Heterologous expression and characterization of a high redox potential laccase from *Coriolopsis polyzona* MUCL 38443

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Abstract: In this study, a novel laccase gene, named as *Cplcc1*, and its corresponding cDNA were isolated and characterized from the *Coriolopsis polyzona* MUCL 38443 strain. The *Cplcc1* gene consists of a 1563-bp open reading frame encoding a protein of 520 amino acids with a 20-residue putative signal peptide. The size of the *Cplcc1* gene is 2106 bp and it contains ten introns and five potential N-glycosylation sites. Additionally, the isolated full-length *Cplcc1* cDNA was successfully expressed in *Pichia pastoris*. The heterologous expression conditions were also optimized and the highest activity value increased to 800 U L^{-1} with 1.5% methanol, 0.8 mM CuSO$_4$, and 0.6% L-alanine supplementation. The recombinant laccase was partially purified and the molecular weight was found as approximately 54 kDa. The maximum oxidation activity was observed for 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at pH 3.0. The optimal temperature was found as 70 °C. On the other hand, at 30 °C, the enzyme was stable for more than a week and its half-life was longer than 8 h. The K$_m$, V$_{max}$, k$_{cat}$, and k$_{cat}$ K$_m$$^{-1}$ values of the recombinant laccase were identified as 0.137 mM, 288.6 µmol min^{-1} L^{-1}, 5.73 × 10^5 min^{-1}, and 4.18 × 10^6 min^{-1} mM^{-1}, respectively. Sodium azide, L-cysteine, and SDS were found as usual inhibitors.

Key words: *Coriolopsis polyzona*, enzyme activity, heterologous expression, laccase, *Pichia pastoris*, purification

1. Introduction

Laccases are attracting proliferating interest due to their ability to degrade a wide range of substrates including aromatics amines, phenols, and recalcitrant compounds. These enzymes (EC 1.10.3.2) are multicopper-containing oxidases catalyzing monoelectronic oxidation of diverse substrates by reducing oxygen to water (Riva, 2006; Giardina et al., 2010; Chandra and Chowdhary, 2015; Wang et al., 2015). Owing to their broad substrate spectrum in biodegradation ability, laccases became desired biocatalysts in many industrial processes including diagnosis and bioremediation approaches and the detoxification of industrial effluents of paper, pulp, textile, and petrochemical industries. Laccases are also employed in the processing of beverages (wine, fruit juice, and beer), gelation of sugar beet pectin, baking, and the design of biosensors (Osma et al., 2010; Gassara-Chatti et al., 2013; Lanzellotto et al., 2014). The promising and valuable applications of laccases in many biotechnological processes have resulted in increased demand for isolation of new laccase genes from different sources (Giardina et al., 2010; Piscitelli et al., 2010).

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

Many laccase genes and cDNAs were cloned from different microorganisms and heterologously expressed in yeasts [*Saccharomyces cerevisiae* (Cassland and Jönsson, 1999), *Pichia pastoris* (O'Callaghan et al., 2002), *Pichia methalonica* (Guo et al., 2006), *Yarrowia lipolytica* (Madzak et al., 2005), and *Kluyveromyces lactis* (Pezzella et al., 2009)], filamentous fungi [*Trichoderma reesei* (Kiiskinen et al., 2004) and *Aspergillus niger* (Téllez-Jurado

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et al., 2006)], bacteria [*Escherichia coli* (Bento et al., 2005) and *Streptomyces lividans* (Dubé et al., 2008)], and plants [*Arabidopsis thaliana* (Wang et al., 2004) and *Oryza sativa* (de Wilde et al., 2008)]. Among those organisms, yeasts are usually more attractive hosts for heterologous protein production because of their faster microbial growth and ease of gene manipulation, along with the ability to perform posttranslational modifications. In particular, *Pichia pastoris*, a methylotrophic yeast, has become a very popular host organism for the production of heterologous proteins with a strong inducible alcohol oxidase 1 (*AOX1*) gene promoter, which results in increased protein bioactivity by reducing proteolytic degradation of the expressed recombinant protein (Macauley-Patrick et al., 2005; Antosova and Sychrova, 2016).

White-rot fungi are the best known organisms that can completely degrade lignin to carbon dioxide and water and can further degrade cellulose and hemicelluloses (Eriksson et al., 1990; ten Have and Teunissen, 2001). The basidiomycete *Coriolopsis polyzona* is a white-rot fungus strain known as a high-yielding ligninolytic enzyme producer (Eriksson et al., 1990; Jaouani et al., 2006). In this study, a novel laccase gene, named as *Cplcc1*, and its cDNA were isolated, sequenced, and characterized from *Coriolopsis polyzona* MUCL 38443. Subsequently, the corresponding *Coriolopsis polyzona* laccase gene was expressed in the yeast *Pichia pastoris* under the control of alcohol oxidase promoter *AOX1*. Catalytic properties of the recombinant laccase were next characterized to explore its suitability as a desirable industrial biocatalyst. This is the first report on the cloning and characterization of the laccase-encoding gene and the corresponding encoding laccase from the white-rot fungus strain *Coriolopsis polyzona* MUCL 38443.

2. Materials and methods

2.1. Organisms and culture conditions
The white-rot fungus *Coriolopsis polyzona* MUCL 38443 was obtained through EU-FP6 SMI-IP (IP: 505899). For the extraction of RNA, 1.0 mL of the spore suspension was added to 100 mL of LSK medium containing 50 g glucose (Merck) L^{-1}, 17 g bacteriological peptone (Acumedia) L^{-1}, 2.5 g KH_2PO_4 (Merck) L^{-1}, 1.027 g $MgSO_4.7H_2O$ (Merck) L^{-1}, 0.03127 g $CuSO_4.5H_2O$ (Merck) L^{-1}, 0.0559 g $MnSO_4.H_2O$ (Merck) L^{-1}, and 10 mg thiamine-HCl (Sigma) L^{-1} in 500-mL flasks. The culture was incubated for 10 days at 28 °C and 150 rpm. *Coriolopsis polyzona* mycelia were collected by filtration, washed with diethylpyrocarbonate (DEPC)-treated water, and immediately frozen in liquid nitrogen. *Escherichia coli* DH5α cells were used for transformation of recombinant vectors. Methylotrophic yeast *Pichia pastoris* wild-type strain X-33 (Invitrogen) was used in heterologous expression. The pGEM®-T

Easy vector (Promega) was used for subcloning of PCR fragments and the pPICZC shuttle vector (Invitrogen) was used for gene expression in *Pichia pastoris*. All media and protocols related to *Pichia pastoris* were performed according to the EasySelect™ *Pichia* Expression Kit Manual (Invitrogen). Buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY) contained (per liter): 1.34% yeast nitrogen base (Sigma), 1.0% yeast extract (Sigma), 2.0% mycological peptone (Bio-Chemika), 100 mM potassium phosphate (pH 6.0), 4 × 10⁻⁵% biotin, and 1% glycerol (Fluka) for BMGY or 0.5% methanol (Fluka) for BMMY. Buffered minimal methanol medium (BMM) contained (per liter): 1.34% yeast nitrogen base (Sigma), 100 mM potassium phosphate (pH 6.0), 4 × 10⁻⁵% biotin, and 0.5% methanol. Yeast extract peptone dextrose medium (YPD) contained (per liter): 1.0% yeast extract, 2.0% mycological peptone, and 2.0% dextrose (D-glucose).

2.2. Screening of laccase genes
The total RNA extracted from *Coriolopsis polyzona* MUCL 38443 mycelia by using the RNeasy Plant Mini Kit (QIAGEN) was used for cDNA synthesis. Laccase-specific first-strand cDNA was synthesized using the Transcriptor First Strand Synthesis Kit (Roche) according to the manufacturer's instructions. The first-strand mixture was used as a template for further PCR amplification using the Expand High Fidelity PCR System (Roche) with LAC-N1 and LAC-C1 primers (Table 1) designed based on the conserved sequences in copper-binding regions I and IV. PCR conditions were set as 94 °C for 3 min for initial denaturation, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2.5 min with a final extension at 72 °C for 10 min. Approximately 1.2-kb PCR products were amplified and cloned into the pGEM®-T Easy Vector (Promega) after purification for sequencing.

2.3. Cloning of full-length *Cplcc1* laccase cDNA
The 5' and 3' rapid amplification of cDNA ends (RACE) protocols were employed for the identification of the full-length *Cplcc1* laccase cDNA from *Coriolopsis polyzona* MUCL 38443 by using the BD SMARTer™ RACE cDNA Amplification Kit (Clontech) with 10 mM concentrations of the gene-specific RACE and RACE nested primers listed in Table 1.

The Transcriptor High Fidelity cDNA Synthesis Kit with total RNA and anchored-oligo(dT)18 primer was used for full-length *Cplcc1* cDNA synthesis. PCR amplification of full-length *Cplcc1* laccase cDNA was performed with the Expand High Fidelity PCR System (Roche) using the *Cplcc1* forward primer (10 mM) and *Cplcc1* reverse primer (10 mM) (Table 1). PCR conditions were set as 94 °C for 2 min for initial denaturation; 5 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.5 min; 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2.5 min; and a final extension at 72 °C for 10 min.

Table 1. List of primers used in this study.

Primer	Sequence
LAC-N1	5'-CAYTGGCAYGGNTTYTTYCA-3'
LAC-C1	5'-TGRAARTCDATRTGRCARTG-3'
5'-RACE reverse	5'-AGCCTGGTCAGGGACCTGGAAGTCGT-3'
5'-RACE reverse nested	5'-ATGAACGCGGGACCATCGGCCCAGTT-3'
3'-RACE forward	5'-CGACAACCCGATCTTCCGCGACGTCG-3'
3'-RACE forward nested	5'-CACGATCCGCTTCCGCACCGACAACC-3'
Cplcc1 forward	5'-CCGGAATTCAGCATGTCGCGGTTCAACCAGCTC-3'
Cplcc1 reverse	5'-AACGCGGCCGCTTACTGGTCGTTGGGGTCGAG-3'

All PCR products were purified from agarose gel using the MinElute Gel Extraction Kit (QIAGEN) and cloned into the pGEM®-T Easy Cloning Vector (Promega) for sequencing.

2.4. Isolation of the *Cplcc1* gene from *Coriolopsis polyzona* MUCL 38443

Genomic DNA isolation from *Coriolopsis polyzona* MUCL 38443 was carried out with the Fast Prep Instrument and Kit (MPBio) using 100 mg of *Coriolopsis polyzona* mycelium. *Pfu* DNA polymerase enzyme (Fermentas) was used for the amplification of the *Cplcc1* gene. PCR conditions were set as 95 °C for 3 min for initial denaturation, followed by 35 cycles of 95 °C for 30 s, 70 °C for 30 s, and 72 °C for 4.5 min with a final extension at 72 °C for 15 min.

2.5. DNA sequence analysis

Sequence similarities were determined by alignment of laccase gene sequences from the GenBank-EMBL-DDBJ-PDB databases using the BLAST homology search. The putative amino acid sequence encoded by *Cplcc1* was determined with the ExPASy-Translate tool. The molecular weight of the CpLcc1 laccase protein was determined with a theoretical MW (Compute pI/MW, ExPasy) calculator using the full-length amino acid sequence. While multiple alignment was achieved using ClustalW2, Molecular Evolutionary Genetics Analysis (MEGA) Version 6 and GenAlEx 6.41 software programs were used for distance-based analysis of laccase-specific cDNA sequences. Phylogenetic analysis was performed using MEGA 6.0 software. The phylogenetic tree was constructed using a neighbor-joining method that incorporated a Poisson correction model.

2.6. Heterologous expression of *Cplcc1* in *Pichia pastoris*

After double digestion with *Eco*RI and *Not*I, the full-length *Cplcc1* cDNA fragment from the recombinant pGEM®-T Easy cloning vector was subcloned into the *Eco*RI and *Not*I digested pPICZC shuttle vector (Invitrogen). The resulting recombinant pPICZC shuttle vector including *Cplcc1* cDNA was sequenced to confirm that the laccase cDNA was in frame with the C-terminal peptide before *Pichia pastoris* transformation. The recombinant pPICZC was then linearized within the 5' *AOX1* region with *Pme*I to integrate into the 5' *AOX1* region of the host's genome and used for the transformation of chemically competent *Pichia pastoris* strain X-33 cells for Zeocin resistance via the EasyComp™ Transformation Kit (Invitrogen). Transformants were plated onto yeast extract peptone dextrose (YPD) agar containing 100 µg Zeocin (Invitrogen) mL^{-1} and incubated at 30 °C for 4 days. Putative transformants were then transferred onto minimal methanol (BMM) agar plates supplemented with 0.2 mM ABTS (Sigma) and 100 µg Zeocin mL^{-1} and incubated for 3 days at 30 °C to select the laccase-producing transformants, whereby laccase-producers were identified by dark green halos around the colonies. In order to select the best laccase-producing transformant, the laccase-positive transformants were assayed for extracellular laccase production by growing them in BMM liquid medium at 28 °C and 250 rpm with 0.5% methanol (Merck) added daily for 7 days. Laccase activity was assayed every day.

To optimize the laccase production, the selected best laccase-producing transformant was inoculated in 25 mL of BMGY medium in a 250-mL flask and grown at 28 °C with shaking at 250 rpm until the culture reached OD_{600} = 2.0–6.0 for preculture. After harvesting cells by centrifugation, pellets were resuspended in 25 mL of BMMY medium to OD_{600} = 1.0 and incubated at 28 °C with shaking at 250 rpm, and the effects of various methanol concentrations (0%, 0.5%, 0.75%, 1.0%, and 1.5% methanol), $CuSO_4$ concentrations (0 to 4 mM $CuSO_4$) (Sigma), and L-alanine concentrations (0% to 1.0% L-alanine) (AppliChem) were investigated. The effects of different cultivation temperatures (23 °C and 28 °C) on laccase expression were also determined.

2.7. Assay of laccase activity

Laccase activity of the culture supernatant was determined by measuring the oxidation of 2.5 mM ABTS in 100 mM

sodium citrate buffer (pH 3.0) incubated for 3 min at room temperature ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}). One unit (U) of laccase activity was defined as the amount of enzyme oxidizing 1 µmol of ABTS per minute under the assay conditions. All assays were carried out in triplicate.

2.8. Partial purification of recombinant laccase
Culture supernatants were collected by centrifugation at 5000 × ***g*** for 20 min. Protein precipitation was carried out by 90% $(NH_4)_2SO_4$ saturation at 4 °C and the mixture was pelleted by centrifugation at 5000 × ***g*** for 1 h. The collected sample was resuspended in 50 mM sodium phosphate buffer at pH 7.35 and desalted by ultrafiltration with Amicon Ultra-15 centrifugal filter units (molecular mass cut-off of 10 kDa) (Millipore). The sample was first applied onto Q-Sepharose resin (GE Healthcare Life Sciences) preequilibrated with 50 mM sodium phosphate buffer at pH 7.35 and eluted at a flow rate of 2 mL min⁻¹. A gradient was formed with 0.5 M NaCl. Active fractions were pooled, concentrated by ultrafiltration, and loaded onto a Superdex 75 prep gel column (GE Healthcare Life Sciences) preequilibrated with 20 mM sodium acetate buffer containing 0.15 M NaCl at pH 5.35 (flow rate of 0.3 mL min⁻¹). At the final step, pooled fractions were concentrated with ultrafiltration and stored at 4 °C.

2.9. Protein determination
Protein concentration was determined by the Bradford protein assay using bovine serum albumin (Thermo Scientific) as a standard.

2.10. Polyacrylamide gel electrophoresis (PAGE) and zymogram analysis
Native-PAGE and SDS-PAGE were performed on 12% polyacrylamide gels by the method of Laemmli (1970). In SDS-PAGE, the Unstained Protein MW Marker (26610, Pierce) was used. The constructed SDS-PAGE molecular weight standard curve: log MW of the used SDS-protein marker versus R_f values is given in the Supplementary Materials. PAGE gels were stained with Coomassie Brilliant Blue G-250 (Merck) after electrophoresis. The protein sample used for the SDS-PAGE zymogram was not denatured; that is, there was no sample boiling, nor the addition of reducing agent 2-mercaptoethanol. Zymograms were obtained by soaking the gels with 100 mM sodium citrate buffer at pH 3.0 three times for every 15 min and then incubating in 2.5 mM ABTS in the assay buffer at room temperature.

2.11. Characterization of recombinant laccase
The effect of temperature on the recombinant enzyme was analyzed at different temperatures ranging from 20 °C to 90 °C using 2.5 mM ABTS as the substrate. The thermal stability of the enzyme was also assayed by incubating the enzyme at the same temperatures up to 8 h prior to activity assay, except for 30 °C, at which the enzyme was incubated

for 1 week. The optimum pH of the partially purified recombinant laccases was determined with 2.5 mM ABTS as the substrate in 100 mM HCl-KCl buffer (pH 2.0), 100 mM citrate buffers (pH 3.0 to 6.0), and 100 mM sodium phosphate buffers (pH 7.0 to 8.0). Oxidation of different substrates by recombinant laccases was examined by analyzing laccase activity for 5 mM ABTS, 5 mM DMP (Sigma), and 5 mM guaiacol (Merck) as substrates. The effect of various potential inhibitors on the recombinant laccase activity was tested using 0.1–1 mM sodium azide (Merck), 0.1–1 mM L-cysteine (Merck), and 1 mM SDS (Merck) after incubation of the enzyme at 30 °C for 5 min. Inhibition percentage was calculated by comparing retaining activity with the control sample.

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

2.12. Nucleotide sequence accession numbers
The nucleotide sequences of cDNA and genomic DNA of *Cplcc1* have been deposited in the GenBank database under accession numbers KT802746 and KT802747, respectively.

3. Results and discussion
3.1. Screening of laccase genes from *Coriolopsis polyzona* MUCL 38443
To screen laccase gene-specific sequences, we performed the reverse transcription-polymerase chain reaction (RT-PCR) technique by using degenerate primers designed based on the highly conserved copper-binding regions I and IV of known fungal laccases (D'Souza et al., 1999; Hoshida et al., 2001) and total RNA as a template. As a result, a single 1.2-kb DNA band was amplified and cloned into the pGEM®-T Easy Cloning Vector (Promega) to construct the partial cDNA library of *C. polyzona* MUCL 38443. From the constructed cDNA library, nucleotide sequences obtained from the 52 clones were aligned using ClustalW2. The sequences were classified into three distinct groups, namely *Cplcc1*, *Cplcc2*, and *Cplcc3*. We next compared these sequences with the previously reported sequences in the National Center for Biotechnology Information (NCBI) database using a BLAST search. They exhibited approximately 80% homology to known laccases (Table 2). In the partial cDNA library, the most frequently obtained cDNA sequences belonged to *Cplcc1*, which appeared to be the major expressed laccase gene

Table 2. Blast analysis of the partial cDNA sequences of *Cplcc1*, *Cplcc2*, and *Cplcc3* from *Coriolopsis polyzona* MUCL 38443.

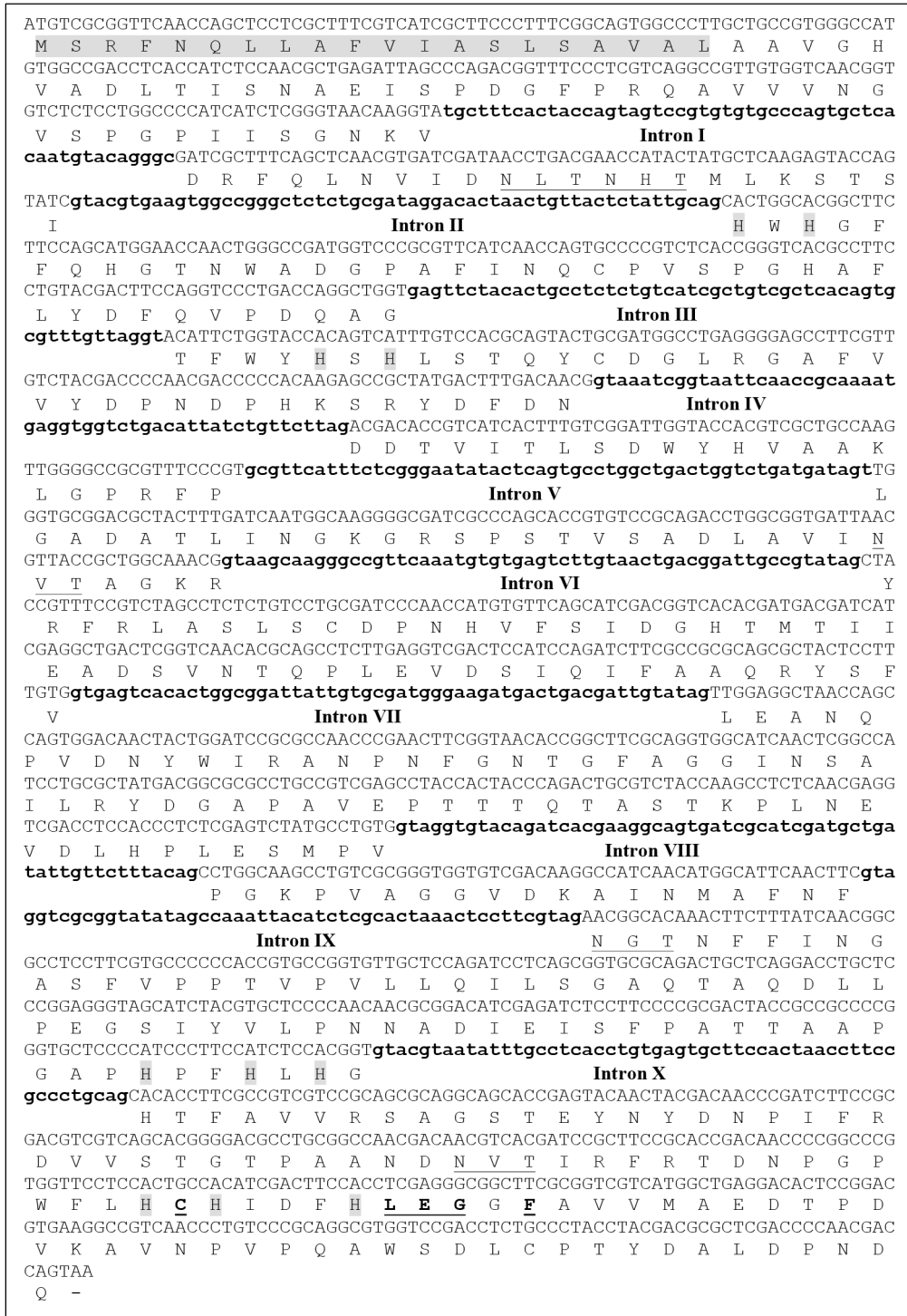| *Cplcc1*			
Accession number	Description	Score	Homology
XM_008034423.1	*Trametes versicolor* FP-101664 SS1 laccase B precursor (TvLac3), partial mRNA	778	79%
KP027478.1	*Trametes hirsuta* laccase A mRNA, complete cds	756	79%
Y18012.1	*Trametes versicolor* mRNA for laccase	756	79%
Cplcc2			
Accession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

in *Coriolopsis polyzona* MUCL 38443 under the applied conditions.

3.2. Isolation and characterization of genomic and cDNA sequences encoding the laccase CpLcc1

To isolate the full-length cDNA of *Cplcc1*, 5'- and 3'-RACE techniques were performed. An approximately 1.6-kb full-length *Cplcc1* cDNA product was amplified and sequenced after cloning into the pGEM®-T easy cloning vector (results of agarose gel electrophoresis belonging to cloning studies are given in the Supplementary Materials). The complete cDNA sequence of *Cplcc1* from start codon ATG to the TAA stop codon is 1563 bp and it encodes a protein precursor of 520 amino acids. The first 20 amino acids correspond to a putative signal sequence (Figure 1). Subsequently, the *Cplcc1* gene was also isolated by PCR and sequenced after cloning into the pGEM®-T Easy cloning vector. The *Cplcc1* gene consists of 2106 bp with 57% GC content. The coding regions of fungal laccases are generally interrupted by 8–13 introns of about 50–90 bp in length and splicing junctions usually adhere to the 5'-GT….AG-3' rule (Galhaup et al., 2002). In the literature, the *lap2* gene of *Trametes pubescens* and the *lcc1* gene of *Trametes villosa* contain 8 introns (Yaver et al., 1996; Galhaup et al., 2002), *lcc2* of *Trametes villosa* has 10 introns (Yaver et al., 1996), and *CVLG1* of *Coriolus versicolor* and *Cs-Lcs1* of *Ceriporiopsis subvermispora* possess 11 introns (Mikuni and Morohoshi, 1997; Karahanian et al., 1998). Consistently, sequence comparison of the genomic and cDNA sequences of *Cplcc1* revealed 10 introns with

sizes ranging from 51 to 58 bp (Figure 1). There are five potential N-glycosylation sites (Asn-X-Thr/Ser) and five cysteine residues present in the deduced protein sequence of *Cplcc1* (Figure 1).

Cu^{+2} ligand domains containing ten histidine residues and one cysteine residue, which are highly conserved in most laccases (Messerschmidt, 1997; Temp et al., 1999), are detected in the *Cplcc1* coding region. Comparison of the amino acid sequences of *Cplcc1* with the other available laccases in the database revealed that Cys474 is assumed to be the conserved laccase signature acting as a ligand to the Type 1 copper domain. Additionally, H416 and H479 seem to coordinate the Type 1 atom; H85 and H419 seem to coordinate the Type 2 copper atom; and H87, H130, H132, H421, H473, and H475 seem to coordinate the Type 3 copper atom (Figure 1). Another critical residue, involved in the coordination of Type 1 copper atoms and located ten amino acids downstream of the conserved Cys residue, can be Leu, Met, or Phe. However, generally high redox potential fungal laccases contain a Phe residue at this position (Temp et al., 1999). In addition to that, the Lue–Glu–Ala tripeptide motif adjacent to the last conserved His residue in high redox potential versus the Val–Ser–Gly in low potential fungal laccases has been identified as essential for the reactivity of laccases (Xu et al., 1998). This motif was found as Leu–Glu–Gly in the *Cplcc1* coding region, as in the case of *Trametes hirsuta* laccase A (AIZ72721.1) and *Trametes hirsuta* laccase (ACC43989.1). The Phe residue (Phe484) is present in the

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ATGTCGCGGTTCAACCAGCTCCTCGCTTTCGTCATCGCTTCCCTTTCGGCAGTGGCCCTTGCTGCCGTGGGCCAT
  M  S  R  F  N  Q  L  L  A  F  V  I  A  S  L  S  A  V  A  L  A  A  V  G  H
GTGGCCGACCTCACCATCTCCAACGCTGAGATTAGCCCAGACGGTTTCCCTCGTCAGGCCGTTGTGGTCAACGGT
  V  A  D  L  T  I  S  N  A  E  I  S  P  D  G  F  P  R  Q  A  V  V  V  N  G
GTCTCTCCTGGCCCCATCATCTCGGGTAACAAGGTAtgctttcactaccagtagtccgtgtgtgcccagtgctca
  V  S  P  G  P  I  I  S  G  N  K  V                           Intron I
caatgtacagggcGATCGCTTTCAGCTCAACGTGATCGATAACCTGACGAACCATACTATGCTCAAGAGTACCAG
             D  R  F  Q  L  N  V  I  D  N  L  T  N  H  T  M  L  K  S  T  S
TATCgtacgtgaagtggccgggctctctgcgataggacactaactgttactctattgcagCACTGGCACGGCTTC
  I                     Intron II                              H  W  H  G  F
TTCCAGCATGGAACCAACTGGGCCGATGGTCCCGCGTTCATCAACCAGTGCCCCGTCTCACCGGGTCACGCCTTC
  F  Q  H  G  T  N  W  A  D  G  P  A  F  I  N  Q  C  P  V  S  P  G  H  A  F
CTGTACGACTTCCAGGTCCCTGACCAGGCTGGTgagttctacactgcctctctgtcatcgctgtcgctcacagtg
  L  Y  D  F  Q  V  P  D  Q  A  G                     Intron III
cgtttgttaggtACATTCTGGTACCACAGTCATTTGTCCACGCAGTACTGCGATGGCCTGAGGGGAGCCTTCGTT
            T  F  W  Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  A  F  V
GTCTACGACCCCAACGACCCCCACAAGAGCCGCTATGACTTTGACAACgtaaatcggtaattcaaccgcaaaat
  V  Y  D  P  N  D  P  H  K  S  R  Y  D  F  D  N                Intron IV
gaggtggtctgacattatctgttcttagACGACACCGTCATCACTTTGTCGGATTGGTACCACGTCGCTGCCAAG
                            D  D  T  V  I  T  L  S  D  W  Y  H  V  A  A  K
TTGGGGCCGCGTTTCCCGTgcgttcatttctcgggaatatactcagtgcctggctgactggtctgatgatagtTG
  L  G  P  R  F  P                     Intron V                           L
GGTGCGGACGCTACTTTGATCAATGGCAAGGGGCGATCGCCCAGCACCGTGTCCGCAGACCTGGCGGTGATTAAC
  G  A  D  A  T  L  I  N  G  K  G  R  S  P  S  T  V  S  A  D  L  A  V  I  N
GTTACCGCTGGCAAACGgtaagcaagggccgttcaaatgtgtgagtcttgtaactgacggattgccgtatagCTA
  V  T  A  G  K  R                     Intron VI                           Y
CCGTTTCCGTCTAGCCTCTCTGTCCTGCGATCCCAACCATGTGTTCAGCATCGACGGTCACACGATGACGATCAT
  R  F  R  L  A  S  L  S  C  D  P  N  H  V  F  S  I  D  G  H  T  M  T  I  I
CGAGGCTGACTCGGTCAACACGCAGCCTCTTGAGGTCGACTCCATCCAGATCTTCGCCGCGCAGCGCTACTCCTT
  E  A  D  S  V  N  T  Q  P  L  E  V  D  S  I  Q  I  F  A  A  Q  R  Y  S  F
TGTGgtgagtcacactggcggattattgtgcgatgggaagatgactgacgattgtatagTTGGAGGCTAACCAGC
  V                     Intron VII                          L  E  A  N  Q
CAGTGGACAACTACTGGATCCGCGCCAACCCGAACTTCGGTAACACCGGCTTCGCAGGTGGCATCAACTCGGCCA
  P  V  D  N  Y  W  I  R  A  N  P  N  F  G  N  T  G  F  A  G  G  I  N  S  A
TCCTGCGCTATGACGGCGCGCCTGCCGTCGAGCCTACCACTACCCAGACTGCGTCTACCAAGCCTCTCAACGAGG
  I  L  R  Y  D  G  A  P  A  V  E  P  T  T  T  Q  T  A  S  T  K  P  L  N  E
TCGACCTCCACCCTCTCGAGTCTATGCCTGTGgtaggtgtacagatcacgaaggcagtgatcgcatcgatgctga
  V  D  L  H  P  L  E  S  M  P  V                     Intron VIII
tattgttctttacagCCTGGCAAGCCTGTCGCGGGTGGTGTCGACAAGGCCATCAACATGGCATTCAACTTCgta
               P  G  K  P  V  A  G  G  V  D  K  A  I  N  M  A  F  N  F
ggtcgcggtatatagccaaattacatctcgcactaaactccttcgtagAACGGCACAAACTTCTTTATCAACGGC
         Intron IX                               N  G  T  N  F  F  I  N  G
GCCTCCTTCGTGCCCCCCACCGTGCCGGTGTTGCTCCAGATCCTCAGCGGTGCGCAGACTGCTCAGGACCTGCTC
  A  S  F  V  P  P  T  V  P  V  L  L  Q  I  L  S  G  A  Q  T  A  Q  D  L  L
CCGGAGGGTAGCATCTACGTGCTCCCCAACAACGCGGACATCGAGATCTCCTTCCCCGGACTACCGCCGCCCCG
  P  E  G  S  I  Y  V  L  P  N  N  A  D  I  E  I  S  F  P  A  T  T  A  P
GGTGCTCCCCATCCCTTCCATCTCCACGGTgtacgtaatatttgcctcacctgtgagtgcttccactaaccttcc
  G  A  P  H  P  F  H  L  H  G                     Intron X
gccctgcagCACACCTTCGCCGTCGTCCGCAGCGCAGGCAGCACCGAGTACAACTACGACAACCCGATCTTCCGC
         H  T  F  A  V  V  R  S  A  G  S  T  E  Y  N  Y  D  N  P  I  F  R
GACGTCGTCAGCACGGGGACGCCTGCGGCCAACGACAACGTCACGATCCGCTTCCGCACCGACAACCCCGGCCCG
  D  V  V  S  T  G  T  P  A  A  N  D  N  V  T  I  R  F  R  T  D  N  P  G  P
TGGTTCCTCCACTGCCACATCGACTTCCACCTCGAGGGCGGCTTCGCGGTCGTCATGGCTGAGGACACTCCGGAC
  W  F  L  H  C  H  I  D  F  H  L  E  G  G  F  A  V  V  M  A  E  D  T  P  D
GTGAAGGCCGTCAACCCTGTCCCGCAGGCGTGGTCCGACCTCTGCCCTACCTACGACGCGCTCGACCCCAACGAC
  V  K  A  V  N  P  V  P  Q  A  W  S  D  L  C  P  T  Y  D  A  L  D  P  N  D
CAGTAA
  Q  -
```

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

position 10 amino acids downstream of the conserved Cys residue (Cys474) (Figure 1), suggesting that the laccase encoded by *Cplcc1* is a high redox potential laccase.

The deduced amino acid sequence of *Cplcc1* was compared with the sequence of other laccases reported in GenBank, revealing that the CpLcc1 laccase was closely related to *Trametes hirsuta* laccase A (AIZ72721.1) and *Trametes hirsuta* laccase (ACC43989.1) by 88% identity. This is followed by 87% with *Lenzites gibbosa* (AEP71394.1), 86% with *Trametes versicolor* (AAL93622.1), 86% with *Trametes* sp. (ADK55593.1), 84% with *Pycnoporus coccineus* (AKE14488.1), 82% with *Trametes sanguinea* (ACZ37083.1), and 81% with *Trametes cinnabarina* (O59896.1) laccases. To investigate the evolutionary relationships with the other laccases, a phylogenetic tree was constructed based on the amino acid sequence of *Cplcc1* and 45 laccase sequences derived from GenBank, and the analysis showed that *Cplcc1* and *Trametes versicolor* laccases belonged to the same phylogenetic group (Figure 2).

3.3. Heterologous expression of the laccase CpLcc1 in *Pichia pastoris*

For heterologous expression, the isolated full-length *Cplcc1* cDNA was subcloned into the yeast shuttle expression vector pPICZC (Invitrogen) with its own natural secretion signal sequence. Chemically competent *Pichia pastoris* strain X-33 cells were transformed with the recombinant pPICZC vector bearing *Cplcc1* cDNA. Twenty-five of the green halo-forming laccase-positive transformants (Figure 3) were then screened for extracellular laccase production by growing them in BMM liquid medium at 28 °C with 0.5% methanol added daily to induce laccase expression for 7 days. The *Pichia pastoris* transformant expressing the highest level of laccase (76.5 U L^{-1}) was selected for further investigations.

The level of recombinant CpLcc1 production in *Pichia pastoris* was further improved by optimizing the methanol concentration, copper concentration, L-alanine concentration, and cultivation temperature. Methanol was added daily during cultivation to induce laccase expression via the AOX1 promoter. Accurate regulation of the methanol feed is critical not only to keep the induction of the genes under the control of the AOX1 promoter but also to hinder methanol accumulation at levels that are toxic to the cells. For this, the effects of methanol concentrations ranging from 0.5% to 1.5% on laccase expression were first investigated to optimize methanol concentration. The highest laccase activity of 211.29 U L^{-1} was achieved in the presence of 1.5% methanol concentration (Figure 4a).

Cu^{2+} ions positively affect the activity and stability of laccases (Baldrian and Gabriel, 2002). Thus, there is a positive correlation between copper availability and laccase activity. In this study, to test the effect of Cu^{2+} concentrations on the recombinant laccase production, various concentrations of $CuSO_4$ ranging from 0 mM to 4.0 mM were added to the cultivation medium. It was found that the 0.8 mM $CuSO_4$ concentration enhanced the highest laccase activity of 380.52 U L^{-1} with a 2.2-fold increase in laccase production (Figure 4b). However, in the studies of O'Callaghan et al. (2002) and Liu et al. (2003), the optimal concentrations of copper atoms were reported as 0.2 mM and 0.4 mM, respectively, confirming that copper concentration is one of the important variables for optimization of laccase production.

Control of pH is usually required during the cultivation process, because some acidic proteases can be activated at a lower pH and may cause a detrimental effect on laccase production (Soden et al., 2002). It was shown that metabolism of alanine by *Pichia pastoris* can stabilize the culture pH because of the ammonia produced by alanine metabolism and consequently neutralize the acidic end products of methanol metabolism (O'Callaghan et al., 2002). In our study, the effect of L-alanine concentrations ranging from 0% to 1.0% on recombinant laccase expression was also investigated. The highest laccase activity of 591.53 U L^{-1} was achieved in the presence of 0.6% L-alanine with a 1.7-fold increase in laccase production (Figure 4c).

Cultivation temperature is another important parameter to improve the recombinant protein production. Although recombinant proteins are usually expressed at 28–30 °C in *Pichia pastoris*, some studies have demonstrated that activity of recombinant laccases in *Pichia pastoris* could be improved by lowering the cultivation temperature to approximately 16–20 °C, especially for laccases suffering from instability and misfolding at high temperatures (Cassland and Jönsson, 1999; Hong et al., 2002; Liu et al., 2003). Accordingly, we tested the effect of two cultivation temperatures by incubating the cultures in BMMY expression medium containing 1.5% methanol, 0.8 mM $CuSO_4$, and 0.6% L-alanine at 23 °C and at 28 °C with shaking at 250 rpm for 8 days. Inconsistent with previous studies, in our study, the lower cultivation temperature (23 °C) did not improve the activity of recombinant laccase CpLcc1 compared to 28 °C. The culture incubated at 23 °C provided about 24% lower laccase activity than that incubated at 28 °C (Figure 4d).

Finally, optimized expression conditions for recombinant laccase CpLcc1 in *Pichia pastoris* were defined as follows: growth in BMMY expression medium supplemented with 1.5% methanol, 0.8 mM $CuSO_4$, and 0.6% L-alanine at 28 °C with shaking at 250 rpm for 8 days. Under those optimized conditions, activity of recombinant laccase CpLcc1 in *Pichia pastoris* was achieved as 800 U L^{-1} with a 9.8-fold increase. Although this enzyme yield is found to be convenient for biochemical characterization, it is still rather low for industrial applications. Production
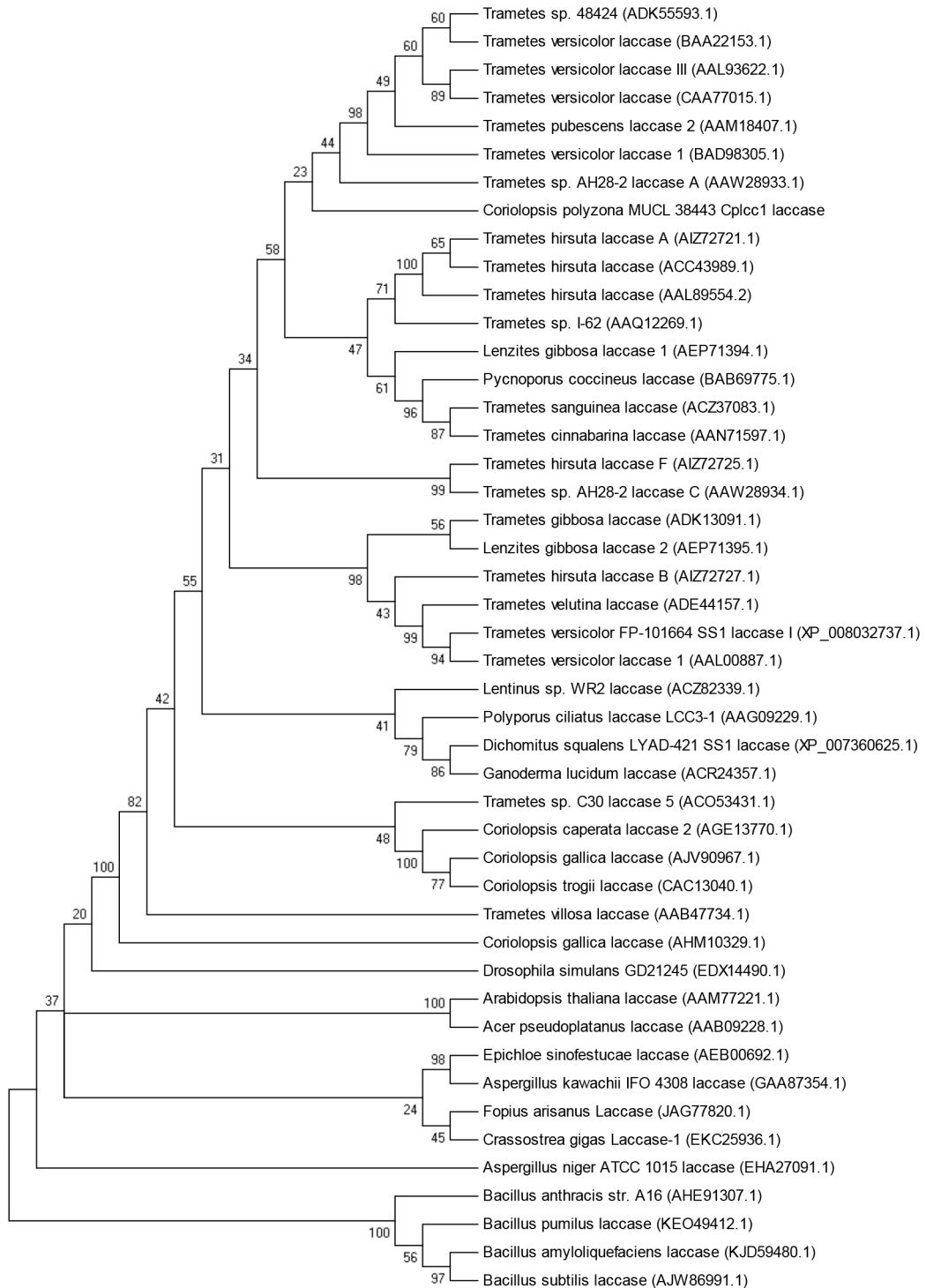
Figure 2. Phylogenetic tree of the deduced amino acid sequence of *Cplcc1* and other laccases from GenBank. The tree is calculated with p-distances using Mega ver. 6, based on a ClustalW alignment with a Poisson correction model.

level of CpLcc1 in *Pichia pastoris* could be further improved by employing several strategies such as the use of native promoters and multiple gene copies, codon optimization, and alteration of secretory signal sequences, as reported previously (Piscitelli et al., 2010; Antosova and Sychrova, 2016).
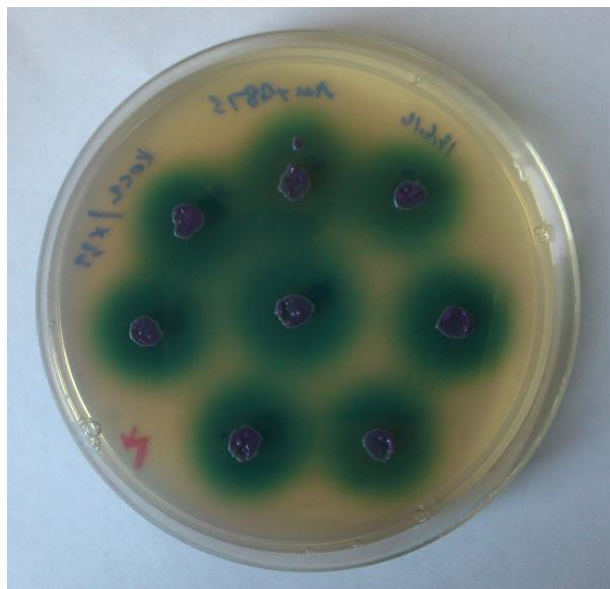
Figure 3. The agar plate assay to select *Pichia pastoris* X-33 transformants containing amplified *Cplcc1* cDNA on minimal methanol medium supplemented with 0.2 mM ABTS.

3.4. Partial purification of the recombinant laccase CpLcc1

Recombinant laccase CpLcc1 was partially purified by performing 90% ammonium sulfate precipitation followed by Q-Sepharose anion exchange chromatography and Superdex-75 prep gel filtration chromatography (Table 3; Figure 5). As a result, recombinant laccase CpLcc1 was purified 27.52-fold; the specific activity of the partially purified enzyme was 157.14 U mg^{-1}. The laccase activity of partially purified CpLcc1 was confirmed by not only the native-PAGE zymogram (given in the Supplementary Materials) but also SDS-PAGE zymogram analysis using ABTS as a substrate, visualized as a green-colored band (Figure 5). Together with the SDS-PAGE zymogram, we also employed standard SDS-PAGE by using the Unstained Protein MW Marker (26610, Pierce) (Figure 5). Thus, based on SDS-PAGE, the relative molecular mass of the partially purified recombinant laccase CpLcc1 was estimated as 54 kDa (Figure 5), which is in the range reported for most fungal laccases. The majority of laccases from fungi were reported to have molecular weights in the range of 50 to 100 kDa (Baldrian, 2006; Riva, 2006; Giardina et al., 2010). However, it must be taken into account that the molecular mass of recombinant laccases expressed in *Pichia* has often been found to be higher than that of the native form, due to the increased glycosylation in *Pichia* (Gu et al., 2014; Kalyani et al., 2015).

3.5. Effect of substrate on the activity of the recombinant laccase CpLcc1

Substrate specificity of the recombinant laccase was examined for different compounds including ABTS, DMP, and guaiacol. Among them, ABTS is the most common substrate for laccases, as it is not pH-dependent and does not form quinones (Giardina et al., 2010; Majeau et al., 2010). Like other laccases, recombinant CpLcc1 displayed the maximum activity towards ABTS while DMP was oxidized with 15.14% relative activity. When using guaiacol, no particular activity was detected (Table 4).

3.6. Effect of temperature and pH on the activity of the recombinant laccase CpLcc1

Optimum temperature of fungal laccases generally ranges from 50 °C to 80 °C (Baldrian, 2006). Thus, in the present study, effect of temperature on the enzyme activity was analyzed against ABTS between 20 °C and 90 °C. Consistent with a previous report (Kalyani et al., 2015), the optimum temperature of the recombinant enzyme CpLcc1 was found as 70 °C (Figure 6a). The half-life of its activity (t$_{1/2}$) was longer than 8 h at both 30 °C and 40 °C and it was 30 min at 50 °C (Figure 6b). CpLcc1 kept its stability for more than a week at 30 °C, but rapidly lost its stability at 60 °C, 70 °C, and 80 °C (Figure 6b). It was not surprising to see its rapid activity loss over 50 °C since fungal laccases generally quickly lose their activity at high temperatures (Viswanath et al., 2014). In fact, the thermal stability profile of recombinant CpLcc1 was similar to that of a white laccase from *Pleurotus ostreatus*, POXA2, as the half-life of its activity was 12 min at 60 °C (Palmieri et al., 1997), and a laccase from *Trametes versicolor*, which was completely inactivated at 70 °C in 20 min (Stoilova et al., 2010). Thus, the thermal behavior of recombinant CpLcc1 can be found very promising as a biocatalyst for the newly found laccase. The optimum pH value of recombinant CpLcc1 was determined by using ABTS as a substrate over the pH range of 2.0–8.0. The optimum pH was found to be 3.0 for ABTS as the substrate (Figure 6c). This is also comparable to the laccases of fungal origin, such as two laccase isoenzymes from *Coprinus comatus* (Lac3 and Lac4, pH 3.0) (Gu et al. 2014) and a laccase enzyme from *Ganoderma lucidum* TR6 (pH 3.5) (You et al. 2014).

3.7. Effect of inhibitors on the activity of the recombinant laccase CpLcc1

Inhibitory effects of known laccase inhibitors, sodium azide, L-cysteine, and SDS, were analyzed following the incubation of the purified enzyme with those compounds at 30 °C for 5 min. Recombinant CpLcc1 laccase was strongly inhibited by 1 mM sodium azide and 1 mM SDS by 90.12% and 84.90%, respectively, while 0.1 mM sodium azide and 1 mM L-cysteine also caused 55.62% and 25.34% inhibition, respectively (Table 5). These results were in accordance with the results from previous reports (Garcia et al., 2007; More et al., 2011; Kalyani et al., 2015).

### 3.8. Catalytic properties of the recombinant laccase CpLcc1

Catalytic properties of the recombinant enzyme were determined under optimized conditions using ABTS. The
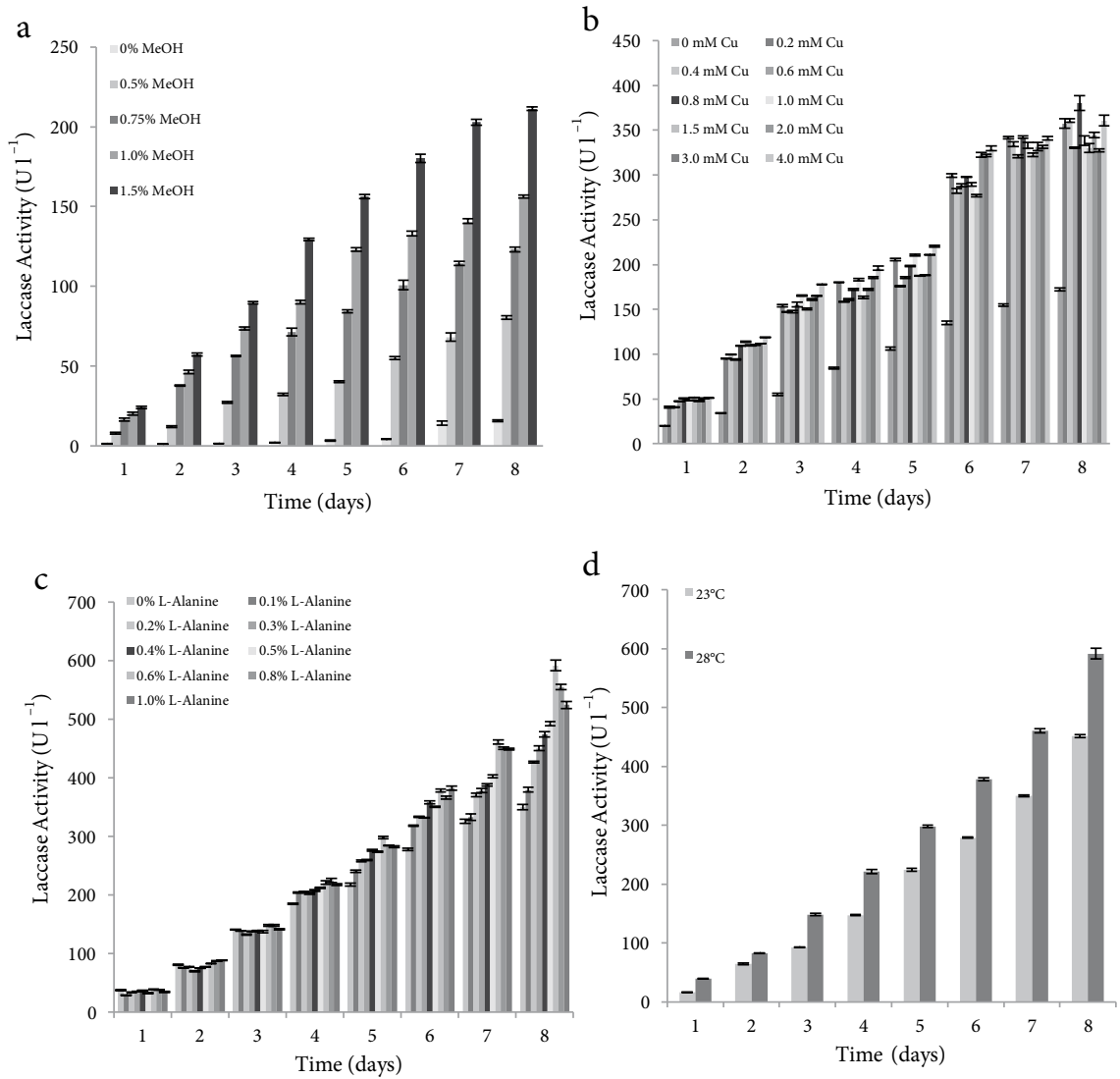
Figure 4. Optimization of expression conditions for the recombinant laccase CpLcc1 in *Pichia pastoris*. Effect of methanol concentrations (a), copper concentrations (b), L-alanine concentrations (c), and cultivation temperature (d) on the activity of the recombinant laccase CpLcc1.

Table 3. Summary of steps involved in the purification of the recombinant CpLcc1.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Culture	600.00	105.12	5.71	100.00	1.00
$(NH_4)_2SO_4$ precipitation	512.55	86.58	5.92	85.42	1.04
Ultrafiltration	466.82	77.89	5.99	77.80	1.05
Anion exchange chromatography	227.59	6.43	35.40	37.93	6.20
Gel filtration chromatography	127.60	0.81	157.14	21.27	27.52
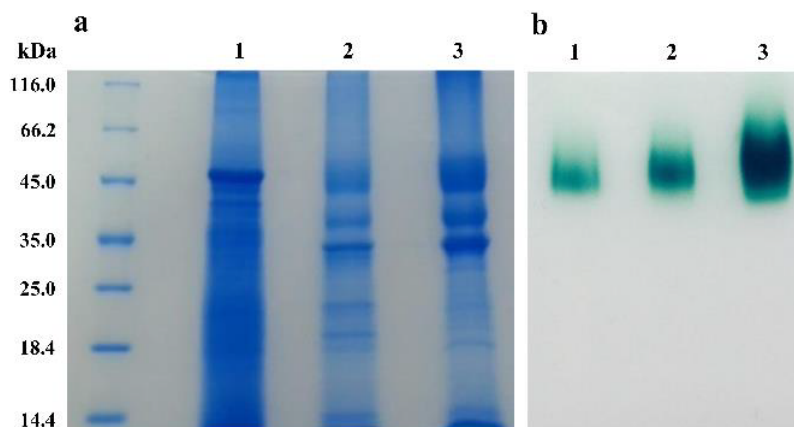
Figure 5. The 12% SDS-PAGE (a) and SDS-PAGE zymogram analysis (b) of partially purified recombinant laccase CpLcc1. Lane 1: Sample from 90% ammonium sulfate fraction desalted by ultrafiltration; Lane 2: sample from Q-Sepharose anion exchange chromatography; Lane 3: sample from Superdex-75 prep gel filtration chromatography. SDS-PAGE gel was stained with Coomassie Brilliant Blue G-250. Zymogram analysis for laccase activity was performed after SDS-PAGE by using 2.5 mM ABTS in 100 mM sodium citrate buffer (pH 3.0).

Table 4. Laccase activity of the recombinant CpLcc1 with respect to different substrates.

Substrate	ε_{max} (M^{-1} cm^{-1})	Wavelength	Concentration (mM)	Relative activity (%)
ABTS	36,000	420	5.0	100
DMP	35,565	470	5.0	15.14
Guaiacol	6400	436	5.0	-

K_m and V_{max} values of the recombinant CpLcc1 were found to be 0.137 mM and 288.6 µmol min^{-1} L^{-1}, respectively. The turnover (k_{cat}) and catalytic efficiency (k_{cat} K_m^{-1}) values of the recombinant CpLcc1 were determined as 5.73×10^5 min^{-1} and 4.18×10^6 min^{-1} mM^{-1}, respectively. When compared to other laccases, the kinetic parameters of recombinant CpLcc1 for ABTS seem to be promising. The K_m value of recombinant CpLcc1 was 7-fold lower than that of a laccase from *Ganoderma lucidum* in *Pichia pastoris* (0.9665 mM) (Sun et al., 2012) and 2-fold lower than the laccase POXA3 from *Pleurotus ostreatus* (0.280 mM) (Palmieri et al., 1997), a laccase from *Pleurotus* sp. (0.250 mM) (More et al., 2011), and a fungal laccase from *M. purpureofusca* (0.296 mM) (Sun et al., 2013). Similarly, the turnover (k_{cat}) value of recombinant CpLcc1 was found to be 7.7-, 14-, and 40-fold higher than that for the laccase Lac4 from *Pleurotus sajor-caju* (Soden et al., 2002), the laccase LAP2 from *Trametes pubescens* (Galhaup et al., 2002), and a laccase from *P. sanguineus* BRFM 902 (Uzan et al., 2010), respectively. Consequently, the resulting specificity constant (k_{cat}/K_m) value of the recombinant CpLcc1 for ABTS was also remarkably higher than previously reported laccases.

In summary, in the present study, genomic and cDNA sequences encoding a novel laccase, namely CpLcc1 from *Coriolopsis polyzona* MUCL 38443, were isolated and characterized. The isolated full-length Cplcc1 cDNA was successfully overexpressed in the yeast *Pichia pastoris* under the control of the AOX1 promoter and secreted into the culture. This is the first report on the cloning and the characterization of a laccase-encoding gene and the corresponding encoding laccase from the white-rot fungus strain *Coriolopsis polyzona* MUCL 38443. Recombinant CpLcc1 expressed in *Pichia pastoris* was partially purified and characterized with a molecular weight of 54 kDa, optimum temperature of 70 °C, and optimum pH value of pH 3.0. Thermal stability and kinetic parameters of the recombinant CpLcc1 were found as promising. Moreover, it is worthy to note that sequence analysis indicated that CpLcc1 is a high redox potential laccase, which makes it a good candidate for industrial applications because laccases with high redox potential provide a broader substrate range without the use of mediators, which makes them more attractive for biotechnological applications. Finally, a heterologous expression system of this newly found laccase could permit rational protein engineering approaches to
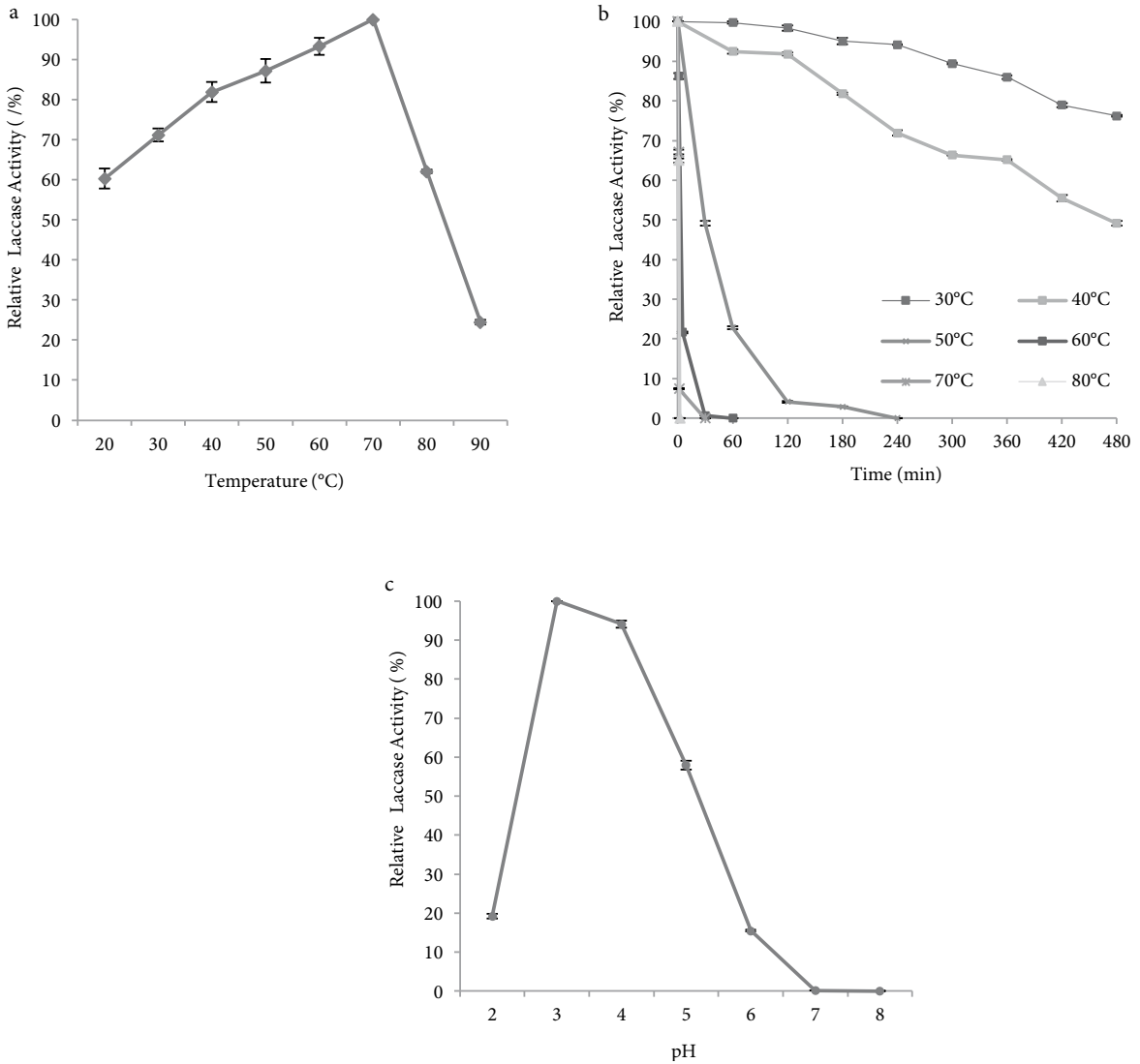
Figure 6. Effect of temperature on the activity (a) and thermal stability (b) of the recombinant laccase CpLcc1. Effect of pH (c) on the activity of the recombinant laccase CpLcc1.

Table 5. Effect of typical laccase inhibitors on the activity of the recombinant CpLcc1.

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

further improve the properties of the enzyme. Extending the laccases derived from different sources is critical to explore them as novel enzymes for low-cost productions and wide usage in industrial applications.

Acknowledgments

References

Antosova Z, Sychrova H (2016). Yeast hosts for the production of recombinant laccases: a review. Mol Biotechnol 58: 93-116.

Baldrian P (2006). Fungal laccases–occurrence and properties. FEMS Microbiol Rev 30: 215-242.

Baldrian P, Gabriel J (2002). Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. FEMS Microbiol Lett 206: 69-74.

Bento I, Martins LO, Lopes GG, Carrondo MA, Lindley PF (2005). Dioxygen reduction by multi-copper oxidases; a structural perspective. Dalton Trans 21: 3507-3513.

Cassland P, Jönsson LJ (1999). Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Appl Microbiol Biotechnol 52: 393-400.

Chandra R, Chowdhary P (2015). Properties of bacterial laccases and their application in bioremediation of industrial wastes. Environ Sci Process Impacts 17: 326-342.

de Wilde C, Uzan E, Zhou Z, Kruus K, Andberg M, Buchert J, Record E, Asther M, Lomascolo A (2008). Transgenic rice as a novel production system for *Melanocarpus* and *Pycnoporus* laccases. Transgenic Res 17: 515-527.

D'Souza TM, Merrit CS, Reddy CA (1999). Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*. Appl Environ Microbiol 65: 5307-5313.

Dubé E, Shareck F, Hurtubise Y, Daneault C, Beauregard M (2008). Homologous cloning, expression, and characterisation of a laccase from *Streptomyces coelicolor* and enzymatic decolourisation of an indigo dye. Appl Microbiol Biotechnol 79: 597-603.

Eriksson KEL, Blanchette RA, Ander P (1990). Biodegradation of lignin. In: Timmel TE, editor. Microbial and Enzymatic Degradation of Wood and Wood Components. Berlin, Germany: Springer-Verlag, pp. 237-247.

Galhaup C, Goller S, Peterbauer CK, Strauss J, Haltrich D (2002). Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. Microbiology 148: 2159-2169.

Garcia TA, Santiago MF, Ulhoa CJ (2007). Studies on the *Pycnoporus sanguineus* CCT-4518 laccase purified by hydrophobic interaction chromatography. Appl Microbiol Biotechnol 75: 311-318.

Gassara-Chatti F, Brar SK, Ajila CM, Verma M, Tyagi RD, Valero JR (2013). Encapsulation of ligninolytic enzymes and its application in clarification of juice. Food Chem 137: 18-24.

Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G (2010). Laccases: a never-ending story. Cell Mol Life Sci 67: 369-385.

Gu C, Zheng F, Long L, Wang J, Ding S (2014). Engineering the expression and characterization of two novel laccase isoenzymes from *Coprinus comatus* in *Pichia pastoris* by fusing an additional ten amino acids tag at N-terminus. PLoS One 9: e93912.

Guo M, Lu F, Du L, Pu J, Bai D (2006). Optimization of the expression of a laccase gene from *Trametes versicolor* in *Pichia methanolica*. Appl Microbiol Biotechnol 71: 848-852.

Hong F, Meinander NQ, Jönsson LJ (2002). Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. Biotechnol Bioeng 79: 438-449.

Hoshida H, Nakao M, Kanazawa H, Kubo K, Hakukawa T, Morimasa K, Akada R, Nishizawa Y (2001). Isolation of five laccase gene sequences from the white-rot fungus *Trametes sanguinea* by PCR, and cloning, characterization and expression of the laccase cDNA in yeasts. J Biosci Bioeng 92: 372-380.

Jaouani A, Tabka MG, Penninckx MJ (2006). Lignin modifying enzymes of *Coriolopsis polyzona* and their role in olive oil mill wastewaters decolourisation. Chemosphere 62: 1421-1430.

Kalyani D, Tiwari MK, Li J, Kim SC, Kalia VC, Kang YC, Lee JK (2015). A highly efficient recombinant laccase from the yeast *Yarrowia lipolytica* and its application in the hydrolysis of biomass. PLoS One 10: e0120156.

Karahanian E, Corsini G, Lobos S, Vicuña R (1998). Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim Biophys Acta 1443: 65-74.

Kiiskinen LL, Kruus K, Bailey M, Ylösmäki E, Siika-Aho M, Saloheimo M (2004). Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology 150: 3065-3074.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lanzellotto C, Favero G, Antonelli ML, Tortolini C, Cannistraro S, Coppari E, Mazzei F (2014). Nanostructured enzymatic biosensor based on fullerene and gold nanoparticles: preparation, characterization and analytical applications. Biosens Bioelectron 15: 430-437.

Liu W, Chao Y, Liu S, Bao H, Qian S (2003). Molecular cloning and characterization of a laccase gene from the basidiomycete *Fome lignousus* and expression in *Pichia pastoris*. Appl Microbiol Biotechnol 63: 174-181.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005). Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22: 249-270.

Madzak C, Otterbein L, Chamkha M, Moukha S, Asther M, Gaillardin C, Beckerich JM (2005). Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. FEMS Yeast Res 5: 635-646.

Majeau JA, Brar SK, Tyagi RD (2010). Laccases for removal of recalcitrant and emerging pollutants. Bioresour Technol 101: 2331-2350.

Messerschmidt A (1997). Spatial structures of ascorbate oxidase, laccase and related proteins: implications for the catalytic mechanism. In: Messerschmidt A, editor. Multi-Copper Oxidases. 1st ed. Singapore: World Scientific, pp. 23-80.

Mikuni J, Morohoshi N (1997). Cloning and sequencing of a second laccase gene from the white-rot fungus *Coriolus versicolor*. FEMS Microbiol Lett 155: 79-84.

More SS, Renuka PS, Pruthvi K, Swetha M, Malini S, Veena SM (2011). Isolation, purification, and characterization of fungal laccase from *Pleurotus* sp. Enzyme Res 2011: 248735.

O'Callaghan J, O'Brien M, McClean K, Dobson AD (2002). Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*. J Ind Microbiol Biotechnol 29: 55-59.

Osma JF, Toca-Herrera JL, Rodríguez-Couto S (2010). Uses of laccases in the food industry. Enzyme Res 2010: 918761.

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G (1997). A novel white laccase from *Pleurotus ostreatus*. J Biol Chem 272: 31301-31307.

Pezzella C, Autore F, Giardina P, Piscitelli A, Sannia G, Faraco V (2009). The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. Curr Genet 55: 45-57.

Piscitelli A, Pezzella C, Giardina P, Faraco V, Sannia G (2010). Heterologous laccase production and its role in industrial applications. Bioeng Bugs 1: 252-262.

Riva S (2006). Laccases: blue enzymes for green chemistry. Trends Biotechnol 24: 219-226.

Soden DM, O'Callaghan J, Dobson AD (2002). Molecular cloning of a laccase isozyme gene from *Pleurotus sajor-caju* and expression in the heterologous *Pichia pastoris* host. Microbiology 148: 4003-4014.

Stoilova I, Krastanov A, Stanchev V (2010). Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. Adv Biosci Biotechnol 1: 208-215.

Sun J, Peng RH, Xiong AS, Tian Y, Zhao W, Xu H, Liu DT, Chen JM, Yao QH (2012). Secretory expression and characterization of a soluble laccase from the *Ganoderma lucidum* strain 7071-9 in *Pichia pastoris*. Mol Biol Rep 39: 3807-3814.

Sun S, Zhang Y, Que Y, Liu B, Hu K, Xu L (2013). Purification and characterization of fungal laccase from *Mycena purpureofusca*. Chiang Mai J Sci 40: 151-160.

Téllez-Jurado A, Arana-Cuenca A, Becerra AG, Viniegra-González G, Loera O (2006). Expression of a heterologous laccase by *Aspergillus niger* cultured by solid-state and submerged fermentations. Enzyme Microb Tech 38: 665-669.

Temp U, Zierold U, Eggert C (1999). Cloning and characterization of a second laccase gene from the lignin degrading basidomycete *Pycnoporus cinnabarinus*. Gene 236: 169-177.

ten Have R, Teunissen JMP (2001). Oxidative mechanisms involved in lignin degradation by white-rot fungi. Chem Rev 101: 3397-3413.

Uzan E, Nousiainen P, Balland V, Sipila J, Piumi F, Navarro D, Asther M, Record E, Lomascolo A (2010). High redox potential laccases from the ligninolytic fungi *Pycnoporus coccineus* and *Pycnoporus sanguineus* suitable for white biotechnology: from gene cloning to enzyme characterization and applications. J Appl Microbiol 108: 2199-2213.

Viswanath B, Rajesh B, Janardhan A, Kumar AP, Narasimha G (2014). Fungal laccases and their applications in bioremediation. Enzyme Res 2014: 1-21.

Wang GD, Li QJ, Luo B, Chen XY (2004). Ex planta phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase. Nat Biotechnol 22: 893-897.

Wang J, Feng J, Jia W, Chang S, Li S, Li Y (2015). Lignin engineering through laccase modification: a promising field for energy plant improvement. Biotechnol Biofuels 8: 145.

Xu F, Berka RM, Wahleithner JA, Nelson BA, Shuster JR, Brown SH, Palmer AE, Solomon EI (1998). Site-directed mutagenesis in fungal laccases: effect on redox potential, activity and pH profile. Biochem J 334: 63-70.

Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dalboge H (1996). Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62: 834-841.

You LF, Liu ZM, Lin JF, Guo LQ, Huang XL, Yang HX (2014). Molecular cloning of a laccase gene from *Ganoderma lucidum* and heterologous expression in *Pichia pastoris*. J Basic Microbiol 54: S134-S141.
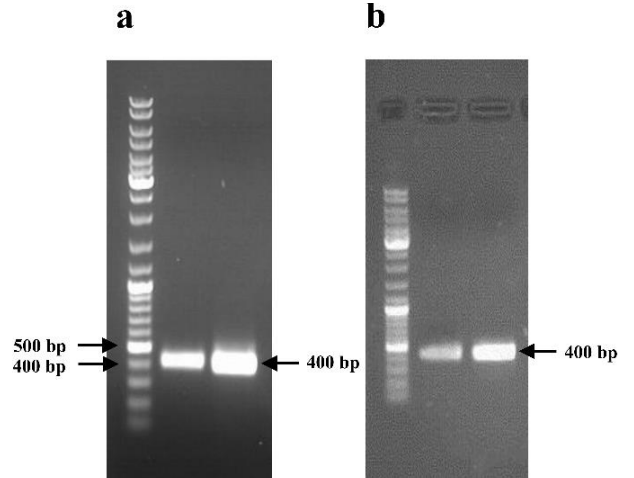
Supplementary Materials

**1. Results of agarose gel electrophoresis belonging to cloning studies.**
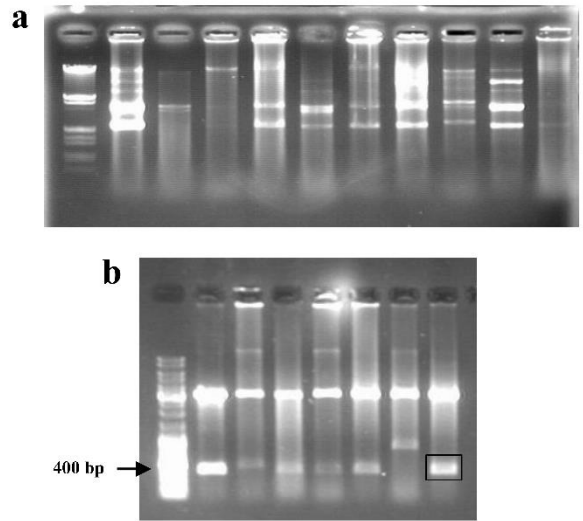
5'-RACE gel photos
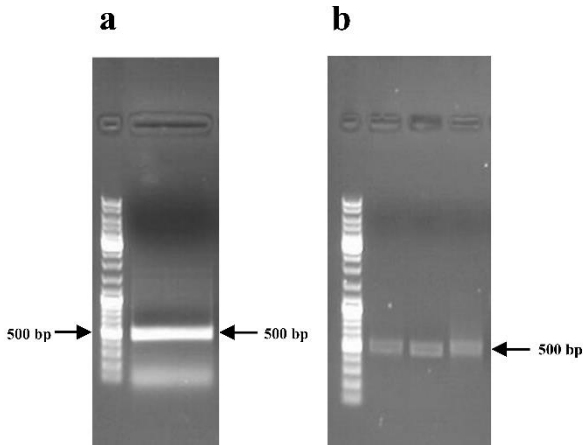
Figure S1. a) 5'-RACE PCR product before gel extraction, b) 5'-RACE PCR product after gel extraction. Marker: GeneRuler DNA Ladder Mix (Fermentas).

Figure S3. a) 5'-Nested RACE PCR product before gel extraction, b) 5'-nested RACE PCR product after gel extraction. Marker: GeneRuler DNA Ladder Mix (Fermentas).
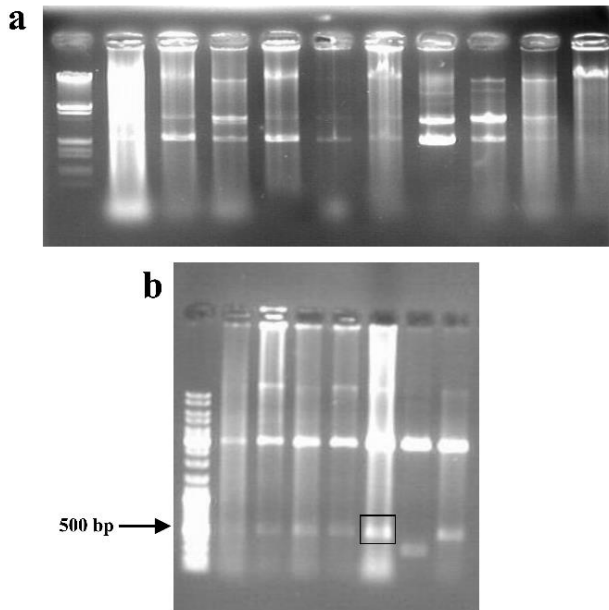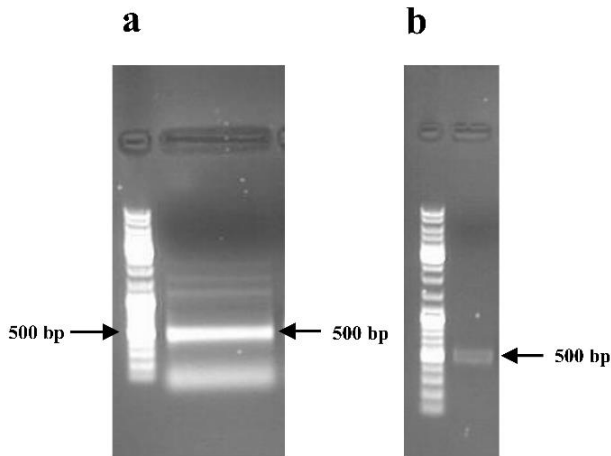

Figure S2. a) Plasmid DNAs isolated from *E. coli* DH5α Amp^R transformants containing 5'-RACE fragments. Marker: Lambda DNA/*Eco*RI+*Hind*III Marker (Fermentas). b) *Eco*RI restriction analysis of plasmid DNAs isolated from *E. coli* DH5α Amp^R transformants. Marker: GeneRuler DNA Ladder Mix (Fermentas).

Figure S4. a) Plasmid DNAs isolated from *E. coli* DH5α Amp^R transformants containing 5'-nested RACE fragments. Marker: Lambda DNA/*Eco*RI+*Hind*III Marker (Fermentas). b) *Eco*RI digestion of plasmid DNAs isolated from *E. coli* DH5α Amp^R transformants. Marker: GeneRuler DNA Ladder Mix (Fermentas).

3'-RACE gel photos

Figure S5. a) 3'-RACE PCR product before gel extraction, b) 3'-RACE PCR product after gel extraction. Marker: GeneRuler DNA Ladder Mix (Fermentas).
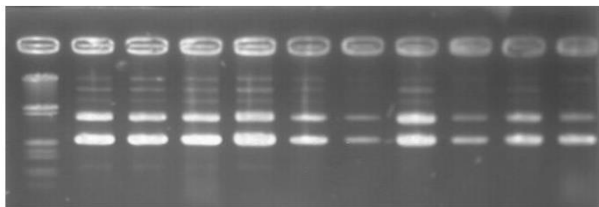

Figure S6. Plasmid DNAs isolated from positive colonies including 3'-RACE fragment. Marker: Lambda DNA/*Eco*RI+*Hind*III Marker (Fermentas).
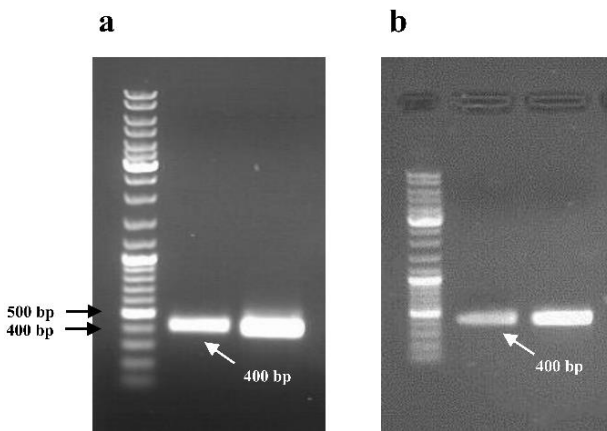

Figure S7. a) 3'-Nested RACE PCR product before gel extraction, b) 3'-nested RACE PCR product after gel extraction. Marker: GeneRuler DNA Ladder Mix (Fermentas).
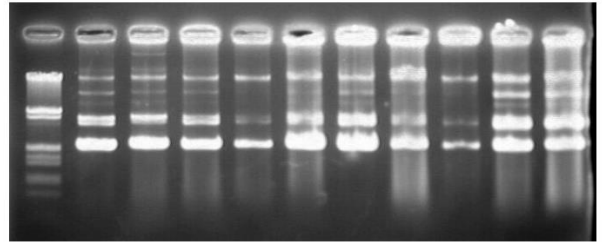
Figure S8. Plasmid isolated from positive colonies including 3'-nested RACE fragment. Marker: Lambda DNA/*Eco*RI+*Hind*III Marker (Fermentas).
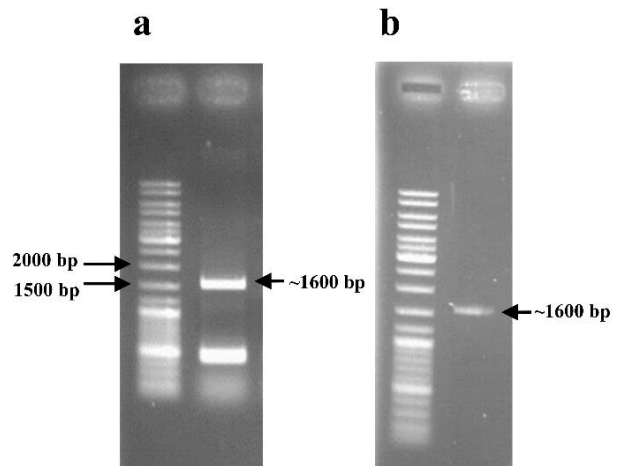
Figure S9. a) Full-length *Cplcc1* cDNA amplified with *Cplcc1* forward primer and *Cplcc1* reverse primer before gel extraction, b) full-length *Cplcc1* cDNA amplified with *Cplcc1* forward primer and *Cplcc1* reverse primer. Marker: GeneRuler DNA Ladder Mix (Fermentas).
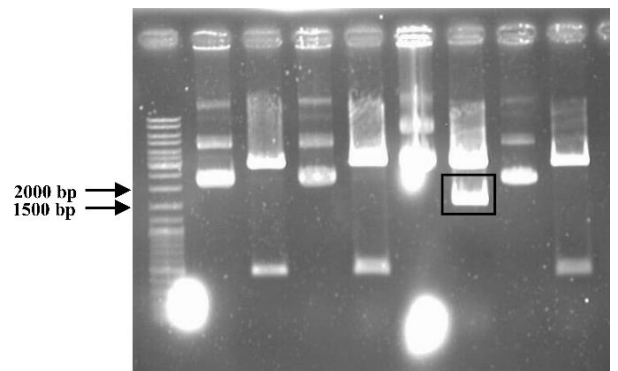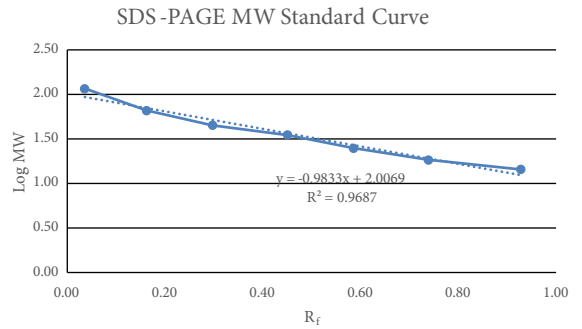

Figure S10. Digested with restriction enzyme and nondigested forms of digestion results of *Cplcc1*/pGEM®-T plasmids. Marker: GeneRuler™ DNA Ladder Mix (Fermentas).
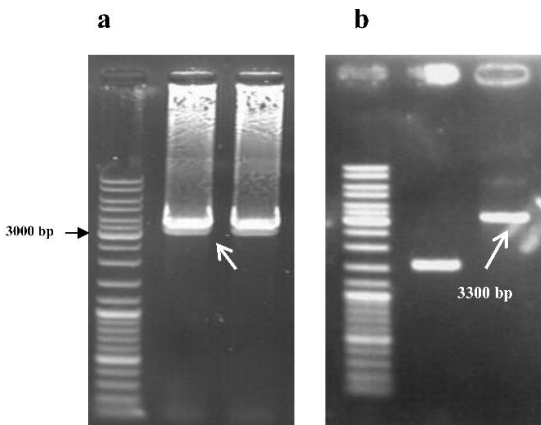
a   **b**
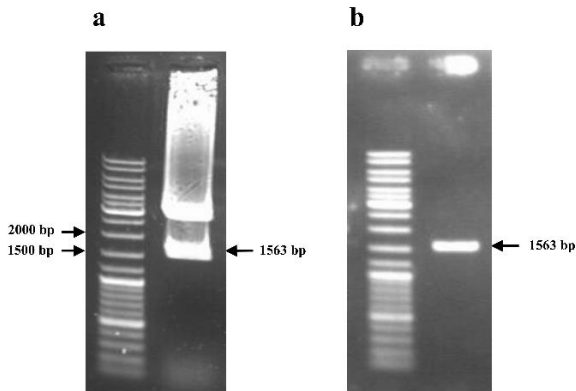
Figure S11. a) *Not*I and *Eco*RI double digestion of pGEM®-T cloning vector including PCR amplified full-length *Cplcc1* cDNA, b) PCR amplified full-length *Cplcc1* cDNA after gel extraction. Marker: GeneRuler DNA Ladder Mix (Fermentas).

a   **b**

Figure S12. a) *Not*I and *Eco*RI double digestion of pPICZC shuttle expression vector, b) pPICZC shuttle expression vector after gel extraction method. Marker: GeneRuler DNA Ladder Mix (Fermentas).

a   **b**

Figure S13. a) Plasmid isolation from positive clones with pPICZC including PCR amplified full-length *Cplcc1* cDNA, b) *Not*I and *Eco*RI double digestion of positive clones with pPICZC including PCR amplified full-length *Cplcc1* cDNA. Marker: GeneRuler DNA Ladder Mix (Fermentas).

2. SDS-PAGE molecular weight standard curve constructed for CpLcc1.

SDS-PAGE MW Standard Curve
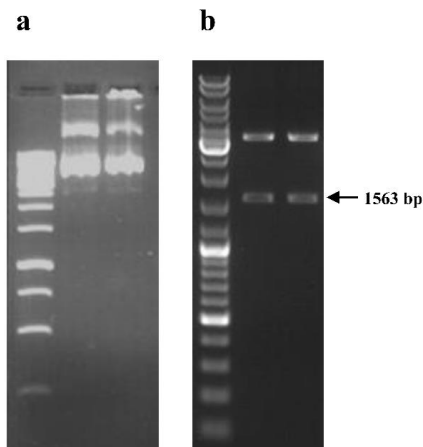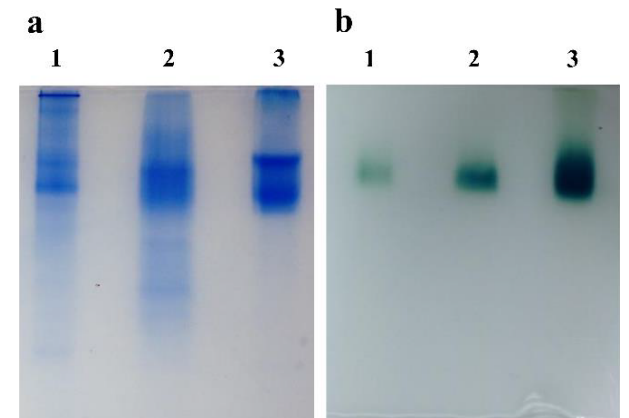
$y = -0.9833x + 2.0069$
$R^2 = 0.9687$

Marker standards (kDa)	Log	Migration (cm)	R_f
116.0	2.06	0.4	0.04
66.2	1.82	1.8	0.16
45.0	1.65	3.3	0.30
35.0	1.54	5.0	0.45
25.0	1.40	6.5	0.59
18.4	1.26	8.2	0.74
14.4	1.16	10.3	0.93
Dye front (cm) = 11.1			
Migration distance of CpLcc1 (cm) = 3.1			
Rf of CpLcc1 = 0.2793			

3. Native PAGE and Native-PAGE zymogram images.

a   **b**

 **1**   **2**   **3**   **1**   **2**   **3**

Native-PAGE (a) and Native-PAGE-zymogram analysis (b) of partially purified recombinant laccase CpLcc1. Lane 1: Sample from 90% ammonium sulfate fraction desalted by ultrafiltration; Lane 2: sample from Q-Sepharose anion exchange chromatography; Lane 3: Sample from Superdex-75 prep gel filtration chromatography. Native-PAGE gel was stained with Coomassie Brilliant Blue G-250. Zymogram analysis for laccase activity was performed after SDS-PAGE by using 2.5 mM ABTS in 100 mM sodium citrate buffer (pH 3.0).

4. Determination of kinetic constants of CpLcc1 (Michaelis–Menten plot of the recombinant CpLcc1 obtained by using GraphPad Prism version 5.00 (GraphPad Software)).

For determination of the K_m and V_{max} parameters, ABTS with concentration ranging from 0.1 to 0.6 mM was used as a substrate for 3 min in the presence of 100 mM sodium citrate buffer at pH 3.0 and 25 °C. All activity assays were carried out in triplicate. Standard deviation did not exceed 5% of the average values. Kinetic constants (K_m and V_{max} values) were calculated by Michaelis–Menten method using GraphPad Prism version 5.00 (GraphPad Software). Catalytic efficiency was explained by k_{cat} (turnover number) and specificity constant was indicated by $k_{cat} K_m^{-1}$ value. The k_{cat} value was also calculated as V_{max}/enzyme concentration (μM). The $k_{cat} K_m^{-1}$ value was also calculated using k_{cat} and K_m values. For determination of the K_m and V_{max} parameters, ABTS with concentration ranging 0.1 to 0.6 mM was used as a substrate for 3 min in the presence of 100 mM sodium citrate buffer at pH 3.0 and 25 °C. All activity assays were carried out in triplicate. Standard deviation did not exceed 5% of the average values. Catalytic efficiency was explained by k_{cat} (turnover number) and specificity constant was also indicated by $k_{cat} K_m^{-1}$ value. The k_{cat} value was also calculated as V_{max}/enzyme concentration (μM). The $k_{cat} K_m^{-1}$ value was also calculated using k_{cat} and K_m values.
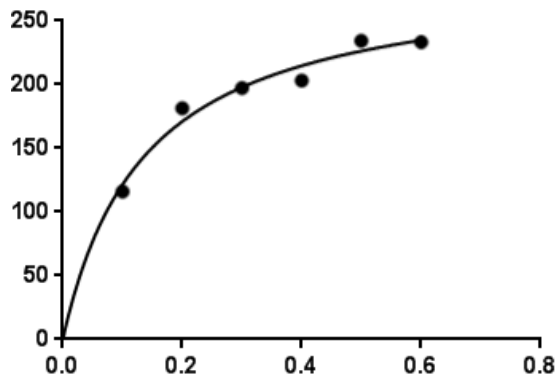
Michaelis–Menten plot of CpLcc1 constructed by using GraphPad Prism version 5.00 (GraphPad Software); Y-axis: laccase activity (U L^{-1}), X-axis: ABTS concentrations (mM).