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Comparison of Two Laccases from *Trametes versicolor* for Application in the Decolorization of Dyes

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It has been previously demonstrated that laccases exhibit great potential for use in several industrial and environmental applications. In this paper, two laccase isoenzyme genes, *lccB* and *lccC*, were cloned and expressed in *Pichia pastoris* GS115. The sequence analysis indicated that the *lccB* and *lccC* genes consisted of 1,563 and 1,584 bp, and their open reading frames encoded 520 and 527 amino acids, respectively. They had 72.7% degree of identity in nucleotides and 86.7% in amino acids. The expression levels of LccB and LccC were up to 32,479 and 34,231 U/l, respectively. The recombinant laccases were purified by ultrafiltration and $(NH_4)_2SO_4$ precipitation, showing a single band on SDS-PAGE, which had a molecular mass of 58 kDa. The optimal pH and temperature for LccB were 2.0 and 55°C with 2,2'-azino-bis-[3-ethylbenzthiazolinesulfonic acid (ABTS) as a substrate, whereas LccC exhibited optimal pH and temperature at 3.0 and 60°C. The apparent kinetic parameters of LccB were 0.43 mM for ABTS with a V_{max} value of 51.28 U/mg, and the K_m and V_{max} values for LccC were 0.29 mM and 62.89 U/mg. The recombinant laccases were able to decolorize five types of dyes. Acid Violet 43 (100 g/ml) was completely decolorized by LccB or LccC (2 U/ml), and the decolorization of Reactive Blue KN-R (100 g/ml) was 91.6% by LccC (2 U/ml). Thus, the study characterizes useful laccase isoenzymes from *T. versicolor* that have the capability of being incorporated into the treatment of similar azo and anthraquinone dyes from dyeing industries.

Keywords: Laccase, heterologous expression, *T. versicolor*, decolorization

Introduction

It is known that reactive dyes, including Reactive Blue KN-R and Reactive Red 7, are most commonly used in cotton dyeing industries, which have an effective bonding in a typical dyeing process, but the remaining dyes without adhesion are lost into the wastewater because they are uneasily removed owing to their complex structures. It is evident that textile dyes can have negative impacts on the entire surroundings of an ecosystem. In order to combat these negative effects, several physical and chemical methods such as coagulation-flocculation, adsorption, oxidation, ion-exchange, and electronchemistry [22] have been utilized to remove the remaining dyes from the effluent after printing and dyeing. However, under the physical and chemical conditions, these current approaches are unsafe and uneconomical. By contrast, laccases, which come from the white-rot basidomycetes, act oxidatively and less specifically on aromatic rings; thus, they have the potential to degrade a wider range of compounds [24].

Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) belong to a family of multi-copper oxidases, which have a very broad substrate specificity with respect to the electron donor. They can catalyze the four-electron reduction of O_2 to H_2O with the concomitant oxidation of phenolic compounds [14]. Oxidation of methoxyhydroquinones during lignin degradation, followed by the autooxidation of the resulting methoxysemiquinones, results in the formation of superoxide anion radicals that can undergo further reactions [11]. The rather broad substrate specificity of laccases may be additionally expanded by the addition of redox mediators, such as ABTS (2,2′-azino-bis-[3-

ethylbenzthiazolinesulfonic acid), 1-hydroxybenzotriazole, or compounds secreted by lignolytic fungi [2, 12, 27].

Laccases are widely distributed in fungi such as *Pleurotus ostreatus*, *Melanocarpus albomyces*, *T. versicolor*, and *Aeromonas hydrophila* [1, 19, 25]. Currently, laccases are also found to be widespread among bacteria, and actual laccase activity has been found in *Escherichia coli*, *Bacillus subtilis*, *Bacillus halodurans*, *Thermus thermophilus*, and several streptomycetes [17, 18, 21]. However, fungal laccases probably play important roles in several industrial applications, including decoloring different types of dyes, degrading lignocellulosic material, paper pulping, bioremediation, as well as other biotechnological applications [28]. Consequently, laccase proves to be a valuable enzyme with the potential to degrade a vast range of compounds. *T. versicolor*, a typical white-rot fungus with excellent performance for laccase production [13], was previously shown to secrete several laccase isoenzymes. The laccase isoenzyme LccA has been cloned and overexpressed in *P. pastoris* X33, and its biochemical characteristics have been studied extensively (data not shown).

In this study, we report the sequence analysis, cloning, expression, biochemical characterization, and applications of LccB and LccC from *T. versicolor*. This research lays a good foundation for the further application of laccase from *T. versicolor* in the biodegradation of industrial dyes.

Materials and Methods

Fungus Strains, Plasmids, and Growth Media

The white-rot fungus *T. versicolor* was preserved by the Microbial Technology Research Laboratory, Nanjing Forestry University (NJFU) and was screened from Purple Mountain located in Nanjing, China. *Escherichia coli* TOP10F, *P. pastoris* GS115, and expression vector pPICZαB and pPICZαC were purchased from Invitrogen (Carlsbad, CA, USA). The cloning vector pMD19-T, restriction endonucleases *Eco*RI, *Xba*I, T_4 DNA ligase, and Ex-Taq DNA polymerase were purchased from TaKaRa Biotechnology (Da Lian, China). ABTS was purchased from Sigma (St. Louis, MO, USA). YPD plates, YPDS plates, minimal methanol plates, and buffered glycerol-complex medium (BMGY) were prepared according to the operation manual of the *Pichia* Expression Kit (Invitrogen, USA). Final concentrations of 0.2 mM ABTS and 0.3 mM $CuSO_4$ were added to MM plates to prepare MM+ABTS+Cu^{2+} plates.

T. versicolor was grown at 28°C in Kirk salts medium: K_2PO_4 20 g/l, $MgSO_4{\cdot}7H_2O$ 5 g/l, $CaCl_2$ 1.3 g/l, Thiamin sol. 0.1 g/l, and Kirk trace elements 100 ml/l. Kirk trace elements (pH 4.5) contained $N(CH_2COOH)_3$ 9 g/l, $CoSO_4{\cdot}7H_2O$ 1.1 g/l, $ZnSO_4{\cdot}7H_2O$ 1.1 g/l, $CuSO_4{\cdot}5H_2O$ 0.06 g/l, $AlK(SO_4){\cdot}12H_2O$ 0.11 g/l, H_3BO_3 0.06 g/l, $MnSO_4{\cdot}5H_2O$ 4.3 g/l, $FeSO_4{\cdot}7H_2O$ 0.6 g/l, NaCl 6 g/l, $Na_2MoO_4{\cdot}2H_2O$ 0.072 g/l, $MgSO_4{\cdot}7H_2O$ 18 g/l, and $CaCl_2{\cdot}2H_2O$ 0.6 g/l.

RNA and DNA Manipulation

Qiagen plasmid kit and PCR purification kit (Qiagen, USA) were used for the purification of plasmids and PCR products. RNA Prime Script RT reagent Kit and the DNA restriction and modification enzymes were purchased from TaKaRa. DNA transformation was performed by electroporation using a GenePulser (Bio-Rad, USA).

Cloning of Laccase Genes

Total RNA was isolated from a culture of *T. versicolor* using Gromroff *et al*'s method [10]. PCR primers for cDNA amplification of *lccB* were designed based on the sequence of *lac*3 (GenBank Accession No. AB212732) and *lccC* based on *lac*4 (GenBank Accession No. 212733). The first-strand cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit (TaKaRa). PCR was carried out using exTaq DNA polymerase (TaKaRa), and the cDNA was used as the template. The 1.5 kb PCR products of *lccB* and *lccC* were cloned into vector pMD19-T and sequenced. All the primers are shown in Table 1.

Expression of Laccase Genes

PCR products *lccB* and *lccC* were digested with *EcoR*I and *Xba*I, ligated with the digested expression vectors pPICZαC and pPICZαB, and sequenced. *P. pastoris* GS115 cells were transformed with *Sac*I-linearized plasmids according to the *Pichia* expression vectors manual (Invitrogen). Transformants were picked from YPDS plates supplemented with zeocin (100 μg/ml) at 28°C for 3 days and screened for production of laccase by transferring the colonies onto MM plates containing $CuSO_4$ (0.3 mM) and ABTS (0.2 mM). Laccase-producing transformants were identified by the presence of a dark green color around the *Pichia* colonies.

Production of the Two Laccases

The transformants were inoculated into 20 ml of BMGY liquid

Table 1. Primer sequences for amplification and expression of laccase genes.

Name	Sequence (5′-3′)
*lccB*N	GGGATGGGCAGGGTCTCATCTCTCTG
*lccB*C	GGGTTAGAGGTCGGATGAGTCAAGAGCG
*lccC*N	GGGATGGGCAAGTTTCACTCTTTTGTGAA
*lccC*C	GGGTCAGAGGTCGGACGAGTCCAAA
*lccB*_pPICZαCF	GGGGAATTCATGGGCAGGGTCTCATCTCTC
*lccB*_pPICZαCR	GGGTCTAGATTAGAGGTCGGATGAGTCAAG
*lccC*_pPICZαBF	GGGGAATTCATGGGCAAGTTTCACTCTTT
*lccC*_pPICZαBR	GGGTCTAGATCAGAGGTCGGACGAGTCCA

medium containing 2% peptone, 1% yeast extract, 0.02% biotin, 1% glycerol, and 1% yeast nitrogen base in pH 6.0 100 mM phosphate buffer for 24 h at 28°C with constant shaking at 180 rpm. When OD_{600} reached values between 3.0 and 5.0, the cultures were harvested by centrifugation (1,500 ×*g*, 5 min). The pellets were resuspended in 50 ml of BMGY with 0.5 mM $CuSO_4$ to an OD_{600} of 1.0. The cultures were grown in 250 ml flasks at 28°C, 180 rpm, and with 0.6% (v/v) methanol being added daily. Laccase activity was assayed every 2 days.

Assay of Laccase Activity and Protein

The laccase activity assay system contained 500 µl of 10 mM ABTS and 4,350 µl of 50 mM sodium citrate buffer (under the optimal pH). Maintaining the mixture at 50°C, the change in absorbance at 420 nm was recorded once every minute for 5 min using a spectrophotometer. One enzyme activity unit (1 U) is defined as the amount of enzyme that oxidizes 1 µmol of substrate per minute [7]. For every sample, the activity was measured three times, and the average result was used to determine the laccase activity.

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard [3].

Characterization of Recombinant Laccases

The transformant with the highest laccase activity was inoculated to BMGY medium. After 16 days, the liquid supernatant was collected as the crude laccase. SDS-PAGE was carried out according to Laemmli's protocol with 5% stacking gel and 10% resolving gel. The molecular mass of the laccase was determined by calculating the relative mobility of a low molecular mass marker. The recombinant laccases' biochemical characteristics were investigated using ABTS as the substrate. The impact of temperature was examined over a range of temperatures (30–80°C) in 50 mM sodium citrate buffer, pH 4.5, for 5 min. For thermal stability analysis, the laccases were kept at a certain temperature for different pre-incubation times (10, 20, 30, 60, 90, and 120 min at a range of temperatures of 30–70°C); afterwards, the residual activities of the laccases were determined at 50°C , pH 4.5. The effects of pH on laccases were evaluated over a pH range of 2.0–8.0 at 50°C using sodium citrate buffer. The studies on the pH stability of the two laccases were carried out by pre-incubating the enzyme solutions at 4°C for 12 h in the aforementioned buffer systems in the absence of substrate. After 12 h, samples were taken and ABTS oxidation activity was measured. The effects of adding different ions on the recombinant laccases' activity levels were examined by adding 0.5 mM of different metal ions into the sodium citrate buffer (50 mM, pH 4.5). The activity was measured in 50 mM sodium citrate buffer, pH 4.5, with ABTS as substrate.

Every experiment was performed in triplicate.

Kinetic Parameters and Substrate Specificity

Kinetic parameters of the two purified enzymes were determined at room temperature using different concentrations of ABTS, guaiacol, syringaldazine, 2,6-dimethyl phenol, and Ledtolidine. The Michaelis-Menten constant was determined in 50 mM sodium cirtrate buffer, pH 4.5, at 50°C.

Nucleotide Sequence Analysis of *lccB* and *lccC*

The nucleotide sequences of *lccB*, *lccC*, and other known laccase genes were analyzed by the software Clustal 2.1. Then, the NJ Evolutionary tree was constructed by the software Mega 5.0. The glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Purification of Recombinant Laccases

The supernatants were harvested from the cultures of the recombinant *P. pastoris* GS115-LccB and GS115-LccC after 16 days. The supernatants were obtained from the cultured samples by centrifugation at 6,000 ×*g* for 10 min; afterwards, the centrifuged samples were purified by ultrafiltration using a CXA-50 30000 ultrafiltration membrane (Shanghai Yadong Nuclear Grade Resins Limited Company, China). The resulting filtrates were precipitated by adding ammonium sulfate to a final mass concentration of 80%. The proteins were dissolved in 50 mM sodium cirtrate buffer (pH4.5) and dialyzed for 12 h against the same buffer.

Decolorization of Dye by Recombinant Laccases

The involvement of *T. versicolor* laccases in dye decolorization was investigated using the purified enzymes. Decolorization was determined by measuring the decrease of dye absorbance, as its maximum visible wavelength and results are expressed in terms of a percentage. Laccase-catalyzed decolorization of synthetic dyes was carried out in a 10 ml reaction mixture containing oxalic acid buffer (20 mM, pH 4.5). A certain amount of purified enzyme and 100 mg/l dyes were reacted statically at 50°C for 28 h. During incubation, the time course of decolorization was detected every 4 h by measuring the absorbance. In this study, two anthraquinonic dyes (Reactive Blue KN-R and Acid Violet 43) and three azo dyes (Acid Red 35, Direct Turquoise Blue 5B, and Reactive Orange 7) (Zhejiang Runtu Chemical Group Limited Company, China) were used as specified in a previous study. Control samples without the enzyme were processed in parallel with the test samples. The decolorization of the five dyes was performed in triplicate as described above.

The absorption wavelengths of the five dyes were 610 nm for Reactive Blue KN-R, 600 nm for Acid Violet 43, 505 nm for Acid Red 35, 599 nm for Direct Turquoise Blue 5B, and 485 nm for Reactive Orange 7. (The structures of the five dyes are shown in Fig. 1.) The decolorization efficiency of each dye is shown as dye decolorization (%). The decolorization of dyes was calculated by means of the formula

$$\text{Decolorization (\%)} = [(C_i - C_t)/C_i] \times 100$$

where C_i is the initial concentration of the dye, and C_t is the dye concentration along the time [15]. The concentration of the dye was calculated according to the value of absorbance.

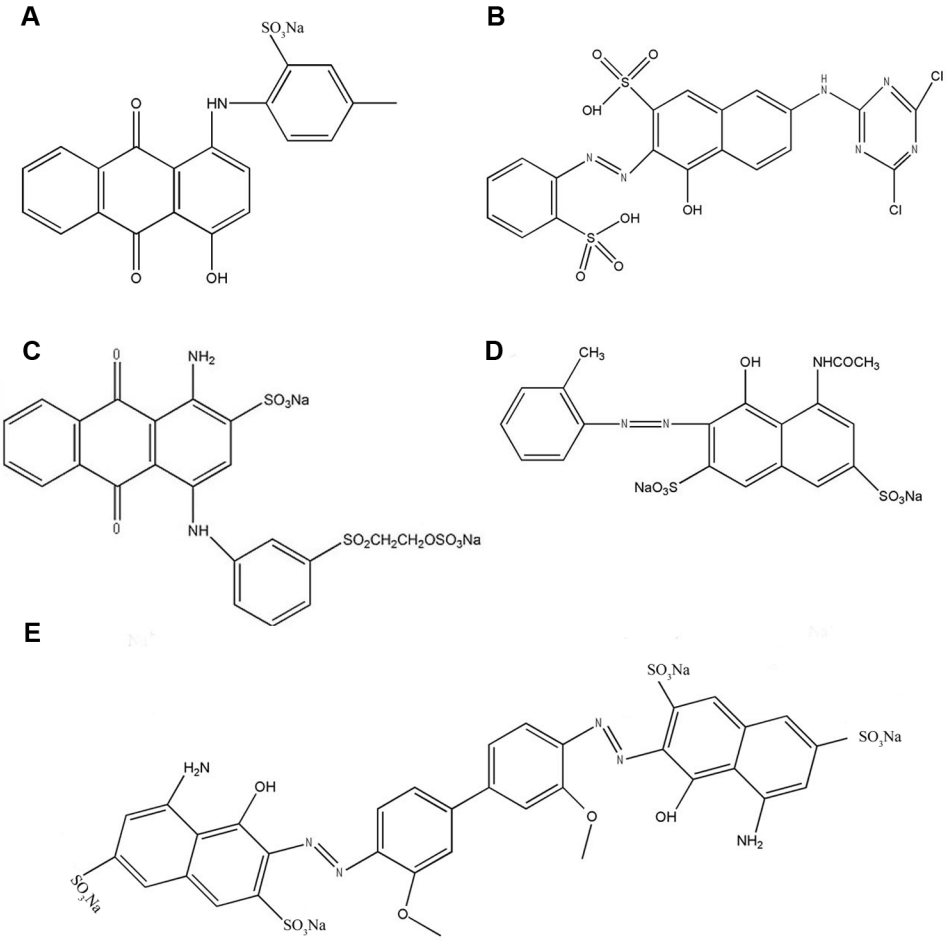

Fig. 1. The structures of the five dyes.
(**A**) Acid Violet 43; (**B**) Reactive Orange 7; (**C**) Reactive Blue KN-R; (**D**) Acid Red 35; (**E**) Direct Turquoise Blue 5B.

Results and Discussion

Cloning and Analysis of Laccase Genes *lccB* and *lccC*

According to previous studies, most fungi produce more than one laccase isoenzyme, which have an amino acid identity of between 60% to 80%, and different patterns of isoenzymes coded by genes families are differentially regulated by growth conditions and physiological states [16]. Different white-rot fungi are generally considered to be a good producer with a high yield of laccase [8, 9]. The structures of the laccase gene sequences are diverse in various promoter regions, which should contribute to the differential expression of laccase isoenzymes. Several promoter sequences of fungal laccases have been cloned and analyzed [6, 23]. In this work, a highly novel laccases-producing fungus, *T. versicolor*, was screened. Genes, namely *lccB* and *lccC*, were cloned and expressed in the heterologous host *P. pastoris* GS115. Laccase cDNA *lccB* and *lccC* were cloned by RT-PCR from total RNA of *T. versicolor* according to laccase sequences released in GenBank (GenBank Accession No. AB212733 for *lccB* and GenBank Accession No. AB212734 for *lccC*). The laccase cDNA was isolated from the *T. versicolor*. Two single PCR products of 1,563 and 1,584 bp were obtained for *lccB* and *lccC*, respectively (Fig. 2). The deduced amino acid sequences of LccB and LccC encode proteins of 521 and 526 amino acids, respectively. The similarity of amino acid sequences between LccB and LccC is 72%. The *lcc*B is closest to *Coriolus versicolor* laccase (GenBank: HM137002) with 93% identity. The *lccC* is closest to *T. versicolor* laccase IV (GenBank: U44431.1) with 95% identity. To gain insight into the evolutionary relationship among the laccases, we constructed the phylogenetic trees of 17 candidate sequences by using the NJ method and the MP method, respectively,

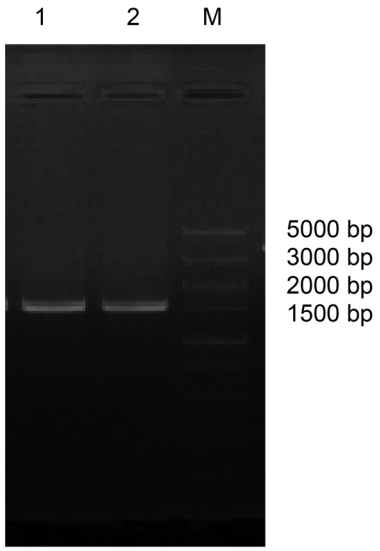

Fig. 2. Agarose gel of RT-PCR products.
Lane 1: lccB; Lane 2: lccC; Lane3: Marker (DL5000).

both supporting almost the same topology. The deduced amino acid sequences of the *T. versicolor* laccases were compared with 15 other kinds of laccases available in the GenBank database (Table 2). The results revealed that *lccB* and *lccC* from *T. versicolor* have a relatively distant relationship, and they have a close affinity with *Coriolus versicolor* (Fig. 3).

Expression and Purification of Laccase Genes *lccB* and *lccC* in *P. pastoris*

Laccase production from parent strains acquired from the optimization of culture medium and conditions does not meet the modern-day industry's demands. For production of the recombinant protein, genetic engineering is the first choice because it is easy, fast, and cheap [26]. In the past decade, the yeast *P. pastoris* has become a common system for the expression of heterologous proteins.

PCR products *lccB* and *lccC* were digested with *Eco*RI and *Xba*I and ligated with the digested expression vector pPICZαC and pPICZαB, respectively. The recombinant plasmids pPICZαC-lccB and pPICZαB-lccC constructed by our laboratory were linearized with *Sac*I, and then transformed into the recombinant *P. pastoris* GS115. The *P. pastoris* GS115 contained pPICZαC-lccB and pPICZαB-lccC competent cells that had been constructed, and expressed the laccase by using a Gene Pulser Xcell Electroporation System (Bio-Rad, USA) at 2,000 V and 5.1 msec according to the *Pichia* expression vectors manual (Invitrogen). The multicopy recombinant *P. pastoris* strains were screened and identified on YPDS plate containing zeocin (100 μg/ml) at 28°C for 3–4 days. The transformants were screened for production of

Table 2. The highest levels of identity between *lccB* and *lccC* and other fungal laccase genes.

Gene	Percent identity to gene															
	lccB	*lccC*	*lcc1*	*lcc2*	*lacA*	*lacB*	*lacC*	*lacD*	*lacE*	*T.C30*	*T.I62*	*C.G*	*C.R*	*F.V*	*G.L*	*T.H*
lccA	66.4	69.0	66.3	68.4	65.6	65.7	66.1	59.7	71.8	71.2	86.5	74.0	71.6	71.0	75.3	78.4
lccB		74.1	93.6	73.0	69.5	62.1	62.8	65.7	64.5	68.7	68.2	66.5	65.0	61.9	66.8	67.2
lccC			73.0	95.4	67.5	64.2	64.9	64.8	64.4	74.1	70.5	68.5	65.8	65.6	67.3	68.6
lcc1				70.6	68.3	61.9	62.6	64.7	63.4	68.6	67.9	66.0	68.5	61.3	66.0	70.6
lcc2					65.2	63.2	63.8	63.4	63.3	72.9	70.0	67.4	68.9	64.7	66.7	71.9
lacA						62.3	62.7	67.9	65.5	73.7	67.5	69.9	65.3	63.4	67.9	69.0
lacB							97.8	57.3	61.5	63.5	65.0	64.7	66.3	69.0	63.0	65.9
lacC								61.7	63.5	65.1	65.2	66.7	69.8	63.4	65.7	70.5
lacD									64.4	74.8	65.2	65.5	64.1	63.4	64.6	68.1
lacE										71.2	71.5	75.8	75.5	69.8	73.1	77.4
T.C30											73.4	80.9	76.5	68.9	72.8	76.5
T.I62												75.4	75.3	71.7	75.2	80.8
C.G													76.5	70.6	75.0	80.8
C.R														69.6	72.2	80.2
F.V															77.7	79.8
F.V																72.5

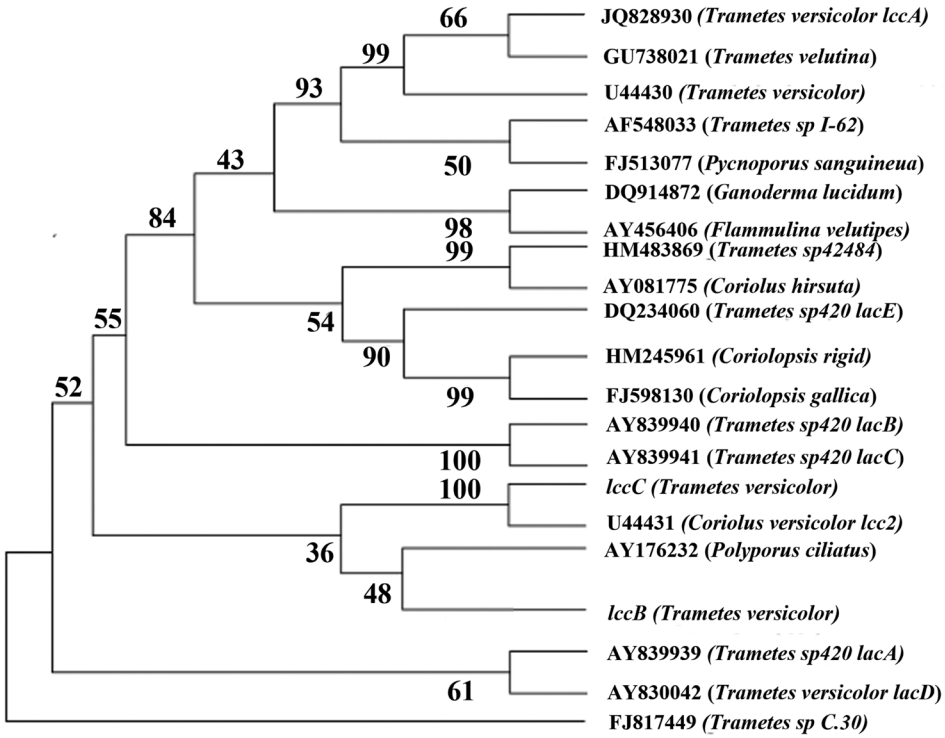

Fig. 3. The NJ tree of the two recombined laccases LccB and LccC.

laccase by transferring the colonies on MM plates containing 0.3 mM $CuSO_4$ and 0.2 mM ABTS. Laccase-producing transformants were identified by the presence of a dark green color around the *Pichia* colonies. Then positive transformants were cultured in BMGY medium with methanol (100%) added daily (final concentration 0.6% (v/v)) to maintain induction. The two laccase genes were expressed in *P. pastoris* GS115 with the highest activities of 32,479 U/l (LccB) and 34,231 U/l (LccC). The profile showed a peak of laccase activity at the 16th day (Fig. 4).

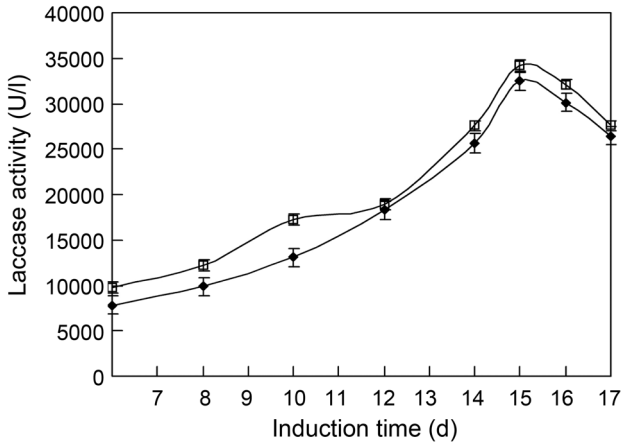

Fig. 4. Production of recombinant laccases LccB and LccC.
LccB (filled square); LccC (hollow square).

The transformants GS115-3.5K-lccB-38 and GS115-3.5K-lccC-50 had the highest laccase-secreting abilities. Based on the fermentation profile, the liquid cultures were harvested after 16 days. The crude laccases were subsided with 80% ammonium sulfate and dialyzed using 50 mM sodium citrate buffer 2 to 3 times. The purified laccases showed a single band on the SDS-PAGE gel (Fig. 5). The recombinant laccases LccB and LccC showed a molecular mass of about 58 kDa on 10% SDS-PAGE, without undesired bands (Fig. 5), indicating that *P. pastoris* secretes very low levels of native protein. The molecular mass was greater than the theoretic molecular mass 55 and 56 kDa, which may be the glycosylation of the enzyme in *P. pastoris*. By the analysis of amino acid sequence of LccB and LccC from *T. versicolor*, 4 and 11 glycosylation sites were found, respectively, using the software SignalP 4.0. These may normally be relatively highly glycosylated.

Characterization of LccB and LccC

The biochemical properties of the laccases were investigated by using the purified enzyme. The optimum pH for LccB

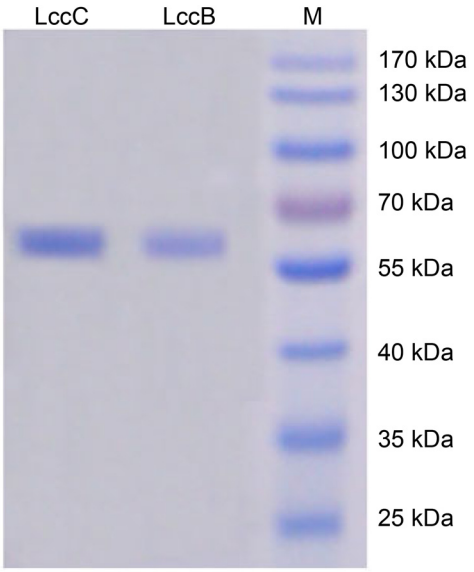

Fig. 5. SDS-PAGE of the recombinant laccases.
Lane 1–2: Purified heterologous laccases (1: LccC; 2: LccB). Lane 3: Prestained protein marker (10–170 kDa).

and LccC for ABTS was determined to be 2.0 and 3.0, respectively, and both isoenzymes showed higher than 50% of the maximum activity at the pH range of 2.0 to 4.0 (Fig. 6A). The two laccases showed good stability after being treated in sodium citrate buffers of different pH levels for 12 h (Fig. 6B). The optimum temperature of LccB and LccC was 55°C and 60°C, respectively (Fig. 6C). Thermostability assays indicated that LccB residual activity was more that 80% after being incubated at 50°C for 1 h, and LccC residual activity was more that 60% after being incubated at 55°C for 1 h. In practical applications, the high thermostability of the enzyme is desirable because the longer active life means less consumption of the enzyme. The laccase showed excellent stability after being incubated at 30°C and 50°C for 2 h (Fig. 6D). The recombinant laccases have satisfactory pH stability and thermostability, which are favorable factors for the potential applications of the enzyme. Meanwhile, the different enzymatic properties between the two laccases demonstrate that even the same source of isoenzyme may have different properties.

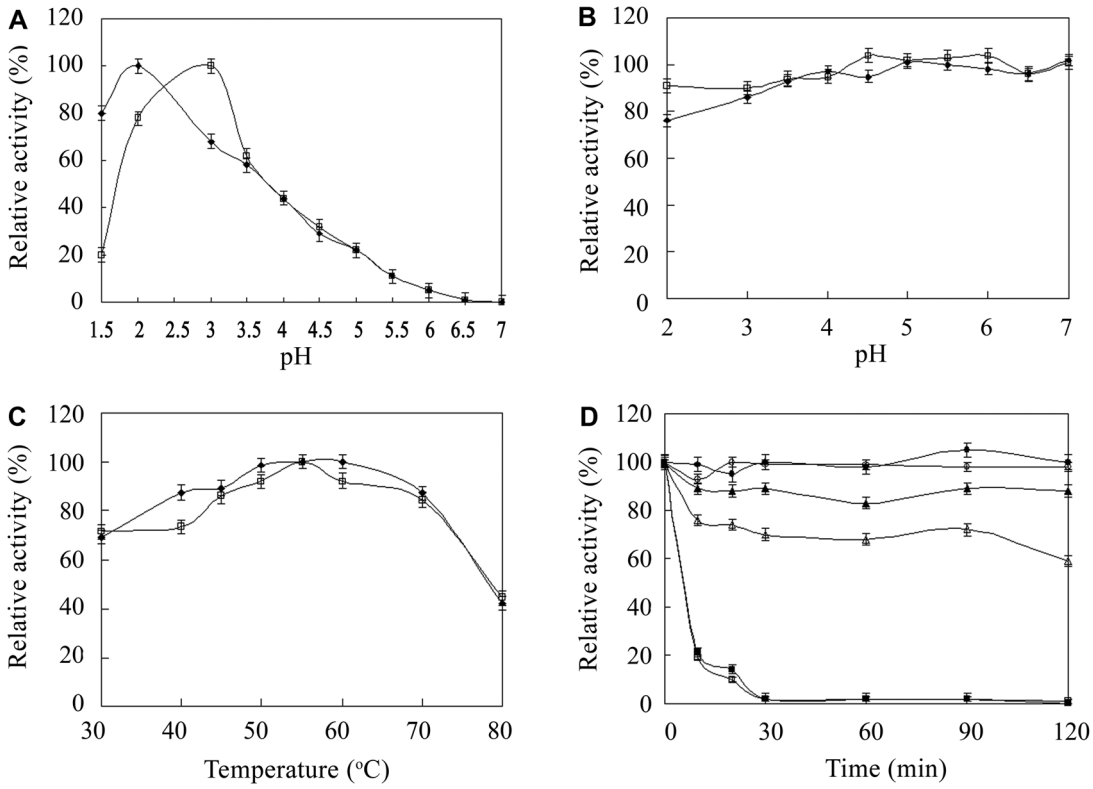

Fig. 6. Effects of pH and temperature on the activity and stability of the two laccases.
(**A**) Optimum pH of recombined laccases LccB and LccC. LccB (filled square); LccC (hollow square). (**B**) pH stability of recombined laccases LccB and LccC. LccB (filled square); LccC (hollow circle). (**C**) Optimum temperature of recombined laccases LccB and LccC. LccB (filled square); LccC (hollow square). (**D**) Thermostability of recombined laccase LccB: 30°C (filled circle), 50°C (filled triangle), 70°C (filled square); and LccC: 30°C (*hollow* circle), 50°C (hollow triangle), 70°C (hollow square).

Table 3. Effects of metal ions on laccases LccB and LccC.

Ions	Relative enzyme activity (%)	
	LacB	LacC
Control	100	100
EDTA	93.6	93.9
Al^{3+}	126.9	133.3
Cu^{2+}	113.4	107.1
Zn^{2+}	66	63.6
Ca^{2+}	62.3	67.7
Fe^{2+}	1.4	0
Fe^{3+}	43.2	44.7
Na^{+}	80.1	78.6
K^{+}	71.6	76.8
Li^{+}	84.4	76.8
Mg^{2+}	60.3	62.9
NH_4^{+}	93.5	98
Mn^{2+}	60.3	62.6
Ba^{2+}	73	65.7
Hg^{+}	98.4	94.5
Co^{2+}	66.5	73.9
Ni^{2+}	102.9	111.3

Metal ions' effects on enzymatic activity were characterized by the ability of Cu^{2+} and Al^{3+} to significantly enhance the enzyme activity, whereas the activity was completely inactivated by Fe^{2+}. The effects of NH_4^{+}, Li^{+}, Mg^{2+}, and EDTA on the enzyme activity were not so significant (Table 3). The kinetic parameter of LccB was 0.43 mM for ABTS with a V_{max} value of 51.28 U/mg, and K_m and V_{max} of LccC were 0.29 mM and 62.89 U/mg under optimal conditions. As shown in Table 4, the K_m values of LccB and LccC under other substrates were all higher than ABTS, which suggests that these two laccases have good substrate specificity for ABTS.

Decolorization of Dyes by LccB and LccC

Laccase is known to have the ability to decolorize dyes of various classes [20]. Different laccase isoenzymes from various sources are known to be diverse in the efficiency of decolorization of a certain dyestuff [4, 5]. It is thus important to search for the laccase isoenzymes LccB and LccC from *T. versicolor* with ideal characteristics and to understand what specific dyes can be decolorized by them. In this paper, the capability of the purified recombinant laccases LccB and LccC from the recombinant *P. pastoris* was assessed by using two anthraquinonic dyes (Reactive Blue KN-R and Acid Violet 43) and three azo dyes (Acid Red 35, Direct Turquoise Blue 5B, and Reactive Orange 7). The Acid Violet 43 was decolorized completely with a final decolorization degree of above 99% by LccB or LccC within half an hour. For the other four dyes, however, the decolorization rates were only between 20% and 85%. In addition, LccC was better than LccB in decolorizing Reactive Blue KN-R (anthraquinonic dye), but performed worse than LccB in decolorizing Acid Red 35 (azo dye); thus, it can further enable laccase to decolorize real textile industry wastewater containing synthetic dyes of different chemical structures such as anthraquinone, azo, *etc*. It also can be concluded that laccases that belong to the same source have different substrate specificity with different substrates of dyes.

To improve the decolorization of the other four dyes, except the Acid Voilet 43, conditions of the reaction were

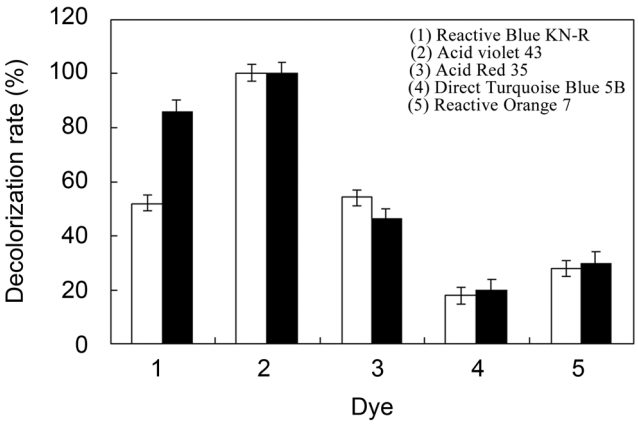

Fig. 7. Decolorization efficiencies of laccases LccB and LccC for five dyes.
LccB (*hollow column*); LccC (*filled column*).

Table 4. The K_m values of different substrate for LccB and LccC.

Type	K_m value (mM/l)				
	ABTS	Guaiacol	Ledtolidine	Syringaldazine	2,6-Dimethyl phenol
LccB	0.44	7.84	2.09	0.56	7.14
LccC	0.29	6.46	2.11	0.43	4.06

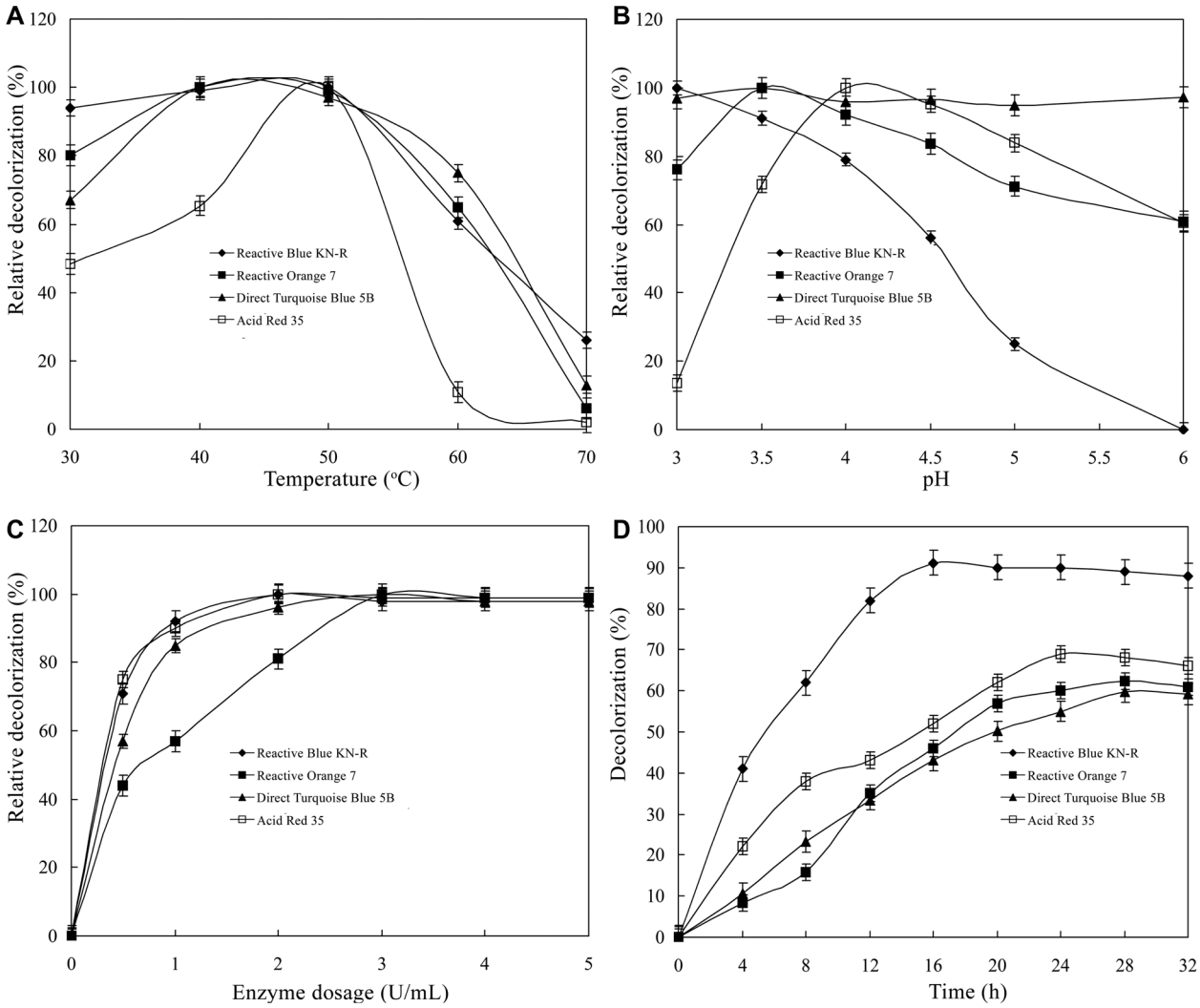

Fig. 8. Optimization of conditions for the decolorization of the four dyes.
(**A**) Optimum temperature of the decolorization efficiencies of four dyes. Reactive Blue KN-R (filled diamond); Acid Red 35 (filled square); Direct Turquoise Blue 5B (filled triangle); Reactive Orange 7 (hollow square). (**B**) Optimum pH of the decolorization efficiencies of four dyes. Reactive Blue KN-R (filled diamond); Acid Red 35 (filled aquare); Direct Turquoise Blue 5B (filled triangle); Reactive Orange 7 (hollow square). (**C**) Optimum enzyme activity of the decolorization efficiencies of four dyes. Reactive Blue KN-R (filled diamond); Acid Red 35 (filled square); Direct Turquoise Blue 5B (filled triangle); Reactive Orange 7 (hollow square). (**D**) Optimum incubation time of the decolorization efficiencies of four dyes. Reactive Blue KN-R (filled diamond); Acid Red 35 (filled square); Direct Turquoise Blue 5B (filled triangle); Reactive Orange 7 (hollow square).

optimized by the single-factor experiments. The optimum temperatures for decolorizing the Reactive Blue KN-R, Direct Turquoise Blue 5B, and Reactive Orange 7 with LccC and the Acid Red 35 with LccB were 40°C and 50°C, respectively (Fig. 8A). The optimum pH levels were 3.0 (LccC for the Reactive Blue KN-R and Direct Turquoise Blue 5B), 3.5 (LccC for Reactive Orange 7), and 4.5 (LccB for the Acid Red 35) (Fig. 8B). The higher the activity of the laccase, the faster the speed required for completing the process of the decolorization (Fig. 8C). The optimum enzyme dosages of the four dyes were 2 U/ml for Reactive Blue KN-R and Acid Red 35 and 3 U/ml for Direct Turquoise Blue 5B. The decolorization efficiency for 16 h of incubation was detected to be 91.5% for Reactive Blue KN-R in 40°C, pH 3.0 by LccC and for 24 h of incubation was detected to be 68.9% for Acid Red 35 in 50°C, pH 4.5 by LccB with enzyme dosage of 2 U/ml. For the Direct Turquoise Blue 5B and Direct Orange 7, the decolorization

efficiencies were 59.7% and 68.2% after 28 h of incubation in 40°C by LccB with enzyme dosage of 3 U/ml with pH 3.0 and 3.5, respectively (Fig. 8D). After optimization, the decolorization rates of the other four dyes increased in different degrees.

Summary

In the present work, two laccase isoenzyme genes *lccB* and *lccC* were derived and expressed in *P. pastoris* GS115 with the highest activity of 32,497 U/l, and 34,231 U/l, respectively. The optimum pH of LccB and LccC for ABTS was 2.0 and 3.0, respectively. The optimum temperature of LccB and LccC was 55°C and 60°C, respectively. Both laccases showed good pH and temperature stability, which play an important role in the decolorization of different dyes. Owing to these characteristics, *T. versicolor* and its laccases possess great potential and promising application in the process of decolorizing industrial dyes.

However, the yield of the heterologous enzymes produced by the new laccase genes was lower than expected. To improve the expression system, future studies should focus on the optimization of the culture condition for heterologous expression to achieve industrial-scale production of the heterologously expressed laccase. Additional research such as directed evolution should be also carried out in order to make the two laccase isoenzymes ready for application.

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