İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ISOLATION AND CHARACTERIZATION OF GENOMIC AND cDNA SEQUENCES ENCODING THE LACCASE CpLcc2 FROM *Coriolopsis polyzona* MUCL 38443

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Coriolopsis polyzona MUCL 38443 SUŞUNDAKİ CpLcc2 LAKKAZINI KODLAYAN GENOMİK VE cDNA DİZİLERİNİN İZOLASYONU VE KARAKTERİZASYONU

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HAZİRAN 2016

Dicle MALAYMAR, a M.Sc. student of İTU Graduate School of Science Engineering and Technology student ID 521131106, successfully defended the thesis entitled "Isolation And Characterization Of Genomic And cDNA Sequences Encoding The Laccase CpLcc2 From *Coriolopsis polyzona* MUCL 38443", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Ayten KARATAŞ** İstanbul Technical University	....
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Date of Submission : 2 May 2016
Date of Defense : 2 June 2016

To my father and grandparents,

FOREWORD

I would like to express my sincerest appreciation and special thanks for my thesis advisor Prof. Dr. Ayten Yazgan Karataş for giving me the opportunity of conducting this research, her invaluable guidance and continued advices throughout my research.

I would like to thank my all colleagues in MOBGAM especially Hasan Demirci, Soner Torun, Sinem Sarıtaş, Ayşe Buse Özdabak and Naciye Durmuş İşleyen for creating a pleasant working atmosphere and their valuable friendship.

I am especially grateful to my mentor and my fiancé Orkun Pinar for sharing his great knowledge with me, endless support and deep love. I have learned from him how to think and study like a scientist. Thank you for always being with me whenever I need, for encouraging, recovering my morale every time I am faced with failure and hopelessness. I cannot imagine a life without you.

I am also greatly indepted to my mother Özden Malaymar and my sister Deniz Malaymar who have always been there for me with their endless love, believed in me and supported me to make my own way. There are no words that can really express my appreciation to my family.

Special thanks to The Scientific and Technological Research Council of Turkey (TUBITAK), 2210-A National Scholarship Programme for Graduate Students which have been really important financial support for me to move towards an academic career.

June 2016 | Dicle MALAYMAR
Bioengineer, MSc.

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ABBREVIATIONS

1-HBT	: 1-hydrobenzotriazole
3-HAA	: 3-hydroxyanthranilic acid
µg	: Microgram
µl	: Microliter
ABTS	: 2-2’-azinobis-(3-ethylbenzthiazoline-6-sulphonate)
Amp	: Ampicillin
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
cDNA	: Complementary Deoxyribonucleicacid
DNA	: Deoxyribonucleicacid
dATP	: Deoxyadenosine triphosphate
dNTP	: Deoxynucleoside triphosphate
DTT	: Dithiothreitol
EB	: Elution buffer
EDTA	: Ethylene diamine tetra-acetic acid
FA	: Formaldehyde
gDNA	: Genomic Deoxyribonucleicacid
kb	: Kilobase
kDa	: Kilodalton
LB	: Luria-Bertani
Ml	: Milliliter
mM	: Millimolar
mRNA	: Messenger RNA
MUCL	: Mycotheque de l'Universite catholique de Louvain
NCBI	: National Center for Biotechnology Information Database
ng	: Nanogram
NUP	: Nested Universal Primer
PAH	: Polycyclic aromatic hydrocarbon
PDA	: Potato Dextrose Agar
PCR	: Polymerase chain reaction
RACE	: Rapid amplification of cDNA ends
RNA	: Ribonucleicacid
rpm	: Rounds per minute
rRNA	: Ribosomal RNA
RT-PCR	: Reverse transcriptase-polymerase chain reaction
TAE	: Tris-Acetic acid-EDTA
TNT	: 2,4,6-trinitrotoluene
Tm	: Melting temperature
U	: Units
UPM	: Universal Primer Mix
UV	: Ultraviolet

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ISOLATION AND CHARACTERIZATION OF GENOMIC AND cDNA SEQUENCES ENCODING THE LACCASE CpLcc2 FROM *Coriolopsis polyzona* MUCL 38443

SUMMARY

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are members of the protein superfamily which includes multicopper oxidases (MCO). These enzymes are able to catalyze the oxidation of phenolic and non-phenolic lignin related compounds. Thanks to these characteristics, laccases have great industrial importance because of their useful applications for several biotechnological processes such as the detoxification of industrial effluents (mostly from the paper, pulp, textile and wastes of petroleum chemical industry), medical diagnosis, bioremediation of pesticides, herbicides and certain explosives in soil, processing of the beverages (wine, fruit juice and beer), determination of ascorbic acid, the gelation of the sugar beet pectin, bakery, cosmetics and construction of biosensors.

Previously, three different partial cDNA sequences described as *Cplcc1*, *Cplcc2* and *Cplcc3*, belonging to laccase isoenzymes were identified from white-rot fungus *Coriolopsis polyzona* MUCL 38443 strain by our research group. Those laccase-specific cDNA sequences showed around 80% similarity with previously submitted laccase encoding sequences in National Center for Biotechnology Information (NCBI) database using BLAST search. As a further work, the present research aimed at isolation and characterization of the genomic DNA and full-length cDNA, encoding the laccase CpLcc2 from *Coriolopsis polyzona* MUCL 38443.

Principally, the genomic DNA encoding the laccase Cplcc2 was isolated and characterized. The corresponding open reading frame for full length *Cplcc2* cDNA from ATG start codon to TGA stop codon is 1563 bp coding for 520 amino acids with a putative 20-residue signal sequence. BLAST homology search results of *Cplcc2* cDNA revealed 82% similarity with laccase genes of *Trametes* species. *Cplcc2* open reading frame encodes deduced CpLcc2 protein which gives 86% identity with laccases of *Trametes* species. Theoretical molecular weight and isoelectric point of CpLcc2 were calculated as 56.4366 kDa and 6.33, respectively. Additionally, the genomic DNA sequence of *Cplcc2* is 2145 bp in length and interrupted with 10 introns vary between 52 to 77 base pairs in length. *Cplcc2* gene shows maximum 73% identity with laccase genes from *Trametes* species.

The deduced amino acid sequence of CpLcc2 has five potential N-glycosylation sites (Asn-X-Thr/Ser). Conserved copper ligands comprised of one cysteine residue and ten histidine residues were identified in the deduced aminoacid sequence of CpLcc2 that are crucial for the coordination of copper atoms on the active site of laccase. On the other hand, Cys 474 and Phe 484 residues were also found which are important for T1 copper domain and high redox potential of laccases, respectively. Phylogenetic analyses were performed using the MEGA version 6.0 software. Deduced aminoacid sequences of CpLcc2 were aligned using the Clustal W program

in MEGA 6.0. Neighbor joining tree which was constructed with other laccases from GenBank, indicated that CpLcc2 and *Trametes hirsuta* laccase belong to same phylogenetic family.

Coriolopsis polyzona MUCL 38443 SUŞUNDAKİ CpLcc2 LAKKAZINI KODLAYAN GENOMİK VE cDNA DİZİLERİNİN İZOLASYONU VE KARAKTERİZASYONU

ÖZET

Lignoselüloz, selüloz, hemiselüloz ve lignin polimerlerinden oluşan ve dünya üzerinde en çok bulunan biyokütledir. Odunsu bitkilerin en dış katmanında bulunan ve heterojen yapısı nedeniyle parçalanması çok zor olan lignin, selüloz ve hemiselülozu sararak mikrobiyal depolimerizasyondan korur. Global karbon döngüsünde ve lignoselülozik hammaddelerin kullanıldığı endüstriyel uygulamalarda ligninin biyodegredasyonu çok önem arz eden bir aşamadır.

Doğada, ligninin biyodegredasyonunu gerçekleştirebilen bazı özelleşmiş mantar türleri bulunmaktadır. Mantarlar arasında beyaz çürükçül küf mantarları, lignin peroksidaz (LiP), manganez peroksidaz (MnP) ve lakkaz enzimleriyle lignoselülozları parçalayabilmektedir. Peroksidazlar elektron alıcısı olarak H_2O_2 kullanırken, lakkazlar atmosferik oksijeni elektron alıcısı olarak kullanarak delignifikasyonu gerçekleştirmektedir.

Lakkazlar (benzendiol:oksijen oksidoredüktaz; EC 1.10.3.2) çoklu mavi bakır oksidazları (ÇBO) içeren protein süper ailesinin üyeleridir. Bu enzimler moleküler oksijeni elektron alıcısı olarak kullanıp suya indirgeyerek fenolik ve fenolik olmayan lignin içerikli bileşiklerin de oksidasyonunu katalizleyebilmektedir. Bu özellikleri ile endüstriyel atıkların (özellikle kağıt, tekstil ve petrokimya endüstrisi atıkları) detoksifikasyonu, tıbbi tanı çalışmaları, pestisitlerin, herbisitlerin ve topraktan belirli patlayıcıların temizlenmesi, içeceklerin (meyve suyu, şarap, bira) işlenmesi, askorbik asit belirlemesi, şeker pancarı pektininden jel eldesi, fırıncılık, kozmetik ve biyosensör yapımı gibi birçok farklı biyoteknolojik alanda kullanılabilen, endüstriyel öneme sahip enzimlerdir. Şimdiye kadar, bitkilerden ve küflerden pek çok lakkaz izole edilip karakterize edilmesine rağmen hali hazırda biyoteknolojik uygulamalarda çoğunluklar küf kökenli lakkaz enzimleri kulanılmaktadır.

Birçok fungus türü, farklı büyüme koşulları ve çevre şartlarında birden fazla lakkaz izoenzimi üretebilmektedir. Aynı suş tarafından üretilen lakkaz izoenzimlerinin biyokimyasal özellikleri ve enzim aktiviteleri birbirinden farklılık göstermektedir. Bu nedenle, yeni bir lakkaz olma potansiyeli barındıran her bir lakkaz izoenziminin izole edilerek fizyolojik ve biyokimyasal özelliklerinin detaylı bir şekilde araştırılması gerekmektedir. Bu çalışmalar, hem lakkazların organizmadaki fizyolojik rollerinin anlaşılmasına katkıda bulunacağı için hem de her biyoteknolojik uygulamaya yönelik en uygun lakkaz enziminin kullanılabilmesine olanak sağlayacağı için önemlidir.

Araştırma grubumuz tarafından gerçekleştirilen önceki çalışmada, beyaz çürükçül küf mantarı *Coriolopsis polyzona* MUCL 38443 suşunda, lakkazların korunmuş bölgelerine özgü dejenere primerler kullanılarak cDNA kütüphanesi oluşturulmuş ve lakkaz izoenzimlerine ait üç farklı kısmi cDNA dizisi, *Cplcc1*, *Cplcc2* ve *Cplcc3*

olarak tanımlanmıştır. Bu lakkaza özgü cDNA dizileri, National Center for Biotechnology Information (NCBI) veritabanında yapılan BLAST araması sonucunda, önceden tanımlanmış lakkaz kodlayan dizilerle, yaklaşık olarak %80 benzerlik göstermiştir.

İleriki çalışma olarak gerçekleştirilen bu mevcut araştırmada ise *Coriolopsis polyzona* MUCL 38443 suşundaki CpLcc2 lakkazını kodlayan genomik DNA ve tam boyuttaki cDNA'nın izolasyonu ve karakterizasyonu amaçlanmıştır.

Cplcc2 kısmi cDNA'sı kalıp alınarak dizayn edilen RACE primerleriyle *Coriolopsis polyzona* MUCL 38443 total RNA'sı kullanılarak 5' ve 3' RACE reaksiyonları gerçekleştirilmiştir. Reaksiyon ürünleri dizilenerek analizleri gerçekleştirilmiş ve *Cplcc2* geninin 5' ve 3' uçları elde edilerek gen spesifik primer dizaynı yapılmıştır. Dizayn edilen primerler ile tam boyuttaki *Cplcc2* geni ve cDNA'sı izole edilmiş ve karakterize edilmiştir.

Tam boyuttaki *Cplcc2* cDNAsı, ATG başlama kodonundan TGA bitiş kodonuna kadar, teorik olarak 20 amino asitlik sinyal dizisi taşıyan ve 520 aminoasit kodlayan, 1563 baz çifti içermektedir. Buna göre *Cplcc2* cDNA'sı National Center for Biotechnology Information (NCBI) veritabanında yapılan BLAST homoloji araması sonucunda, *Trametes* türlerine ait lakkaz genleriyle %82 benzerlik göstermiştir.

Cplcc2 açık okuma çerçevesinin kodladığı CpLcc2 proteini ise BLAST analizleri yapıldığında *Trametes* türlerine ait lakkazlar ile %86 benzerlik göstermiştir. Ayrıca yapılan protein BLAST analizleri sonucunda CpLcc2 lakkazının üzerinde, teorik olarak korunmuş T1 bakır bağlanma bölgesi, T2/T3 trinükleer bakır bağlanma bölgeleri gösterilmiştir.

CpLcc2 türetilmiş amino asit dizisinden yola çıkılarak, CpLcc2 proteininin teorik moleküler ağırlığı 56.4366 kDa ve teorik izoelektrik noktası (pI) 6.33 olarak hesaplanmıştır.

Buna ek olarak, gen spesifik primerlerle *Coriolopsis polyzona* MUCL 38443 genomik DNA'sından izole edilen *Cplcc2* genomik DNA dizisi, 2145 baz çifti uzunluğunda olup, 52 ile 77 baz çifti arasındaki uzunluklarda değişen 10 intron içermektedir. *Cplcc2* geninin BLAST analizleri yapıldığında ise *Trametes* türlerine ait lakkaz genleri ile maksimum %73 benzerlik gösterdiği belirlenmiştir.

Cplcc2 genine ait türetilmiş amino asit dizisinin, beş adet potansiyel N-glikolizasyon bölgesine (Asn-X-Thr/Ser) sahip olduğu gösterilmiştir. Lakkaz aktif bölgesinde yer alan ve bakır atomlarının koordinasyonu için büyük önem taşıyan, bir sistein (Cys 474) ve on histidin (His85, His87, His130, His132, His416, His419, His421, His473, His475, His479) kalıntısından oluşan korunmuş bakır ligandları, CpLcc2 türetilmiş amino asit dizisi üzerinde belirlenmiştir. Diğer yandan, T1 bakır bölgesi açısından önemli Cys 474 ile yüksek redoks potansiyeli için esansiyel olan Phe 484 kalıntıları da ayrıca bulunmuştur. Bunlara ek olarak, CpLcc2 lakkazını kodlayan amino asit dizisi beş sistein kalıntısı içermektedir.

CpLcc2 enziminin filogenetik analizleri ise MEGA 6.0 yazılımı ile gerçekleştirilmiştir. CpLcc2'ye ait türetilmiş amino asit dizisi, diğer lakkazları kodlayan amino asit dizileriyle MEGA 6.0 yazılımı içerisinde yer alan ClustalW programı kullanılarak hizalanmıştır. GenBank'taki diğer fungus, bakteri, bitki ve böcek lakkazlarıyla kurulan Neighbor joining ağacı ile, CpLcc2 ve *Trametes hirsuta* lakkazının aynı filogenetik ailede yer aldıkları gösterilmiştir.

Sonuç olarak, bu tez kapsamında *Coriolopsis polyzona* MUCL 38443 suşunda CpLcc2 lakkazını kodlayan cDNA ve gen dizilerinin izolasyonu ve karakterizasyonu başarıyla gerçekleştirilmiştir. Bu lakkaz izoenziminin filogenetik analizleri yapılmış, enzimin teorik molekül ağırlığı ve izoelektrik noktası hesaplanmıştır. Türetilmiş CpLcc2 aminoasit dizisi lakkazlara özgü motifler saptanmış ve CpLcc2 lakkazının yüksek redoks potansiyeline sahip bir lakkaz olduğuna işaret eden amino asit kalıntıları belirlenmiştir.

1. INTRODUCTION

1.1 Wood Structure

Total forest area has been recently estimated to be approximately 31% of the world's land surface (Sedjo and Lyon, 2015). Wood is one of the most significant lignocellulosic product acquired from forests. Basicly, lignocellulose consists of three main polymeric components which are cellulose, hemicellulose and lignin (Martinez et al., 2005). Lignocellulose is the most abundant biomass on Earth and the dominant element of woody plant (Figure 1.1) (Kües, 2015).

The structure of wood cell walls is comprised of the cellulose which is around 45% of the weight of wood. Cellulose is a linear polymer of anhydrocellobiose units linked by β-1.4-glycosidic bonds and its molecules are held together by hydrogen bonding interactions and Van der Waals forces which makes natural cellulose structurally complex. The individual cellulose molecules seem like bundles called microfibrils, each of them contains around 40 individual cellulose molecules. The cellulose

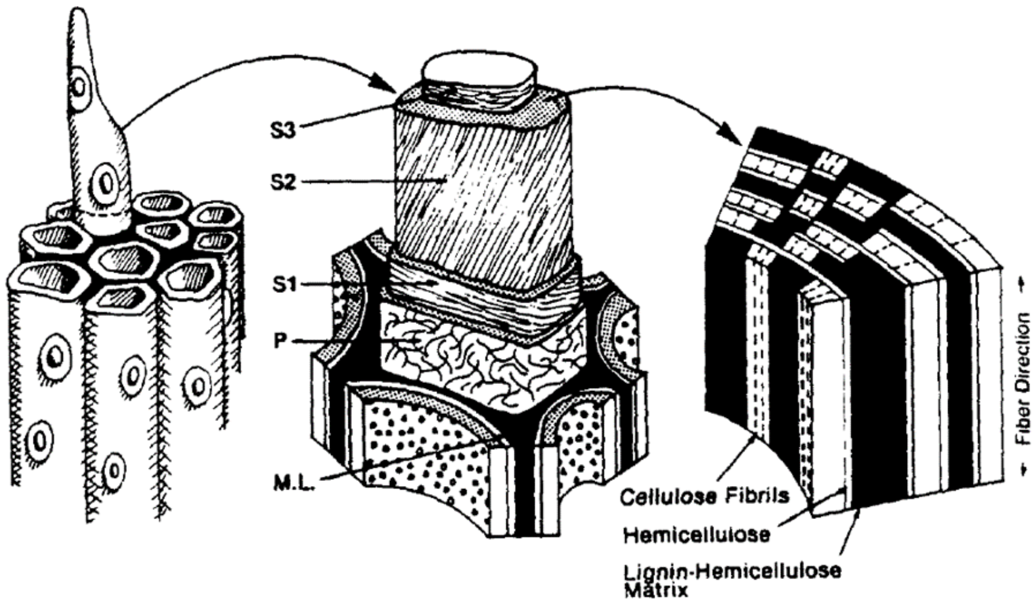

Figure 1.1 : Lignocellulosic Wood Structure (Kirk and Cullen, 1998).

microfibrils are seen as encircled by a matrix of hemicelluloses and lignin (Kirk and Cullen, 1998).

Hemicelluloses form a coating on the cellulose microfibrils and this hemicellulose coating is connected with the lignin. Hemicellulose is 25 to 30% of the weight of wood which consists of linear β-1.4-linked monosaccharide polymers. When compared to cellulose, hemicellulose molecules have a shorter, random and amorphous structure. On the other hand, lignin has completely different structure from cellulose and hemicellulose (Kirk and Cullen, 1998). Lignin is a branched polymer formed by a random polymerization reaction of p-coumaryl alcohol, 3-(4-hydroxyphenyl)propen-1-ol, radicals, and their methoxy-substituted derivatives. Lignin polymers consist of monomeric units are known as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. It surrounds the hemicellulose and cellulose, which is called holocellulose. By this way it protects them from microbial depolymerization which make it difficult to be degraded since it has a heterogeneous nature of lignin, However, lignin's biodegradation plays a crucial role in the global carbon cycle. It has been suggested that lignin degradation is a rate-limiting step of carbon recycling (Palonen, 2004; Kirk and Cullen, 1998; ten Have and Teunissen, 2001; Eggert et al., 1996; Okhuma et al., 2001; Munk et al., 2015).

Degradation of lignin barrier that protects cellulose and hemicellulose could be achieved only by some specialized members of Basidiomycota and Ascomycota phylums. This degradation process is crucial for the mineralization of wood and provides carbohydrates for other organisms such as arthropods, bacteria and non-ligninolytic fungi etc (Arnstadt et al., 2016).

1.2 White Rot Fungi

Lignin degradation is a challenge for both carbon recycling and industrial applications. However, various fungi have the ability to mineralize and degrade lignin in nature (Yaver et al., 1996).

According to their degradation strategies, saprotrophic wood-inhabiting fungi can be classified as white rot, brown rot and soft rot according to the type of rot they cause (Hatakka and Hammel 2011). Whereas white rot fungi primarily degrade hemicelluloses and lignin, brown rot fungi preferably target cellulose and hemicelluloses for degradation (Bouws et al., 2008).

The only microorganisms that are able to degrade lignin in wood to CO_2 and H_2O with enzymatic reaction are basidiomycetes which cause white rot decay. These kinds of fungi are called white rot fungi. Basidiomycete fungi have various ecological roles performing conversion of lignocellulosic materials into the simple sugars, oligosaccharides and humic substances in soil such as endophytes and symbionts as in lichens, plant root mycorrhizas, leaves, needles, animal and plant parasites or pathogens and saphrotrophs. The white rot basidiomycetes such as brackets, corticioids or cap fungi have the ability to decompose all lignocellulose components, both in angiosperm hardwoods and coniferous softwood, including the lignin structure with enzymatic hydrolysis (Lundell et al., 2010).

The first step in this enzymatic process is the removal of an electron from a subunit of lignin by a ligninolytic enzyme. These lignin-modifying enzymes (LMEs) responsible of lignin degradation are lignin peroxidase (LiP), manganese peroxidase (MnP) and a copper containing phenoloxidase which is known as laccase. While the peroxidases use H_2O_2 as an electron acceptor, the oxidases (laccases) use O_2 for the same purpose (Lundell et al., 2010; Saloheimo et al., 1991).

Basidiomycete white rot fungus *Coriolopsis polyzona* is a member of *Coriolaceae* family and high-yield ligninolytic enzyme producer (Eriksson et al., 1990; Jaouani et al., 2006).

1.3 Laccases

Laccases (EC 1.10.3.2 benzenediol: oxygen oxidoreductase) are multinuclear copper-containing oxidases which catalyze the monoelectronic oxidation of aromatic substrates by using molecular oxygen as an electron acceptor. In addition, they are able to oxidize wide range of substrates including ortho- and paradiphenols, aminophenols, polyphenols, polyamines, lignin as well as some inorganic ions coupled to the reduction of oxygen to water (Riva, 2006; Giardina et al., 2010).

Catalytic center of laccases contains three different types of four reactive copper atoms. Organization of copper atoms can be described as mononuclear T1 site and trinuclear copper cluster which contains one type 2 and two type 3 copper atoms

(Figure 1.2). T1-type copper atom which gives the characteristic blue color to enzyme, has a characteristic absorbance around 610 nm. T1-type copper lacking laccase enzymes named as “yellow” or “white” laccases. Oxidation of the substrate starts in T1-type copper site. T1 Copper-containing cavity on the enzyme surface is quite large, so wide range of substrate molecules can be bound. The primary electron acceptor site (T1 site) catalyzes four 1e^{-} oxidations of a reducing substrate molecule. The reduction of T1 copper is rate-limiting step in the whole catalytic process. Received electrons from substrate molecule are transferred via a highly conserved His–Cys–His tripeptide motif to the T2/T3 trinuclear cluster. Dioxygen reduction to water takes place in T2/T3 trinuclear center. While the type 3 coppers have a maximum absorption at 330 nm, the type 2 copper has no visible absorbance. (Wong, 2009; Morozova et al., 2007; Baldrian, 2006; Mayer and Staples, 2002; Giardina et al., 2010).

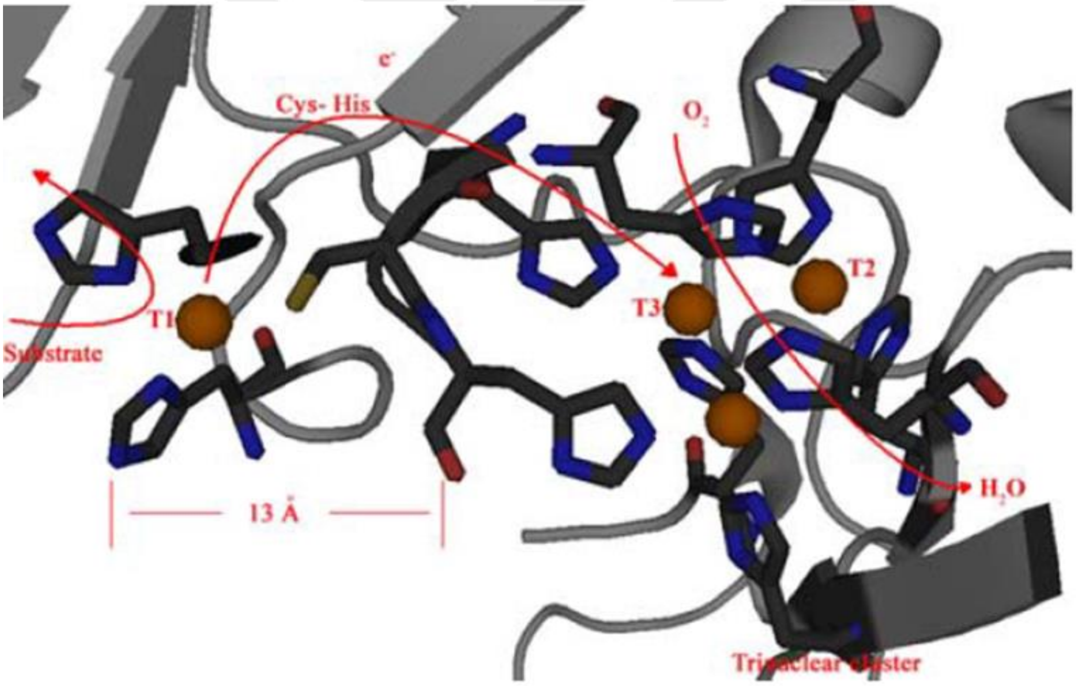

Figure 1.2 : The structure of the laccase active site, red arrows represent the flow of electrons (Giardina et al., 2010).

First laccase enzyme was identified by Yoshida as a component of the resin ducts of the lacquer tree *Rhus vernicifera* at the end of the 19th century (Yoshida et al., 1883). Although laccase enzyme was discovered in plants, it is widely distributed in fungi, bacteria and insects. In fungi, laccases participate in several physiological processes including lignin degradation, morphogenesis, stress defense and fungal plant pathogen/host interaction. In plants, laccases are involved in lignin biosynthesis. However, bacterial laccases have important roles in morphogenesis, pigment formation, copper homeostasis, biosynthesis of the brown pigment in bacterial spore coat and also in protection against UV light and hydrogen peroxide. In insects, it has been proposed that laccases have a role in cuticle sclerotization by oxidizing catechols. (Thurston, 1994; Sato et al., 2001; Sharma et al., 2007; Giardina et al., 2010; Dittmer et al., 2004).

According to structure and properties of copper center, laccases are categorized into two groups, low-redox potential and high-redox potential laccases. Redox potential is an important parameter for substrate specificity. Higher redox potential means that enzyme is able to oxidize wider range of substrates. While white rot fungi laccases have high redox potential, bacteria plant and insect laccases' redox potential is low. Low redox potential laccases are able to oxidize phenolic units directly (Figure 1.3). On the other hand, high redox potential laccases need some small electron transfer mediator molecules such as native 3-HAA or synthetic ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), 1-HBT (1-hydrobenzotriazole) for the oxidation of non-phenolic substrates (Figure 1.4). Moreover, in the presence of both natural and synthetic mediators, the substrate range of laccase may be expanded to non-phenolic substrates that are very recalcitrant and hardly oxidized by laccase alone (e.g. polycyclic aromatic hydrocarbons (PAH), azo-dyes, polychlorinated biphenyls, organophosphate pesticides). These oxidation reactions are important in biotechnological applications. Unlike other lignin modifying enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), laccases use molecular oxygen not hydrogen peroxide. (Antorini et al., 2002; Martinez et al., 2005; Bulter et al., 2003; Burton, 2003; Piontek et al., 2002; Couto and Herrera, 2006; Mikolasch and Schaur, 2009; Jeon at al., 2012; Mate and Alcalde, 2015).

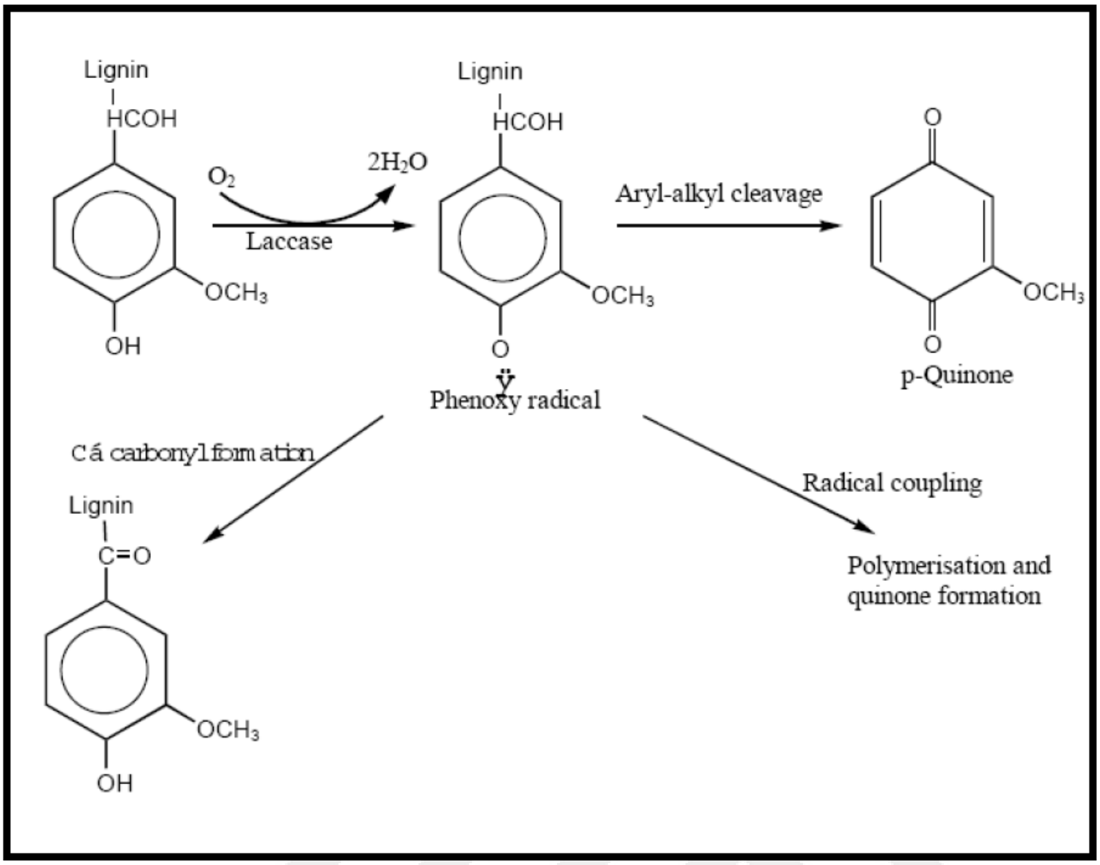

Figure 1.3: Laccase-catalyzed oxidation of phenolic subunits of lignin (Archibald et al., 1997).

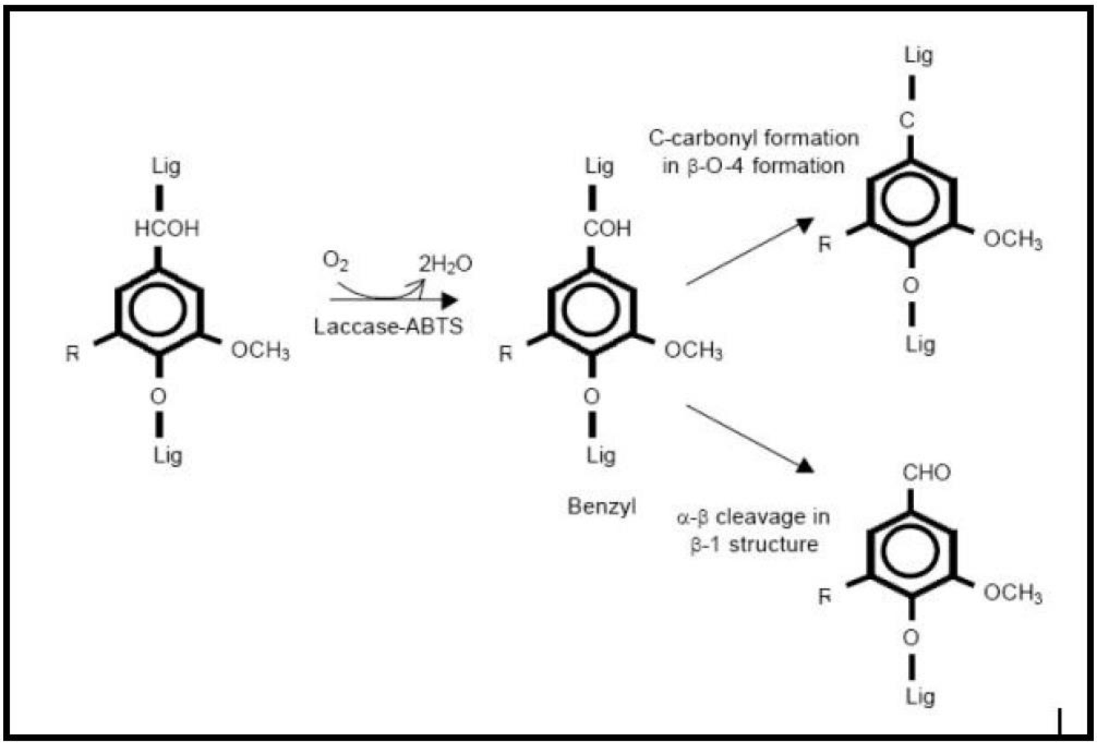

Figure 1.4 : Laccase mediator system-catalyzed oxidation of non-phenolic subunits of lignin (Archibald et al., 1997).

Most of biotechnologically important laccases are from fungal origin (Kalmiş et al., 2008). The catalytic site of laccases is highly conserved in different species of fungi, but the remaining part of structure shows high diversity (Gochev and Krastanov, 2007). This diversity have been observed in the physicochemical characteristics and induction mechanism such as molecular weight, carbohydrate content, optimum pH and temperature, isoelectric point and kinetic properties in different fungal laccases (Duran et al., 2002). Generally, fungal laccases are extracellular, inducible, monomeric glycoproteins with 10-20% (w/w) carbohydrate contents which may make a contrubition to the enzyme stability (Mayer and Staples, 2002). The open reading frame of laccase contains about 520-550 aminoacids including a N-terminal secretion peptide (Gianfreda et al., 1999).

Laccase enzymes are usually larger than other lignin modifying peroxidases, having an average molecular weight 60-70 kDa. Like other extracellular enzymes, laccases are glycosylated to increase hydrophilicity (ten Have and Teunissen, 2001; Jeon at al., 2012). Most of fungal species produce more than one laccase isoenzyme depending on the growth conditions and multiplicity of laccase encoding genes (Ng, 2004). Many parameters affects laccase synthesis and secretion, including nutrient levels, culture conditions and developmental stage. Some lignin-derived aromatic compounds like ferulic acid, xylidine and veratric acid and some heavy metals such as Cu^{2+}, Ag^{+} and Mn^{2+} enhance production of laccase (Soden and Dobson, 2001; Jeon at al., 2012).

1.4 Industrial Applications of Laccases

Even though most of the conventional oxidation technologies have many disadvantages like undesirable, non-specific side-reactions and the usage of toxic chemicals, oxidation is an essential reaction in several industrial applications. To eliminate these disadvantages, new oxidation technologies have been searched for years. Enzymes are specific catalysts and reactions can be performed in mild conditions. Laccase is a promising enzyme because of its capability to oxidize wide range of toxic and environmentally problematic substrates in various industrial fields such as the pulp and paper, textile and food industries (Couto and Herrera, 2006).

1.4.1 Food and beverages industry

Laccases can be used in some processes which enhance or change the appearance of food or beverage by eliminating undesirable phenolics causing browning, haze formation and turbidity in clear fruit juice, beer and wine (Shraddha et al., 2011; Couto and Herrera, 2006).

In food and beverages industry, there are several pre- and post treatments to prevent post-turbidity and discoloration of fruit juices such as browning which is one of the main problems in beverages. Utilization of laccase for the fruit juice stabilization have been proposed especially for apple juice (Minussi et al., 2002).

Determination of ascorbic acid, baking, sugar beet pectin gelation and the treatment of olive mill wastewater are also carried out with laccases. Selinheimo et al. (2006) suggested that a laccase of white-rot fungus *Trametes hirsuta* increases the dough resistance and decreases the dough extensibility (Brijwani et al., 2010; Selinheimo et al., 2006; Pannu and Kapoor, 2015).

Laccase is also commonly used for treatment of wastewater in food industry with the ability to prevent possible environmental damages resulted from beer factory wastewater containing polyphenols and dark-brown color. Laccase producer white-rot fungus *Coriolopsis gallica* is able to degrade this high-tannin-containing wastewater. Laccases were also studied for bioremediation of olive mill wastewaters which is a by-product of olive-oil production and represents a major environmental problem in the Mediterranean area (Minussi et al., 2002; Yague et al., 2000).

Sugar beet pectin which is a food ingredient forms gels by an oxidative cross-linking of ferulic acid. It has been showed that cross-linking of beet pectin can be carried out through the oxidative coupling of the feruloyl groups using laccase. On the other hand, peroxidases also can perform the oxidation of gelation as well. While peroxidases require addition of hydrogen peroxide, laccase is able to use oxygen present in the sample (Norsker et al., 2000; Minussi et al., 2002).

Whereas alcohol and organic acids are responsible for the aroma of wine, the taste and the color depend on the phenolic compounds found in different types of wines. As a specific and mild technology, laccase treatments have been suggested for the removal of phenolics causing discoloration, haze, flavor changes and clouding. Moreover, a laccase has been commercialized (Suberzyme®) for preparing cork stoppers for wine bottles (Kunamneni et al., 2008).

Laccases are also used to enhance storage life of beer by removing unwanted oxygen and some of the polyphenols in the finished beer (Minussi et al., 2002; Kunamneni et al., 2008).

1.4.2 Pulp and paper industry

Wood components cellulose and lignin are rigid organic polymers which are used as a primary source for paper industry. Pulping and bleaching of woody substrates are two main steps for paper manufacturing. Separation and degradation of lignin in wood pulp is a crucial step for manufacturing of paper pulp. Conventionally, this process is carried out using chlorine- or oxygen-based chemical oxidants. Instead of conventional methods which decrease cellulose fiber strength, laccases are able to depolymerize lignin and delignify wood pulps, kraft pulp fibers by breaking aromatic and aliphatic C–C bonds. Laccase-based delignification provides cleaner and milder strategies also prevents integrity of cellulose.Furthermore, Laccases are able to delignify pulp more easily when they are used together with mediators. The mediator is oxidized by laccase, then the oxidized mediator molecule oxidizes subunits of lignin in order to prepare for laccase oxidation. (Brijwani et al., 2010; Pannu and Kapoor, 2015; Minussi et al., 2002)

Pulp bleaching is currently achieved by treating pulps with chlorine-based chemicals. As a result of this treatment, toxic chlorinated aliphatic and aromatic compounds could be form. Because of that, there have been intensive studies to develop environmentally friendly enzymatic bleaching technologies. Laccase can be used as a biobleaching agent since it degrades the residual lignin in pulp and decolorize it. Laccase is also an ideal oxidative enzyme for pulp bleaching thanks to its ability of usage readily available atmospheric oxygen as an electron acceptor during the process. Laccase of *Trametes versicolor* has been intensively studied for paper pulp biobleaching, treatment of effluents, and other industrial applications (Arora and Sharma, 2010; Couto and Herrera, 2006; Widsten and Kandelbauer, 2007).

1.4.3 Textile industry

The textile industry corresponds to two-thirds of the total dyestuff market and use up great volumes of water and chemicals in wet processing of textiles. Dyes are chemicals in different chemical structures. There are more than one hundred dyes and greater part of dyes are resistant to light exposure, chemical substances or water. The effluents of textile industry are also controlled by governments due to environmental concerns. Laccase applications are important in several parts of textile industry as a "green tools". These enzymes have been integrated in processes such as fiber bleaching, dyeing and modifying or improving textile properties. Bioremediation of textile wastewater also occupies a big part of laccase applications (Riu et al., 1998; Fernández-Fernández et al., 2013; Couto and Herrera, 2006).

Textile dye degradation by several fungal laccases has been reported. For example, *Phlebia* species laccases have been employed for decolorization of many synthetic and industrial dyes. Malachite green was successfully degraded by *Ganoderma lucidum* laccase (Arora and Sharma, 2010). *Trametes hirsuta* laccase degraded triarylmethane, indigoid, azo, and athraquinonic dyes used in textile industry (Mayer et al., 2002).

Laccase has the ability to bleach released dye in the washing solution and prevent back staining of dyed or printed textiles. In 1996, Novozyme (Novo Nordisk, Denmark) developed a new industrial application of laccases in denim finishing: DeniLite®. This was the first industrial laccase and the first bleaching enzyme acting with the help of a mediator molecule. (Couto and Herrera, 2006).

1.4.4 Bioremediation

The ability of fungi to depolymerize several hazardous chemicals has gained an increasing interest to use them in bioremediation. Enzymatic treatment is currently regarded as an alternative way for the removal of toxic xenobiotics from the environment. Due to their catalytic properties, laccases have an important role in green biodegradation. Laccases can be used in order to degrade many substances such as undesirable contaminants, byproducts, discarded materials, plastic waste having olefin units. Laccases may be also applied to eliminate odor emitted from garbage disposal sites, livestock farms or pulp mills. Phenolic compounds are existed in industrial wastes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others. Degradation of soil contaminants such as polycyclic aromatic hydrocarbons (PAHs) and some xenobiotics e.g. 2,4,6-trinitrotoluene (TNT) can be carried out with laccases. Laccase from white-rot fungus *Trametes hirsuta*, has been used for oxidation of alkenes. *Coriolopsis gallica* laccase has the ability to oxidize carbozole, N-ethylcarbozole, fluorine, and dibenzothiophene in presence of 1-hydroxybenzotriazole (HBT) and 2.2′-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) as mediators (Pointing, 2001; Durán and Esposito, 2000; Couto and Herrera, 2006; Kunamneni et al., 2008; Viswanath et al., 2014).

1.4.5 Nanobiotechnology

Enviromental pollution whether accidental or as a by product of industrial processes requires monitoring (Messerschmidt, 1997). The principle of many laccase-based biosensors is monitoring oxygen consumption during oxidation of the analyte resulting from reduction of oxygen to water (Kudanga and Le Roes-Hill, 2011). Due to numerous applications of laccases, the biotechnologists have used several types of solid supports and gels for developing effective catalyst. Immobilization increases the thermostability because maintenance of three-dimensional structure prevents denaturation and loss of catalytic activities of protein. Immobilized laccase in hydrophobic xerogels can stay stable in a wide range of temperature (50–80°C) than free laccase. This method makes laccase more suitable candidate for industrial applications. Amperometric principles are widely used in biosensor design to measure polyphenols in food products. Biosensors which utilize laccase include an electrode can be applied for the detection of phenols, such as catechols in tea, phenolic compounds in wine, lignins and phenols in wastewater without additional cofactors. Laccases in biosensor design are mainly obtained from *Trametes*, *Aspergillus* and *Ganoderma* genera (Couto and Herrera, 2006; Viswanath et al., 2014; Rodríguez-Delgado et al., 2015).

Laccase catalysis can be used to assay other enzymes, like amylase, amino-peptidase, glucosidase, chymotripsin; thrombin and plasmin. Laccase covalently conjugated to a bio-binding molecule (antigen or antibody) can be used as a reporter for immunochemical (ELISA, Western blotting), cytochemical, histochemical or nucleic acid-detection applications (Arora and Sharma, 2010; Kunamneni et al., 2008).

Besides, laccase has a significant potential for the applications of biofuel cells. The main reason of this interest is the usage of molecular oxygen as a substrate, which is converted into water. This property of laccase provide an advantage in nanotechnology for medical applications in living animals. In this biofuel cell application, oxygen can be scavenged from the bloodstream and water can be benign as a byproduct (Viswanath et al., 2014; Rodríguez-Delgado et al., 2015).

1.4.6 Cosmetic

Cosmetic is a novel application area for laccases. For example, laccases can be used in hair dye formulation as an oxidising agent instead of H_2O_2. These laccase based hair dyes could be less irritant and easier to handle than current hair dyes. Futhermore, protein containing cosmetic and dermatological preparations for skin lightening have been studied. Laccases may be used in personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers. Allergenicity of laccases can be reduced by protein engineering studies (Golz-Berner et al., 2004; Couto and Herrera, 2006; Kunamneni et al., 2008).

1.5 Aim of Thesis

The white-rot fungus *Coriolopsis polyzona* MUCL 38443 is a promising industrial enzyme producer (Jaouani et al., 2006). Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are one of the most important enzyme group among those enzymes. Previously, in our research group, laccase gene specific sequences in *Coriolopsis polyzona* MUCL 38443 were screened by performing Reverse Transcription-Polymerase Chain Reaction (RT-PCR) technique via using degenerate primers designed based on the highly conserved copper binding regions I and IV of known fungal laccases (D’souza et al., 1970; Hoshida et al., 2001) and total RNA as a template. This study was revealed three different laccase specific partial cDNA sequences entitled as *Cplcc1*, *Cplcc2* and *Cplcc3*. They share 80% similarity with previously submitted laccase encoding sequences in National Center for Biotechnology Information (NCBI) database. (Pinar et al., 2012). Among those cDNA sequences, *Cplcc2* partial cDNA sequence (637 bp) (Figure 1.5) exhibited 82% homology with *Trametes versicolor* strain NL-1 laccase (lccA) gene, complete cds (JQ828930.1), *Trametes versicolor* FP-101664 SS1 laccase I (TvLac1), partial mRNA (XM_008034546.1), *Trametes versicolor* laccase 1 (lac1) mRNA, complete cds (AY049725.1), *Trametes versicolor* laccase 1 (lcc1) mRNA, complete cds (AY693776.1) etc. As a further work, the present study aimed at isolation and characterization of the genomic DNA and the full-length cDNA sequences encoding the laccase CpLcc2 from *Coriolopsis polyzona* MUCL 38443.

>*Cplcc2* Partial cDNA Sequence

```
5'ACGGGTTTTTCCAGGCAGGCACCAACTGGGCAGACGGCCCCGCGTTCGTGAATCAGTGTC
CTATCGCGTCCGGGCACTCGTTCTTGTACGACTTCCGTGTGCCGGACCAAGCCGGTACCTTC
TGGTATCATAGTCATCTTTCGACCCAGTACTGCGACGGGCTGAGGGGGCCATTCGTGGTCTA
TGACCCGAAGGACCCCCATGCCAGTCGTTACGATGTTGACGACGAGAGCACGGTGATCACCC
TTGCTGACTGGTACCACACCGCGGCTCGCCTCGGGCCTAGGTTCCCATTGGGGGCGGACTCC
ACGCTCATCAATGGTCTCGGTCGATCGTCCTCGACCCCTACAGCCCCTTTGGCGGTCATCAA
TGTGCAACGTGGAAAACGCTATCGCTTCCGCCTCGTGTCGCTCTCATGCGACCCGAACCACG
TGTTCAGCATCGACGGTCACAACATGACCATCATCGGGGTCGACGGCGTCAACAGCAAGCCT
CTCACTGTGGACTCCATCCAAATCTTCGCTGCGCAACGCTACCTCCTTGTTTTGAAAGCAAA
TCAACCGGGGGGGCAACTACTGGGATCCGTGGCAACCCCAACTTCGGGACGACCGGGTTCGC
GGGCGGGATTAACTCCCCC-3'
```

Figure 1.5 : Partial cDNA sequence of *Cplcc2* from *Coriolopsis polyzona* MUCL 38443

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains

The source organism of this study is a laccase producer white rot fungus *Coriolopsis polyzona* MUCL 38443 was provided through EU-FP6 SMI-IP (IP: 505899). In order to activate frozen cells, the organism was firstly grown on Potato Dextrose Agar (PDA) at 28°C for 7 days. For subcloning studies *Escherichia coli* DH5α strain was used and bacteria were grown overnight on Luria- Bertani (LB) medium at 37°C.

2.1.2 Plasmid

Promega pGEM®-T Easy Vector System is used for the cloning of PCR products obtained in different steps of this study.

2.1.2.1 pGEM®-T Easy cloning vector

Direct-cloning of PCR products were performed into the linear pGEM®-T Easy Vector System (Promega) (Figure 2.1). The pGEM®-T Easy Vector has been linearized with *Eco*RV at base 60 and designed with a single thymidine residue at both 3′-ends. The 3′terminal-T overhangs significantly improve the ligation efficiency of PCR product into the vector by preventing vector recircularization and providing a compatible overhang for PCR products generated by certain thermostable PCR polymerases which add a single deoxyadenosine to the 3′-ends of the amplified products in a template-independent manner. *Eco*RV site of vector will not be recovered until the ligation of the vector and PCR fragment. Inserts cloned into the pGEM®-T Easy Vector can be released by a single digest with *Bst*ZI, *Eco*RI or *Not*I restriction enzymes. Alternatively double digest may be performed to excise the insert. pGEM®-T Easy Vector is a high-copy number vector which includes T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase. Successful cloning of the PCR product into the pGEM®-T Easy Vector interrupts the coding sequence of βgalactosidase. Insertional inactivation of the α-peptide allows identification of recombinant clones by blue/white screening on indicator plates.

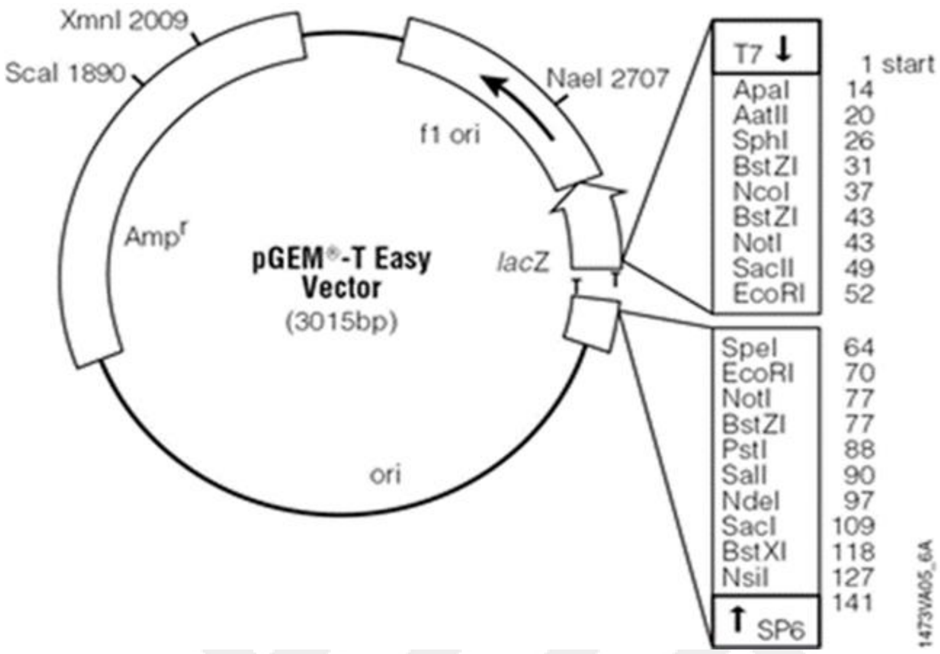

Figure 2.1 : pGEM®-T Easy Vector Map including multiple cloning region and lac operon sequences (Promega).

2.1.3 Culture media

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

2.1.4 Buffers and solutions

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

2.1.5 Chemicals and enzymes

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

2.1.6 Laboratory equipment

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

2.2 Methods

2.2.1 Cultivation of fungal and bacterial strains

Laccase producer white rot fungus *Coriolopsis polyzona* MUCL 38443 was kindly provided through EU-FP6 SMI-IP (IP: 505899). The fungus on the wood chips was grown on Potato dextrose agar (PDA) plates at 28ºC for 5 days. These PDA plates were used as inoculum for the spore production. For this step on long-grain rice was washed with distilled water for 30 minutes and autoclaved for 15 minutes in Fernbach Erlenmeyer flasks. Then, this sterilized rice was inoculated with the mycelial block. Steril distilled water was added in order to maintain rice moist for spore formation during incubation at 28°C for 8 days. The fungal spores were harvested by extraction with sterile 0.9% NaCl solution and filtered through six layers of sterilized cheesecloth. After washing, concentration of spore suspension was adjusted to $1x10^8$ spores/ml and 1 ml spore suspension was inoculated in 100 ml liquid LSK medium on rotary shaker at 150 rpm at 28ºC for 10 days to grow vegetative *Coriolopsis polyzona* MUCL 38443.

During this study, *E. coli* DH5α strain was grown in Luria-Bertani (LB) liquid or solid medium for 16 hours at 37°C. 10% glycerol stock of the bacteria was prepared for long term storage at -80ºC. In the cloning experiments with pGEM®-T Easy vector, ampicillin (Amp) antibiotic (100 µg/ml) was used as the selective agent for *E.coli* DH5α strain. Before inoculation in liquid mediums, bacteria strains were activated on their proper solid medium plates from -80ºC stocks.

2.2.2 Genomic DNA isolation

Genomic DNA isolation from *Coriolopsis polyzona* MUCL 38443 was performed by using Fast Prep Instrument and Fast DNA Spin Kit (MPBio). Firstly, frozen 100 mg *Coriolopsis polyzona* pellet from -80°C stock was weighed and resuspended in 1 ml cell lysis solution CLS-TC. The sample was transferred into Lysing Matrix A. Fastprep Instrument was then used at speed level 4 for 20 seconds for homogenization. Centrifugation of sample was carried out at 14,000 rpm for 10 minutes to discard pellet debris. Supernatant (700 µl) was carefully transferred into new clean 1.5 mL microcentrifuge tube. 500 µl Binding matrix was added for binding of DNA to the matrix and inverted to mix. Tubes were incubated with gentle agitation for 5 minutes at room temperature on a rotator. To pellet the Binding Matrix, samples were centrifuged at 14,000 rpm for 1 minutes and supernatant was discarded. 500 µl of prepared SEWS-M was added and gently resuspend the pellet using the force of the liquid from the pipet tip. Resuspended Binding Matrix was transferred into SPIN Module (Spin filter and catch tube) and centrifugation was performed at 14,000 rpm for 1 minutes. Then, tubes were centrifuged a second time at 14,000 rpm for 1 minute without any addition of liquid. Catch tube contents were discarded and replaced the Catch Tube with a new, clean recovery tube. For the elution of DNA, Binding Matrix above the SPIN filter was gently resuspended in 100 µl of DES and incubated for 5 minutes at 55°C in heat block. Following incubation, centrifugation was performed at 14,000 rpm for 1 minute to bring eluted DNA into the recovery tube and spin filter was discarded. Isolated genomic DNA was aliquoted and stored at -20°C for further experiments.

2.2.3 Total RNA isolation

White rot fungus *Coriolopsis polyzona* MUCL 38443 was grown in LSK medium at 28ºC for 7 days, in which laccase activity is reached to maximum level in the culture. Fresh cells were harvested and total RNA was immediately isolated by using "Qiagen RNeasy Plant Mini Kit". To prevent degradation of RNA, isolation procedure steps were performed as quickly as possible at room temperature. Before starting, 10 µl β-Mercaptoethanol was added to lysis buffer RLC, containing guanidine hydrochloride. Fungal biomass were centrifuged at 12000 rpm for 30 minutes. Supernatant was completely removed and cell pellet was kept at -80°C. 100 mg *Coriolopsis polyzona* pellet was placed in liquid nitrogen, and ground thoroughly with a RNase-free mortar and pestle. Tissue powder and liquid nitrogen were transfered into an RNase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube and allowed the liquid nitrogen to evaporate, but aviod the sample thaw. For the lysis, 450 µl Buffer RLC was added to sample and vortex vigorously. In order to disrupt the tissue, incubation was carried out at 56°C for 3 minutes and the lysate was transferred into a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuged for 2 minutes at full speed. The supernatant of the flow-through was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. 0.5 volume of ethanol (96–100%) was added to the supernatant, and mixed immediately by pipetting. Sample was transferred to the RNeasy spin column (pink) and centrifuged for 30 seconds at 11500 rpm. After centrifugation flow-through was discarded and collection tube was reused. To wash the membrane of spin column 350 µl Buffer RW1 was added to the spin column and centrifuged for 30 seconds at 11500 rpm. In order to prepare DNase I incubation mix, 10 µl DNase I stock solution was added to 70 µl Buffer RDD and mixed by gently inverting the tube. 80 µl DNase I incubation mix was added directly to the RNeasy spin column membrane, and incubated on the benchtop (20–30°C) for 15 min. After incubation, 350 µl Buffer RW1 was added to the spin column and centrifuged at 11500 rpm for 30 seconds. RNeasy Spin column was carefully removed from the collection tube so as not to contact the flow-through. Spin column was placed on a new, clean collection tube and 500 µl Buffer RPE was added to the spin column. Column was centrifuged for 30 seconds at 11500 rpm to wash the column membrane and the flow-through was discarded. The spin column membrane was washed again with 500 µl Buffer RPE and centrifuged for 2 minutes at 11500 rpm. The old collection tube was discarded with flow-through and spin column was placed into a new collection tube. Column was centrifuged at full speed for 1 minute to get rid of residual ethanol. RNeasy spin column was placed to a 1.5 ml RNase-free microcentrifuge tube and 40 µl RNase-free water was added directly to the spin column membrane. After incubation of the column on the benchtop for 1 minute, centrifugation was performed for 1 minute at 11500 rpm to elute the total RNA. Eluted total RNA was aliquoted and kept at -80°C for short term storage.

2.2.4 Formaldehyde denaturing gel

Formaldehyde denaturing agarose gel electrophoresis was performed to check isolated total RNA purity and integrity. Gel was run at 5–7 V/cm in 1X FA gel running buffer prepared according to RNeasy Plant Mini Kit Manuel. SYBR Green I Nucleic Acid Gel Stain (Thermo) was used for visualizing total RNA. Eukaryotic subunits 28S and 18S ribosomal RNAs should be seen as sharp bands. Generally 28S rRNA to 18S RNA ratio should be 2:1. In the case of RNA degradation, the ribosomal bands are not bright and sharp, also seems like a smear with smaller sized RNAs.

2.2.5 Plasmid DNA isolation

Plasmid DNA isolation from *E.coli* transformants was performed with the procedure of the “QIAquick Plasmid DNA Isolation Kit” (QIAGEN). Plasmid isolation buffers were prepared according to the kit manual and compositions of buffers described in the Appendix A2.

E.coli DH5α culture was pelleted by centrifugation at 13000 rpm for 5 minutes. The medium was removed and the cell pellet was resuspended in 330 µl P1 buffer by pipeting. 330 µl P2 buffer was added to tube, mixed by inverting gently and incubated at benchtop for 5 minutes. 330 µl P3 buffer was added and mixed through inverting the tube 4-6 times. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated white material contains proteins, genomic DNA, cell debris. Tube was incubated on ice for 15 minutes. After incubation, centrifugation was applied at 13000 rpm for 15 minutes. Then Supernatant was transferred to a new 1.5 ml eppendorf tube without disturbing white precipitate and plasmid DNA was assembled after adding 0.7 volume of isopropanol to the supernatant containing plasmid. After that, plasmid DNA was pelleted by centrifugation at 13000 rpm for 30 minutes and 1 ml of 70% ethanol added to wash the plasmid DNA pellet and centrifugation performed at 13000 rpm for 5 minutes. The supernatant was discarded without disturbing pellet and residual ethanol was dried out at 37°C for 1 hour. Finally, air dried pellet was dissolved in 15 ml of elution buffer (EB), incubated at 37°C for 30 minutes, aliquoted and stored at -20°C. The isolated plasmid DNA was checked on 1% agarose gel.

2.2.6 Agarose gel electrophoresis

Agarose gel system was composed of 1% agarose gel prepared with 1xTAE buffer (Appendix A2). 1xTAE buffer also used as running buffer in electrophoresis tank. SYBR Green I Nucleic Acid Gel Stain (Thermo) and RedSafe™ Nucleic Acid Staining Solution (iNtRON) were used for visualizing the samples of DNA. The gel was run at 4 V/cm and the gels were visualized in UV transilluminator. Agarose gel concentration was adjusted according to size of the nucleic acid molecules.

2.2.7 Polymerase chain reaction (PCR)

Polymerase chain reaction technique was used in several steps of this study. Identification of 5' and 3' ends laccase encoding cDNA with RACE-PCRs and the full-length *Cplcc2* cDNA and genomic DNA synthesis were performed with PCR using different primer sets and thermal protocols. All PCR conditions were described in the relevant parts. The annealing temperatures were determined according to Tm values of primer sets. The final extension time was adjusted with considering the expected size of the fragment that will be amplified.

2.2.8 *Cplcc2* gene isolation from *Coriolopsis polyzona* MUCL 38443 genomic DNA

In order to amplify highly accurate sequence of *Cplcc2* gene, *Pfu* polymerase (Fermentas) which is a proofreading DNA polymerase isolated from *Pyrococcus furiosus*, was used for PCR reaction. 50 µl of PCR reaction mix containing 5 µl 10X Pfu Buffer (with $MgSO_4$), 1 µl *Cplcc2* Forward primer (10 mM) and 1 µl *Cplcc2* Reverse primer (10 mM), 5 µl dNTP mix (2 mM each), 1 µl *Pfu* DNA polymerase, 0.5 µl *Coriolopsis polyzona* genomic DNA (approximately 300 ng) as PCR template and 36.5 µl PCR grade dH_2O was prepared. Polymerase chain reaction was carried out according to the following thermal profile; initial denaturation at 95°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 70°C for 30 seconds, 72°C for 4.5 minutes and a final extension at 72°C for 15 minutes.

Cplcc2 gene PCR product was polyadenylated with Taq polymerase (iNtRON) to be able to perform TA cloning procedure. For polyadenylation reaction, 2 µl 10X PCR buffer, 5 µl purified *Cplcc2* gene PCR product, 0.5 µl dATP (10 mM), 0.5 µl i-Taq™ DNA Polymerase (5U/µl) and 12 µl PCR Grade dH_2O were mixed. Reaction tubes were incubated at 72°C for 30 minutes.

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

Reverse Transcriptase PCR (RT-PCR) and following 5'- 3' RACE (Rapid Amplification of cDNA ends) reactions were used to determine full-length *Cplcc2* cDNA of *Coriolopsis polyzona* MUCL 38443 starting with total RNA. Rapid Amplification of cDNA Ends (RACE) technique is a simple, efficient PCR based cDNA cloning strategy that overcomes the problems encountered in obtaining full-length cDNA clones of low-abundance mRNAs. This technique has been used for amplification and cloning of rare mRNAs besides applied to existing cDNA libraries (Frohman et al., 1988). While PCR reaction requires two sequence-specific primers for the amplification of interested sequence, RACE method enables amplification of unkown nucleic acid sequences from a mRNA template between a defined internal site and either the 3´ or the 5´ end of template (Yaver et al.,1996; Hoshida et al., 2001).

In this study, the identification of 5' and 3'- ends of *Cplcc2* cDNA was performed by BD SMARTer™ RACE cDNA Amplification Kit (Clontech). Principle of RACE experiments is summarized in the Figure 2.2 and Figure 2.3.

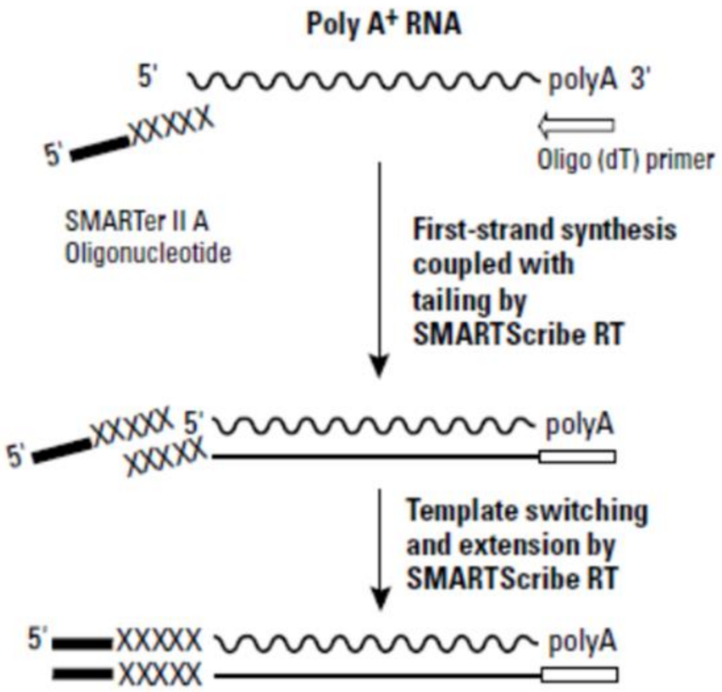

Figure 2.2 : Mechanism of SMARTer™ cDNA synthesis (Clontech).

First-strand synthesis is started with a modified oligo (dT) primer which anneals polyA tail. When SMARTScribe Reverse Transcriptase (RT) reaches the end of the mRNA template, it adds several nontemplate residues. Then SMARTer II A Oligonucleotide anneals to this added residues of the cDNA and serves as a template for SMARTScribe RT (SMARTer™ RACE cDNA Amplification Kit Manual).

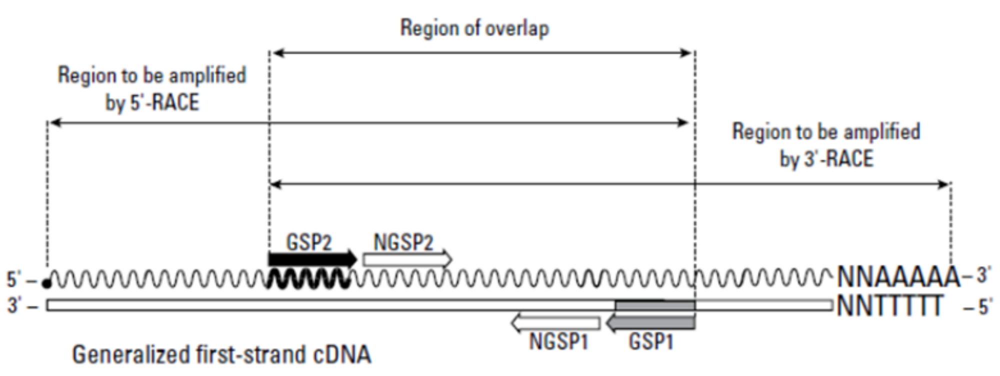

Figure 2.3 : Schematic representation of designed gene-specific primers (GSPs) and Nested gene-specific primers (NGSPs) organization on the first-strand cDNA template (Clontech).

Gene-specific RACE and Nested RACE primers needed for amplification of the cDNA ends were designed based on the sequence of the partial cDNA *Cplcc2* laccase/the sequence of the conserved copper binding region of laccases. Two set of gene specific primers (GSP) are required for 5’-3’ RACE protocol, one sense primer for the 3'-RACE reaction and one antisense primer for the 5'-RACE reaction. Primers should be 23–28 nucleotides in length and have 50–70% GC content. Tm value should be at least 65°C. Also 3'-terminal complementarity should be minimized to prevent primer-dimer artifacts. Since the RACE-PCR reactions are performed using only one gene specific primer (GSP), exact primer design is important for efficient PCR reaction. Primers used in RACE experiments are introduced in Table 2.1.

Table 2.1 : Primers used in the RACE reactions.

Primer	**Sequence**
Cplcc2 RACE Reverse	5’-AAGGTACCGGCTTGGTCCGGCACACG-3’
Cplcc2 Nested RACE Reverse	5’-CACTGATTCACGAACGCGGGGCCGTC-3’
Cplcc2 RACE Forward	5’- GCGACCCGAACCACGTGTTCAGCATC -3’
Cplcc2 Nested RACE Forward	5’-GCAACTACTGGGATCCGTGGCAACCC-3’
3'-RACE CDS Primer A	5'–AAGCAGTGGTATCAACGCAGAGTAC(T)30V N–3' (N = A, C, G, or T; V = A, G, or C)
5'-RACE CDS Primer A	5'–(T)25V 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’-CTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT-3' Short (0.2 µM): 5’-CTAATACGACTCACTATAGGGC-3’
Nested Universal Primer A (NUP)	5'–AAGCAGTGGTATCAACGCAGAGT–3'

2.2.10 Determination of 5’ end of the *Cplcc2* cDNA

2.2.10.1 5' RACE ready cDNA synthesis

5' RACE ready cDNA was synthesized from total RNA in order to be used as a template in further 5’ RACE reaction. In a RNase-free PCR tube 2 µl of total RNA, 1 µl 5'-CDS primer A and 0.75 µl sterile RNase-free dH_2O added for final volume 3.75 µl. Contents was mixed and spined in a microcentrifuge before incubation at 72°C for 3 minutes then at 42°C for 2 minutes. After cooling of mixture, 1 µl SMARTer IIA oligo was added per reaction. Then, 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/µl) and 1 µl SMARTScribe™ Reverse Transcriptase (100 U) were added up to 10 µl final volume. Next, tubes were incubated at 42°C for 90 minutes and 70°C for 10 minutes in a hot-lid thermal cycler. Finally, synthesized 5’ RACE Ready cDNA diluted with 40 µl Tricine-EDTA Buffer, aliquoted and stored at -20°C.

2.2.10.2 5’ RACE PCR reaction

Amplification of 5’ end of *Cplcc2* cDNA was fulfilled by using *Cplcc2* RACE Reverse primer as GSP. For this PCR reaction 5 µl 10X Advantage 2 PCR Buffer, 1 µl dNTP mix (10 mM), 5 µl Universal Primer Mix (10 X), 1 µl *Cplcc2* RACE Reverse primer (10 µM), 2.5 µl 5’ RACE ready cDNA, 1 µl 50X Advantage 2 Polymerase Mix and 34.5 µl PCR grade water were added and mixed for 50 µl final volume. Touchdown PCR thermal profile was used for 5’ RACE reaction. In the case of gene specific primer Tm value over 70°C, Touchdown PCR protocol (Program 1) was recommended by SMARTer™ RACE cDNA Amplification Kit Manual.

Touchdown PCR program; 5 cycles of 94°C for 30 seconds, 72°C for 2 minutes, 5 cycles of 94°C for 30 seconds, 70°C for 30 seconds, 72°C for 2 minutes and 25 cycles of 94°C for 30 seconds, 68°C for 30 seconds, 72°C for 2 minutes. Amplified 5’ RACE product was cloned into the pGEM®-T Easy Cloning Vector (Promega). Transformants were selected and positive plasmids were sequenced with ABI PRISM® 3100 Genetic Analyzer.

2.2.10.3 5’ RACE Nested PCR reaction

For the 5’ RACE Nested PCR Reaction, 5 µl 10X Advantage 2 PCR Buffer, 1 µl dNTP mix (10 mM), 5 µl 5’ RACE product (1:50 dilution), 1 µl Nested Universal Primer Mix (10 X), 1 µl *Cplcc2* Nested RACE Reverse primer (10 µM), 1 µl 50X Advantage 2 Polymerase Mixture and 36 µl PCR grade water were added for 50 µl final volume. Thermal protocol of reaction; 15 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes. Amplified 5’ Nested RACE fragment was cloned into the pGEM®-T Easy Cloning Vector (Promega). Transformants were selected and positive plasmids were sequenced with ABI PRISM® 3100 Genetic Analyzer.

2.2.11 Determination of 3’ end of the *Cplcc2* cDNA

2.2.11.1 3' RACE ready cDNA synthesis

3' RACE ready cDNA was synthesized from total RNA in order to use as template in further 3’ RACE reactions. In a RNase-free PCR tube 2 µl of total RNA, 1 µl 5'-CDS primer A and 1.75 µl sterile RNase-free dH_2O added for final volume 4.75 µl. Contents was mixed and spined in a microcentrifuge before incubation at 72°C for 3 minutes then at 42°C for 2 minutes. After cooling of mixture, 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/µl) and 1 µl SMARTScribe™ Reverse Transcriptase (100 U) were added up to 10 µl final volume. Tubes were incubated at 42°C for 90 minutes and 70°C for 10 minutes in a hot-lid thermal cycler. Synthesized 3’ RACE Ready cDNA diluted with 40 µl Tricine-EDTA Buffer, aliquoted and stored at -20°C.

2.2.11.2 3’ RACE PCR reaction

Amplification of 3’ end of *Cplcc2* cDNA was performed by using *Cplcc2* RACE Forward primer as GSP. For this PCR reaction 5 µl 10X Advantage 2 PCR Buffer, 1 µl dNTP mix (10 mM), 5 µl Universal Primer Mix (10 X), 1 µl *Cplcc2* RACE Reverse primer (10 µM), 2.5 µl 3’ RACE ready cDNA, 1 µl 50X Advantage 2 Polymerase Mix and 34.5 µl PCR grade water were added and mixed for 50 µl final volume. Touchdown PCR protocol was used for 3’ RACE reaction program; 5 cycles of 94°C for 30 seconds, 72°C for 2 minutes, 5 cycles of 94°C for 30 seconds, 70°C for 30 seconds, 72°C for 2 minutes and 25 cycles of 94°C for 30 seconds, 68°C for 30 seconds, 72°C for 2 minutes. Amplified 3’ RACE product was cloned into the pGEM®-

T Easy Cloning Vector (Promega). Transformants were selected and positive plasmids were sequenced with ABI PRISM® 3100 Genetic Analyzer.

2.2.11.3 3' RACE Nested PCR reaction

For the 3' RACE Nested PCR Reaction, 5 µl 10X Advantage 2 PCR Buffer, 1 µl dNTP mix (10 mM), 5 µl 3'RACE product (1:50 dilution), 1 µl Nested Universal Primer Mix (10 X), 1 µl *Cplcc2* Nested RACE Forward primer (10 µM), 1 µl 50X Advantage 2 Polymerase Mixture and 36 µl PCR grade water were mixed for 50 µl final volume. Thermal profile of reaction; 20 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes. Amplified 5' Nested RACE fragment was cloned into the pGEM®-T Easy Cloning Vector (Promega). Transformants were selected and positive plasmids were sequenced with ABI PRISM® 3100 Genetic Analyzer.

2.2.12 The amplification of full length *Cplcc2* laccase cDNA

Full-length *Cplcc2* laccase cDNA was obtained by using a high fidelity PCR enzyme, SeqAmp DNA Polymerase (Clontech). For this reaction, 5' RACE-ready cDNA was used as a template. 5' RACE-ready cDNA was synthesized according to the procedure mentioned in 5' RACE ready cDNA synthesis part. 50 µl PCR mixture containing 25 µl 2X SeqAmp PCR Buffer (includes $Mg^{2+,}$ dNTPs), 1 µl Cplcc2 Forward primer (10 mM), 1 µl Cplcc2 Reverse primer (10 mM), 5 µl 5' RACE-ready cDNA as a template, 1 µl SeqAmp DNA Polymerase and 17 µl PCR grade water was prepared. Thermal cycling was initiated with 94°C for 1 minute following 30 cycles of 98°C for 10 seconds, 58°C for 15 seconds, 68°C for 1 minute. Purified PCR product was cloned into the pGEM®-T Easy Cloning Vector (Promega), transferred into the *E.coli* DH5α cells. At last, transformants were selected and positive plasmids were sequenced with ABI PRISM® 3100 Genetic Analyzer.

2.2.13 Gel extraction

For the purpose of purification and extraction of PCR products and linear plasmids from agarose gel NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) was used. The DNA fragment was excised from the gel with a clean scalpel and the weight of gel slice was determined. According to the weight of DNA fragment contining gel piece, 2 volumes of NT1 buffer was added.then, the sample was incubated for 5-10 minutes at 50°C and mixed by vortexing every 2-3 minutes until the gel was completely dissolved. 1 volume of isopropanol was added, the sample was loaded to NucleoSpin® Gel and PCR Clean-up Column up to 700 ul. For DNA binding to column membrane, centrifuged for 30 seconds at 11000 x g. The flow through was discarded and the column placed back into the collection tube. 700 µL Buffer NT3 was added to the NucleoSpin® Gel and PCR Clean-up Column to wash the silica membrane and was centrifuged at 11000 g for 30 seconds and flow-through was discarded and the column was placed back into the collection tube. In order to minimize chaotropic salt carry-over and low A_{260}/A_{230} previous washing step was repeated. Empty column centrifuged at 11000 x g for 1 minute to dry silica membrane. The column was placed into a new 1.5 ml microfuge tube, 15-30 µl EB buffer was applied to the center of the silica membrane, incubated for 1 minute at benchtop. After incubation, DNA fragment was eluted with centrifugation at 11000 x g for 1 minute. Purified DNA fragment was checked on 1% agarose gel and stored at -20°C for further analysis.

2.2.14 Enzymatic digestion

Digestion reactions were performed according to the restriction enzymes manufacturer's protocols. Enzymatic digestion of PCR products, cloning and expression vectors were carried out by using 5U of enzyme for each µg of DNA and 1X Digestion buffer. Reactions were incubated at 37°C for 1 to 4 hours for different DNA templates.

2.2.15 Ligation of the PCR products into the pGEM®-T Easy cloning vector

After gel extraction purified PCR fragments were ligated into pGEM®-T Easy Cloning Vector (Promega) according to the pGEM®-T Easy Vector Systems Instructions. Ligation reaction prepared as following protocol; 5 µl 2X Rapid ligation buffer, 1 µl pGEM-T Easy vector, 2-3 µl PCR product, 1 µl T4 DNA ligase enzyme (5 U/µl) and PCR grade dH_2O up to 10 ml final volume and mixed. Reaction mixture was incubated at 22°C for 1 hours then overnight at 4°C in order to obtain maximum number of transformants. Before transformation, the reaction was incubated at 65°C for 10 minutes to inhibit T4 ligase. Then ligation mix was used for transformation of chemically competent *E. coli* DH5α cells.

2.2.16 Transformation of bacteria

Following ligation reaction, obtained recombinant plasmids were cloned into chemically competent *Escherichia coli DH5α* cells

2.2.16.1 Preparation of chemically competent *E. coli* DH5α cells

Overnight *E. coli* DH5α culture in 2xYT broth was inoculated into 50 ml 2xYT broth in 500 ml flask by adjusting OD_{600} value 0.1. Flask was incubated in shaker at 37^{o}C and 180 rpm until OD_{600} value of culture reached 0.4-0.6. Cells were seperated into precooled sterile falcons and incubated on ice for 10 minutes. Chilled cultures were centrifuged at 4000 rpm, 4°C for 10 minutes. After that, supernatant was discarded and cell pellet was resuspended with 1:2 volume of culture cold 100 mM $CaCl_2$ and centrifuged at 4000 rpm, 4°C for 10 minutes. Supernatant was discarded and cell pellet was resuspended with 1:20 volume cold 100 mM $CaCl_2$ resuspended and transferred into precooled 2 ml tubes at 100 µl volume. For long term storage at -80°C, 11 µl cold and sterile 100% glycerol was added into the tubes.

2.2.16.2 Transformation of chemically competent *E. coli* DH5α cells

Recombinant plasmids obtained from ligation reactions were transferred into *E.coli* DH5α cells. Chemically competent *E.coli* cells were thawed on ice.Subsequently, 5-10 µl of ligation product was added into competent cells tube, mixed gently and incubated on ice for 45 minutes. After incubation, competent cells and tube containing ligation product were incubated at 37°C waterbath for 5 minutess. Following heat shock, tube was placed on ice for 2 minutes. Then, 1 ml LB broth was added into reaction tube and incubated at 37°C, 180 rpm for 1 hour. Lastly, transformation mix was centrifugated at 10000 rpm for 5 minutes. Cell pellet was resuspended with 100 µl saline solution (0.85% NaCl) and was spread on LB agar plates containing Amp/X-gal/IPTG for The pGEM®-T Easy cloning vector. Colonies were observed after overnight incubation at 37°C.

2.2.17 DNA sequencing

The cloned fragments within cloning and expression vectors were sequenced using *Cplcc2* and vector specific primers (Table 2.2) with ABI PRISM 3100 Avant automated sequencer. All cDNA and gDNA sequences were compared with National

Center for Biotechnology Information (NCBI) database using the BLAST search at the web site. Multiple alignment was performed using Clustal Omega.

Table 2.2 : *Cplcc2* and vector specific primers used in DNA sequencing.

Primer	Sequence
Cplcc2 Forward	5’-CCGGGAATTCAGCATGGTTGGCTTGCAGCGTTTCGGA-3’
Cplcc2 Reverse	5’-AATGCGGCCGCCTACCCCGTCCGCGTTCACTG-3’
M13 Forward	5'-GTTTTCCCAGTCACGAC-3'
M13 Reverse	5'-AACAGCTATGACCATG-3'
Cplcc2 RACE Forward	5’- GCGACCCGAACCACGTGTTCAGCATC -3’
Cplcc2 RACE Reverse	5’-AAGGTACCGGCTTGGTCCGGCACACG-3’

3. RESULTS AND DISCUSSION

3.1 Total RNA isolation from *Coriolopsis polyzona* MUCL 38443

Total RNA from *Coriolopsis polyzona* MUCL 38443 cells was isolated by using "Qiagen RNeasy Plant Mini Kit" according to instructions of manufacturer. The integrity of purified total RNA was checked by formaldehyde (FA) agarose gel electrophoresis (Figure 3.1).

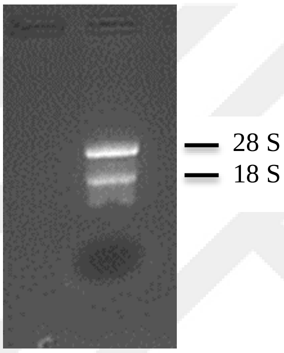

Figure 3.1 : Isolated total RNA from *Coriolopsis polyzona* MUCL 38443

3.2 Rapid Amplification of cDNA Ends (RACE)

Laccase cDNAs isolation by using RACE (Rapid Amplification of cDNA Ends) technique has been reported in different studies (Hoshida et al., 2001; Kiiskinen and Saloheimo, 2004; Liu et al., 2003). Amplification of unknown sequences causes a limitation for designing PCR primers but this limitation can be overcomed by using 5'- and 3'-RACE procedures.

Strategy to design the primers used in RACE procedure performed for cloning of laccase genes is shown in the Figure 3.3. (Hoshida et al., 2001; Kiiskinen and Saloheimo, 2004; Liu et al., 2003). Amplification of unknown sequences causes a limitation for designing PCR primers but this limitation can be overcome by using 5'- and 3'-RACE procedure. Primer design strategy applied for cloning of laccase genes is shown in the Figure 3.2.

In order to obtain 5’ and 3’ ends of *Cplcc2* cDNA with RACE (Rapid Amplification of cDNA Ends) reaction, gene specific RACE primers primers used in the 5’- and 3’-RACE protocols were designed by using partial *Cplcc2* cDNA sequence as template (Table 2.1).

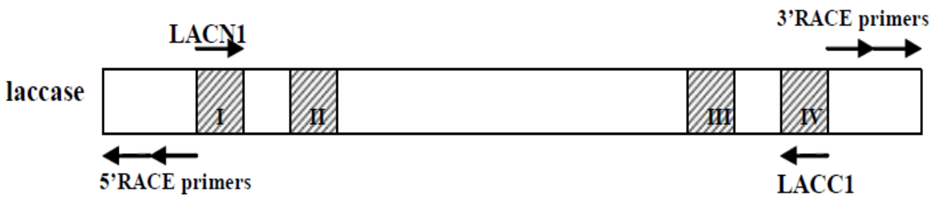

Figure 3.2 : Schematic representation of designing degenerate PCR primers and 5’ and 3’ RACE-Nested RACE primers.

3.2.1 Determination of 5’ end of Cplcc2 cDNA

By using total RNA as template, first strand cDNA was synthesized with “Smart II A Oligonucleotide”. This 5’ RACE ready cDNA was used as template for 5’ RACE reaction. As a result, 550 bp 5’ RACE products was obtained by using the gene specific reverse primer *“Cplcc2* RACE Reverse”and the universal primer (UPM), which recognizes the Smart II A oligonucleotide (Figure 3.3).

Specificity of obtained 550 bp 5’ RACE product was increased by constructed Nested PCR by using the gene specific reverse primer “*Cplcc2* Nested RACE Reverse” and Nested Universal primer. This nested PCR yielded 500 bp DNA fragment. The 550 bp 5’ RACE and 500 bp 5’-Nested RACE products were then cloned into pGEM®-T Easy TA cloning vector and used to transform chemically competent *E.coli* DH5-α cells. Transformants were selected with Blue/White screening method. After plasmid isolation from white colonies, *Eco*RI restriction enzyme digestion was performed to verify the cloning of that fragments (Figure 3.4, Figure 3.5). Plasmid DNAs including the 550 bp 5’ RACE and 500 bp 5’ Nested RACE products were, then, sequenced (Figure 3.6).

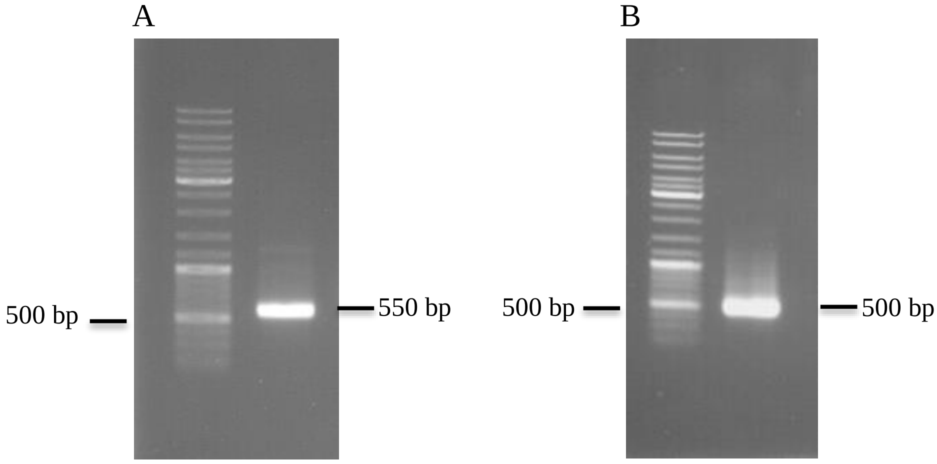

Figure 3.3 : A) 550 bp 5' RACE products obtained by using the gene specific reverse primer *"Cplcc2* RACE Reverse"and the universal primer (UPM), **B)** 500 bp 5' Nested RACE products obtained by using the gene specific reverse primer "*Cplcc2* Nested RACE Reverse" Nested Universal primer. **Marker DNA:** Gene Ruler™ DNA Ladder Mix (Thermo Scientific)

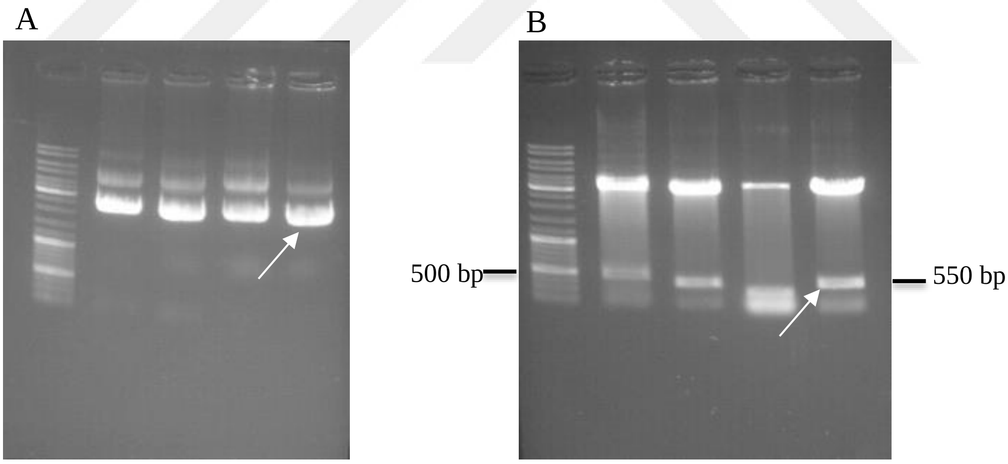

Figure 3.4 : A) Plasmid DNAs isolated from selected white clones. **B)** Plasmid DNAs digested with *EcoR*I. **Marker DNA:** Gene Ruler™ DNA Ladder Mix (Thermo Scientific).

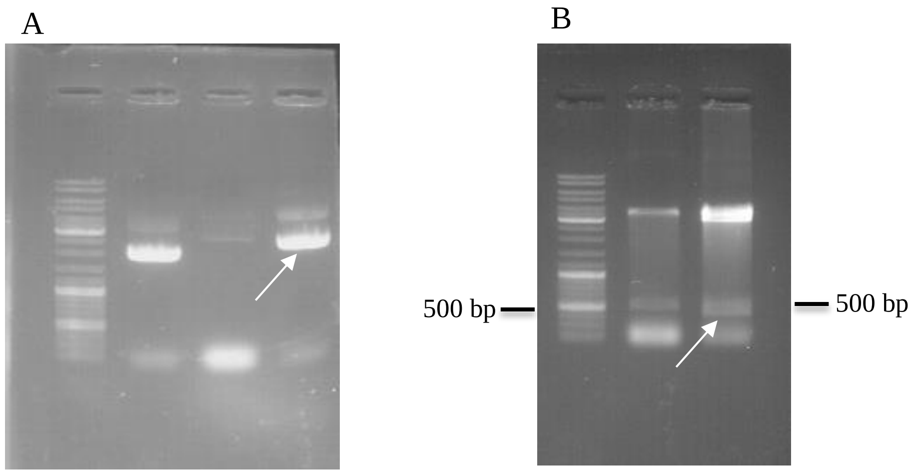

Figure 3.5 : A) Plasmid DNAs isolated from selected white clones. **B)** Plasmid DNAs digested with *EcoR*I. **Marker DNA:** Gene Ruler™ DNA Ladder Mix (Thermo Scientific).

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5′…GAATTCGATTAAGCAGTGGTATCAACGCAGAGTACATGGGGAAGCTCGAGCTCG
TCTTGAGCTCCGATCCCCAGGAATTCGCCTTCCAATCCCTTCCTCCCGACCTGTACA
CTCAGAGATATGGTTGGCTTGCAGCGTTTCGGATTGTTTGTTACCCTTGCGCTCGTC
TCTGGCACCCTTGGTGCTGTGGGGCCGAGGGCTGACCTCGTCATTGCCAACGGCCCC
GTGTCTCCCGACGGCTTCACGCGCGAGGCCATTACCGTCAACGGCGCCTTCCCGAAC
CCGCTCATCGTTGGCAAGAAGGGTGACCGCTTCCGGCTCAACGTGGTCGACAATCTA
ACCAATCACACTATGCTGAAGTCCACTAGCATCCATTGGCACGGGTTTTTCCAGGCA
GGCACCAACTGGGCAGACGGCCCCGCGTTCGTGAATCAGTGTCCTATCGCGTCCGGG
CACTCGTTCTTGTACGACTTCCGTGTGCCGGACCA…3′
```

Figure 3.6 : The sequence of 5'-end of *Cplcc2* cDNA. Gene-specific forward, 5' RACE Nested RACE and Nested Universal primer are highlighted with gray, red and purple colors, respectively. Start codon (ATG) and Kozak (bold and underlined) sequence were determined. Start codon takes part in Kozak sequence.

Obtained 5' end sequences were analysed with NCBI highly similar sequences (megablast) program. Alignment analysis exhibited approximately 80% similarity with previously identified laccases (Table 3.1).

The start codon "ATG" has been found as a part of Kozak consensus sequence and followed by another guanine (Figure 3.6). Kozak consensus sequence, gccRccAUGG

(R=A,G) is located at three base upstream of the start codon on eukaryotic mRNAs. This pattern has an important role in the translation initiation with recognition by ribosome (Kozak, 1986).

Table 3.1 : Sequences producing significant alignments with 5'end sequence of *Cplcc2* cDNA.

Accession number	Description	Score	Query coverage	Maximum identity
AY693776.1	*Trametes versicolor* laccase 1 (lcc1) mRNA, complete cds	279	73%	81%
XM_008034546.1	*Trametes versicolor* FP-101664 SS1 laccase I (TvLac1), partial mRNA	268	73%	80%
FJ469151.1	*Trametes versicolor* laccase protein mRNA, complete cds	268	73%	80%
AY049725.1	*Trametes versicolor* laccase 1 (lac1) mRNA, complete cds	268	73%	80%
AB212732.1	*Trametes versicolor* lac2 mRNA for laccase2, complete cds	268	73%	80%
KT211485.1	*Trametes ochracea* laccase B mRNA, complete cds	265	69%	81%
U44430.1	*Trametes versicolor* laccase I (lccI) mRNA, complete cds	263	73%	80%
KR492189.1	*Trametes versicolor* isolate K4 laccase gene, complete cds	257	73%	80%
GU738021.1	*Trametes velutina* strain 5930 laccase (lac5930-1) mRNA, complete cds	254	75%	79%
JQ828930.1	*Trametes versicolor* strain NL-1 laccase (lccA) gene, complete cds	241	73%	79%
FJ465129.1	*Trametes versicolor* strain 1.028 laccase-like protein mRNA, complete cds, alternatively spliced	224	64%	80%
AF548033.1	*Trametes sp.* I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	182	68%	76%
AF548032.1	*Trametes sp.* I-62 laccase (pox1) mRNA, complete cds	171	61%	77%
AY839938.1	*Trametes sp.* AH28-2 laccase D (lacD) gene, partial cds	148	23%	90%
EU678777.1	*Trametes suaveolens* strain BRFM 13 laccase gene, partial cds	137	23%	88%
AY243863.1	*Xylaria sp.* HKUCC 2782 isolate Lcc11 laccase gene, partial cds	135	20%	90%
EU678778.1	*Trametes versicolor* strain CIRM-BRFM 75 laccase gene, partial cds	132	23%	87%
U44851.1	*Trametes versicolor* laccase I (lccI) gene, complete cds	132	23%	87%
U65399.1	*Basidiomycete CECT* 20197 phenoloxidase (pox1) gene, complete cds	99.0	14%	92%

3.2.2 Determination of 3’ End of Cplcc2 cDNA

To determine the nucleotide sequence of 3’ end of *Coriolopsis polyzona* MUCL 38443 laccase Cplcc2 cDNA, 3’-RACE procedure was used. In this procedure, poly(A) tail found in mRNA serves as a generic priming site for RACE PCR reaction. 3’ RACE ready cDNA is synthesized using a traditional reverse transcription, but total RNA was converted into the first strand cDNA with a special oligo(dT)-anchor primer which anneals poly(A) tail of mRNAs. This 3’ RACE ready cDNA was used as template for 3’ RACE reaction. First, 3’ RACE PCR was set up with *Cplcc2* 3’ RACE primer and Universal Primer Mix (UPM). This reaction resulted in 1200 bp PCR product (Fig. 3.7). The specificity of 3’-end amplification was also confirmed by Nested RACE-PCR reaction by using the gene specific reverse primer “*Cplcc2* Nested RACE Forward” and Nested Universal primer. This Nested PCR yielded 550 bp PCR fragment.(Figure 3.7).

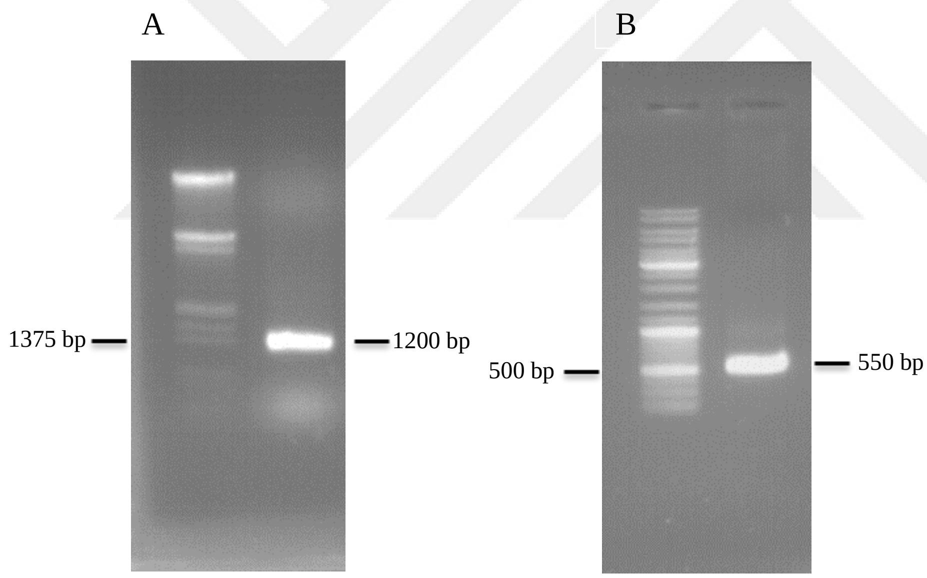

Figure 3.7 : A) 1200 bp 3’ RACE products obtained by using *Cplcc2* RACE Forward primer and Universal Primer Mix (UPM). **B)** 550 bp 3’ Nested RACE products obtained by using the gene specific reverse primer *Cplcc2* Nested RACE Reverse and Nested Universal primer. **Marker DNA:** Gene Ruler^{TM} DNA Ladder Mix (Thermo Scientific)

Obtained 1200 bp 3’-RACE and 550 bp 3’-Nested RACE products were cloned into pGEM®-T Easy TA cloning vector and transferred into chemically competent *E.coli* DH5α cells. Transformants were selected with Blue/White screening method. After plasmid isolation from white colonies, restriction digestion with *Eco*RI was performed to verify the cloning of that fragments (Figure 3.8 and Figure 3.9). Plasmid DNAs containing the inserts were, then, sequenced. While obtained 3’ cDNA end sequences BLAST homology analysis was performed with other laccases and revealed 88% homology (Table3.2), 5’ cDNA end sequences were analysed with NCBI highly similar sequences (megablast) program. Alignment analysis exhibited approximately 80% similarity with previously identified laccases.

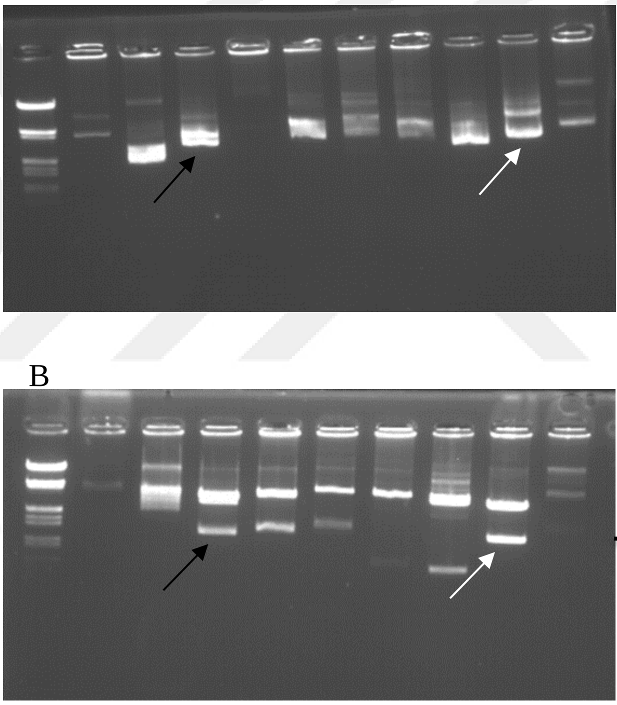

Figure 3.8 : A) Plasmid DNAs isolated from selected white clones. **B)** Plasmid DNAs digested with *Eco*RI. Marker DNA: Lambda DNA/*Eco*RI+*Hind*III Marker (Thermo Scientific)

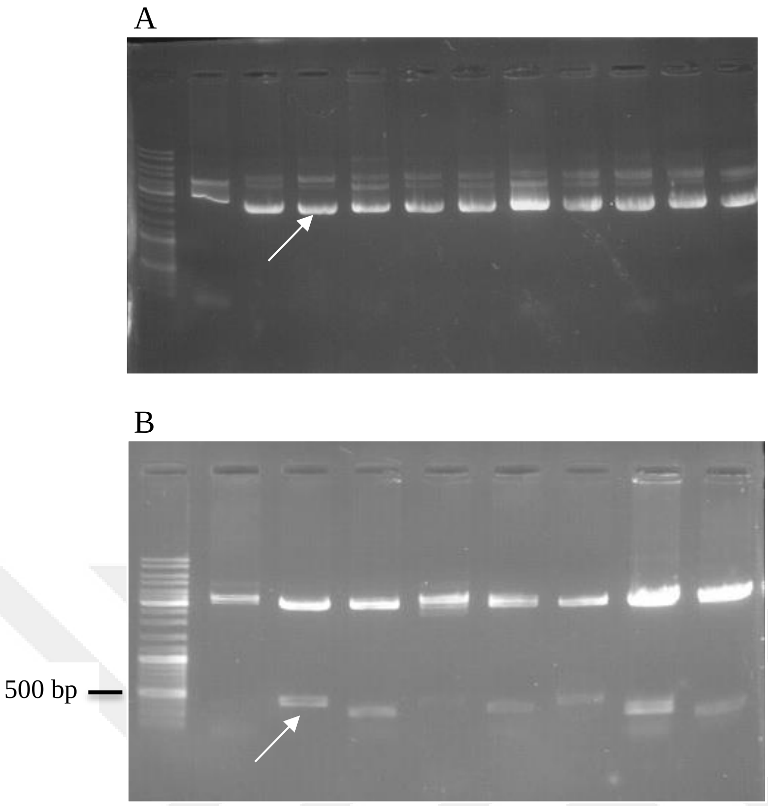

Figure 3.9 : A) Plasmid DNAs isolated from selected white clones. **B)** Plasmid DNAs digested with *Eco*RI. Marker DNA: Gene RulerTM DNA Ladder Mix (Thermo Scientific).

"*Cplcc2* RACE Forward" primer sequence is underlined and stop codon "TGA" was found at the upstream of the polyadenylation site (Figure 3.10).

CGGCGTCAACAGCAAGCCTCTCACTGTGGACTCCATCCAAATTTTCGCCGCGCAGCG
CTACTCCTTTGTTTTGAACGCAAATCAACCGGTGGGCAACTACTGGGATCCGTGCGA
ACCCCGTGCCGAAGGCGTGGTCGGACTTGTGTCCGATTTACGACAGGCTCCCGGAGG
ACGAGCAG**TGA**ACGCGGACGGGGTAGCGAGGTTTATGACGTGGATTGTACGGTTATC
GGTGAAGTACTCGGATCGGAGTGCTTGTTCAGTATCCCGGAGAAGCTGACTTTCGCA
CATTCGTTGTAACTGAGGGGGTCGATTTGGGAAGTTCGGTTTACGAATGTACAATAT
AGTATAACGGAATAGCGGTAAAGCGTAGCCCTATGCGTACGGAAGTCCGAGGTATAC
GAATATCCAAGAGATCGACCAAACTGTACATAAATACGAAACGACGACGCAACACGA
TTTTACAGTCCGTCGCAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.10 : The sequence of 3'end of *Cplcc2* cDNA. Used gene-specific reverse primer, *Cplcc2* RACE Forward and *Cplcc2* Nested RACE Forward primers are highlighted with red, gray and green colors, respectively. Stop codon (TGA) (bold and underlined) and Polyadenylation site is also highlighted with yellow color.

Table 3.2 : Sequences producing significant alignments with 3'end sequence of *Cplcc2* cDNA.

Accession Number	Description	Score	Query Coverage	Maximum Identity
HM106994.1	Pycnoporus sanguineus strain MUCL 38531 laccase (lcc1) mRNA, complete cds	140	23%	87%
AY458017.1	Pycnoporus sanguineus laccase mRNA, complete cds	140	23%	87%
KR492188.1	Trametes versicolor isolate 10E laccase gene, complete cds	202	31%	88%
KR492187.1	Trametes versicolor isolate 6C laccase gene, complete cds	202	31%	88%
KR492186.1	Trametes versicolor isolate 7E laccase gene, complete cds	202	31%	88%
KR492185.1	Trametes versicolor isolate 3A laccase gene, complete cds	202	31%	88%
XM_008034546.1	Trametes versicolor FP-101664 SS1 laccase I (TvLac1), partial mRNA	215	35%	88%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	213	37%	88%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	197	34%	86%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	197	34%	86%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	213	37%	88%
AY693776.1	Trametes versicolor laccase 1 (lcc1) mRNA, complete cds	208	31%	88%
KR492189.1	Trametes versicolor isolate K4 laccase gene, complete cds	202	31%	87%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	202	37%	87%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	131	23%	85%
JQ828930.1	Trametes versicolor strain NL-1 laccase (lccA) gene, complete cds	199	31%	86%
KT211485.1	Trametes ochracea laccase B mRNA, complete cds	184	30%	84%
KF317949.1	Cerrena sp. HYB07 laccase 7 precursor, mRNA, complete cds	120	23%	83%
AB244273.1	Spongipellis sp. FERM P-18171 mRNA for laccase 1 precursor, complete cds	120	23%	83%
KP027484.2	Trametes hirsuta laccase B mRNA, complete cds	175	32%	82%
JN367286.1	Trametes gibbosa laccase 2 mRNA, partial cds	116	22%	83%

3.3 Isolation of Full-length *Cplcc2* laccase cDNA and gDNA

Determination of 5’ end and 3’ end allowed primer design to amplify full length *Cplcc2* cDNA and genomic DNA. Forward and reverse primers involving the initiation and stop codons and also restriction sites for further cloning studies were designed and listed in Table 3.3.

Table 3.3 : Primers used in the full length PCR reactions.

Primer	Sequence
Cplcc2 Forward	5’-CCGGGAATTCAGCATGGTTGGCTTGCAGCGTTTCGGA-3’
Cplcc2 Reverse	5’-AATGCGGCCGCCTACCCCGTCCGCGTTCACTG-3’

3.3.1 Amplification of the full-length *Cplcc2* cDNA of *Coriolopsis polyzona* MUCL 38443

The full length cDNA of *Cplcc2* was obtained with SeqAmp DNA polymerase which is a high fidelity PCR enzyme. PCR reaction was set up with *Cplcc2* Forward and *Cplcc2* Reverse primers and a fragment approximately 1600 bp in length was amplified (Figure 3.11). Purified fragment was cloned into pGEM®-T Easy TA cloning vector and transformed chemically competent *E.coli* DH5 cells. Transformants were selected on LB plates containing Ampicillin and Xgal-IPTG with Blue/White screening method. After plasmid isolation from white colonies restriction enzyme analysis with *Eco*RI was performed (Figure 3.12). Plasmid DNAs containing the inserts were, then, sequenced.

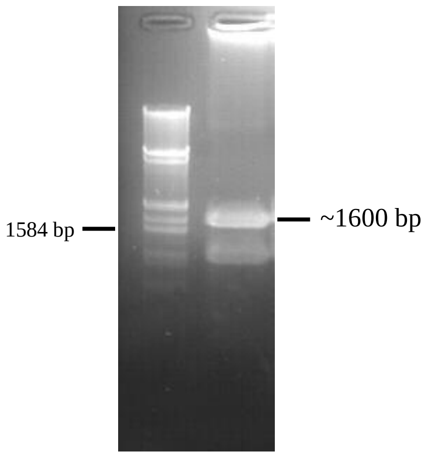

Figure 3.11 : Full-length *Cplcc2* cDNA PCR Product. **Marker DNA:** Lambda DNA/*Eco*RI+*Hind*III Marker (Thermo Scientific) (Lane 1).

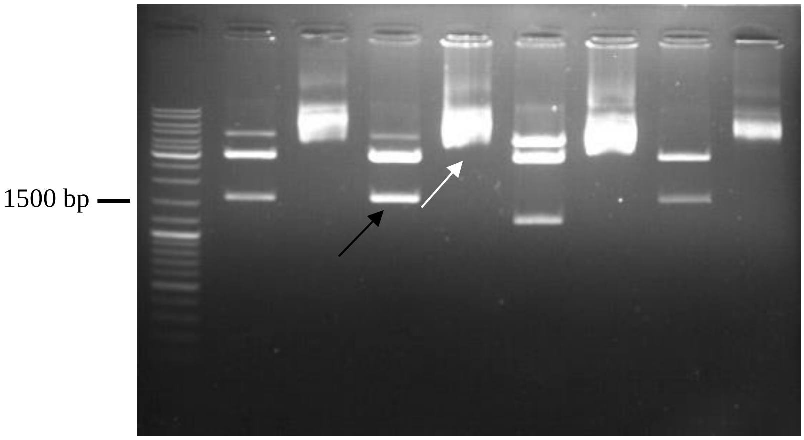

Figure 3.12 : *Eco*RI digestion of plasmids isolated from *E.coli* DH5α including *Cplcc2* cDNA products **Marker DNA:** Gene Ruler™ DNA Ladder Mix (Thermo Scientific) (Lane 1).

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ATGGTTGGCTTGCAGCGTTTCGGATTGTTTGTTACCCTTGCGCTCGTCTCTGGCACCCTTGGTGCTG
TGGGGCCGAGGGCTGACCTCGTCATTGCCAACGGCCCCGTGTCTCCCGACGGCTTCACGCGCGAGGC
CATTACCGTCAACGGCGCCTTCCCGAACCCGCTCATCGTTGGCAAGAAGGGTGACCGCTTCCGGCTC
AACGTGGTCGACAATCTAACCAATCACACTATGCTGAAGTCCACTAGCATCCATTGGCACGGCTTCT
TCCAGGCAGGCACCAACTGGGCAGACGGCCCCGCGTTCGTGAATCAGTGTCCCATCGCGTCCGGGCA
TTCGTTCTTGTACGACTTCCGTGTGCCGGACCAAGCCGGTACCTTCTGGTATCATAGTCATCTTTCG
ACCCAGTACTGCGACGGGCTGAGGGGGCCATTCGTAGTTTATGACCCGAAGGACCCTCATGCCAGTC
GTTACGATGTTGACGACGAGAGCACGGTGATCACCCTTGCTGACTGGTACCACACCGCGGCTCGCCT
CGGACCCAGGTTCCCATTGGGGGCGGACTCTACGCTCATCAATGGTCTCGGTCGATCGTCCTCGACC
CCTACAGCCCCTTTGGCCGTCATCAATGTGCAACGTGGGAAACGCTATCGGTTCCGCCTCGTGTCGC
TCTCATGCGACCCGAACCACGTGTTTAGTATCGACGGGCACAACATGACGATCATCGAGGTCGACGG
TGTCAACAGCAAGCCTCTCACCGTGGACTCCATCCAGATCTTCGCTGCGCAGCGCTACTCCTTCGTT
TTGAACGCAAATCGACCTGTGGGCAACTACTGGATCCGTGCAAACCCCAACTTCGGCACGACCGGGT
TCGCGGGCGGGATCAACTCCGCCATCCTGCGCTACAAGGGAGCCCCGGCTATTGAGCCGACGACCAC
TCAAACACCCTCCGTCAACCCACTCGTTGAGACGAACCTCCACCCACTCACACGCACGCCGGTGCCT
GGTCGTCCCACACCCGGAGGCGTGGACAAGGCGATCAACCTCGCTTTCAACTTTAATGGGTCCAACT
TCTTCATCAACGACGCGACGTTCACACCACCCAGCGTCCCCGTGCTGCTCCAAATTCTGAGCGGTGC
GCAGACCGCTCAGGACCTCCTGCCCCCGGGCTCCGTCTACCCTCTCCCGGGCCACTCCTCCATTGAG
CTCACGCTGCCCGCGACGGCGATGGCCCCAGGCGCGCCCCACCCCTTTCACCTGCATGGTCACGTCT
TCGCCGTCGTCCGCAGCGCGGGCAGCACCGAGTACAACTACAACGACCCGATCTTCCGCGACGTCGT
GAACACGGGCACGCCCGCGGCCAACGACAACGTCACGATCCGCTTCCGGACGGACAACCCCGGCCCG
TGGTTCCTCCACTGCCACATCGACTTCCACCTCGAGGCCGGGTTCGCGGTCGTGTTCGCGGAGGACG
TGCCGCGCGTCGCGCTCGCGAACCCCGTGCCGAAGGCGTGGTCGGACTTGTGCCCGATTTACGACAG
GCTCCCGGAGGACGACCAGTGA
```

Figure 3.13 : Open reading frame of *Cplcc2* from *Coriolopsis polyzona* MUCL 38443. Start codon and stop codon are highlighted with green and red, respectively.

Full length cDNA sequence of *Cplcc2* from ATG start codon to TGA stop codon is 1563 bp (Figure 3.13). This open reading frame encodes deduced CpLcc2 protein consisting of 520 amino acids (Figure X). BLAST homology search results of *Cplcc2* cDNA has 82% similarity with laccase gene of *Trametes versicolor* isolate 7E (KR492186.1), laccase (lccA) gene of *Trametes versicolor* strain NL-1 (JQ828930.1), laccase gene of *Trametes versicolor* isolate 3A (KR492185.1) and laccase 1 (lcc1) mRNA from *Trametes versicolor* (AY693776.1). Homology analysis results are listed in Table 3.4.

This 1563 bp open reading frame encodes deduced CpLcc2 protein consisting of 520 amino acids (Figure 3.14). Putative conserved copper binding domains of CpLcc2 laccase were found using NCBI Protein Blast analysis (Figure 3.15). The deduced amino acid sequence of CpLcc2 gives 86% homology with *Trametes velutina* laccase (ADE44157.1), laccase I of *Trametes versicolor* FP-101664 SS1 (XP_008032737.1) and *Trametes versicolor* laccases (AFM31222.1, AMT85330.1, AMT85329.1)(Tablo 3.5). Theoretical molecular weight and isoelectric point of CpLcc2 were calculated as 56.4366 kDa and 6.33, respectively (http://web.expasy.org/protparam/).

Phylogenetic analyses were performed using the MEGA version 6.0 software. Deduced aminoacid sequences of CpLcc2 were aligned using the Clustal W program in MEGA 6.0. Bootstrapping was performed with 1000 replications. Neighbor joining tree which was constructed with other laccases from GenBank showed that CpLcc2 and *Trametes hirsuta* laccase B (AIZ72727.1) are belong to same phylogenetic groups (Figure 3.16).

In fungal laccases, conserved copper ligands comprised of one cysteine residue and ten histidine residues ensure the coordination of copper atoms on the active site of enzyme. (Messerschmidt, 1997; Temp 1999). Ten histidine residues His85, His87, His130, His132, His416, His419, His421, His473, His475, His479 and Cys474 were located in the amino acid sequence of *Cplcc2*. Deduced amino acid sequence of *Cplcc2* has five potential N-glycosylation sites (Asn-X-Thr/Ser) (http://www.cbs.dtu.dk/services/NetNGlyc/). Additionally five Cystein residues are present in the deduced amino acid sequence *Cplcc2*. Cys 474 is thought to be the conserved laccase signature acting like a ligand to T1 copper domain. Phe482 is located at the position mentioned in deduced amino acid sequence of *lcc1* and is considered to be essential for the high redox potential of laccases (Temp et al. 1999).

However in the open reading frame of *Cplcc2*, phenylalanine is found at 484th position. Phe 484 of amino acid sequence of *Cplcc2* might be essential for the high redox potential laccase production (Figure 3.19).

Table 3.4 : Laccase sequences producing significant alignments with *Cplcc2* cDNA sequence (Rest of the table is presented in Appendix A5).

Accession Number	Description	Score	Query Coverage	Maximum Identity
KR492186.1	Trametes versicolor isolate 7E laccase gene, complete cds	1293	98%	82%
JQ828930.1	Trametes versicolor strain NL-1 laccase (lccA) gene, complete cds	1293	98%	82%
KR492185.1	Trametes versicolor isolate 3A laccase gene, complete cds	1288	98%	82%
AY693776.1	Trametes versicolor laccase 1 (lcc1) mRNA, complete cds	1288	98%	82%
XM_008034546.1	Trametes versicolor FP-101664 SS1 laccase I (TvLac1), partial mRNA	1279	99%	82%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	1279	99%	82%
KR492189.1	Trametes versicolor isolate K4 laccase gene, complete cds	1271	98%	82%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	1271	98%	82%
GU738021.1	Trametes velutina strain 5930 laccase (lac5930-1) mRNA, complete cds	1267	98%	82%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	1267	99%	81%
KT211485.1	Trametes ochracea laccase B mRNA, complete cds	1264	96%	82%
KR492187.1	Trametes versicolor isolate 6C laccase gene, complete cds	1262	98%	82%
AB212732.1	Trametes versicolor lac2 mRNA for laccase2, complete cds	1249	98%	81%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	1042	95%	79%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	1037	95%	79%
JF817353.1	Lenzites gibbosa strain CB-1 laccase 2 mRNA, complete cds	1035	94%	79%
HM243485.1	Trametes gibbosa strain LS01 laccase (lac1) mRNA, complete cds	965	86%	80%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	835	90%	78%
KF836751.1	Lentinus sp. WR2 laccase (lcc4) mRNA, complete cds	804	93%	77%
KP027478.1	Trametes hirsuta laccase A mRNA, complete cds	754	91%	76%
KT211484.1	Trametes ochracea laccase A mRNA, complete cds	734	88%	77%
XM_008034423.1	Trametes versicolor FP-101664 SS1 laccase B precursor (TvLac3), partial mRNA	717	92%	76%

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atggttggcttgcagcgtttcggattgtttgttacccttgcgctcgtctctggcaccctt
 M  V  G  L  Q  R  F  G  L  F  V  T  L  A  L  V  S  G  T  L
ggtgctgtggggccgagggctgacctcgtcattgccaacggccccgtgtctcccgacggc
 G  A  V  G  P  R  A  D  L  V  I  A  N  G  P  V  S  P  D  G
ttcacgcgcgaggccattaccgtcaacggcgccttcccgaacccgctcatcgttggcaag
 F  T  R  E  A  I  T  V  N  G  A  F  P  N  P  L  I  V  G  K
aagggtgaccgcttccggctcaacgtggtcgacaatctaaccaatcacactatgctgaag
 K  G  D  R  F  R  L  N  V  V  D  N  L  T  N  H  T  M  L  K
tccactagcatccattggcacggcttcttccaggcaggcaccaactgggcagacggcccc
 S  T  S  I  H  W  H  G  F  F  Q  A  G  T  N  W  A  D  G  P
gcgttcgtgaatcagtgtcccatcgcgtccgggcattcgttcttgtacgacttccgtgtg
 A  F  V  N  Q  C  P  I  A  S  G  H  S  F  L  Y  D  F  R  V
ccggaccaagccggtaccttctggtatcatagtcatctttcgacccagtactgcgacggg
 P  D  Q  A  G  T  F  W  Y  H  S  H  L  S  T  Q  Y  C  D  G
ctgagggggccattcgtagtttatgacccgaaggaccctcatgccagtcgttacgatgtt
 L  R  G  P  F  V  V  Y  D  P  K  D  P  H  A  S  R  Y  D  V
gacgacgagagcacggtgatcacccttgctgactggtaccacaccgcggctcgcctcgga
 D  D  E  S  T  V  I  T  L  A  D  W  Y  H  T  A  A  R  L  G
cccaggttcccattgggggcggactctacgctcatcaatggtctcggtcgatcgtcctcg
 P  R  F  P  L  G  A  D  S  T  L  I  N  G  L  G  R  S  S  S
acccctacagcccctttggccgtcatcaatgtgcaacgtgggaaacgctatcggttccgc
 T  P  T  A  P  L  A  V  I  N  V  Q  R  G  K  R  Y  R  F  R
ctcgtgtcgctctcatgcgacccgaaccacgtgtttagtatcgacgggcacaacatgacg
 L  V  S  L  S  C  D  P  N  H  V  F  S  I  D  G  H  N  M  T
atcatcgaggtcgacggtgtcaacagcaagcctctcaccgtggactccatccagatcttc
 I  I  E  V  D  G  V  N  S  K  P  L  T  V  D  S  I  Q  I  F
gctgcgcagcgctactccttcgttttgaacgcaaatcgacctgtgggcaactactggatc
 A  A  Q  R  Y  S  F  V  L  N  A  N  R  P  V  G  N  Y  W  I
cgtgcaaaccccaacttcggcacgaccgggttcgcgggcgggatcaactccgccatcctg
 R  A  N  P  N  F  G  T  T  G  F  A  G  G  I  N  S  A  I  L
cgctacaagggagccccggctattgagccgacgaccactcaaacaccctccgtcaaccca
 R  Y  K  G  A  P  A  I  E  P  T  T  T  Q  T  P  S  V  N  P
ctcgttgagacgaacctccacccactcacacgcacgccggtgcctggtcgtcccacaccc
 L  V  E  T  N  L  H  P  L  T  R  T  P  V  P  G  R  P  T  P
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 G  G  V  D  K  A  I  N  L  A  F  N  F  N  G  S  N  F  F  I
aacgacgcgacgttcacaccacccagcgtccccgtgctgctccaaattctgagcggtgcg
 N  D  A  T  F  T  P  P  S  V  P  V  L  L  Q  I  L  S  G  A
cagaccgctcaggacctcctgcccccgggctccgtctaccctctcccgggccactcctcc
 Q  T  A  Q  D  L  L  P  P  G  S  V  Y  P  L  P  G  H  S  S
attgagctcacgctgcccgcgacggcgatggccccaggcgcgccccacccctttcacctg
 I  E  L  T  L  P  A  T  A  M  A  P  G  A  P  H  P  F  H  L
catggtcacgtcttcgccgtcgtccgcagcgcgggcagcaccgagtacaactacaacgac
 H  G  H  V  F  A  V  V  R  S  A  G  S  T  E  Y  N  Y  N  D
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 P  I  F  R  D  V  V  N  T  G  T  P  A  A  N  D  N  V  T  I
cgcttccggacggacaaccccggcccgtggttcctccactgccacatcgacttccacctc
 R  F  R  T  D  N  P  G  P  W  F  L  H  C  H  I  D  F  H  L
gaggccgggttcgcggtcgtgttcgcggaggacgtgccgcgcgtcgcgctcgcgaacccc
 E  A  G  F  A  V  V  F  A  E  D  V  P  R  V  A  L  A  N  P
gtgccgaaggcgtggtcggacttgtgcccgatttacgacaggctcccggaggacgaccag
 V  P  K  A  W  S  D  L  C  P  I  Y  D  R  L  P  E  D  D  Q
                              tga
                               -
```

Figure 3.14 : Deduced aminoacid sequence of *Cplcc2* cDNA.

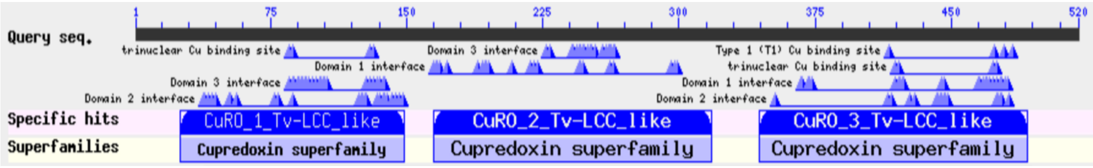

Figure 3.15 : Putative conserved domains found in CpLcc2 laccase.

Table 3.5 : The deduced aminoacid sequence of *Cplcc2*, compared with previously reported laccases (Rest of the table is presented in Appendix A5).

Accession Number	Description	Score	Query Coverage	Maximum Identity
ADE44157.1	laccase [Trametes velutina]	937	100%	86%
AFM31222.1	laccase [Trametes versicolor]	935	99%	86%
XP_008032737.1	laccase I [Trametes versicolor FP-101664 SS1]	934	99%	86%
AMT85330.1	laccase [Trametes versicolor]	933	99%	86%
Q99046.1	RecName: Full=Laccase-2; AltName: Full=Benzenediol:oxygen oxidoreductase 2; AltName: Full=Diphenol oxidase 2; AltName: Full=Urishiol oxidase 2; Flags: Precursor	932	99%	86%
AMT85329.1	laccase [Trametes versicolor]	931	99%	86%
AAC49828.1	laccase I [Trametes versicolor]	931	99%	86%
AAW29420.1	laccase 1 [Trametes versicolor]	931	99%	86%
ALT22025.1	laccase B [Trametes ochracea]	931	99%	86%
Q12718.1	RecName: Full=Laccase-2; AltName: Full=Benzenediol:oxygen oxidoreductase 2; AltName: Full=Diphenol oxidase 2; AltName: Full=Laccase I; AltName: Full=Urishiol oxidase 2; Flags: Precursor	928	99%	86%
AMT85333.1	laccase [Trametes versicolor]	925	99%	85%
ACK77785.1	laccase protein [Trametes versicolor]	922	99%	85%
AAL00887.1	laccase 1 [Trametes versicolor]	920	99%	85%
BAD98306.1	laccase2 [Trametes versicolor]	919	99%	85%
AAQ12268.1	laccase [Trametes sp. I-62]	916	99%	85%
AAQ12267.1	laccase [Trametes sp. I-62]	915	99%	85%
1GYC_A	Chain A, Crystal Structure Determination At Room Temperature Of A Laccase From Trametes Versicolor In Its Oxidised Form Containing A Full Complement Of Copper Ions	910	95%	87%
AJP70335.1	laccase I [synthetic construct]	909	98%	85%
AIZ72727.1	laccase B [Trametes hirsuta]	895	98%	83%
AAB63443.1	phenoloxidase [Trametes sp. I-62]	892	99%	84%
AMT85331.1	laccase [Trametes versicolor]	887	99%	82%
ADK13091.1	laccase [Trametes gibbosa]	874	100%	81%
AEQ28164.1	laccase 2 [Lenzites gibbosa]	870	100%	82%

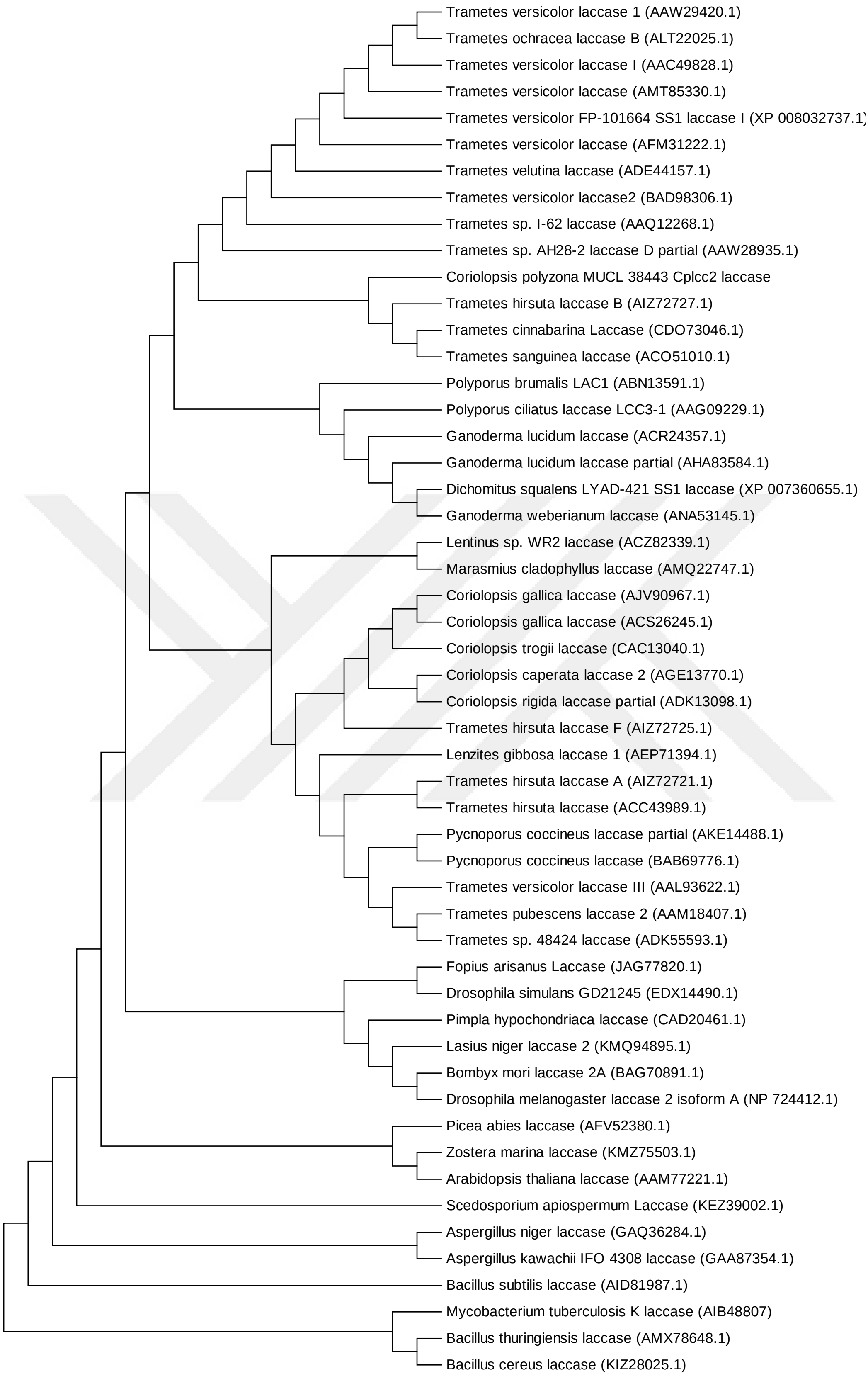

Figure 3.16 : Neighbor-joining tree of the deduced amino acid sequences of *Coriolopsis polyzona* MUCL 38443 CpLcc2 laccase with other laccases from GenBank.

3.3.2 Amplification of genomic DNA encoding the laccase CpLcc2 from *Coriolopsis polyzona* MUCL 38443

PCR reaction was set up with *Cplcc2* Forward and *Cplcc2* Reverse primers by using *Coriolopsis polyzona* MUCL 38443 genomic DNA as template. and a fragment approximately 2200 bp in length was amplified (Figure 3.17). Purified fragment was cloned into pGEM®-T Easy TA cloning vector and transformed chemically competent E.coli DH5α cells. Transformants were selected on LB plates containing Ampicillin and Xgal-IPTG with Blue/White screening method. After plasmid isolation from white colonies, restriction analysis with *Eco*RI was performed (Figure 3.18). Subsequently, plasmid DNAs containing the inserts were sequenced.

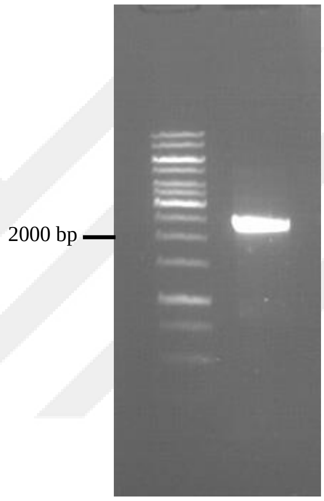

Figure 3.17 : *Cplcc2* gDNA amplified by PCR. **Marker DNA:** GeneRuler 1 kb DNA Ladder Marker (Thermo Scientific) (Lane 1).

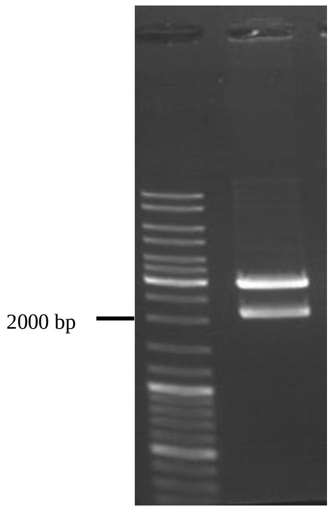

Figure 3.18 : *Eco*RI digestion of Plasmid carrying *Cplcc2* gDNA fragment **Marker DNA:** GeneRuler™ DNA Ladder Mix Marker (Thermo Scientific)(Lane 1).

Firstly, deduced sequence of *Cplcc2* gene aligned with previously identified laccases in the literature. *Cplcc2* gene shows %73 identity with laccase gene from *Trametes villosa* (clone LCC2) (L49377.1), 72% identity with laccase I (lccI) gene from *Trametes versicolor* (U44851.1), 71% identity with laccase 2 gene of *Lenzites gibbosa* strain CB-1 (JF906787.1) and laccase D (lacD) gene of *Trametes* sp. AH28-2 (AY839938.1) (Table 3.6).

Cplcc2 gene is 2145 bp in length and has an open reading frame of 1563 bp with 59% GC content. The coding sequence of *Cplcc2* is interrupted by 10 introns (Figure 3.19). The length of introns vary between 52 to 77 base pairs and similar to other fungal laccase genes intron number in the literature. Generally, open reading frames of fungal laccases are interrupted by 8-13 introns of around 50-90 bp in length. Usually splicing junctions adhere to the 5'- GT….AG-3' rule (Galhaup et al., 2002). *Trametes pubescens* lap2 gene and *Trametes villosa* lcc1 gene contain 8 introns (Galhaup et al., 2002; Yaver et al. 1996), *Trametes villosa* lcc2 gene and lcc2 gene of *Pycnoporus cinnabarinus* contain 10 introns (Temp et al., 1999; Yaver et al., 1996), *Coprinopsis cinerea* lac3 gene have 13 introns (Billette et al., 2011).

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ATGGTTGGCTTGCAGCGTTTCGGATTGTTTGTTACCCTTGCGCTCGTCTCTGGCACCCTTGGTGCTGTGGGGCCG
 M  V  G  L  Q  R  F  G  L  F  V  T  L  A  L  V  S  G  T  L  G  A  V  G  P
AGGGCTGACCTCGTCATTGCCAACGGCCCCGTGTCTCCCGACGGCTTCACGCGCGAGGCCATTACCGTCAACGGC
 R  A  D  L  V  I  A  N  G  P  V  S  P  D  G  F  T  R  E  A  I  T  V  N  G
GCCTTCCCGAACCCGCTCATCGTTGGCAAGAAGGGTccgcgacaagttctaaccgctacttttcagcactgaca
 A  F  P  N  P  L  I  V  G  K  K  G                     Intron I
ttctccgcttagggtGACCGCTTCCGGCTCAACGTGGTCGACAATCTAACCAATCACACTATGCTGAAGTCCACT
                D  R  F  R  L  N  V  V  D  N  L  T  N  H  T  M  L  K  S  T
AGCATCgtgagtgtcaattccgtcgaagagtatcggccggagctgatagcttgtataaagCATTGGCACGGCTTC
 S  I                 Intron II                               H  W  H  G  F
TTCCAGGCAGGCACCAACTGGGCAGACGGCCCCGCGTTCGTGAATCAGTGTCCCATCGCGTCCGGGCATTCGTTC
 F  Q  A  G  T  N  W  A  D  G  P  A  F  V  N  Q  C  P  I  A  S  G  H  S  F
TTGTACGACTTCCGTGTGCCGGACCAAGCCGGtgggtgaatccgtacgctgtccccaagcgacacgctgagatct
 L  Y  D  F  R  V  P  D  Q  A  G                     Intron III
gcatgtttcaacaggTACCTTCTGGTATCATAGTCATCTTTCGACCCAGTACTGCGACGGGCTGAGGGGGCCATT
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  V  V  Y  D  P  K  D  P  H  A  S  R  Y  D  V  D  D          Intron IV
tctacaaaggcgcatgctgattgtgatttcagAGAGCACGGTGATCACCCTTGCTGACTGGTACCACACCGCGGC
                                    E  S  T  V  I  T  L  A  D  W  Y  H  T  A  A
TCGCCTCGGACCCAGGTTCCCAtatgtccgctcgtccataattaataaaacatataatcatgaagctacccagaT
  R  L  G  P  R  F  P                        Intron V
TGGGGGCGGACTCTACGCTCATCAATGGTCTCGGTCGATCGTCCTCGACCCCTACAGCCCCTTTGGCCGTCATCA
L  G  A  D  S  T  L  I  N  G  L  G  R  S  S  S  T  P  T  A  P  L  A  V  I
ATGTGCAACGTGGGAAACGCcagtgcagctatactcgccgtctctcaccgatgctcattcctcccgggtcttgcc
N  V  Q  R  G  K  R                          Intron VI
cagcTATCGGTTCCGCCTCGTGTCGCTCTCATGCGACCCGAACCACGTGTTTAGTATCGACGGGCACAACATGAC
     Y  R  F  R  L  V  S  L  S  C  D  P  N  H  V  F  S  I  D  G  H  N  M  T
GATCATCGAGGTCGACGGTGTCAACAGCAAGCCTCTCACCGTGGACTCCATCCAGATCTTCGCTGCGCAGCGCTA
  I  I  E  V  D  G  V  N  S  K  P  L  T  V  D  S  I  Q  I  F  A  A  Q  R  Y
CTCCTTCGTTTTGgtacgtagctcacaccaatactgcgggctgctgacctcatgccactcgcagttgAACGCAAA
  S  F  V  L                        Intron VII                       N  A  N
TCGACCTGTGGGCAACTACTGGATCCGTGCAAACCCCAACTTCGGCACGACCGGGTTCGCGGGCGGGATCAACTC
  R  P  V  G  N  Y  W  I  R  A  N  P  N  F  G  T  T  G  F  A  G  G  I  N  S
CGCCATCCTGCGCTACAAGGGAGCCCCGGCTATTGAGCCGACGACCACTCAAACACCCTCCGTCAACCCACTCGT
  A  I  L  R  Y  K  G  A  P  A  I  E  P  T  T  T  Q  T  P  S  V  N  P  L  V
TGAGACGAACCTCCACCCACTCACACGCACGCCGGTggtacgtgcccagctgcgcagtgtaggttcaaatcacaa
  E  T  N  L  H  P  L  T  R  T  P  V                       Intron VIII
ctaaggcatattatgcatcttttGCCTGGTCGTCCCACACCCGGAGGCGTGGACAAGGCGATCAACCTCGCTTTC
                          P  G  R  P  T  P  G  G  V  D  K  A  I  N  L  A  F
AACTTTgtgagccagcaccctccttatcacctctccgtcgaatcttgacactcgtctctagtagAATGGGTCCAA
 N  F                   Intron IX                                N  G  S  N
CTTCTTCATCAACGACGCGACGTTCACACCACCCAGCGTCCCCGTGCTGCTCCAAATTCTGAGCGGTGCGCAGAC
  F  F  I  N  D  A  T  F  T  P  P  S  V  P  V  L  L  Q  I  L  S  G  A  Q  T
CGCTCAGGACCTCCTGCCCCCGGGCTCCGTCTACCCTCTCCCGGGCCACTCCTCCATTGAGCTCACGCTGCCCGC
  A  Q  D  L  L  P  P  G  S  V  Y  P  L  P  G  H  S  S  I  E  L  T  L  P  A
GACGGCGATGGCCCCAGGCGCGCCCCACCCCTTTCACCTGCATGGTgtacgtcaccgtcttctatctgctcacct
  T  A  M  A  P  G  A  P  H  P  F  H  L  H  G
ctactttctcatactgacccatcatcactcgccacccatcacccgcagCACGTCTTCGCCGTCGTCCGCAGCGCG
                 Intron X                          H  V  F  A  V  V  R  S  A
GGCAGCACCGAGTACAACTACAACGACCCGATCTTCCGCGACGTCGTGAACACGGGCACGCCCGCGGCCAACGAC
 G  S  T  E  Y  N  Y  N  D  P  I  F  R  D  V  V  N  T  G  T  P  A  A  N  D
AACGTCACGATCCGCTTCCGGACGGACAACCCCGGCCCGTGGTTCCTCCACTGCCACATCGACTTCCACCTCGAG
 N  V  T  I  R  F  R  T  D  N  P  G  P  W  F  L  H  C  H  I  D  F  H  L  E
GCCGGGTTCGCGGTCGTGTTCGCGGAGGACGTGCCGCGCGTCGCGCTCGCGAACCCCGTGCCGAAGGCGTGGTCG
 A  G  F  A  V  V  F  A  E  D  V  P  R  V  A  L  A  N  P  V  P  K  A  W  S
GACTTGTGCCCGATTTACGACAGGCTCCCGGAGGACGACCAGTGA
 D  L  C  P  I  Y  D  R  L  P  E  D  D  Q  -
```

Figure 3.19 : Nucleotide sequence and deduced amino acid sequence of the *Cplcc2* gene from *Coriolopsis polyzona* MUCL 38443. The 10 putative introns are indicated in lowercase type and numbered (I-X). Conserved ten histidine residues highlighted with red. LEA motif, C474 and F484 residues are also highlighted with green, yellow and pink respectively. Five possible N-glycosylation sites are bold and underlined. The putative signal peptide is shaded.

Table 3.6 : Sequences producing significant alignments with *Cplcc2* gene sequence (Rest of the table is presented in Appendix A5).

Accession Number	Description	Score	Query Coverage	Maximum Identity
L49377.1	Trametes villosa (clone LCC2) laccase gene, exons 1-11, complete cds	1122	99%	73%
U44851.1	Trametes versicolor laccase I (lccI) gene, complete cds	1070	98%	72%
JF906787.1	Lenzites gibbosa strain CB-1 laccase 2 gene, complete cds	987	96%	71%
AY839938.1	Trametes sp. AH28-2 laccase D (lacD) gene, partial cds	946	93%	71%
EU678778.1	Trametes versicolor strain CIRM-BRFM 75 laccase gene, partial cds	857	77%	73%
EU678777.1	Trametes suaveolens strain BRFM 13 laccase gene, partial cds	856	77%	73%
EU492907.1	Trametes hirsuta laccase (Lac) gene, complete cds	612	88%	68%
JF906786.1	Lenzites gibbosa strain CB-1 laccase 1 gene, complete cds	609	87%	68%
AF123571.1	Pycnoporus cinnabarinus laccase (lcc3-2) gene, complete cds	562	68%	71%
JF825479.1	Trametes sp. Ha1 laccase I precursor, gene, complete cds	560	89%	67%
DQ234060.1	Trametes sp. 420 laccase E (lacE) gene, complete cds	538	83%	68%
KU886230.1	Marasmius cladophyllus strain UMAS MS8 laccase (Lcc1) gene, complete cds	491	89%	67%
AY839936.1	Trametes sp. AH28-2 laccase A (lacA) gene, complete cds	488	74%	70%
AF388910.1	White-rot fungus AH28-2 laccase gene, partial sequence	486	73%	70%
M60560.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	479	72%	70%
U65399.1	Basidiomycete CECT 20197 phenoloxidase (pox1) gene, complete cds	471	89%	69%
Z12156.1	Basidiomycete gene for laccase	455	74%	70%
M60561.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	455	72%	69%
U65400.1	Basidiomycete CECT 20197 phenoloxidase (pox2) gene, complete cds	452	74%	70%
FJ858751.1	Pycnoporus sanguineus strain BRFM 902 laccase gene, complete cds	443	73%	69%
EU678776.1	Pycnoporus sanguineus strain CIRM-BRFM 906 laccase gene, partial cds	408	63%	69%
EU678773.1	Pycnoporus sanguineus strain CIRM-BRFM 902 laccase gene, partial cds	408	63%	69%
EU678768.1	Pycnoporus sanguineus strain CIRM-BRFM 896 laccase gene, partial cds	408	63%	69%

4. CONCLUSION

The aim of this research was to achieve isolation and characterization of the genomic DNA and and full length cDNA encoding the laccase CpLcc2 from white-rot fungus *Coriolopsis polyzona* MUCL 38443.

Cplcc2 partial cDNA was obtained from previous study in our research group. In the present study, based on its partial cDNA sequence, 5'- and 3'-ends of full length *Cplcc2* cDNA were determined by performing 5' and 3' RACE methods. Following this step, the full-length *Cplcc2* cDNA and the corresponding genomic DNA encoding the laccase CpLcc2 were successfully amplified from fungus *Coriolopsis polyzona* MUCL 38443 and then, characterized.

The corresponding open reading frame for full length *Cplcc2* cDNA from ATG start codon to TGA stop codon is 1563 bp coding for 520 amino acids with a putative 20-residue signal sequence. Additionally, the genomic DNA sequence of *Cplcc2* is 2145 bp in length and interrupted with 10 introns vary between 52 to 77 base pairs in length. The deduced amino acid sequence of CpLcc2 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 CpLcc2 that are crucial for the coordination of copper atoms on the active site of laccase. On the other hand, Cys 474 and Phe 484 residues are also found which are important for T1 copper domain and high redox potential laccase production, respectively. Theoretical molecular weight of CpLcc2 is calculated as 56.4366 kDa and theoretical isoelectric point was achieved as 6.33.

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 CpLcc2 laccase is located close to *Trametes* species.

As a consequence, isolated and characterized *Cplcc2* open reading frame encodes a novel laccase isozyme, may serve as a template for investigation of applicability in biotechnological and industrial area. Heterologous expression of *Cplcc2* in an industrial host organism, determination of optimized expression conditions, purification and characterization of recombinant laccase should be aimed for further studies.

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APPENDICES

APPENDIX A1. Compositions and Preparation of Culture Media

LSK medium (1000 ml)

D-glucose 50g

Bacteriological peptone 17 g,

KH_2PO_4 2.5 g

$MgSO_4.7H_2O$ 1.027 g

$CuSO_4.5H_2O$ 0.03127 g

$MSO_4.H_2O$ 0.0559 g

Components were dissolved in distilled water and sterilized by autoclaving at 121°C for 15 minutes 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

Distilled H_2O was added up to 1000ml and then autoclaved at 121°C for 15 minutes.

Luria Bertani (LB) Medium (1000ml)

Tryptone 10 g

Yeast Extract 5 g

NaCl 5 g

Dissolved in distilled H_2O and completed up to 1000ml and 15 g of agar was added for solid medium. Then autoclaved at 121°C for 15 minutes for sterilization.

2xYT Medium (1000ml)

Tryptone 16 g

Yeast Extract 10 g

NaCl 5 g

Dissolved in distilled H_2O and completed up to 1000ml and 15 g of agar was added for solid medium. Then autoclaved at 121°C for 15 minutes for sterilization.

APPENDIX A2. 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.

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

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

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

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

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

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

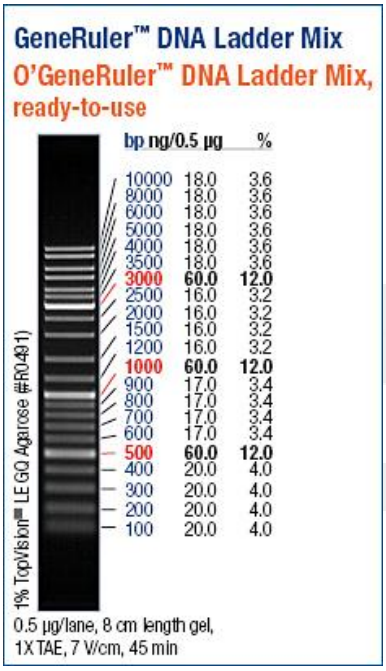
P3 Buffer: 294.5 g potassium acetate dissolved in 500 ml distilled water adn pH was adjusted to 5.5 by adding acetic acid.

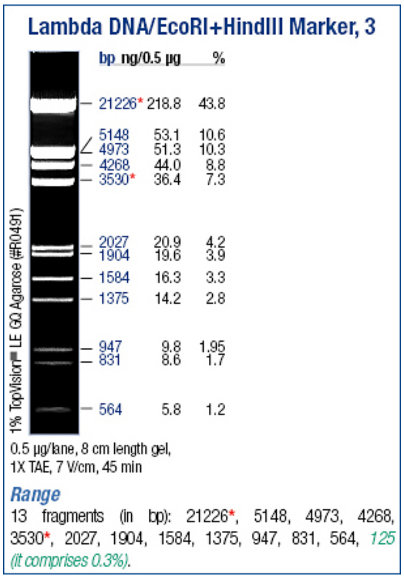
APPENDIX A3. Chemicals and Enzymes

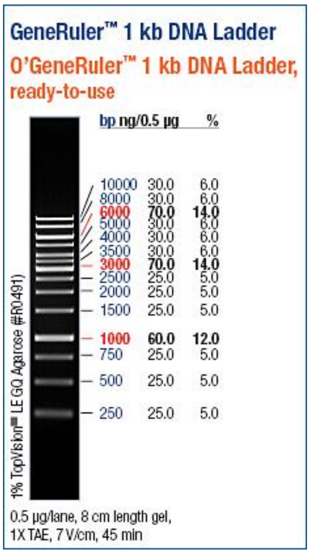
Chemicals	**Supplier**
6X Loading Dye	Fermentas
ABTS	Sigma-Aldrich (Germany)
Acetic acid	Merck (Germany)
Agar	Biolife
Agarose	Peqlab
B-Mercaptoethanol	Merck
Calcium chlorid ($CaCl_2$)	Merck
Copper sulfate pentahydrate	Merck
D-biotin	Fluka
Dextrose	Riedel-de Haën
DEPC	Merck
Dithiothreitol	Merck
EDTA	MP Bio
Formaldehyde	Riedel-deHaen (Germany)
Glycerol	Fluka
Hydrochloric acid	Merck
L- Alanine	Merck
L-Cysteine	Merck
MOPS	Sigma
Natruim hydroxid (NaOH)	Riedel-de Haën
Potassium acetate	Merck
RNase A	Sigma
SDS	BDH
Sodium acetate	J.T.Baker (USA).
Sodium chloride (NaCl)	Riedel-de Haën
Sodium dihydrogen phosphate	Riedel-de Haen
Sodium hydrogen phosphate($Na_2HPO_4.7H_2O$)	Merck
Sodium hydroxide	Riedel-de Haen
SYBR Green I	Lonza
Tris (hydrocymethyl) aminomethane	Merck
Tryptone	Sigma
Yeast Extract	Sigma

Enzymes	**Supplier**
*Eco*RI	Fermentas
Taq polymerase	Intron
Pfu polymerase	Fermentas
SeqAmp Polymerase Kit	Clontech
T4 DNA Ligase	Roche

Ladders







APPENDIX A4. Laboratory Equipments

Autoclave: TOMY SX-700E high pressure steam sterilizer

Centrifuge: Eppendorf, centrifuge 5424

Refrigerator: Bosch +4°C

Deep freeze: -80°C New Brunswick Scientific ultra-low temperature freezer, -20°C Bosch, -20°C Beko

Electrophoresis power supply: Thermo Electron Corporation EC1000-90

Balance: Precisa XB 620C

Water bath: Memmert wb-22

Vortex mixer: Heidolph Reax top

Microbiological Safety Cabinets: Faster BH-EN 2003 class-II

Orbital shaker: Sartorius Certomat SII

Thermomixer: Eppendorf, 1.5 ml thermomixer comfort

pH meter: Hanna Instruments, HI 221 Microprocessor pH meter

Thermal cycler: Labnet International, Multigene gradient TC9600-G-230V

Transilluminator: BIOLAB Laboratory Equipments

Micropipettes: Eppendorf research 10µl, 20µl, 200µl, 1000µl

APPENDIX A5. Tables

Table 3.4 : Laccase sequences producing significant alignments with *Cplcc2* cDNA sequence

Accession Number	Description	Score	Query Coverage	Maximum Identity
KR492186.1	Trametes versicolor isolate 7E laccase gene, complete cds	1293	98%	82%
JQ828930.1	Trametes versicolor strain NL-1 laccase (lccA) gene, complete cds	1293	98%	82%
KR492185.1	Trametes versicolor isolate 3A laccase gene, complete cds	1288	98%	82%
AY693776.1	Trametes versicolor laccase 1 (lcc1) mRNA, complete cds	1288	98%	82%
XM_008034546.1	Trametes versicolor FP-101664 SS1 laccase I (TvLac1), partial mRNA	1279	99%	82%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	1279	99%	82%
KR492189.1	Trametes versicolor isolate K4 laccase gene, complete cds	1271	98%	82%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	1271	98%	82%
GU738021.1	Trametes velutina strain 5930 laccase (lac5930-1) mRNA, complete cds	1267	98%	82%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	1267	99%	81%
KT211485.1	Trametes ochracea laccase B mRNA, complete cds	1264	96%	82%
KR492187.1	Trametes versicolor isolate 6C laccase gene, complete cds	1262	98%	82%
AB212732.1	Trametes versicolor lac2 mRNA for laccase2, complete cds	1249	98%	81%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	1042	95%	79%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	1037	95%	79%
JF817353.1	Lenzites gibbosa strain CB-1 laccase 2 mRNA, complete cds	1035	94%	79%
HM243485.1	Trametes gibbosa strain LS01 laccase (lac1) mRNA, complete cds	965	86%	80%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	835	90%	78%
KF836751.1	Lentinus sp. WR2 laccase (lcc4) mRNA, complete cds	804	93%	77%
KP027478.1	Trametes hirsuta laccase A mRNA, complete cds	754	91%	76%
KT211484.1	Trametes ochracea laccase A mRNA, complete cds	734	88%	77%
XM_008034423.1	Trametes versicolor FP-101664 SS1 laccase B precursor (TvLac3), partial mRNA	717	92%	76%
AB720032.1	Polyporus brumalis pblac1 mRNA for laccase, complete cds	715	87%	76%
EF362634.1	Polyporus brumalis LAC1 mRNA, complete cds	715	87%	76%

Table 3.4 (Continued): Laccase sequences producing significant alignments with *Cplcc2* cDNA sequence

Accession Number	Description	Score	Query Coverage	Maximum Identity
AF414109.1	Trametes versicolor laccase B precursor (lac1) mRNA, complete cds	704	91%	76%
HM483869.1	Trametes sp. 48424 laccase mRNA, complete cds	701	92%	76%
JF817354.1	Lenzites gibbosa strain CB-1 laccase 1 mRNA, complete cds	688	86%	76%
AB006824.1	Ganoderma tsunodae mRNA for bilirubin oxidase, complete cds	678	93%	75%
KC507935.1	Ganoderma lucidum strain CGMCC5.0026 laccase (lcc1) gene, partial cds	671	86%	76%
DQ914872.1	Ganoderma lucidum strain RZ laccase (lac4) mRNA, complete cds	660	86%	76%
Y18012.1	Trametes versicolor mRNA for laccase	656	88%	76%
XM_008038707.1	Trametes versicolor FP-101664 SS1 phenoloxidase (TvLac2), mRNA	654	88%	76%
AF176230.1	Polyporus ciliatus laccase (lcc3-1) mRNA, complete cds	641	87%	75%
KC119400.1	Coriolopsis caperata laccase 2 (Lac2) mRNA, complete cds	632	90%	75%
AF548034.1	Trametes sp. I-62 laccase (pox2) mRNA, complete cds	632	86%	75%
HM245961.1	Coriolopsis rigida strain CECT 20449 laccase (lcc1) mRNA, partial cds	619	87%	75%
AF491759.1	Trametes sp. C30 laccase 1 (lac1) mRNA, complete cds	614	92%	75%
KP135562.1	Coriolopsis gallica strain T906 laccase (Lac1) mRNA, complete cds	592	90%	75%
AY081775.2	Coriolus hirsutus laccase (072-1) mRNA, complete cds	592	91%	75%
KP135563.1	Coriolopsis gallica strain TCK laccase (Lac1) mRNA, complete cds	586	90%	75%
DQ431716.1	Coriolopsis gallica laccase (lac1) mRNA, complete cds	586	90%	75%
FJ598130.1	Coriolopsis gallica laccase mRNA, complete cds	571	88%	75%
AY875867.1	Coriolopsis gallica laccase (lacA) mRNA, partial cds	564	89%	74%
FJ817448.1	Trametes sp. C30 laccase 5 (lac5) mRNA, complete cds	562	92%	74%
AF548035.1	Trametes sp. I-62 laccase (pox3) mRNA, complete cds	560	88%	74%
AM419158.1	Panus tigrinus partial mRNA for laccase (lac1 gene)	555	75%	76%
AB212731.1	Trametes versicolor lac1 mRNA for laccase1, complete cds	555	91%	74%

Table 3.4 (Continued): Laccase sequences producing significant alignments with *Cplcc2* cDNA sequence

Accession Number	Description	Score	Query Coverage	Maximum Identity
KU055621.1	Coriolopsis trogii strain BAFC 463 laccase 1 (lcc1) mRNA, complete cds	542	90%	74%
FJ693715.1	Uncultured Lentinus clone SLF-WR2-L1 laccase 1 mRNA, complete cds	540	86%	74%
XM_007360593.1	Dichomitus squalens LYAD-421 SS1 laccase (DICSQDRAFT_176907), mRNA	525	89%	74%
KP027481.1	Trametes hirsuta laccase E mRNA, complete cds	518	86%	74%
FJ693716.1	Uncultured Lentinus clone SLF-WR2-L2 laccase 2 mRNA, complete cds	516	81%	74%
XM_007360563.1	Dichomitus squalens LYAD-421 SS1 laccase (DICSQDRAFT_142773), partial mRNA	497	91%	73%
KC507946.1	Ganoderma lucidum strain CGMCC5.0026 laccase (lcc12) gene, partial cds	440	58%	76%
FJ817449.1	Trametes sp. C30 laccase hybrid (lac131) mRNA, complete cds	440	92%	73%
AY081188.1	Trametes versicolor laccase III gene, complete cds	418	32%	82%
D13372.1	Coriolus versicolor CVL3 gene for laccase, complete cds	405	32%	81%
L49376.1	Trametes villosa (clone LCC1) laccase gene, exons 1-9, complete cds	403	33%	81%
AF176232.1	Polyporus ciliatus laccase (lcc3-3) mRNA, partial cds	394	83%	73%
XM_008037774.1	Trametes versicolor FP-101664 SS1 laccase-4 (TvLac4), partial mRNA	387	87%	72%
AB212733.1	Trametes versicolor lac3 mRNA for laccase3, complete cds	387	87%	72%
HM137002.1	Trametes versicolor strain njfu-2304 laccase (lcc2304) mRNA, partial cds	381	87%	72%
AF414807.1	Trametes pubescens laccase 2 (lap2) gene, complete cds	377	33%	80%
JF906787.1	Lenzites gibbosa strain CB-1 laccase 2 gene, complete cds	364	25%	91%
AM422387.1	Trametes versicolor mRNA for multicopper oxidase (klc2 gene)	355	87%	72%
KR492188.1	Trametes versicolor isolate 10E laccase gene, complete cds	331	18%	87%
U44851.1	Trametes versicolor laccase I (lccI) gene, complete cds	331	25%	88%
L49377.1	Trametes villosa (clone LCC2) laccase gene, exons 1-11, complete cds	331	25%	88%
FJ972711.1	Coriolopsis gallica laccase 2 (Lac2) mRNA, complete cds	313	83%	72%

Table 3.4 (Continued): Laccase sequences producing significant alignments with *Cplcc2* cDNA sequence

Accession Number	Description	Score	Query Coverage	Maximum Identity
HM569745.1	Ganoderma sp. En3 laccase mRNA, complete cds	311	92%	71%
AY450406.1	Flammulina velutipes laccase mRNA, complete cds	311	92%	71%
KC507939.1	Ganoderma lucidum strain CGMCC5.0026 laccase (lcc5) gene, partial cds	309	92%	71%
AM292415.1	Funalia trogii lcc2 gene for Laccase, strain 201	307	83%	72%
AF491761.1	Basidiomycete C30 laccase 2 (lac2) mRNA, complete cds	302	83%	72%
AY839936.1	Trametes sp. AH28-2 laccase A (lacA) gene, complete cds	300	27%	86%
AF388910.1	White-rot fungus AH28-2 laccase gene, partial sequence	300	27%	86%
M60560.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	294	17%	86%
M60561.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	289	17%	86%
KT211486.1	Trametes ochracea laccase C mRNA, complete cds	285	84%	71%
U65399.1	Basidiomycete CECT 20197 phenoloxidase (pox1) gene, complete cds	283	32%	87%
DQ914876.1	Ganoderma fornicatum strain 0814 laccase (lac1) mRNA, complete cds	272	30%	77%
AF162785.1	Basidiomycete C30 polyphenoloxidase (LAC1) gene, complete cds	268	18%	84%
JF825479.1	Trametes sp. Ha1 laccase I precursor, gene, complete cds	267	27%	84%
DQ431715.1	Coriolopsis gallica laccase (lac1) gene, complete cds	263	18%	83%
JF906786.1	Lenzites gibbosa strain CB-1 laccase 1 gene, complete cds	255	17%	84%
KC507936.1	Ganoderma lucidum strain CGMCC5.0026 laccase (lcc2) gene, partial cds	248	30%	76%
DQ914874.1	Ganoderma tsugae strain 1109 laccase (lac1) mRNA, complete cds	248	30%	76%
KU156698.1	Ganoderma weberianum isolate tzc-1 laccase gene, complete cds	246	16%	84%
Z12156.1	Basidiomycete gene for laccase	246	18%	82%
EU678778.1	Trametes versicolor strain CIRM-BRFM 75 laccase gene, partial cds	244	19%	92%
EU678777.1	Trametes suaveolens strain BRFM 13 laccase gene, partial cds	244	19%	92%
GQ377839.1	Coriolopsis rigida laccase (lcc1) gene, partial cds	243	16%	84%
AY485829.1	Ganoderma lucidum laccase mRNA, complete cds	243	31%	76%

Table 3.4 (Continued): Laccase sequences producing significant alignments with *Cplcc2* cDNA sequence

Accession Number	Description	Score	Query Coverage	Maximum Identity
KT166425.1	Ganoderma tsugae isolate Gps1 laccase mRNA, complete cds	237	30%	76%
AJ294820.1	Trametes trogii lcc1 gene for laccase, exons 1-11	237	18%	82%
U65400.1	Basidiomycete CECT 20197 phenoloxidase (pox2) gene, complete cds	233	17%	83%
FJ465129.1	Trametes versicolor strain 1.028 laccase-like protein mRNA, complete cds, alternatively spliced	230	19%	80%
DQ914875.1	Ganoderma fornicatum strain 0814 laccase (lac1) gene, complete cds	213	17%	81%
DQ914871.1	Ganoderma lucidum strain RZ laccase (lac4) gene, complete cds	213	16%	81%
AY485826.1	Flammulina velutipes laccase gene, complete cds	207	18%	80%

Table 3.5 : The deduced aminoacid sequence of *Cplcc2*, compared with previously reported laccases.

Accession Number	Description	Score	Query Coverage	Maximum Identity
ADE44157.1	laccase [Trametes velutina]	937	100%	86%
AFM31222.1	laccase [Trametes versicolor]	935	99%	86%
XP_008032737.1	laccase I [Trametes versicolor FP-101664 SS1]	934	99%	86%
AMT85330.1	laccase [Trametes versicolor]	933	99%	86%
Q99046.1	RecName: Full=Laccase-2; AltName: Full=Benzenediol:oxygen oxidoreductase 2; AltName: Full=Diphenol oxidase 2; AltName: Full=Urishiol oxidase 2; Flags: Precursor	932	99%	86%
AMT85329.1	laccase [Trametes versicolor]	931	99%	86%
AAC49828.1	laccase I [Trametes versicolor]	931	99%	86%
AAW29420.1	laccase 1 [Trametes versicolor]	931	99%	86%
ALT22025.1	laccase B [Trametes ochracea]	931	99%	86%
Q12718.1	RecName: Full=Laccase-2; AltName: Full=Benzenediol:oxygen oxidoreductase 2; AltName: Full=Diphenol oxidase 2; AltName: Full=Laccase I; AltName: Full=Urishiol oxidase 2; Flags: Precursor	928	99%	86%
AMT85333.1	laccase [Trametes versicolor]	925	99%	85%
ACK77785.1	laccase protein [Trametes versicolor]	922	99%	85%
AAL00887.1	laccase 1 [Trametes versicolor]	920	99%	85%
BAD98306.1	laccase2 [Trametes versicolor]	919	99%	85%
AAQ12268.1	laccase [Trametes sp. I-62]	916	99%	85%
AAQ12267.1	laccase [Trametes sp. I-62]	915	99%	85%

Table 3.5 (Continued) : The deduced aminoacid sequence of *Cplcc2*, compared with previously reported laccases.

Accession Number	Description	Score	Query Coverage	Maximum Identity
1GYC_A	Chain A, Crystal Structure Determination At Room Temperature Of A Laccase From Trametes Versicolor In Its Oxidised Form Containing A Full Complement Of Copper Ions	910	95%	87%
AJP70335.1	laccase I [synthetic construct]	909	98%	85%
AIZ72727.1	laccase B [Trametes hirsuta]	895	98%	83%
AAB63443.1	phenoloxidase [Trametes sp. I-62]	892	99%	84%
AMT85331.1	laccase [Trametes versicolor]	887	99%	82%
ADK13091.1	laccase [Trametes gibbosa]	874	100%	81%
AEQ28164.1	laccase 2 [Lenzites gibbosa]	870	100%	82%
AEP71395.1	laccase 2 [Lenzites gibbosa]	853	100%	81%
BAA28668.1	bilirubin oxidase [Ganoderma tsunodae]	833	100%	78%
3X1B_A	Chain A, Crystal Structure Of Laccase From Lentinus Sp. At 1.8 A Resolution	833	100%	78%
ACN69056.1	multicopper redoxase [Trametes sanguinea]	822	99%	76%
AAB63444.1	phenoloxidase [Trametes sp. I-62]	819	95%	78%
AIZ72721.1	laccase A [Trametes hirsuta]	819	100%	78%
AAM18407.1	laccase 2 [Trametes pubescens]	819	100%	77%
CDO73046.1	Laccase [Trametes cinnabarina]	818	99%	76%
AHA83584.1	laccase [Ganoderma lucidum]	817	100%	74%
ALT22024.1	laccase A [Trametes ochracea]	816	100%	76%
AAQ12269.1	laccase [Trametes sp. I-62]	816	95%	78%
AKE14488.1	laccase [Pycnoporus coccineus]	816	98%	76%
AAG17009.2	laccase [Ganoderma lucidum]	815	100%	74%
ACZ82339.1	laccase [Lentinus sp. WR2]	814	100%	74%
BAB69776.1	laccase [Pycnoporus coccineus]	813	99%	76%
ACR24357.1	laccase [Ganoderma lucidum]	813	100%	74%
BAB69775.1	laccase [Pycnoporus coccineus]	813	99%	76%
ADK55593.1	laccase [Trametes sp. 48424]	812	98%	77%
AHA83586.1	laccase [Ganoderma lucidum]	811	100%	74%
AER26914.1	laccase [synthetic construct]	811	95%	79%
AAB63445.1	phenoloxidase [Trametes sp. I-62]	810	98%	76%
ACC43989.1	laccase [Trametes hirsuta]	810	100%	77%
AAW28935.1	laccase D [Trametes sp. AH28-2]	810	88%	85%
AKE14489.1	laccase [Pycnoporus coccineus]	808	98%	76%
XP_007360655.1	laccase [Dichomitus squalens LYAD-421 SS1]	808	98%	75%
AAQ12270.1	laccase [Trametes sp. I-62]	808	98%	76%
ABN13591.1	LAC1 [Polyporus brumalis]	807	100%	76%
ANA53145.1	laccase [Ganoderma weberianum]	807	99%	73%
AMQ22747.1	laccase [Marasmius cladophyllus]	807	100%	74%
AAF13052.1	laccase [Trametes cinnabarina]	807	99%	75%
O59896.1	RecName: Full=Laccase; AltName: Full=Benzenediol:oxygen oxidoreductase; AltName: Full=Diphenol oxidase; AltName: Full=Ligninolytic phenoloxidase; AltName: Full=Urishiol oxidase; Flags: Precursor	806	99%	76%

Table 3.5 (Continued) : The deduced aminoacid sequence of *Cplcc2*, compared with previously reported laccases.

Accession Number	Description	Score	Query Coverage	Maximum Identity
XP_008036898.1	phenoloxidase [Trametes versicolor FP-101664 SS1]	805	100%	74%
AAN71597.1	laccase [Trametes cinnabarina]	805	99%	76%
XP_007369227.1	laccase [Dichomitus squalens LYAD-421 SS1]	804	98%	75%
CAC13040.1	laccase [Coriolopsis trogii]	804	100%	74%
XP_007360625.1	laccase [Dichomitus squalens LYAD-421 SS1]	803	98%	75%
XP_008032614.1	laccase B precursor [Trametes versicolor FP-101664 SS1]	802	98%	78%
3FPX_A	Chain A, Native Fungus Laccase From Trametes Hirsuta	801	95%	79%
3PXL_A	Chain A, Type-2 Cu-Depleted Fungus Laccase From Trametes Hirsuta	801	95%	79%
AJV90967.1	laccase [Coriolopsis gallica]	800	100%	74%
AJV90966.1	laccase [Coriolopsis gallica]	800	100%	74%
2XYB_A	Chain A, Crystal Structure Of A Fully Functional Laccase From The Ligninolytic Fungus Pycnoporus Cinnabarinus	799	95%	77%
2QT6_A	Chain A, Crystal Structure Determination Of A Blue Laccase From Lentinus Tigrinus	798	95%	76%
ACO51010.1	laccase [Trametes sanguinea]	798	99%	77%
ABD93940.1	laccase [Coriolopsis gallica]	797	100%	74%
Q02497.1	RecName: Full=Laccase; AltName: Full=Benzenediol:oxygen oxidoreductase; AltName: Full=Diphenol oxidase; AltName: Full=Ligninolytic phenoloxidase; AltName: Full=Urishiol oxidase; Flags: Precursor	796	95%	80%
AHA83595.1	laccase [Ganoderma lucidum]	796	100%	74%
AEB97397.1	laccase [Trametes sanguinea]	796	99%	77%
AEP71394.1	laccase 1 [Lenzites gibbosa]	796	100%	77%
AGE13770.1	laccase 2 [Coriolopsis caperata]	795	100%	74%
D0VWU3.1	RecName: Full=Laccase; AltName: Full=Benzenediol:oxygen oxidoreductase; AltName: Full=Diphenol oxidase; AltName: Full=Urishiol oxidase	795	95%	79%
ACS26245.1	laccase [Coriolopsis gallica]	794	100%	74%
ACZ58365.1	laccase 1 [uncultured Lentinus]	794	100%	75%
AAW28933.1	laccase A [Trametes sp. AH28-2]	793	95%	80%
AAA33104.1	ligninolytic phenoloxidase [Trametes hirsuta]	791	95%	80%
AAL93622.1	laccase III [Trametes versicolor]	791	98%	78%
ABK59823.1	laccase [Ganoderma lucidum]	790	100%	73%

Table 3.5 (Continued) : The deduced aminoacid sequence of *Cplcc2*, compared with previously reported laccases.

Accession Number	Description	Score	Query Coverage	Maximum Identity
AAR20864.1	laccase [Trametes sanguinea]	789	99%	76%
AAD49218.1	laccase [Trametes cinnabarina]	789	99%	76%
3DIV_A	Chain A, Crystal Structure Of Laccase From Cerrena Maxima At 1.76a Resolution	789	95%	78%
2HRG_A	Chain A, Crystal Structure Of Blue Laccase From Trametes Trogii Complexed With P-Methylbenzoate	788	95%	76%
ACZ37083.1	laccase [Trametes sanguinea]	788	99%	75%
ABK59822.1	laccase [Ganoderma lucidum]	787	100%	73%
BAA22153.1	laccase [Trametes versicolor]	787	98%	77%
Q99044.1	RecName: Full=Laccase-1; AltName: Full=Benzenediol:oxygen oxidoreductase 1; AltName: Full=Diphenol oxidase 1; AltName: Full=Urishiol oxidase 1; Flags: Precursor	786	98%	77%
AEO20225.1	laccase I precursor [Trametes sp. Ha1]	786	95%	80%
CAA77015.1	laccase [Trametes versicolor]	786	98%	78%
AAL07440.1	laccase B precursor [Trametes versicolor]	786	98%	77%
4A2D_ A	Chain A, Coriolopsis Gallica Laccase T2 Copper Depleted At Ph 4.5	786	95%	76%
4A2F_ A	Chain A, Coriolopsis Gallica Laccase Collected At 12.65 Kev	786	95%	76%
AAG09229.1	laccase LCC3-1 [Polyporus ciliatus]	783	100%	75%
ADK13098.1	laccase [Coriolopsis rigida]	783	95%	76%
ACU29545.1	laccase [Coriolopsis rigida]	783	95%	76%
2H5U_A	Chain A, Crystal Structure Of Laccase From Cerrena Maxima At 1.9a Resolution	782	95%	78%
AIZ72725.1	laccase F [Trametes hirsuta]	781	100%	72%
1KYA_ A	Chain A, Active Laccase From Trametes Versicolor Complexed With 2,5-Xylidine	781	95%	79%
AAF06967.1	polyphenoloxidase [Trametes sp. C30]	780	100%	74%

Table 3.6 : Sequences producing significant alignments with *Cplcc2* gene sequence.

Accession Number	Description	Score	Query Coverage	Maximum Identity
L49377.1	Trametes villosa (clone LCC2) laccase gene, exons 1-11, complete cds	1122	99%	73%
U44851.1	Trametes versicolor laccase I (lccI) gene, complete cds	1070	98%	72%
JF906787.1	Lenzites gibbosa strain CB-1 laccase 2 gene, complete cds	987	96%	71%
AY839938.1	Trametes sp. AH28-2 laccase D (lacD) gene, partial cds	946	93%	71%
EU678778.1	Trametes versicolor strain CIRM-BRFM 75 laccase gene, partial cds	857	77%	73%
EU678777.1	Trametes suaveolens strain BRFM 13 laccase gene, partial cds	856	77%	73%
EU492907.1	Trametes hirsuta laccase (Lac) gene, complete cds	612	88%	68%
JF906786.1	Lenzites gibbosa strain CB-1 laccase 1 gene, complete cds	609	87%	68%
AF123571.1	Pycnoporus cinnabarinus laccase (lcc3-2) gene, complete cds	562	68%	71%
JF825479.1	Trametes sp. Ha1 laccase I precursor, gene, complete cds	560	89%	67%
DQ234060.1	Trametes sp. 420 laccase E (lacE) gene, complete cds	538	83%	68%
KU886230.1	Marasmius cladophyllus strain UMAS MS8 laccase (Lcc1) gene, complete cds	491	89%	67%
AY839936.1	Trametes sp. AH28-2 laccase A (lacA) gene, complete cds	488	74%	70%
AF388910.1	White-rot fungus AH28-2 laccase gene, partial sequence	486	73%	70%
M60560.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	479	72%	70%
U65399.1	Basidiomycete CECT 20197 phenoloxidase (pox1) gene, complete cds	471	89%	69%
Z12156.1	Basidiomycete gene for laccase	455	74%	70%
M60561.1	C.hirsutus ligninolytic phenoloxidase gene, complete cds	455	72%	69%
U65400.1	Basidiomycete CECT 20197 phenoloxidase (pox2) gene, complete cds	452	74%	70%
FJ858751.1	Pycnoporus sanguineus strain BRFM 902 laccase gene, complete cds	443	73%	69%
EU678776.1	Pycnoporus sanguineus strain CIRM-BRFM 906 laccase gene, partial cds	408	63%	69%
EU678773.1	Pycnoporus sanguineus strain CIRM-BRFM 902 laccase gene, partial cds	408	63%	69%
EU678768.1	Pycnoporus sanguineus strain CIRM-BRFM 896 laccase gene, partial cds	408	63%	69%

Table 3.6 (Continued): Sequences producing significant alignments with *Cplcc2* gene sequence.

Accession Number	Description	Score	Query Coverage	Maximum Identity
EU678779.1	Pycnoporus sanguineus strain CIRM-BRFM 893 laccase gene, partial cds	405	63%	69%
AY510604.1	Pycnoporus sanguineus laccase gene, complete cds	405	81%	76%
AF025481.1	Pycnoporus cinnabarinus laccase (lcc3-1) gene, complete cds	401	73%	69%
FJ858750.1	Pycnoporus coccineus strain BRFM 938 laccase gene, complete cds	399	73%	69%
AY147188.1	Pycnoporus cinnabarinus laccase (Lcc1) gene, complete cds	398	73%	69%
HM106997.1	Pycnoporus sanguineus strain MUCL 38531 laccase (lcc1) gene, complete cds	394	90%	75%
KU156698.1	Ganoderma weberianum isolate tzc-1 laccase gene, complete cds	392	75%	76%
AF162785.1	Basidiomycete C30 polyphenoloxidase (LAC1) gene, complete cds	390	62%	76%
EU684158.1	Pycnoporus sanguineus strain MUCL 29375 laccase gene, partial cds	387	62%	69%
FJ232700.1	Pycnoporus sanguineus strain CIRM-BRFM 942 laccase gene, partial cds	385	63%	69%
GQ377839.1	Coriolopsis rigida laccase (lcc1) gene, partial cds	381	61%	76%
DQ431715.1	Coriolopsis gallica laccase (lac1) gene, complete cds	379	62%	76%
FJ232701.1	Pycnoporus sanguineus strain CIRM-BRFM 943 laccase gene, partial cds	378	63%	69%
EU714502.1	Pycnoporus sanguineus strain CIRM-BRFM 979 laccase gene, partial cds	378	63%	69%
EU678780.1	Pycnoporus sanguineus strain CIRM-BRFM 897 laccase gene, partial cds	378	63%	69%
EU678770.1	Pycnoporus sanguineus strain CIRM-BRFM 899 laccase gene, partial cds	378	63%	69%
EU683254.1	Pycnoporus coccineus strain MUCL 38523 laccase gene, partial cds	376	63%	69%
EU714501.1	Pycnoporus coccineus strain MUCL 38525 laccase gene, partial cds	374	63%	69%
EU678767.1	Pycnoporus sanguineus strain CIRM-BRFM 895 laccase gene, partial cds	372	63%	69%
EU684160.1	Pycnoporus cinnabarinus strain MUCL 38420 laccase gene, partial cds	370	63%	69%

Table 3.6 (Continued): Sequences producing significant alignments with *Cplcc2* gene sequence.

Accession Number	Description	Score	Query Coverage	Maximum Identity
EU678771.1	Pycnoporus sanguineus strain CIRM-BRFM 900 laccase gene, partial cds	369	63%	69%
EU678766.1	Pycnoporus sanguineus strain CIRM-BRFM 892 laccase gene, partial cds	369	63%	69%
EU678782.1	Pycnoporus sanguineus strain CIRM-BRFM 905 laccase gene, partial cds	363	63%	69%
EU678781.1	Pycnoporus sanguineus strain BRFM 898 laccase gene, partial cds	363	63%	69%
EU678774.1	Pycnoporus sanguineus strain CIRM-BRFM 903 laccase gene, partial cds	363	63%	69%
EU714503.1	Pycnoporus sanguineus strain CIRM-BRFM 981 laccase gene, partial cds	361	63%	68%
DQ914871.1	Ganoderma lucidum strain RZ laccase (lac4) gene, complete cds	361	66%	75%
EU684161.1	Pycnoporus sanguineus strain CIRM-BRFM 980 laccase gene, partial cds	360	62%	68%
EU678769.1	Pycnoporus sanguineus strain BRFM 894 laccase gene, partial cds	360	63%	68%
KT211485.1	Trametes ochracea laccase B mRNA, complete cds	356	63%	87%
XM_008034546.1	Trametes versicolor FP-101664 SS1 laccase I (TvLac1), partial mRNA	356	63%	87%
FJ473385.2	Ganoderma lucidum strain TR6 laccase gene, complete cds	352	76%	74%
AF185275.2	Ganoderma lucidum strain 7071-9 laccase gene, complete cds	352	76%	74%
KR492189.1	Trametes versicolor isolate K4 laccase gene, complete cds	351	63%	86%
JQ828930.1	Trametes versicolor strain NL-1 laccase (lccA) gene, complete cds	351	65%	86%
GU738021.1	Trametes velutina strain 5930 laccase (lac5930-1) mRNA, complete cds	351	72%	86%
U44430.1	Trametes versicolor laccase I (lccI) mRNA, complete cds	351	63%	86%
AB212732.1	Trametes versicolor lac2 mRNA for laccase2, complete cds	349	66%	86%
AY693776.1	Trametes versicolor laccase 1 (lcc1) mRNA, complete cds	349	66%	86%
AY049725.1	Trametes versicolor laccase 1 (lac1) mRNA, complete cds	347	59%	86%
AF170093.1	Pycnoporus cinnabarinus laccase (Lac1) gene, complete cds	343	67%	67%
FJ469151.1	Trametes versicolor laccase protein mRNA, complete cds	340	66%	86%
KR492187.1	Trametes versicolor isolate 6C laccase gene, complete cds	338	63%	85%

Table 3.6 (Continued): Sequences producing significant alignments with *Cplcc2* gene sequence.

Accession Number	Description	Score	Query Coverage	Maximum Identity
KR492186.1	Trametes versicolor isolate 7E laccase gene, complete cds	338	63%	85%
KR492188.1	Trametes versicolor isolate 10E laccase gene, complete cds	333	54%	85%
KR492185.1	Trametes versicolor isolate 3A laccase gene, complete cds	333	63%	85%
AJ294820.1	Trametes trogii lcc1 gene for laccase, exons 1-11	333	62%	74%
KF836751.1	Lentinus sp. WR2 laccase (lcc4) mRNA, complete cds	331	47%	86%
DQ914875.1	Ganoderma fornicatum strain 0814 laccase (lac1) gene, complete cds	331	54%	74%
HM483869.1	Trametes sp. 48424 laccase mRNA, complete cds	329	40%	85%
KT211484.1	Trametes ochracea laccase A mRNA, complete cds	327	42%	86%
XM_008034423.1	Trametes versicolor FP-101664 SS1 laccase B precursor (TvLac3), partial mRNA	325	42%	85%
AF414807.1	Trametes pubescens laccase 2 (lap2) gene, complete cds	325	75%	86%
AY081188.1	Trametes versicolor laccase III gene, complete cds	325	73%	86%
L49376.1	Trametes villosa (clone LCC1) laccase gene, exons 1-9, complete cds	325	74%	85%
AY485826.1	Flammulina velutipes laccase gene, complete cds	324	55%	73%
AF414109.1	Trametes versicolor laccase B precursor (lac1) mRNA, complete cds	322	42%	84%
D13372.1	Coriolus versicolor CVL3 gene for laccase, complete cds	320	51%	84%
AF548033.1	Trametes sp. I-62 laccase (pox1) mRNA, pox1-lcc1A allele, complete cds	316	65%	84%
AF548032.1	Trametes sp. I-62 laccase (pox1) mRNA, complete cds	316	65%	84%
JF817353.1	Lenzites gibbosa strain CB-1 laccase 2 mRNA, complete cds	313	56%	85%
HM243485.1	Trametes gibbosa strain LS01 laccase (lac1) mRNA, complete cds	313	54%	85%
FJ513077.1	Pycnoporus sanguineus laccase mRNA, complete cds	309	50%	84%
DQ914873.1	Ganoderma tsugae strain 1109 laccase (lac1) gene, complete cds	307	40%	72%
AB212731.1	Trametes versicolor lac1 mRNA for laccase1, complete cds	307	48%	83%
Y18012.1	Trametes versicolor mRNA for laccase	307	45%	83%
FJ598130.1	Coriolopsis gallica laccase mRNA, complete cds	304	44%	84%

Table 3.6 (Continued): Sequences producing significant alignments with *Cplcc2* gene sequence.

Accession Number	Description	Score	Query Coverage	Maximum Identity
AJ586520.2	Ganoderma sp. kk-02 partial gene for laccase	304	40%	72%
AY485825.1	Ganoderma lucidum laccase gene, complete cds	304	40%	72%
AB720032.1	Polyporus brumalis pblac1 mRNA for laccase, complete cds	300	56%	83%
EF362634.1	Polyporus brumalis LAC1 mRNA, complete cds	300	56%	83%
KT254242.1	Ganoderma tsugae strain 1511C0001ACCC51625 laccase gene, partial cds	298	40%	72%
KP135562.1	Coriolopsis gallica strain T906 laccase (Lac1) mRNA, complete cds	298	44%	83%
EU714500.1	Pycnoporus cinnabarinus strain CIRM-BRFM 945 laccase gene, partial cds	298	62%	67%
AB006824.1	Ganoderma tsunodae mRNA for bilirubin oxidase, complete cds	297	55%	83%
EU684157.1	Pycnoporus cinnabarinus strain BRFM 247 laccase gene, partial cds	293	62%	67%
AY875867.1	Coriolopsis gallica laccase (lacA) mRNA, partial cds	293	44%	83%

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