laccase was closely related to *Trametes hirsuta* laccase A (AIZ72721.1) and *Trametes hirsuta* laccase (ACC43989.1) by 88 % similarity. Next, it is followed by 87% with *Lenzites gibbosa*

(AEP71394.1), 86 % with *Trametes versicolor* (AAL93622.1), 86 % with *Trametes* sp. (ADK55593.1), 84 % with *Pycnoporus coccineus* (AKE14488.1), 82 % with *Trametes sanguinea* (ACZ37083.1), 81 % with *Trametes cinnabarina* (O59896.1) laccases. A putative signal peptide of 20 residues, responsible for the secretion of the laccase was also detected in the sequence of *Cplcc1* (Figure 3.29).

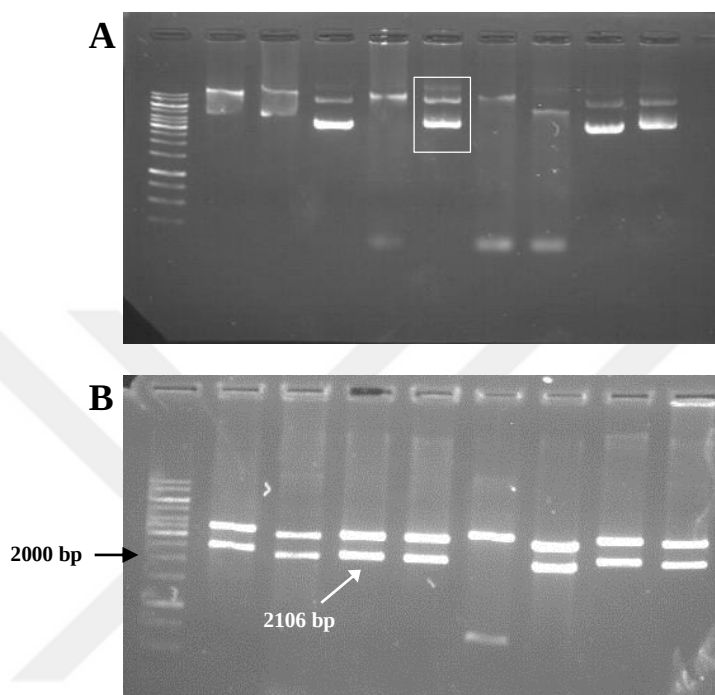


Figure 3.28 : **A.** Plasmid DNAs isolated from positive colonies including *Cplcc1* gene fragment. **B.** *NotI* and *EcoRI* double digest and non-digested forms of *Cplcc1* gene /pGEM[®]-T Easy plasmid DNAs. **Marker:** Generuler DNA Ladder Mix (Fermentas).

Cu²⁺ ligands conserved in laccases with ten histidine residues and one cysteine residue were in the active site of fungal laccases on the basis of coordination with the Type 1, Type 2, or Type 3 Cu²⁺ (Messerschmidt, 1997; Temp, 1999). Ten histidine residues as H85, H87, H130, H132, H416, H419, H421, H473, H475, H479 and C474 were located on the deduced amino acid sequence of *Cplcc1* in addition to five Cysteine residues with five potential N-glycosylation sites (Asn-X-Thr/Ser) (Url-2) are available in the deduced amino acid sequence (Figure 3.29). Comparison of the amino acid sequences of *Cplcc1* with the other available laccases in the database revealed that Cys474 is assumed to be the conserved laccase signature acting as a ligand to Type 1 copper domain. Additionally, H416 and H479 seem to coordinate the Type 1 atom; H85 and H419 seem to coordinate the Type 2 copper atom and H87, H130, H132, H421, H473 and H475 seem to coordinate the Type 3 copper atom. Another critical

residue, involved in the coordination of Type 1 copper atoms and located ten amino acids downstream of the conserved Cys residue, can be Leu, Met or Phe. However, generally high redox potential fungal laccases contain Phe residue at this position (Temp et al, 1999). Phe residue (Phe484) is present in the position 10 amino acids downstream of the conserved Cys residue (Cys474) (Figure 3.29), suggesting that the laccase encoded by *Cplcc1* is a high redox potential laccase. In addition to this fact, a “leucine–glutamate–alanine (LEA)” tripeptide motif adjacent to the last conserved His residue in high redox potential versus the “Val-Ser-Gly” in low potential fungal laccases has been identified as essential for the reactivity of laccases (Xu et al, 1998). This motif was found as “Leu–Glu–Gly” in the *Cplcc1* coding region as in the case of *Trametes hirsuta* laccase A (AIZ72721.1) and *Trametes hirsuta* laccase (ACC43989.1).

To investigate the evolutionary relationships with the other laccases, phylogenetic tree was also constructed by the neighbor-joining method based on the deduced amino acid sequence of the *Cplcc1* and 45 different laccase sequences derived from GenBank. Their analysis showed that *Cplcc1* and *Trametes versicolor* laccases belonged to the same phylogenetic groups (Figure 3.30). As a result of phylogenetic tree, at first, CpLcc1 laccase forms a sister group to the cluster of different *Trametes versicolor* laccases. CpLcc1 is also close to different *Trametes hirsuta*, *Trametes sp.*, *Lenzites gibbosa*, *Pycnoporus coccineus*, *Trametes sanguinea*, *Trametes cinnabarina* laccases as a second degree.

```

ATGTCGCGGTTCAACCAGCTCCTCGCTTTGTCATCGCTTCCCTTTTCGGCAGTGGCCCTTGCTGCCGTGGGCCAT
M S R F N Q L L A F V I A S L S A V A L A A V G H
GTGGCCGACCTCACCATCTCCAACGCTGAGATTAGCCAGACGGTTCCCTCGTCAGGCCGTTGTGGTCAACGGT
V A D L T I S N A E I S P D G F P R Q A V V V N G
GTCTCTCCTGGCCCATCATCTCGGGTAACAAGGTATgctttcactaccagtagtccgtgtgtgcccagtgctca
V S P G P I I S G N K V
caatgtacagggcGATCGCTTTCAGCTCAACGTGATCGATAACCTGACGAACCATACTATGCTCAAGAGTACCAG
D R F Q L N V I D N L T N H T M L K S T S
TATCgtacgtgaagtggcgggctctctcgcataggaacactaactgttactctattgcagCACTGGCACGGCTTC
I
TTCCAGCATGGAACCAACTGGGCGGATGGTCCCGGTTTCATCAACCAAGTGGCCGTTCTACCGGGTCACGCTTC
F Q H G T N W A D G P A F I N Q C P V S P G H A F
CTGTACGACTTCCAGGTCCTGACAGGCTGGTgagttctacactgcctctctgtcatcgctgtcgctcacagtg
L Y D P N D P H K S R Y D F D N
cgttttaggtACATTCTGGTACCACAGTCATTGTCCACGCACTACTGCGATGGCTGAGGGGAGCCTTCGTT
T F W Y H S H L S T Q Y C D G L R G A F V
GTCTACGACCCCAACGACCCCAAGAGCCGCTATGACTTTGACAACGgtaaactcggttaattcaaccgcgaataa
V Y D P N D P H K S R Y D F D N
gaggtggtctgacattatctgttcttagACGACCCGTCATCACTTTGTCGGATTGGTACCACGTCGCTGCCAAG
D D T V I T L S D W Y H V A A K
TTGGGGCCGCTTTCCCGTgcgttcatcttcgggaataatactcagtgccctggctgactggtctgatgatagtTG
L G P R F P
GGTGGGACGCTACTTTGATCAATGGCAAGGGCGATCGCCAGCACCGTGTCCGACAGCTGGCGGTGATTAAC
G A D A T L I N G A G K G R S P S T V S A D L A V I N
GTTACCGCTGGCAACGgtaagcaagggcgttcaaatgtgtgagtccttgtaactgacggattgcccgtatagCTA
V T A G K R
CCGTTTCCGCTAGCCTCTCTGTCCTGCGATCCCAACCATGTGTTTCAGCATCGACGGTCACACGATGACGATCAT
R F R L A S L S C D P N H V F S I D G H T M T I I
CGAGGCTGACTCGGTCAACACGACGCTCTTGAAGTCGACTCCATCCAGATCTTCGCGCGCAGCGCTACTCCTT
E A D S V N T Q P L E V D S I Q I F A A Q R Y S F
TGTGgtgagtcacactggcggtattattgtgcatgggaagatgactgacgattgtatagTTGGAGGCTAACAGC
V
CAGTGGACAACACTACTGGATCCGCGCAACCCGAACCTTCGGTAACACCGGCTTCGCAAGTGGCATCAACTCGGCCA
P V D N Y W I R A N P N F G N T G F A G G I N S A
TCCTGCGCTATGACGGCGCGCTGCGCTGAGCTACCACTACCCAGACTGCGTCTACCAAGCCTCTCAACGAGG
I L R Y D G A P A V E P T T T Q T A S T K P L N E
TCGACCTCCACCTCTCGAGTCTATGCTGTgtaggtgtacagatcacgaaggcagtgatcgcatcgatgctga
V D L H P L E S M P V
tattgttctttacagCCTGGCAAGCCTGTGCGGGTGGTGTGCAAGGCCATCAACATGGCATTCAACTTCgta
P G K P V A G G V D K A I N M A F N F
ggtcgcggtatatagccaaattacatctcgcactaaactccttcgtagAACGGCACAACTCTTTATCAACGGC
GCTCCTTCGTGCCCCCACCCTGCTGCTCCAGATCCTCAGCGGTGCGCAGACTGCTCAGGACCTGCTC
A S F V P P T V P V L L Q I L S G A Q T A Q D L L
CCGGAGGGTAGCATCTACGTGCTCCCAACAACGCGGACATCGAGATCTCCTTCCCGCGACTACCGCGCGCCCG
P E G S I Y V L P N N A D I E I S F P A T T A A P
GGTGTCTCCCATCCCTTCCATCTCCACGGTgtacgtaaatatttgcctcacctgtgagtgcttccactaaacttcc
G A P H P F H L H G
gccttgcagCACACCTTCGCGGTGTCGCGCAGCGCAGCAGCAGTACAACACGACACCCGATCTTCCGC
H T F A V V R S A G S T E Y N Y D N P I F R
GACGTCGTCAGCAGGGGACGCTGCGGCCAAGCAGCAGTACGATCCGCTTCCGACCGACAACCCGCGCCCG
D V V S T G T P A A N D N V T I R F R T D N P G P
TGGTTCCTCCACTGCCACATCGACTTCCACCTCGAGGGCGGCTTCGCGGTGTCATGGCTGAGGACATCCGGAC
W F L H C H I D F H L E G G F A V V M A E D T P D
GTGAAGCGCTCAACCTGTCCCGCAGGCGTGGTCCGACCTTGCCTACCTACGACGCGCTCGACCCCAACGAC
V K A V N P V P Q A W S D L C P T Y D A L D P N D
CAGTAA
Q -

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Figure 3.29 : Nucleotide and deduced amino acid sequences of the *Cplcc1* gene from *C. polyzona* MUCL 38443. The deduced amino acids sequence is shown below the ORF. The introns (I-X) are shown lower case and bold letters. Histidine residues involved in copper binding are highlighted; H416 and H479 coordinated with Type 1 Copper, H85 and H419 with Type 2 Copper, H87, H130, H132, H421, H473 and H475 with two Copper Type 3, C474 and F484 with Type 1 Copper and LEG as important residues for the reactivity of the enzyme are underlined and bold. Possible N-glycosylation sites are also underlined. The putative signal peptide is shaded.

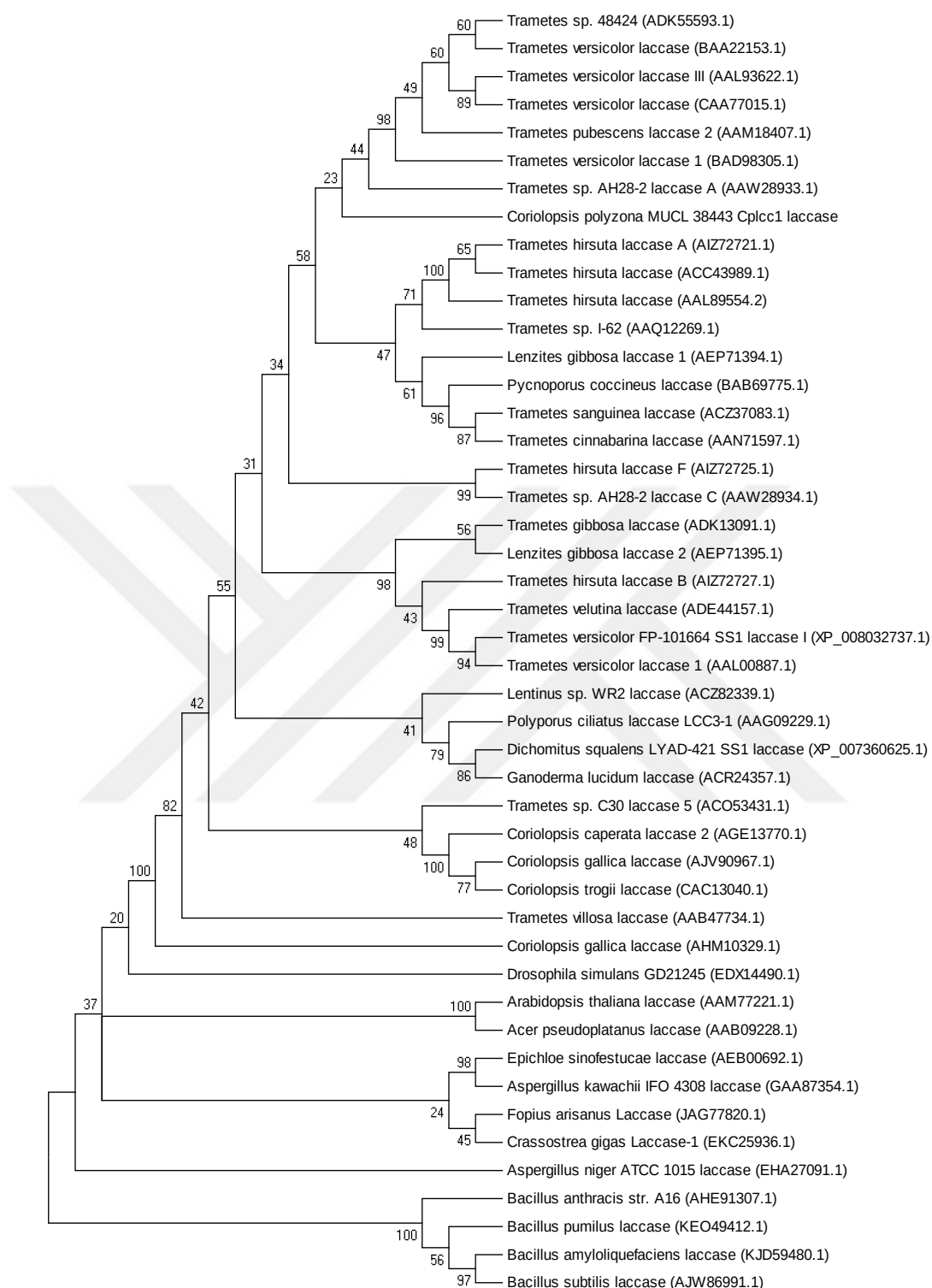


Figure 3.30 : Neighbor-joining tree of the deduced amino acid sequence of *Cplcc1* and other laccases from GenBank. The tree is constructed according to the calculated p-distances using MEGA ver. 6, based on a ClustalW alignment.

3.5.2 Heterologous expression of the laccase CpLcc1 in *Pichia pastoris*

In this study, isolated *Cplcc1* cDNA was successfully expressed in yeast *Pichia pastoris*. Cloning of cDNAs into the shuttle expression vector pPICZC, transformation of the *Pichia pastoris* and the studies performed for optimization of the expression conditions are explained in following part.

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

Cplcc1 laccase cDNA was cloned into yeast shuttle expression vector pPICZC with its own secretion signal sequence. Signal peptides are necessary to direct proteins into the secretory pathway and the own signal sequence of recombinant protein is generally more successful for extracellular secretory expression system in *Pichia pastoris* (Daly and Hearn, 2005).

pGEM®-T cloning vector including PCR amplified 1563 bp full length *Cplcc1* cDNA and pPICZC expression vector were digested with *Eco*RI and *Not*I (Figure 3.31A and Figure 3.32A). After fragments were recovered using gel extraction method (Figure 3.31and Figure 3.32B) and ligated into pPICZC vector. The ligation product was transferred into *E. coli* DH5 α chemically competent cells and transformants are selected on Zeocin-LB agar plates (25 μ g/mL final concentration).

After plasmid DNA isolation from these transformants, *Not*I and *Eco*RI digestion was carried out in order to detect the plasmid DNA containing the 1563 bp full length laccase *Cplcc1* cDNA (Figure 3.33).

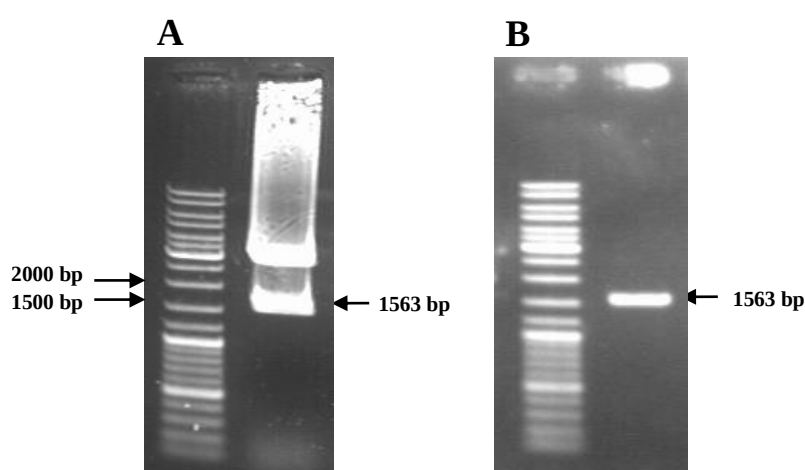


Figure 3.31 : A. *Not*I and *Eco*RI double digestion of pGEM®-T cloning vector including PCR amplified full length *Cplcc1* cDNA **B.** PCR amplified full length *Cplcc1* cDNA after gel extraction, **Marker:** Generuler DNA Ladder Mix (Fermentas).

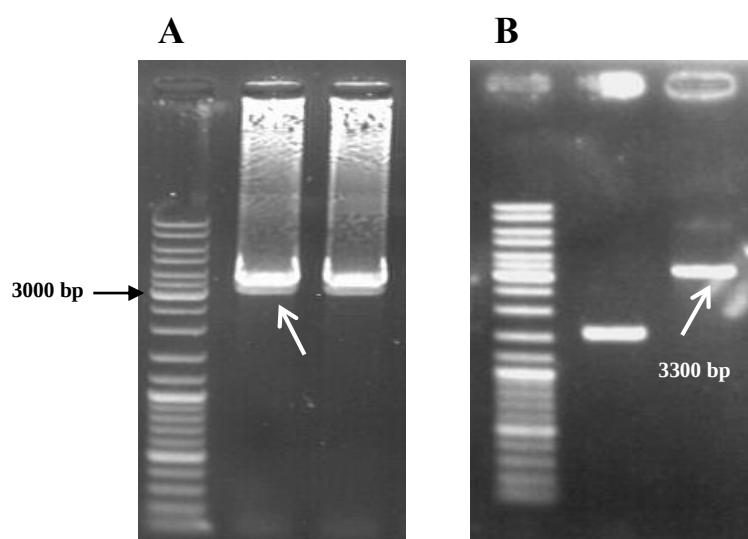


Figure 3.32 : A. *NotI* and *EcoRI* double digestion of pPICZC shuttle expression vector **B.** pPICZC shuttle expression vector after gel extraction method. **Marker:** Generuler DNA Ladder Mix (Fermentas).

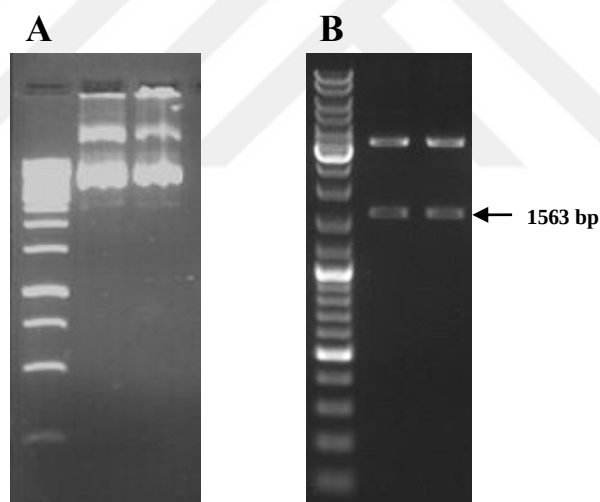


Figure 3.33 : A. Plasmid DNA isolation from positive clones with pPICZC including PCR amplified full length *Cplcc1* cDNA. **B.** *NotI* and *EcoRI* double digest of positive clones with pPICZC including PCR amplified full length *Cplcc1* cDNA. **Marker:** Generuler DNA Ladder Mix (Fermentas).

3.5.3 Transformation of *Pichia pastoris* competent cells

Positive recombinant plasmid DNA should be linearized within the 5'AOX1 region with *SacI*/*PmeI* to integrate into the 5' AOX1 region of *Pichia pastoris* genome via homologous recombination (Daly and Hearn, 2005). *PmeI* was used for linearization of pPICZC including the full length *Cplcc1* cDNA. After gel extraction, linearized pPICZC including the full length *Cplcc1* cDNA was condensed into 4 µg/5µl concentration. Linearized recombinant pPICZC was transferred into chemically competent *Pichia pastoris* strain X-33 cells and approximately 100 transformants were obtained. These transformants were inoculated on minimal methanol plates supplemented with 0.2 mM of ABTS to detect laccase-producing transformants and plates were incubated for 3 days at 30°C. Laccase producing transformants oxidized the ABTS were detected by the green zone around the *Pichia* colonies (Figure 3.34).

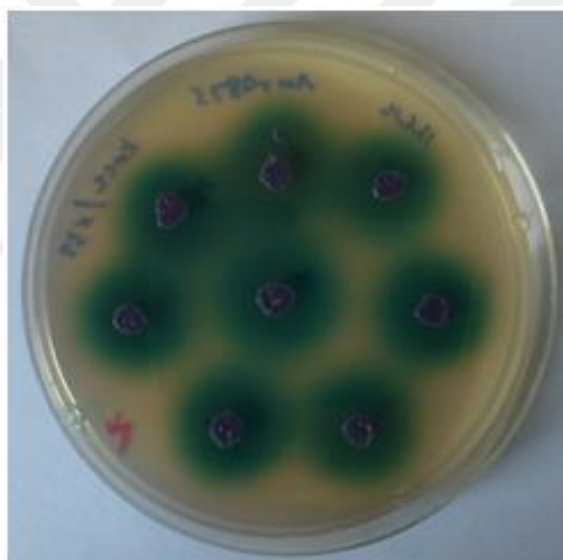


Figure 3.34 : Agar plate assay to select *Pichia pastoris* recombinants containing amplified *Cplcc1* cDNA on minimal methanol media supplemented with 0.2mM ABTS.

25 of well- green halo-forming laccase positive transformants were, then, screened for extracellular laccase production by growing in BMM liquid medium (Appendix A.1) at 28°C with 0.5 % methanol added daily to induce laccase expression for 7 days. Laccase activities of the best three recombinant laccase producers labelled as 33, 41 and 43 were measured as 76.5 U l⁻¹ ,72.3 U l⁻¹ and 71,2 U l⁻¹, respectively (Figure 3.35).

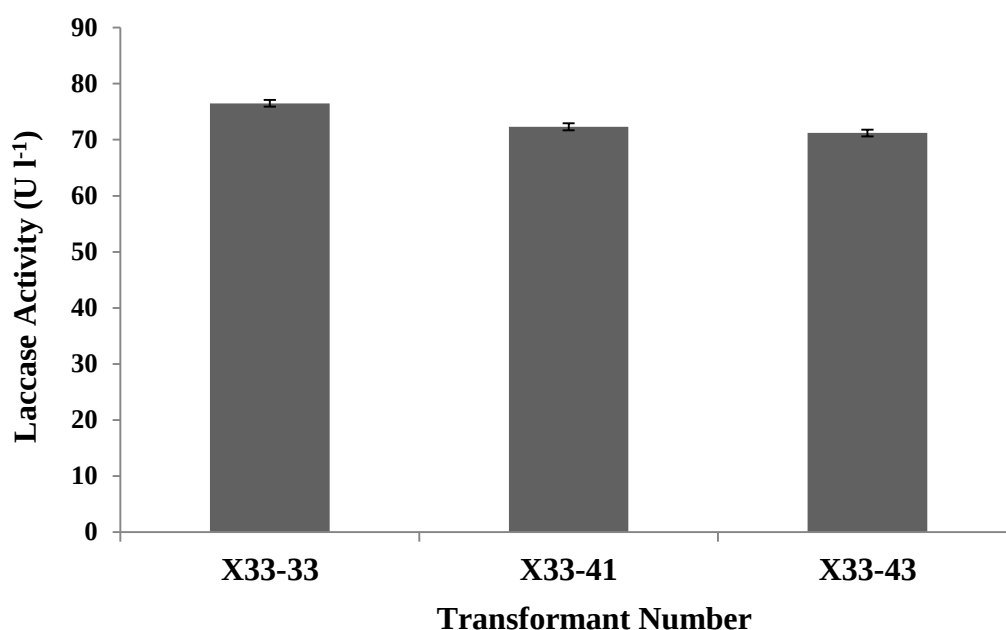


Figure 3.35 : Selection of the laccase positive transformants including *Cplcc1* with highest laccase activity in Buffered Minimal Methanol Medium (BMG/BMM).

In order to choose the best laccase producer laccase activities of the transformants 33, 41 and 43 were also investigated in Buffered Complex Medium (BMGY/BMMY) and measured as 108.2 U l⁻¹, 99.5 U l⁻¹ and 92.0 U l⁻¹, respectively (Figure 3.36). After all activity assay experiments the transformant 33 was selected as best recombinant laccase CpLcc1 producer.

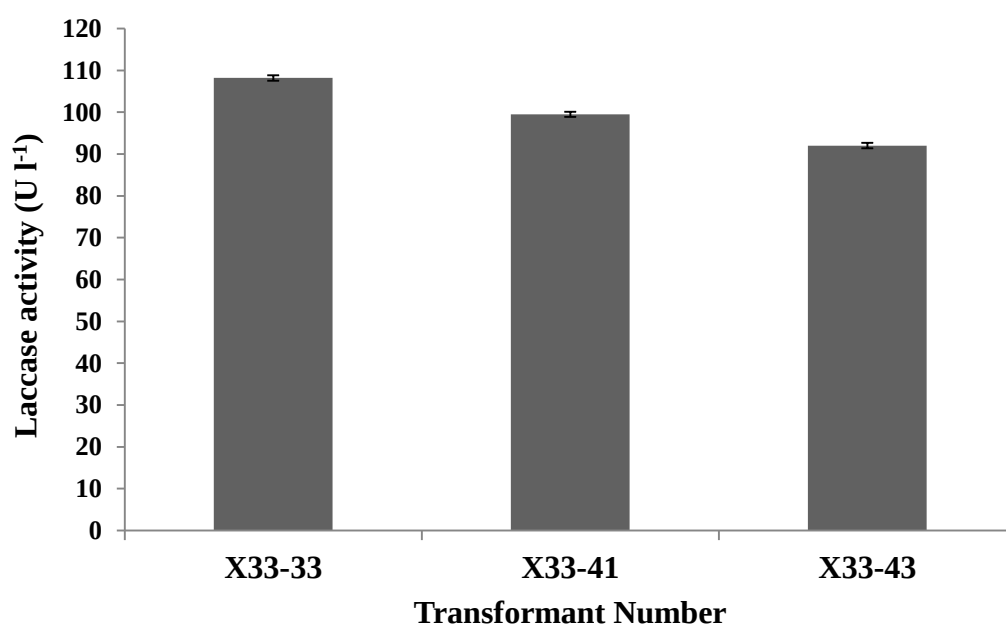


Figure 3.36 : Selection of the laccase positive transformant including *Cplcc1* with highest laccase activity in Buffered Complex Medium (BMGY/BMMY).

3.5.4 Optimization of expression conditions

In this study, the level of recombinant CpLcc1 production in *Pichia pastoris* was further improved by optimizing the methanol concentration, copper concentration, L-Alanine concentration and cultivation temperature

3.5.4.1 Nutrient optimization

The growth conditions, medium compositions and strain type are the three key factors on the expression of laccase. Nitrogen source like tryptone and peptone are important medium components. On the other hand, media compositions supporting high biomass cause decrease in laccase yields and nitrogen limitation is defined as the major factor for enhancing ligninolytic enzyme production in some species. Therefore, 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 (Heinzkill et al, 1998; Xavier et al, 2001; Majeau et al, 2010). For instance, the regulation of laccase expression in *Trametes versicolor* at the transcriptional level by nitrogen, as the increased nitrogen concentration caused the increased levels of laccase mRNA (Collins and Dobson, 1997).

In best laccase recombinant producer selection steps, it was obtained that recombinant *Pichia* cells including *Cplcc1* cDNA showed better growth and higher laccase activity in BMGY/BMMY complex medium over BMG/BMM. Therefore, optimization steps were carried out using BMGY/BMMY complex medium with 25 ml culture in 250 ml flask (10 % total volume) with 250 rpm.

Methanol concentration optimization

Pichia pastoris is 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 *AOX1* is used to drive the expression of high level of recombinant proteins even with the single integrated copy of the gene of interest (Cereghino et al, 2002; Fickers, 2014).

Accurate regulation of the methanol feed is critical not only to keep the induction of the genes under the control of the *AOX1* promoter but also to hinder methanol accumulation at high levels that are toxic to the cells. Methanol optimization was the first step of optimization for this purpose. The effects of methanol concentrations

ranging from 0 % to 1.5 % on laccase expression were first investigated to optimize methanol concentration. Samples were taken from cultures every day during optimization, while growth profiles were determined at 600 nm (Figure 3.37), laccase activities were determined at 420 nm (Figure 3.38).

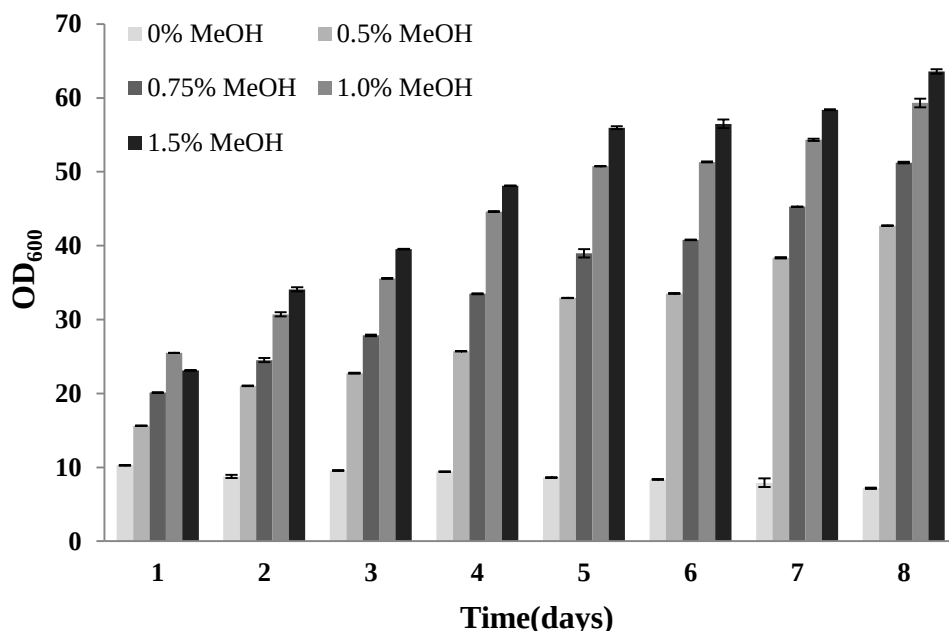


Figure 3.37 : The effect of methanol concentrations on the growth profile of the recombinant *Pichia pastoris*.

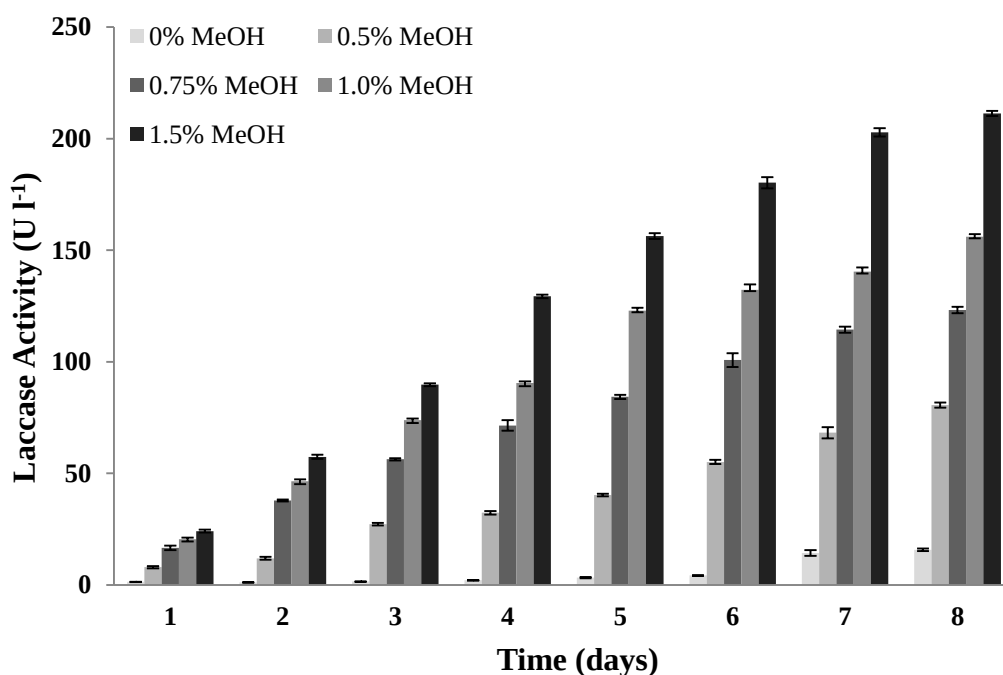


Figure 3.38 : The effect of methanol concentrations on the activity of the recombinant laccase CpLcc1 in *Pichia pastoris*.

According to the effect of different methanol concentrations on laccase activity, the highest laccase activity was observed as 211.29 U l⁻¹ with 1.5 % methanol concentration at the end of 8th day. On the other hand, methanol uninduced culture showed that methanol was very crucial not only for recombinant *Pichia pastoris* growth (Figure 3.38), but also for laccase expression. In previous parts, it was mentioned that best recombinant laccase enzyme producer was selected using BMM with 0.5 % methanol concentration.

As a result of methanol optimization, laccase activity was increased approximately 2.6 fold (80.58 U l⁻¹ to 211.29 U l⁻¹) using BMMY medium supplemented with 1.5 % methanol.

Copper concentration optimization

Previous studies in literature showed the importance of pH and copper availability on the activity of laccase enzyme. In many fungal species, laccase expression is generally regulated by some factors, such as metals, aromatic compounds and nitrogen and carbon sources. Regulation of laccase expression by metals is widespread in fungi such as *T. versicolor*, *C. subvermispora*, *P. ostreatus*, *T. pubescens* and laccase activity was also decreased due to the copper requirement of the enzyme (O'callaghan et al, 2002; Liu et al, 2003; Giardina et al, 2010). Cu²⁺ ions not only affect the activity but also stability of laccases positively (Baldrian and Gabriel, 2002). Collins and Dobson (1997) also reported the effect of copper concentrations on the laccase gene transcription level of *Trametes versicolor*. In this study, 1.5 % methanol concentration was kept constant and various concentrations of copper (0 mM to 4 mM) were tested. Cultures were sampled every day during optimization, growth profiles were determined at 600 nm and enzyme activities were calculated at 420 nm. The growth values of cultures were very similar to each other (Figure 3.39).

Although, laccase activities of cultures were very close to each other, the optimum laccase activity was observed as 380.52 U l⁻¹ at the end of 8th day with 0.8 mM copper concentration which led to a 2.2-fold increase in laccase activity (Figure 3.40). Laccase activity of control culture was approximately calculated as half value of 0.8 mM copper concentration.

As the reports of O'callaghan et al. (2002) and Liu et al. (2003) indicated that the optimal concentrations of copper atoms were reported as 0.2 mM and 0.4 mM,

respectively, confirming that copper concentration is one of the important variables for optimization of laccase production. This data showed that copper is very crucial for laccase activity.

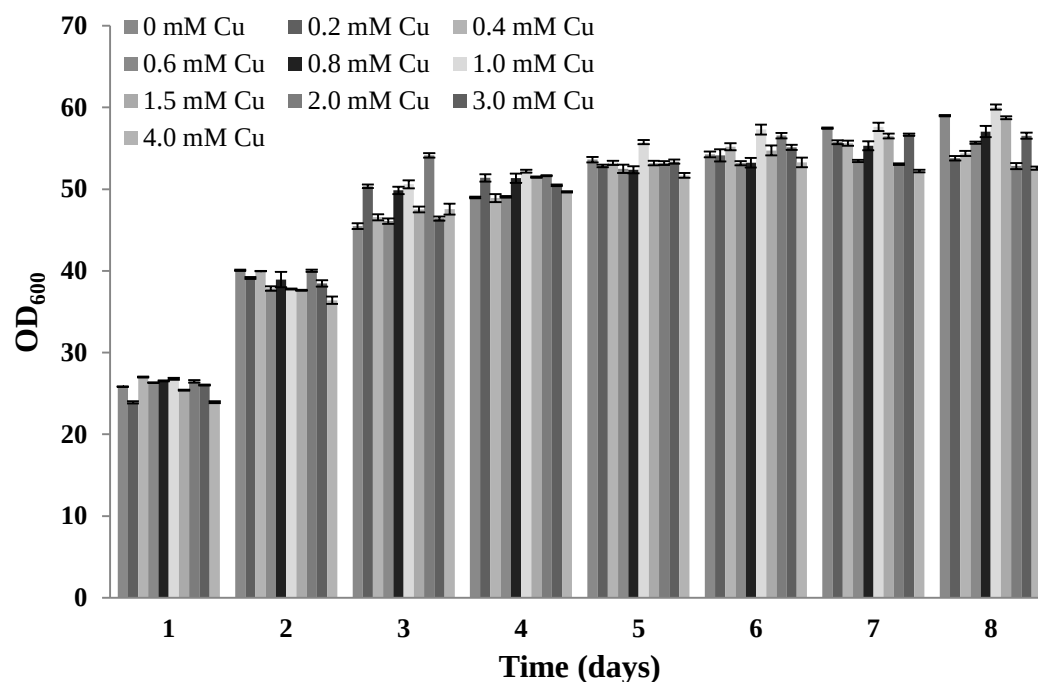


Figure 3.39 : The effect of copper concentration on the growth profile of recombinant *Pichia pastoris*.

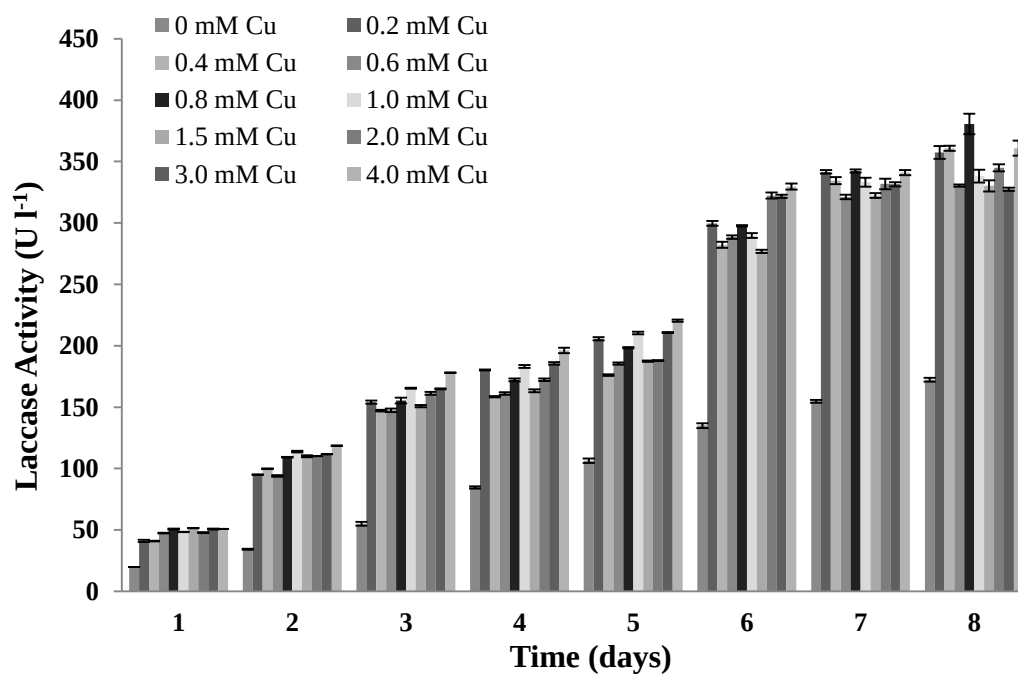


Figure 3.40 : The effect of copper concentrations on the activity of the recombinant laccase CpLcc1 in *Pichia pastoris*.

L-Alanine concentration optimization

Control of pH is usually required during the cultivation process, because some acidic proteases can be activated at a lower pH and may cause a detrimental effect on laccase production (Soden et al, 2002). The importance of pH availability on the activity of laccase enzyme was showed in previous studies (O'callaghan et al, 2002). On the other hand, earlier studies show 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).

Using L-Alanine is very important as stabilizator of laccase production culture. The effect of L-Alanine was supposed that ammonia as a product of the metabolism of L-Alanine, neutralized acidic end products of methanol metabolism and this caused laccase activity increase. The results of the previous studies showed that pH controlled culture by L-Alanine increased the laccase activity as a result of the increased enzyme stability in the L-Alanine-added *Pichia pastoris* cultures producing *Trametes versicolor* laccase (O'callaghan et al, 2002).

In this study, the effect different concentrations of L-Alanine ranging from 0 % to 1.0 % were investigated. The effect of L-Alanine concentration on the growth of cultures indicated that OD₆₀₀ values were similar to each other but higher level of L-Alanine caused an increase in growth (Figure 3.41).

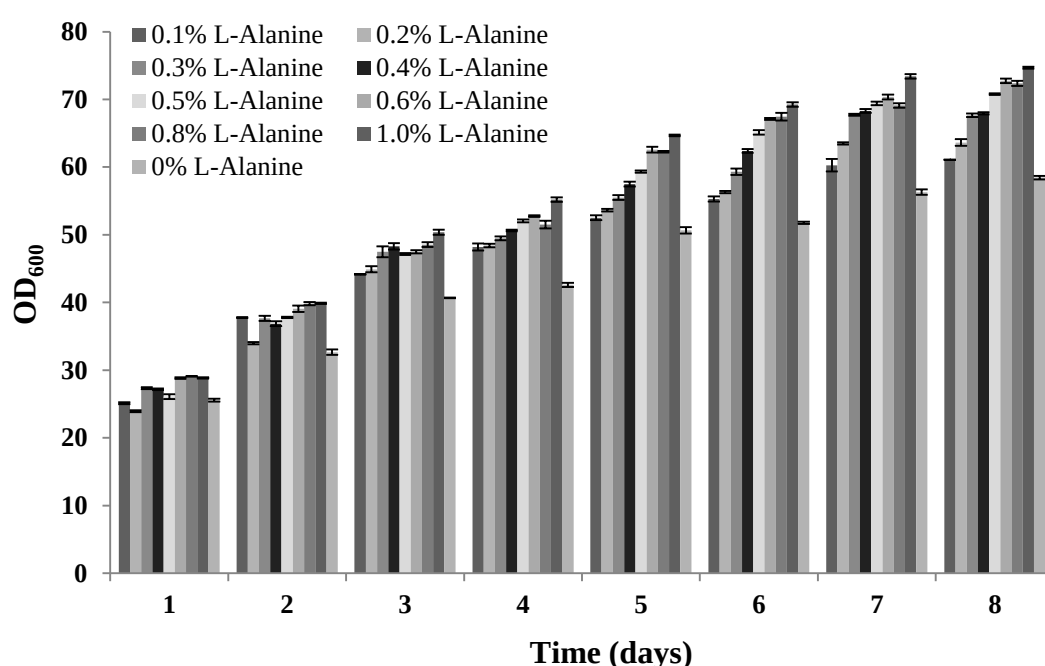


Figure 3.41 : The effect of L-Alanine concentration on the growth profile of recombinant *Pichia pastoris*.

Optimum laccase activity was observed as 591.53 U l⁻¹ at the end of 8th day with 0.6 % L-Alanine concentration (Figure 3.42). Laccase activity of control culture was calculated as 350.16 U l⁻¹. This data indicated that L-Alanine is crucial for laccase activity. As a result of L-Alanine optimization, laccase activity was increased approximately 1.7-fold (350.16 U l⁻¹ to 591.53 U l⁻¹).

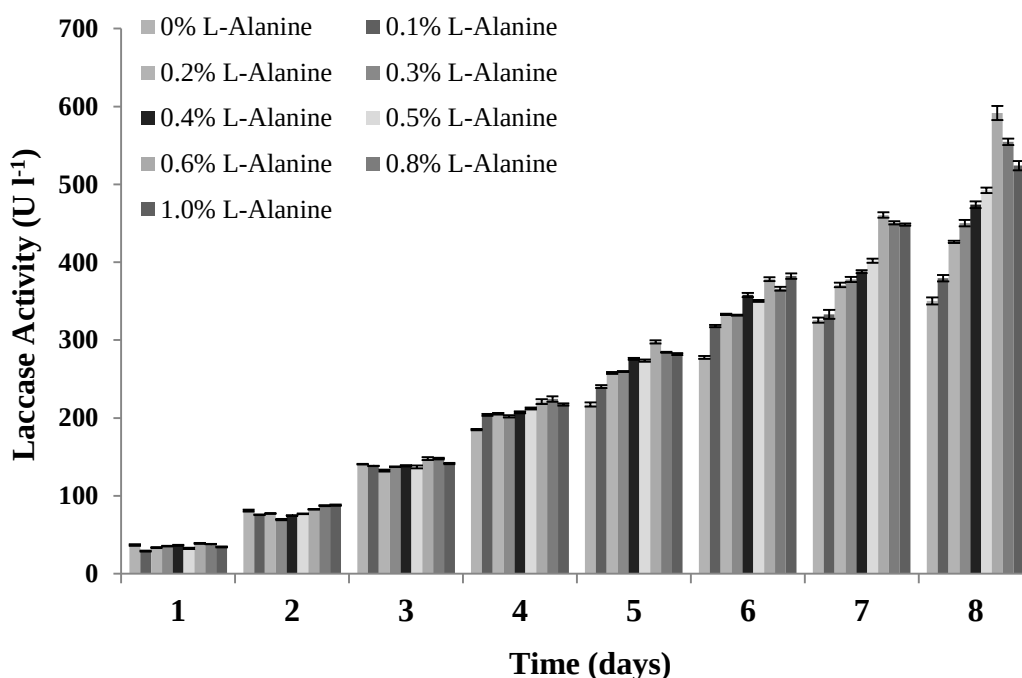


Figure 3.42 : The effect of L-Alanine concentrations on the activity of the recombinant laccase CpLcc1 in *Pichia pastoris*.

3.5.4.2 Optimization of growth temperature

Optimum growth temperature for *P. pastoris* is usually defined as 28-30°C and the previous studies have revealed that a reduction of the growth temperature could improve the recombinant protein production without preventing the growth. Changing the growth temperature affects many cellular processes, including the central carbon metabolism, stress response and protein folding (Gasser et al, 2007). Some studies have demonstrated that activity of recombinant laccases in *Pichia pastoris* could be improved by lowering the cultivation temperature approximately to 16-20°C, especially for laccases suffering from instability and misfolding at high temperature. For example, lower temperature has a positive effect for heterologous expression of *Trametes versicolor* laccase A in *Saccharomyces cerevisiae* (Cassland and Jönsson, 1999; Hong et al, 2002; Liu et al, 2003).

In order to observe the effect of the temperature on the laccase activity, cells were grown at 23°C with 1.5 % methanol, 0.8 mM CuSO₄ and 0.6 % L-Alanine as optimized before. Cultures were sampled every day during optimization, growth profiles were determined at 600 nm and enzyme activities were calculated at 420 nm. Low growth temperature did not cause a dramatical effect on the growth of cultures (Figure 3.43).

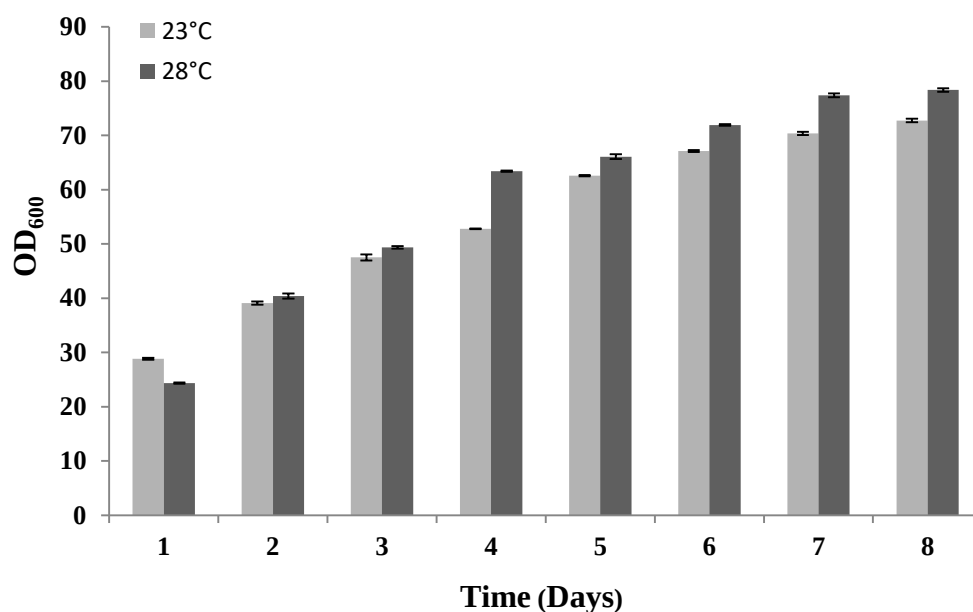


Figure 3.43 : The effect of low temperature (23°C) on the growth profile of recombinant *Pichia pastoris*.

Inconsistently with previous studies, in our study, lower cultivation temperature (23°C) did not improved the activity of recombinant laccase CpLcc1 compared to 28°C. The culture incubated at 23°C provided about 24 % lower laccase activity than 28°C (Figure 3.44).

Finally, optimized expression conditions for recombinant laccase CpLcc1 in *Pichia pastoris* were defined as: growth in BMMY expression medium supplemented with 1.5 % methanol, 0.8 mM CuSO₄ and 0.6 % L-Alanine at 28°C with 250 rpm shaking for 8 days. Under that optimized conditions, activity of recombinant laccase CpLcc1 in *Pichia pastoris* was achieved as 800 U l⁻¹ with 9.8-fold increase. Although this enzyme yield is found to be convenient for biochemical characterization and better for various laccases (e.g. laccase from *Coriolopsis gallica*; 760 U l⁻¹ at 12th days (Calvo et al, 1998)), it is still rather low for industrial applications. Production level of CpLcc1 in *Pichia pastoris* could be further improved by employing several strategies such as use of native promoters and multiple gene copies, codon optimization, altering of

secretory signal sequences as reported previously (Piscitelli et al, 2010; Antosova and Sychrova, 2016).

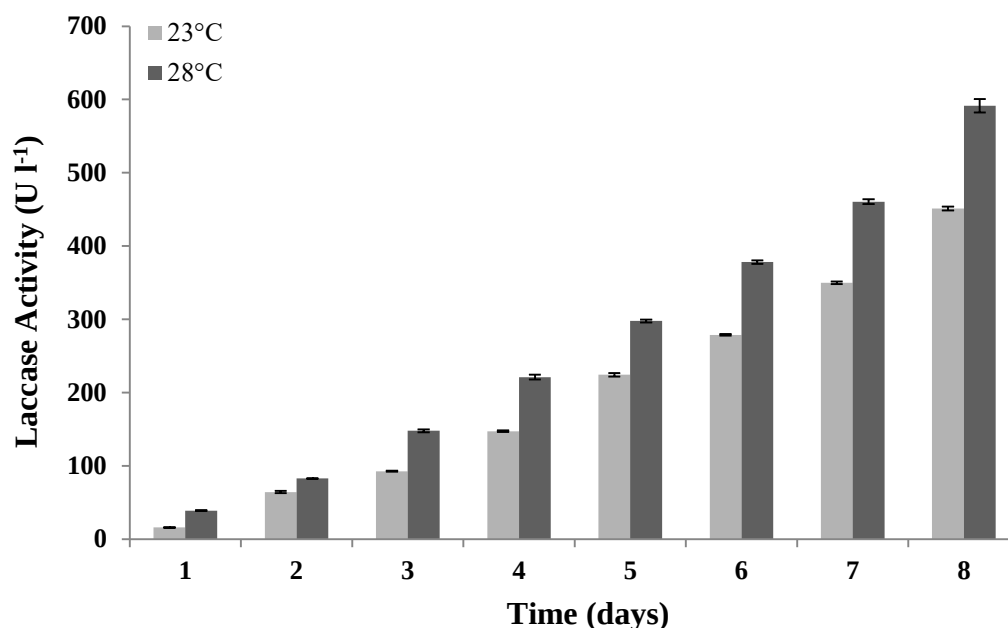


Figure 3.44 : The effect of low temperature (23°C) on laccase activity of the recombinant laccase CpLcc1 in *Pichia pastoris*

3.6 Purification of the Recombinant Laccase from *Pichia pastoris*

As high yielded enzyme production, it is also very important to purify or partially purify the enzymes in order to obtain molecular and biochemical properties for applying them in industrial and biotechnological processes. The general purification techniques used for laccases involve ammonium sulfate precipitation, ultrafiltration, ion exchange, gel filtration and hydrophobic interaction chromatographies (Madhavi and Lele, 2009).

As the first step of recombinant CpLcc1 purification, $(\text{NH}_4)_2\text{SO}_4$ precipitation was applied by adding $(\text{NH}_4)_2\text{SO}_4$ salt between 40 % to 90 % concentrations with 10 % increment at 4°C. to the culture supernatant Laccase activity of the pellets have been tested and saturation with 90 % ammonium sulfate was found as the optimum amount for laccase enzyme precipitation.

Ammonium sulfate precipitates was suspended in 50 mM sodium phosphate buffer at pH 7.35 according to pI value of CpLcc1 which is 5.35. After that, ammonium sulfate salt was removed by ultrafiltration using and centrifugal devices with Amicon Ultra-15 centrifugal filter units (Millipore). The sample was first applied onto the Q-sepharose resin (GE Healthcare Life Sciences) pre-equilibrated with 50 mM sodium

phosphate buffer, at pH 7.35 and eluted proteins at a flow rate of 2 ml/min. The gradient was formed with 0.5 M NaCl. After fractions (5 ml each) were screened with the routine laccase activity assay active fractions (5 ml each) were collected (Figure 3.45) and concentrated with ultrafiltration again. Recombinant laccase CpLcc1 was successfully separated from brown pigment present in the supernatant with the anion exchange chromatography and this procedure on Q-sepharose resin provided 6.2-fold purification with a final enzyme yield of 37.93 % and 35.40 U mg⁻¹ specific activity. Concentrated samples from the anion exchange column was loaded onto the Superdex 75 prep gel column (GE Healthcare Life Sciences) pre-equilibrated with the 20 mM sodium acetate buffer with 0.15 M NaCl at pH 5.35 to separate proteins depending on their sizes and fractions (0.3 ml each) were obtained with the flow rate of 0.3 ml/min. Active fractions (0.3 ml each) between were collected (Figure 3.46) and maximum laccase activity were measured as 47960 U l⁻¹. Active fractions were pooled and concentrated with ultrafiltration. As a result of gel filtration chromatography step, recombinant laccase CpLcc1 was partially purified and gel filtration step provided 27.52-fold purification with a final recovery of 21.27 % and 157.14 U mg⁻¹ specific activity.

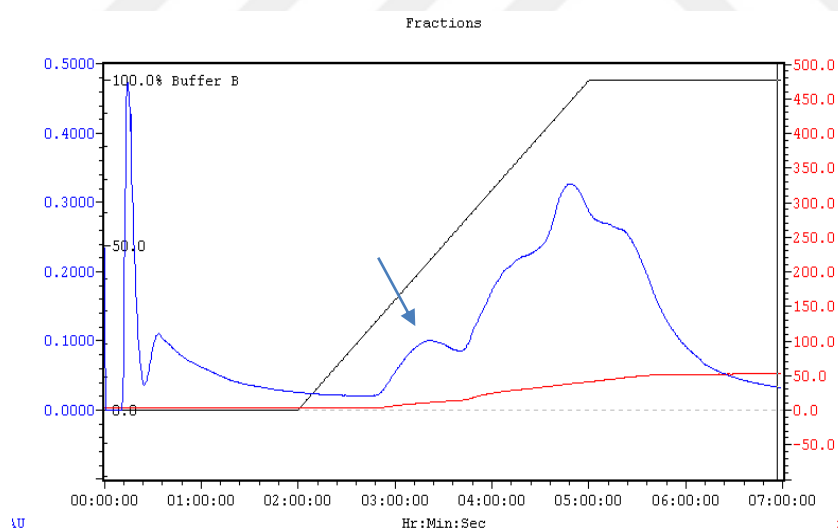


Figure 3.45 : Elution profile of recombinant CpLcc1 laccase applied on anion-exchange chromatography (Q-sepharose resin) column.

The total laccase activity, specific activity, recovery and purification fold values at all purification steps are summarized in Table 3.2.

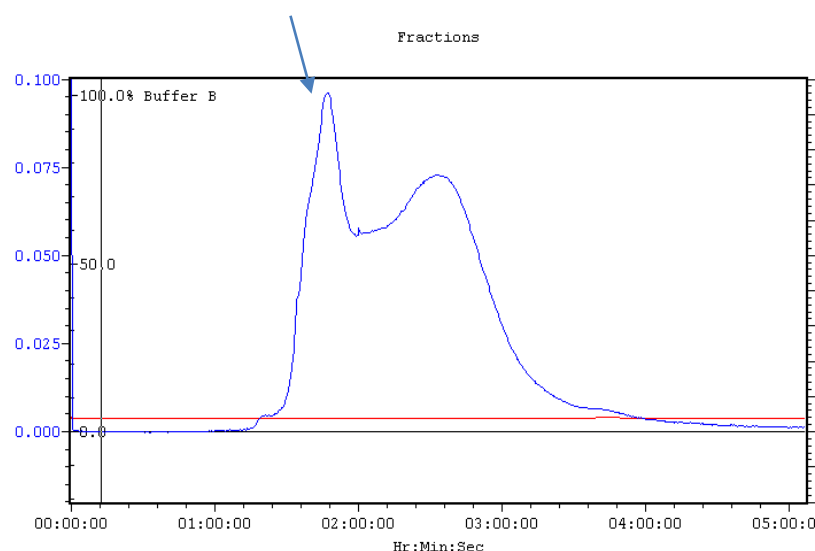


Figure 3.46 : Elution profile of recombinant CpLcc1 laccase applied on gel filtration (Superdex 75 prep gel) column.

Table 3.2 : Summary of recombinant CpLcc1 purification.

Purification steps	Total Activity (U)	Total Protein (mg)	Specific Activity (U mg ⁻¹)	Recovery (%)	Purification fold
Medium	600.00	105.12	5.71	100.00	1.00
(NH ₄) ₂ SO ₄ precipitation	512.55	86.58	5.92	85.42	1.04
Ultrafiltration	466.82	77.89	5.99	77.80	1.05
Anion exchange chromatography	227.59	6.43	35.40	37.93	6.20
Gel filtration chromatography	127.60	0.81	157.14	21.27	27.52

Native-PAGE (Figure 3.47A) and SDS-PAGE (Figure 3.48B) were performed on a 12 % separating and 5 % stacking gel to determine the success of partial purification steps. Gels were stained with Coomassie Brilliant Blue G-250 after electrophoresis. Fungal laccases are copper-containing glycoproteins which have the molecular weight in the range of 50 to 100 kDa (Riva, 2006; Baldrian, 2006; Giardina et al, 2010). However, it must be taken into account that molecular mass of recombinant laccases expressed in *Pichia* has generally been found to be higher than the native form, due to the increased glycosylation in *Pichia* (Kalyani et al, 2015; Gu et al, 2014). Thus, based on the SDS-PAGE, the relative molecular mass of the partially purified recombinant laccase CpLcc1 was estimated as 54 kDa which is in the range reported for most of the fungal laccases. The molecular weight of the protein was also determined as 54 kDa with theoretical MW (Compute pI/MW, ExPasy) calculator using deduced amino acid sequence.

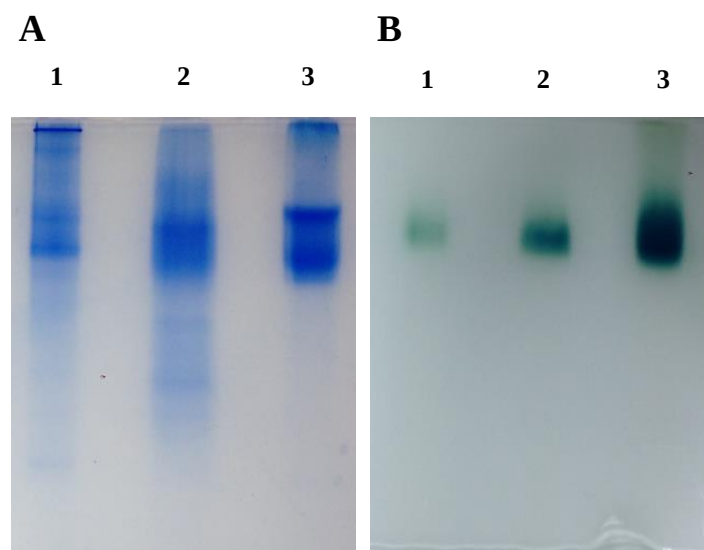


Figure 3.47 : **A.** Native-PAGE gel, **B.** Native-PAGE zymogram analysis of partially purified recombinant laccase CpLcc1. **Lane 1:** Sample from 90 % ammonium sulfate fraction desalted by ultrafiltration; **Lane 2:** Sample from Q-sepharose anion exchange chromatography; **Lane 3:** Sample from Superdex-75 prep gel filtration chromatography.

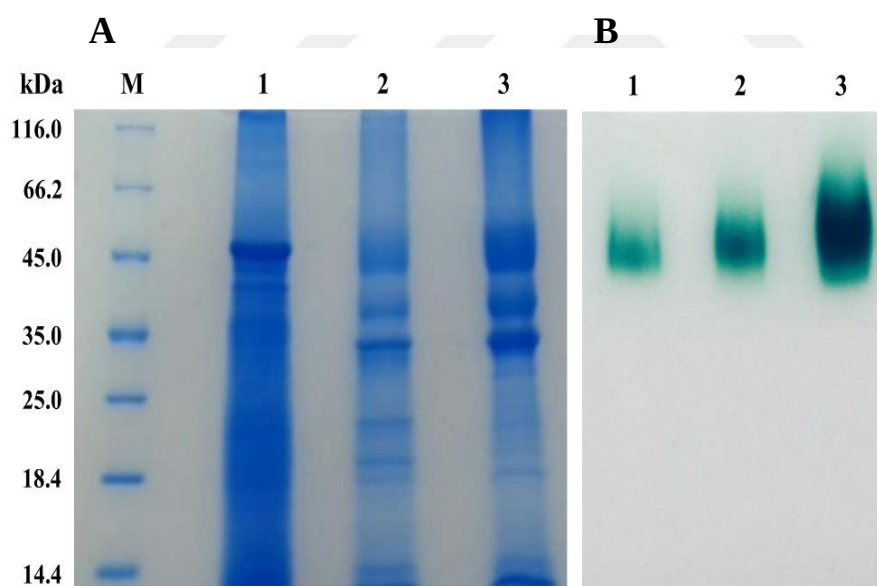


Figure 3.48 : **A.** SDS-PAGE gel, **B.** SDS-PAGE zymogram analysis of partially purified recombinant laccase CpLcc1. **Lane 1:** Sample from 90 % ammonium sulfate fraction desalted by ultrafiltration; **Lane 2:** Sample from Q-sepharose anion exchange chromatography; **Lane 3:** Sample from Superdex-75 prep gel filtration chromatography.

For zymogram step, in order to determine the laccase activity both on the Native-PAGE and SDS-PAGE, samples were loaded without boiling and after electrophoresis gels were soaked with 100 mM sodium citrate buffer, pH 3.0 for 45 min (buffer was changed for every 15 min) and incubated in 2.5 mM ABTS, prepared in the assay

buffer, at room temperature until green color has been observed. The laccase activity of partially purified CpLcc1 was confirmed by not only Native-PAGE-zymogram (Figure 3.47B) but also SDS-PAGE-zymogram (Figure 3.48B) analysis using ABTS as substrate, visualized as a green colored band. Furthermore, the feature of reacting with ABTS on the SDS-PAGE gel indicates that CpLcc1 laccase might be a promising enzyme for textile industry.

3.7 Biochemical Characterization of Recombinant CpLcc1 Laccase

Biochemical characterization of enzymes is very crucial step to determine the properties of enzyme at the molecular and kinetic levels for use of those enzymes in biotechnological and industrial applications. Biochemical characteristics of the laccases have been reported extensively from Basidiomycetes family. In this part of the study, partially purified recombinant laccases of *Coriolopsis polyzona* MUCL 38443 were characterized in terms of determining enzyme activities at different temperatures, pH and assaying effect of different inhibitors on laccase activity for the first time in the literature. Substrate specificity and catalytic properties were also examined.

3.7.1 Determination of optimum temperature and thermal stability

The optimum temperature of fungal laccases is generally ranging from 50°C to 80°C (Baldrian, 2006). Thus, in the present study, temperature on the enzyme activity was analyzed against ABTS between 20°C to 90°C. Consistent with a previous reported YlLac laccase expressed in *Pichia pastoris* (Kalyani et al, 2015), LacI and LacII laccases from *Trametes gallica* (Dong and Zheng, 2004) and a *Coriolopsis gallica* laccase (Calvo et al, 1998), the optimum temperature of the recombinant enzyme CpLcc1 was found as 70°C using ABTS as substrate (Figure 3.49). Thermal stability of enzyme was also assayed by incubating enzyme at those temperatures for 8 hours and measuring the retaining laccase activity at 25°C. The optimum temperature for laccase activity is between 20-37°C and laccases of most white-rot fungi are stable below 50°C (Arora and Sharma 2010; Chandra and Chowdhary, 2015).

Recombinant CpLcc1 was stable at 30°C more than a week and $t_{1/2}$ were higher than 8 hours. Enzyme stability at 40°C for 8 hours and at 50°C for 30 minutes. At 60°C, 70°C and 80°C, recombinant CpLcc1 laccase activity decreased dramatically in few minutes. The half-life of its activity ($t_{1/2}$) was higher than 8 hours both at 30°C and 40°C and it was 30 min at 50°C. CpLcc1 kept its stability more than a week at 30°C, even it was rapidly lost its stability at 60°C, 70°C and 80°C (Figure 3.50). It was not surprised to observe its rapid activity loss over 50°C. Since, generally, fungal laccases quickly loss their activity at high temperature (Viswanath et al, 2014). In fact, thermal stability profile of recombinant CpLcc1 was similar to a white laccase from *Pleurotus ostreatus*, POXA2, half-life of its activity was 12 min at 60°C (Palmieri et al, 1997) and a laccase from *Trametes versicolor* which was completely inactivated at 70°C in 20 min (Stoilova et al, 2010). *Coriolopsis* and *Trametes* species generally have higher stability than other groups of Basidiomycetes. Although they have higher stability, thermal properties of different isozymes and isolates may differ between species (Hilden et al, 2009). Futhermore, thermal behaviour of recombinant CpLcc1 can be found a very promising as a biocatalyst for the newly found laccase.

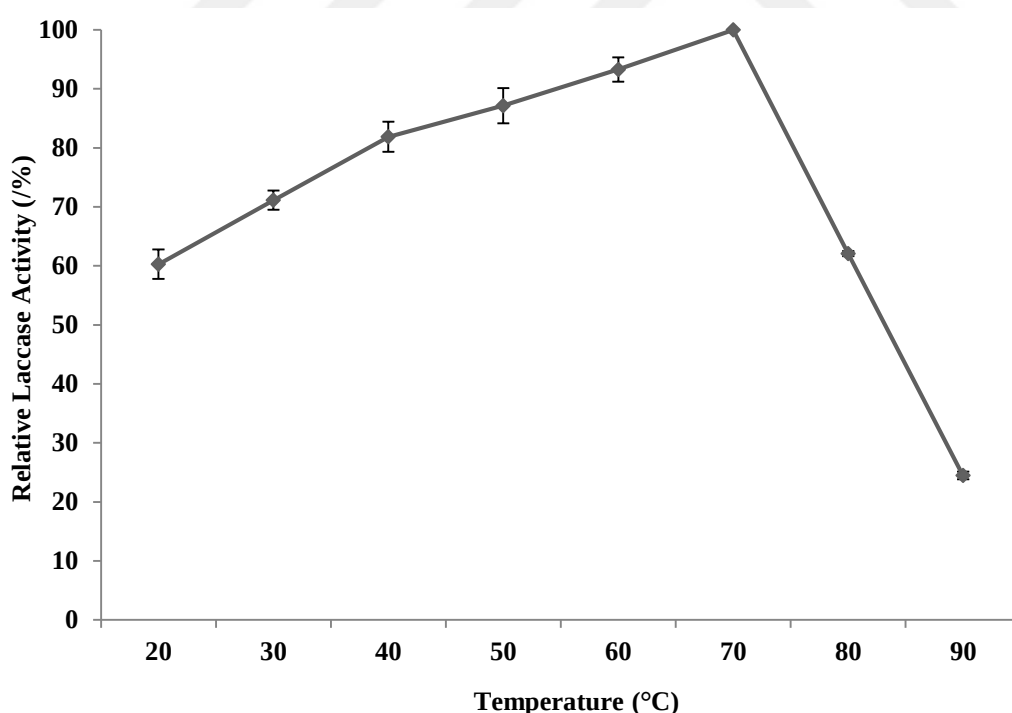


Figure 3.49 : The effect of temperature on the activity of recombinant laccase CpLcc1.

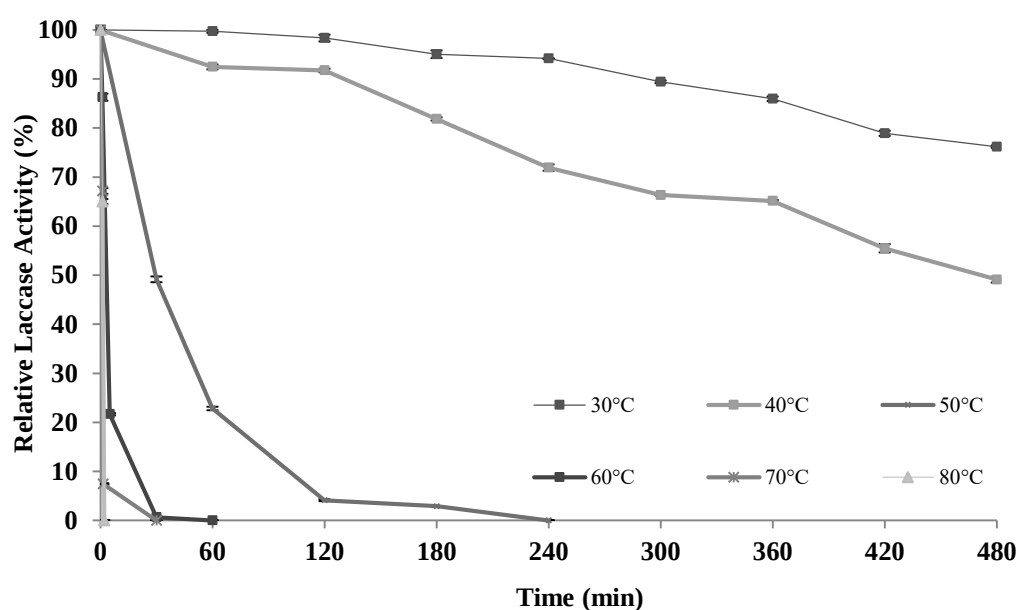


Figure 3.50 : The effect of temperature on the thermal stability of recombinant laccase CpLcc1.

3.7.2 Effect of pH on the laccase activity

Optimum pH could vary with different substrates or with laccase isozymes of the same organism and each substrate of laccase has its own specific optimum pH for its oxidation (Arora and Sharma, 2010). The effect of pH was analyzed against ABTS over the pH range of 2.0-8.0. The pH optima are usually lower than 4.0 for the oxidation of ABTS, whereas phenolic compounds like DMP, guaiacol and syringaldazine show different values of between 4.0 and 7.0 (Baldrian, 2006).

Optimum pH for the recombinant laccase CpLcc1 was observed in the acidic range as pH 3.0 for ABTS as substrate (Figure 3.51). This is also comparable to the laccases with fungal origin such as laccase from *Coriolopsis gallica* (pH 3.0) (Calvo et al, 1998), two laccase isoenzymes from *Coprinus comatus* Lac3 and Lac4 (pH 3.0) (Gu et al, 2014) and a laccase enzyme from *Ganoderma lucidum* TR6 (pH 3.5) (You et al, 2014).

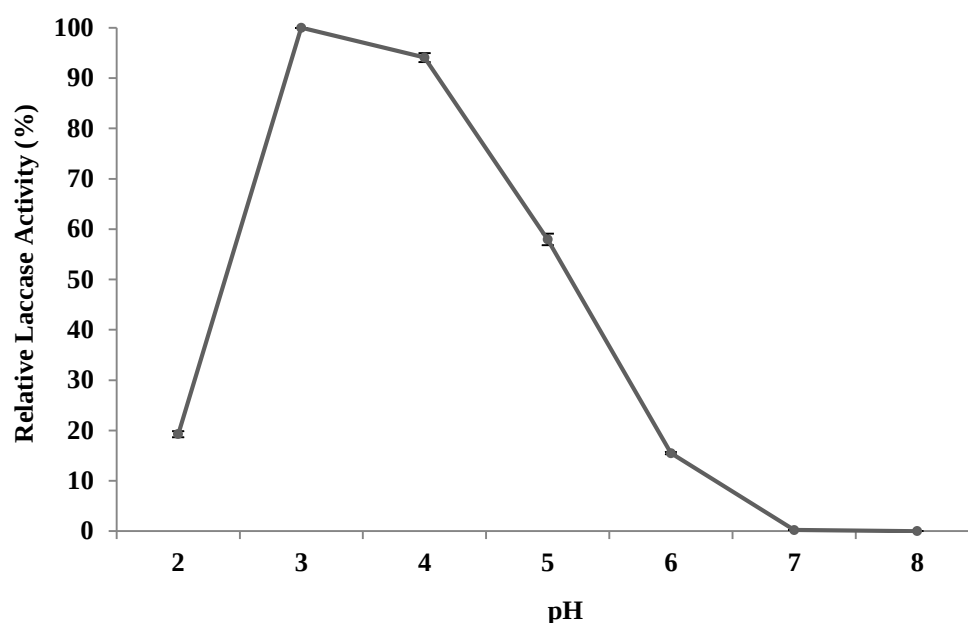


Figure 3.51 : The effect of pH on the activity of recombinant laccase CpLcc1.

3.7.3 Effect of different substrates on laccase activity

Laccases are classified as low-, medium- and high-reduction potentials for the copper sites and fungal laccases have higher reduction potentials than other organisms' laccases (Rodgers et al, 2009). Phenols are typical substrates of laccases and have lower reduction potentials allowing electron abstraction by Type 1 copper. DMP, guaiacol and syringaldazine are common phenolic substrates. Substrate specificity of the recombinant laccase CpLcc1 was examined for different compounds including ABTS, DMP and guaiacol. Among them, ABTS is the most common substrate for laccases, as it is not pH dependent and does not form quinones (Giardina et al, 2010; Majeau et al, 2010). Like other laccases, recombinant CpLcc1 displayed the maximum activity towards ABTS while DMP was oxidized with 15.14 % of relative activity. When using guaiacol, no particular activity was detected (Table 3.3).

Table 3.3 : Laccase activity of recombinant CpLcc1 on different substrates.

Substrate	ϵ_{max} ($\text{M}^{-1} \text{cm}^{-1}$)	Wavelength	Concentration (mM)	Relative Activity (%)
ABTS	36000	420	5.0	100.00
DMP	35565	470	5.0	15.14
Guaiacol	6400	436	5.0	-

3.7.4 Effect of different inhibitors on laccase activity

Inhibitory effects of known laccase inhibitors, sodium azide, L-cysteine, SDS on the laccase activity were analyzed after the incubation of enzyme with those compounds at 30°C for 5 min.

Recombinant CpLcc1 laccase was strongly inhibited by 1 mM sodium azide and 1 mM SDS by 90.12 % and 84.90 %, respectively while 0.1 mM sodium azide and 1 mM L-cysteine also caused 55.62 % and 25.34 % inhibition, respectively (Table 3.4). These results were in accordance with the results from previous reports (Garcia et al, 2007; More et al, 2011; Kalyani et al, 2015).

Table 3.4 : Inhibitory effect of different compounds on laccase activity

Chemical	Concentration (mM)	Inhibition (%)
Sodium Azide	0.1	55.62
Sodium Azide	1.0	90.12
L-cysteine	0.1	-
L-cysteine	1.0	25.34
SDS	1.0	84.90

3.7.5 Catalytic properties of the enzyme

Catalytic performance of the recombinant enzyme was measured by determining Michaelis constant (K_m), turnover number (k_{cat}), and specificity constant (k_{cat}/K_m). Enzyme activity was measured with different concentrations of ABTS (0.1-0.6 mM) (Figure 3.52). Michaelis-Menten constant K_m and V_{max} values were calculated using GraphPad Prism program. K_m , V_{max} , k_{cat} and k_{cat}/K_m values of recombinant CpLcc1 were 0.1374, 288.6, 5.73×10^5 and 4.18×10^6 respectively (Table 3.5).

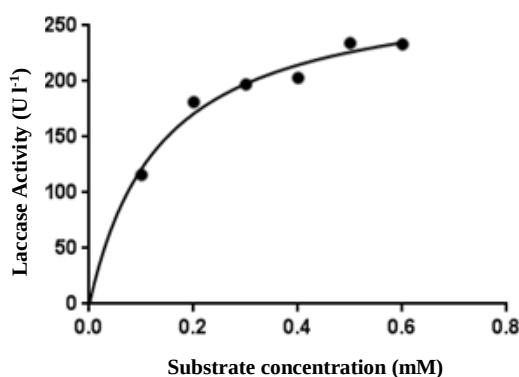


Figure 3.52 : Michaelis-Menten graph for CpLcc1.

Table 3.5 : Catalytic parameters of recombinant CpLcc1 laccase.

CpLcc1	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1}\text{ l}^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\text{ mM}^{-1}$)
ABTS	0.1374	288.6	5.73×10^5	4.18×10^6

When compared to other laccases, kinetic parameters of recombinant CpLcc1 for ABTS were seems to be promising. Since, K_m value of recombinant CpLcc1 was 7-fold lower than that of a laccase from *Ganoderma lucidum* in *Pichia pastoris* (0.9665 mM) (Sun et al, 2012) and 2-fold lower than the laccase POXA3 from *Pleurotus ostreatus* (0.280 mM) (Palmieri et al, 1997), a laccase from *Pleurotus sp.* (0.250 mM) (More et al, 2011) and a fungal laccase from *M. purpureofusca* (0.296 mM) (Sun et al, 2013). Similarly, the turnover (k_{cat}) value of recombinant CpLcc1 was found to be 7.7, 14 and 40 fold higher than that for the laccase Lac4 from *Pleurotus sajor-caju* (Soden et al, 2002), the laccase LAP2 from *Trametes pubescens* (Galhaup et al, 2002) and a laccase from *P. sanguineus* BRFM 902 (Uzan et al, 2010), respectively. Consequently, resulting specificity constant (k_{cat}/K_m) value of the recombinant CpLcc1 for ABTS was also remarkably higher than previously reported laccases.

4. CONCLUSION

In this study isolation and characterization of the genomic and cDNA sequences encoding a novel laccase, namely CpLcc1 were achieved from *Coriolopsis polyzona* MUCL 38443.

Firstly, laccase specific cDNA sequences on the total RNA of *Coriolopsis polyzona* MUCL 38443 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. At least three different laccase cDNA sequences were detected as expressed in the white-rot fungus *Coriolopsis polyzona* MUCL 38443 and named as *Cplcc1*, *Cplcc2* and *Cplcc3*, respectively. Among them, *Cplcc1* was detected as a major expressed laccase gene in *Coriolopsis polyzona* MUCL 38443 under the applied conditions.

In the present study, based on its partial cDNA sequence, 5'- and 3'-ends of full length *Cplcc1* cDNA were determined by performing 5' and 3' RACE methods. Following this step, the full-length *Cplcc1* cDNA and the corresponding genomic DNA encoding the laccase *Cplcc1* were successfully amplified from fungus *Coriolopsis polyzona* MUCL 38443 and then, characterized.

The corresponding open reading frame for full length *Cplcc1* cDNA from ATG start codon to TAA stop codon is 1563 bp with 57 % GC content coding for 520 amino acids with a putative 20-residue signal sequence. Additionally, the genomic DNA sequence of *Cplcc1* is 2106 bp in length and interrupted with 10 introns vary between 51 to 58 base pairs in length. The deduced amino acid sequence of CpLcc1 has five potential N-glycosylation sites. Conserved copper ligands comprised of one cysteine residue and ten histidine residues were determined in deduced aminoacid sequence of CpLcc1 that are necessary for the coordination of copper atoms on the active site of laccase. Moreover, C474 and F484 residues were also found which are crucial for T1 copper domain and high redox potential laccase production, respectively.

Phylogenetic analyses were also performed using the MEGA version 6.0 software. Neighbor-joining tree which was constructed with other laccases from GenBank showed that CpLcc1 laccase is located close to *Trametes* species.

The isolated full-length *Cplcc1* cDNA was successfully overexpressed in the yeast *Pichia pastoris* under the control of *AOX1* promoter and extracellularly secreted into the culture.

For partial purification steps, recombinant CpLcc1 laccase was expressed in 1 L shake flasks containing 100 ml BMGY/BMMY medium supplemented with 1.5 % methanol, 0.8 mM CuSO₄ and 0.6 % L-Alanine as optimized conditions and recombinant CpLcc1 laccase activity was calculated as 800 U l⁻¹. Recombinant CpLcc1 expressed in *Pichia pastoris* was partially purified and characterized with a molecular weight of 54 kDa, optimum temperature as 70°C and optimum pH value as 3.0. Thermal stability and kinetic parameters of recombinant CpLcc1 was found as promising. Furthermore, the sequence analysis indicated that CpLcc1 is a high redox potential laccase and this feature makes CpLcc1 a good candidate for industrial applications. Since laccases with high redox potential provide broader substrate range without the use of mediators which makes them more attractive for biotechnological applications. Finally, heterologous expression system of this newly found laccase CpLcc1 could allow the rational protein engineering approaches to further improve the properties of the enzyme. Extending the laccases derived from different sources are critical to explore them as novel enzymes for low-cost productions and wide usage in industrial applications.

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APPENDICES

APPENDIX A.1: Culture media composition

APPENDIX A.2: Buffers and solutions

APPENDIX A.3: Chemicals and enzymes

APPENDIX A.4: Laboratory equipment

APPENDIX A.5: Supplementary figures





APPENDICES

APPENDIX A.1: Culture media composition

LSK medium (1000 ml)

D-glucose	55g
Bacteriological peptone	17 g
KH ₂ PO ₄	2.5 g
MgSO ₄ .7H ₂ O	1.027 g
CuSO ₄ .5H ₂ O	0.03127 g
MnSO ₄ .H ₂ O	0.0559 g

Components were dissolved in distilled water and sterilized by autoclaving at 121°C for 15 min on liquid cycle. After sterilization 10 mg/L Thiamine-HCl were added to this mixture and completed to 1 liter with sterilized distilled water.

Potato dextrose agar (PDA) medium (1000ml)

Potato dextrose agar	39 g
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Distilled H₂O was added up to 1000ml and then autoclaved at 121°C for 15 min.

Luria Bertani (LB) Medium (1000ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

Dissolved in distilled H₂O and completed up to 1000 ml and 15 g of agar was added for solid medium. Then autoclaved at 121°C for 15 min for sterilization.

2xYT Medium (1000ml)

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g

Dissolved in distilled H₂O and completed up to 1000 ml and 15 g of agar was added for solid medium. Then autoclaved at 121°C for 15 min for sterilization.

Yeast Extract Peptone Dextrose Medium (YPD) (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 (YPDS) 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 at 121°C for 15 min. After cooling to ~60°C, 100 ml of filter-sterilized 20 % D-glucose was added and poured to plates after adding 1.0 ml of 100 mg/ml Zeocin™.

Minimal Dextrose Medium (1000 ml)

Yeast Nitrogen Base	1.34 %
Biotin	4×10^{-5} %
D-glucose	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 at 121°C for 15 min. After cooling to ~60°C, 100 ml of filter-sterilized 20 % dextrose and 2 ml of filter-sterilized 500x biotin were added and poured on plates.

Minimal Methanol Medium (1000 ml)

Yeast Nitrogen Base	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 at 121°C for 15 min. After cooling to ~60°C, 5 ml of filtered methanol and 2 ml of filter-sterilized 500x biotin were added and poured on plates.

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

100 mM potassium phosphate buffer, pH 6.0

Yeast Nitrogen Base 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 at 121°C for 15 min. After cooling 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)

Yeast Nitrogen Base 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 at 121°C for 15 min on liquid cycle. After cooling 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: 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 1000 ml and pH was adjusted to 8.0 by adding HCl.

TBE Buffer (10X): 108 g Tris base, 55 g Boric acid, 40 ml EDTA (0.5M, pH 8.0) mixed, distilled water was added up to 1000 ml 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.2H₂O were mixed 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 and pH was adjusted to 5.5 by adding acetic acid.

1 M potassium phosphate buffer, pH 6.0: 132 ml 1 M K₂HPO₄, 868 ml 1 M KH₂PO₄ 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 0.1 M-citric acid and 180 ml 0.1M-trisodium citrate were mixed.

100 mM sodium-citrate buffer, pH 4.0 (1000 ml): 590 ml 0.1 M-citric acid and 410 ml 0.1M-trisodium citrate were mixed.

100 mM sodium-citrate buffer, pH 5.0 (1000 ml): 350 ml 0.1 M-citric acid and 650 ml 0.1 M-trisodium citrate were mixed.

100 mM sodium-citrate buffer, pH 6.0 (1000 ml): 115 ml 0.1 M-citric acid and 885 ml 0.1 M-trisodium citrate were mixed.

100 mM sodium-phosphate buffer, pH 7.0 (1000 ml): 577 ml 0.1 M- Na_2HPO_4 and 423 ml 0.1 M NaH_2PO_4 were mixed.

100 mM sodium-citrate phosphate, pH 8.0 (1000 ml): 932 ml 0.1 M- Na_2HPO_4 and 68 ml 0.1 M- NaH_2PO_4 were mixed.

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

Native Separating gel (12%) (10 ml): 4 ml Acrylamide/Bis-acrylamide (30 %/0.8 % w/v), 2.5 ml 1.5 M Tris-HCl (pH 8.8), 100 μl APS (10%), 8 μl TEMED, 3.4 ml dH_2O .

Native Stacking Gel (5%) (4 ml): 670 μl Acrylamide/Bis-acrylamide (30 %/0.8 % w/v), 1 ml 0.5 M Tris-HCl (pH 6.8), 40 μl APS (10%), 4 μl TEMED, 2.3 ml dH_2O .

5X Native Sample Buffer: 250 mM Tris-HCl, pH 6.8, 30% glycerol 0.05% Bromophenol Blue.

10X Native Running Buffer: 25 mM Tris and 192 mM glycine.

Native Staining Solution (1000 ml): 1 g Coomassie Brilliant Blue R250, 500 ml Methanol, 100 ml Glacial Acetic acid and 400 ml dH_2O .

Native Destaining Solution (1000 ml): 50 ml Methanol, 100 ml Glacial Acetic acid and 800 ml dH_2O .

1X Separating gel buffer: 1.5 M Tris was prepared and pH was adjusted to 8.8 with HCl solution.

1X Stacking gel buffer for SDS-PAGE: 0.5 M Tris was prepared and pH was adjusted to 6.8 with HCl solution.

5X SDS-PAGE Sample Buffer: 250 mM Tris-HCl pH 6.8, 10 % SDS, 30 % glycerol, 5 % β -mercaptoethanol and 0.02 % bromophenol blue.

5X SDS-PAGE Running Buffer: 15 g Tris, 72 g Glycine, 5 g SDS were completed to 1 L solution with distilled water.

SDS-PAGE Staining Buffer: 1 g Coomassie Brilliant Blue R-250, 500 ml methanol, 100 ml acetic acid were completed to 1 L solution with distilled water.

SDS-PAGE Destaining Solution: 50 ml methanol, 100 ml Glacial Acetic acid dissolved in 1 L of distilled water.

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

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





APPENDIX A.3: Chemicals and enzymes

Chemicals and Supplier

6X Loading Dye	Fermentas
ABTS	Sigma-Aldrich
2,6-dimethoxyphenol (DMP)	Fluka
Acetic acid	Merck
Acrylamide	Sigma –Aldrich
Agar	Acumedia
Agarose	Peqlab
Ammonium persulphate	Merck
Ammonium sulfate	Merck
Ampicillin	Sigma
β-mercaptoethanol	Merck
Bisacrylamide	Sigma-Aldrich
Boric acid	Merck
Bovine serum albumine	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
Calcium chloride (CaCl ₂)	Merck
Citric Acid Monohydrate	Merck
Coomassie Brilliant Blue	Bio-Rad (USA).
Copper sulfate	Merck
dATP	Fermentas
D-biotin	Fluka
D-glucose	Riedel-de Haën
D-Sorbitol	Sigma
Dithiothreitol	Merck
Di-potassium hydrogen phosphate	Merck
Disodium hydrogen phosphate	Riedel-de Haen
DMP	Sigma-Aldrich

EDTA	MP
Ethidium bromide	Sigma
Formaldehyde	Riedel-deHaen
Glycerol	Fluka
Glycine	Merck
Guaiacol	Merck
Hydrochloric acid	Merck
L- Alanine	Merck
L- Cysteine	Merck
Methanol	Merck
Mycological peptone	Bio-Chemika
MOPS	Sigma
Potassium dihydrogen phosphate	Riedel-de Haën
Q Sepharose ® Fast Flow	GE Healthcare
Sodium acetate	J.T. Baker
Sodium azide	Merck
Sodium chloride	Merck
Sodium dodecyl sulphate	BDH
Sodium dihydrogen phosphate	Riedel-de Haen
Sodium hydrogen phosphate	Merck
Sodium hydroxide	Merck
Superdex 75 prep	GE Healthcare
TEMED	Fluka
tri-Sodium Citrate Dihydrate	Merck
Tris	MP
Tryptone	Biolife
Yeast Extract	Merck
Yeast nitrogen base with ammonium sulfate and w/o amino acids	Sigma
Zeocin	Invitrogen

Enzymes Supplier

<i>EcoRI</i>	Fermentas
<i>NotI</i>	Fermentas
<i>SacI</i>	Fermentas

Taq polymerase

Intron

Pfu polymerase

Fermentas

T4 DNA Ligase

Fermentas

Kits

BD SMARTer™ RACE cDNA Amplification Kit

Clontech

RNeasy Plant Mini Kit

Qiagen

Expand High Fidelity PCR System

Roche

Transcriptor First Strand Synthesis Kit

Roche

Pichia EasyComp™ Transformation Kit

Invitrogen

QIAquick Gel Extraction Kit

Qiagen

Fast Prep Instrument and FastDNA™ Spin Kit

MPBio



APPENDIX A.4: Laboratory equipment

Autoclave: TOMY SX-700E high pressure steam sterilizer

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 New Brunswick Scientific Ultra-low temperature freezer, -20°C Bosch, -20°C Beko

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

Electrophoresis power supply: Thermo Electron Corporation EC1000-90

Fast Prep Instrument: MPBio Fast Prep 24

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: 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 International GmbH, Thermo Electron Corporation 430 (USA)

pH meter: Hanna Instruments, HI 221 Microprocessor pH meter

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

Refrigerator: Bosch +4°C

UV-Visible Spectrophotometers: Shimadzu UV-Pharmaspec 1700 (Japan)

Thermocycler: Labnet International, Multigene gradient TC9600-G-230V, Techne FTGENE 5D

Thermomixer: Eppendorf thermomixer comfort (1.5ml)

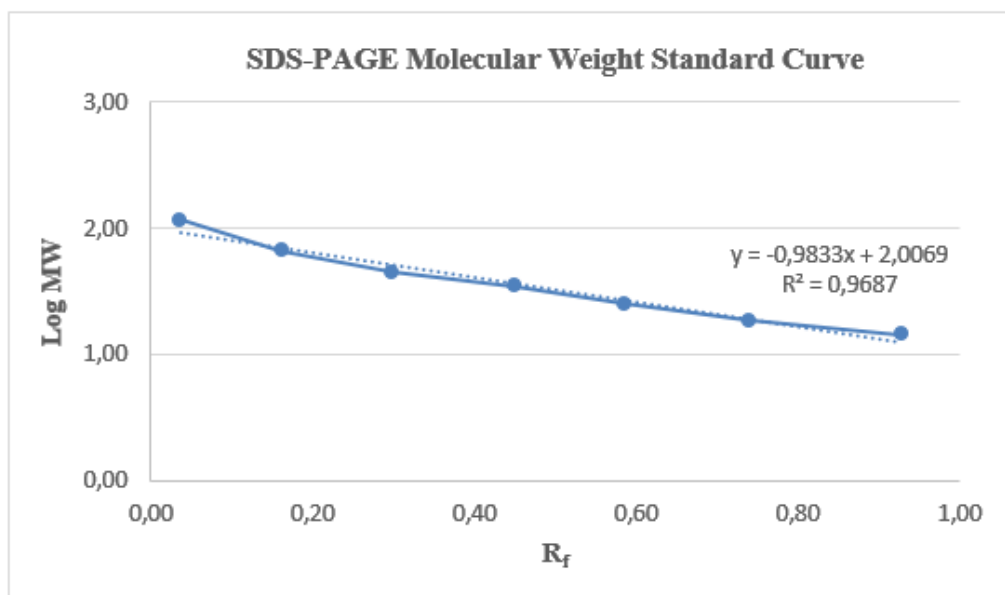
Transilluminator: Biorad UV transilluminator 2000

Vortex machine: Heidolph Raax top (Germany)

Waterbaths: Memmert wb-22



APPENDIX A.5: Supplementary figures



Dye front (cm)= 11,1

Migration Distance of CpLcc1 (cm)=3,1

R_f of CpLcc1= 0,2793

Marker Standards (kDa)	Log MW	Migration (cm)	R _f
116,0	2,06	0,4	0,04
66,2	1,82	1,8	0,16
45,0	1,65	3,3	0,30
35,0	1,54	5,0	0,45
25,0	1,40	6,5	0,59
18,4	1,26	8,2	0,74
14,4	1,16	10,3	0,93



CURRICULUM VITAE



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EDUCATION :

- **B.Sc.** : 2006, Ege University, Faculty of Engineering, Bioengineering Department
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PROFESSIONAL EXPERIENCE AND REWARDS:

- March 2011 - Still, Marmara University, Faculty of Engineering, Bioengineering Department, Research assistant.
- January 2009 - February 2011, TÜBİTAK Marmara Research Center, Genetic Engineering and Biotechnology Institute (GEBI), Enzyme and Microorganism Molecular Genetics Laboratory, Researcher.
- “Technology Development” award with “Production of Industrial Enzymes for Feed Industry” Project. TUBITAK Marmara Research Center in the context of Research and Support Awards.

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

Publications

- **Orkun Pinar**, Candan Tamerler, Ayten Y. Karataş, Heterologous expression and characterization of a high redox potential laccase from *Coriolopsis polyzona* MUCL 38443 (2016) (Accepted: Turkish Journal of Biology).

Oral Presentations

- **Pinar, O.**, Tetik, B., Varışlı, E., Tamerler, C., Yazgan-Karatas, A. 2012. “Beyaz Çürükçül Küf Mantarı *Coriolopsis polyzona* MUCL 38443’den Lakkaza Özgü Sekansların Tanımlanması”, 17th National Biotechnology Congress, 23–26 September 2012, İstanbul, Turkey (in Turkish).

Poster Presentations

- **Pinar, O.**, Tetik, B., Varışlı, E., Tamerler, C., Yazgan-Karatas, A. 2012. “Identification of laccase gene-specific sequences from the white-rot fungus *Coriolopsis polyzona* MUCL 38443”, 15th European Congress on Biotechnology (*New Biotechnology*, Volume 29), 23–26 September 2012, İstanbul, Turkey.
- **Pinar, O.**, Tetik, B., Kurt-Gur, G., Tamerler, C., Yazgan-Karatas, A. Isolation and Cloning of a Laccase specific gene from the White-Rot Fungus *Coriolopsis polyzona* MUCL 38443. The 5th Congress of European Microbiologists (FEMS 2013). 21-25 July 2013, Leipzig, Germany.

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

Publications

- Eda Bal, **Orkun Pinar**, Dilek Kazan, Hasan Umit Öztürk, and Ahmet Alp Sayar. "Immobilization of α -amylase from *Bacillus subtilis* SDP1 isolated from the Rhizosphere of *Acacia cyanophylla* Lindey." *Electronic Journal of Biology* (2016).

Poster Presentations

- **Pinar, O.**, Gür, K., G., İrigül, Ö., Karataş, Y., A. “The Effect of *ywfI* Null Mutation on the Expression of *ywfH* gene in *Bacillus subtilis*”, 33rd FEBS Congress & 11th IUBMB Conference, 28 June-3 July 2008, Athens, Greece.
- Gür, K. G., **Pinar, O.**, Karataş, Y., A., Tamerler, C, “Optimization of *Pycnoporus sanguineus* Methylophilic Yeast *lcc1* gene Expression in *Pichia pastoris*”, XII. International Congress of Bacteriology and Applied Microbiology, International Unions of Microbiological Society (IUMS), 5-9 August 2008, İstanbul, Turkey.

- **Pinar, O.**, Ünlü, E., C., İrigül, Ö., Gür, K., G., Köroğlu, T., E., Karataş, Y., A., “The effects of insertional inactivation of *Spo0A* and *AbrB* genes on the expression of *ywfH* gene in *B. subtilis*”, XII. International Congress of Bacteriology and Applied Microbiology, International Unions of Microbiological Society (IUMS), 5-9 August 2008, İstanbul, Turkey.
- Tayran, H., **Pinar, O.**, İrigül, Ö., Gür, K., G., Köroğlu, T., E., Karataş, Y., A., “The effects of *codY* regulatory gene on the expression profile of *B. subtilis*”, XII. International Congress of Bacteriology and Applied Microbiology, International Unions of Microbiological Society (IUMS), 5-9 August 2008, İstanbul, Turkey.
- Melis S. Sogut, Nurcin C. Ozturk, H. Umit Ozturk, **Orkun Pinar**, Fatih M. Ipek, Ebru Cayir, Abdulmecit Gokce, Deniz Karasu, Dilek C. Ozturk, Yavuz Ozturk, A. Akin Denizci, Bengu Ozturk, Banu Bahar, Naze G. Avci, Burak Ayan, Dilek Kazan, Yonca A. Duman, Sayit Sargin, Sevkett Karacanli, Cengizhan Mizrak, A. Ugur Catli, Kamil Kucukyilmaz, Hatice K. Guvenmez, Burhan Arikan, Mehmet Bozkurt, Ferda Seyhan, Berna S. Akbulut, A. Altan Erarslan, Gaye Ongen, Sadik Dincer, Engin Yenice, Sevnur Mandaci. 2012. “Production of industrial enzymes for feed industry”. 15th European Congress on Biotechnology (*New Biotechnology*, Volume 29), 23-26 September 2012, İstanbul, Turkey.
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- Kübra Karaosmanoğlu, **Orkun Pinar**, Nihat A. Sayar, Dilek Kazan. Hydrolysis of Hazelnut Husk by Using Commercial Enzymes for the Production of Fermentable Sugar, VIth International Bioengineering Congress (IBEC 2013), 12-15 November 2013, Kuşadası, Turkey.
- Ipek, FM., **Pinar, O.** Sogut, MS., Karasu, D., Cayir, E., Ozturk, NC., Denizci, AA., Ozturk, DC., Kazan, D. And Mandaci, S. “Cloning and Expression of *xynA* Gene from Xylanase Secreting *Bacillus pumilus* YMNX25”, International Enzymes for Biocatalysis Workshop”, 03-05 June 2014, Arel University, İstanbul, Turkey.
- **Orkun Pinar**, Başak Koçhan, Nihat Alpagu Sayar, Kübra Karaosmanoğlu, Dilek Kazan. “Enzyme mixture production from *Pycnoporus sanguineus* DMSZ 3024 using a lignocellulosic waste, Hazelnut husk: A case study for laccase and cellulose”. 16th European Congress on Biotechnology, 13-16 July 2014, Edinburgh, Scotland.

- Ozgoren T., **Pinar O.**, Denizci A.A., Utkan G., Kazan D. "Response of novel *Bacillus marmarensis* GMBE 72T to extreme conditions: Poly (3-hydroxybutyrate)", 4-9 July 2015, 40th FEBS Congress, Berlin, Germany.
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- Ecem Kaplan Çoban, Ceren Gençoğlu, Doğançan Kirman, **Orkun Pinar**, Dilek Kazan, Ahmet Alp Sayar. "Assessment of the effects of medium composition on growth, lipid accumulation and lipid profile of *Chlorella vulgaris* as a biodiesel feedstock", 4th International Conference on Renewable Energy Research and Applications, 22-25 November 2015 Palermo, Italy.
- T. Ozgoren, B. Sirin, Ç. Çimenoglu, **O. Pinar**, A.A. Denizci, Kazan D. "Utilisation of poly(3-hydroxybutyrate) from *Bacillus marmarensis* GMBE 72T (DSM21297) as scaffold", 41st FEBS Congress (The FEBS Journal 283), Molecular and Systems Biology for a Better Life, 3-8 September, 2016, Ephesus/Kuşadası, Turkey.