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Research Article

Differential Expression of Laccase Genes in *Pleurotus ostreatus* and Biochemical Characterization of Laccase Isozymes Produced in *Pichia pastoris*

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Abstract In this study, transcriptome analysis of twelve laccase genes in *Pleurotus ostreatus* revealed that their expression was differentially regulated at different developmental stages. *Lacc5* and *Lacc12* were specifically expressed in fruiting bodies and primordia, respectively, whereas *Lacc6* was expressed at all developmental stages. *Lacc1* and *Lacc3* were specific to the mycelial stage in solid medium. In order to investigate their biochemical characteristics, these laccases were heterologously expressed in *Pichia pastoris* using the pPICHOLI-2 expression vector. Expression of the laccases was facilitated by intermittent addition of methanol as an inducer and sole carbon source, in order to reduce the toxic effects associated with high methanol concentration. The highest expression was observed when the recombinant yeast cells were grown for 5 days at 15°C with intermittent addition of 1% methanol at a 12-hr interval. Investigation of enzyme kinetics using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as a substrate revealed that the primordium-specific laccase *Lacc12* was 5.4-fold less active than *Lacc6* at low substrate concentration with respect to ABTS oxidation activity. The optimal pH and temperature of *Lacc12* were 0.5 pH units and 5°C higher than those of *Lacc6*. *Lacc12* showed maximal activity at pH 3.5 and 50°C, which may reflect the physiological conditions at the primordiation stage.

Keywords Heterologous expression, Laccase, Mushroom, Primordia

Laccases (EC 1.10.3.2) are multi-copper polyphenol oxidases produced by fungi, bacteria, and plants. These enzymes are involved in the formation of plant cell wall by catalyzing the polymerization of monolignols to lignin through the generation of free radical via Cu^{2+}-mediated single-electron transfer [1]. Contrary to plants, fungi and bacteria use laccases to mineralize recalcitrant lignin in dead wood making the microbial laccases key players in recycling of carbon-based materials in nature. In addition, laccases have attracted a great deal of attention in biotechnological applications due to their high catalytic activity toward a broad range of aromatic substrates. Laccases, which are potentially useful for bioremediation of aqueous environments polluted by endocrine disruptors [2], polycyclic aromatic hydrocarbons [3, 4], and organic dyes [5], have also been used for denim bleaching in the textile industry [6] and pulp delignification in paper pulp manufacturing processes [7]. Recent studies have also demonstrated the potential utility of laccases in organic synthesis [8] and biotechnological applications, particularly in the fabrication of biosensors and biofuel cells [9, 10].

In fungi, laccase is involved in a variety of cellular physiological events. This enzyme catalyzes the production of pigments, such as melanin in *Lentinula edodes* [11] and *Cryptococcus neoformans* [12], and cinnabarin in *Pycnoporus* mushrooms [13]. Melanins play important roles in the pathogenesis of *C. neoformans* in human [12], as well as in primordiation of *L. edodes* [11]. Cinnabarin, synthesized by *Pycnoporus coccineus*, exhibits antimicrobial activity [13]. Moreover, white rot fungi, such as *Trametes versicolor* and *Pleurotus ostreatus*, appear to produce laccases as a defense mechanism against environmental microorganisms [14]. An example of this defense mechanism is the degradation

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of aflatoxin by laccase from *P. ostreatus* [15].

Genomes of filamentous fungi, particularly basidiomycetes, contain multiple copies of laccase genes [16, 17]. Laccase genes are induced by substrate analogs [18-20], copper ions [4, 20, 21], and nutritional factors [22]; however, the presence of an inducer does not result in all the copies of the laccase genes being expressed. *P. coccineus* expresses a single laccase gene, encoding a major extracellular protein, upon induction by copper ions, despite the presence of 7~9 isogenes in the genome [4]. Similarly, *P. ostreatus* has 12 homologous laccase genes but produces only two major laccase proteins, LCC1 and LCC2, in the culture supernatant [23]. Recent studies using chemically defined medium have demonstrated that the expression of laccase genes in *P. ostreatus* is rigorously regulated in response to different environmental conditions, in a strain-dependent manner, at the transcription level [24]. In particular, two laccases, *Lacc2* and *Lacc10*, which were highly overexpressed in the inducible medium, were mainly responsible for the laccase activity observed in the culture supernatant [24]. *Lacc2*, characterized under the name of POXA3, was found to form heterodimer with a 16-kDa small subunit protein [25, 26]. *Lacc9* and *Lacc10* were also characterized under the names POX1 [27] and POX2 or POXC [28, 29], respectively.

This study was conducted to extend our knowledge of the physiological and biochemical characteristics of *P. ostreatus* laccases. In order to accomplish this, we investigated the expression of 12 laccase genes at the transcription level during different developmental stages. We next constructed a heterologous expression system in *Pichia pastoris* and optimized the culture conditions for maximal laccase production. Finally, the biochemical characteristics of two laccase enzymes were examined.

MATERIALS AND METHODS

Strains and culture media. A commercial strain of *Pleurotus ostreatus* Chunchu was obtained from Culture Collection of Wild Mushroom, Incheon University, Korea. *P. ostreatus* was grown in a polypropylene bottle with a solid substrate consisting of poplar tree sawdust (50%), beet pulp (30%), and cottonseed hull (20%). The water content of the solid substrate was adjusted to 75%. *Pichia pastoris* GS115 (Life Technology, San Diego, CA, USA) was used as the host strain for laccase expression. The yeast cells harboring the expression vector were grown in buffered methanol-complex medium (BMMY) consisting of yeast extract (10 g/L), tryptone (10 g/L), yeast nitrogen base (13.4 g/L), biotin (0.4 mg/L), histidine (40 mg/L), 0.1 M potassium phosphate buffer (pH 6.0), and zeocin (100 mg/mL).

Preparation of mRNA from mycelia, primordia, and fruiting bodies of *P. ostreatus* for transcriptome analysis. In order to obtain mycelia, primordia, and fruiting bodies of *P. ostreatus*, mycelia were grown in the substrate bottle by incubating at 25°C for 30 days in the dark. Fruiting was induced by shifting the temperature to 18°C after physical shock. For mRNA preparation, mycelia, primordia, and fruiting bodies were collected from the culture bottle and frozen in liquid nitrogen. The frozen samples were ground with a pestle and mortar. Total RNA was extracted from the ground samples using TRIzol reagent (Solgent Co., Daejeon, Korea). RNA-Seq was conducted using a commercial sequencing service (Macrogen, Seoul, Korea). The mRNA level determined by RNA-Seq was expressed as Reads Per Kilobase per Million mapped reads (RPKM).

Construction of expression vectors and transformation. The DNA sequences for *P. ostreatus* laccase genes were obtained from the *P. ostreatus* genome sequence in the Comparative Fungal Genomics Platform site (http://cfgp.snu.ac.kr/ [30]) by BLAST search with known fungal laccase gene sequences. The laccase genes were synthesized, with codon optimization, for *Pichia* expression using a commercial gene synthesis service (Bioneer Co., Daejeon, Korea). The synthesized genes were amplified using a forward primer (5′-GGCTTAGTCGACATGGCCGTCTCTGTC-3′) and reverse primer (5′-CATAATGCGGCCGCTGCGGGCAG-ACGATG-3′). The PCR products were inserted between the *Sal*I and *Not*I sites of a *Pichia* expression vector, pPICHOLI-2 (MoBiTec GmbH, Göttingen, Germany). Transformation of *P. pastoris* was performed by electroporation using competent yeast cells grown to OD600 = 1. Laccase gene expression in pPICHOLI-2 was placed under the control of the methanol-inducible alcohol oxidase I (AOX1) promoter.

Laccase activity assay. Laccase activity was measured using 2 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma-Aldrich, St. Louis, MO, USA) in 50 mM sodium acetate buffer (pH 4.0). The reaction mixture, containing 0.99 mL substrate solution and 0.01 mL enzyme, was incubated for 3 min at 25°C. The reaction was tracked using a UV-Vis spectrophotometer (Model Ultraspec 2100 Pro; Amersham Biosciences, Piscataway, NJ, USA) at 420 nm. The absorbance at 420 nm was converted into the amount of product using the molecular extinction coefficient of the oxidized ABTS ($\varepsilon_{420\,nm}$ = 36,000/M/cm). One unit of enzyme activity was defined as the amount of laccase required to produce 1 μmole of the product per minute.

Purification of the laccase protein. The culture supernatant of *P. pastoris* harboring pPICHOLI-*Lacc* was collected by centrifugation at 2,500 ×g for 10 min. The supernatant was precipitated by ammonium sulfate fractionation. The precipitant was dissolved in phosphate buffered saliine buffer and then loaded onto a DEAE-Sephadex column (Amersham Biosciences). The bound protein was eluted by NaCl gradient in 30 mM sodium phosphate buffer (pH 7.0). The flow rate was 1 mL/min.

RESULTS

Laccase genes in *P. ostreatus* and their differential expression. The *P. ostreatus* genome contains 12 homologous laccase genes (Table 1). Comparison of the translated protein sequences with known laccase sequences from four mushroom species revealed that the *P. ostreatus* laccases are clustered into three distinct groups (Fig. 1). *P. ostreatus* laccase proteins commonly contain a highly conserved mononuclear copper center, T1, and a trinuclear copper center, T2/T3, with conserved sequence signatures, including HXHG, HXH, HXXHXH, and HCHXXXHXXXXL/F (Fig.

Table 1. Laccase genes in *Pleurotus ostreatus*

Gene	Synonym	Sequence name	Length of protein (aa)	Reference
Lacc1[a]		e_gw1.6.1115.1	532	-
Lacc2	POXA3	e_gw1.8.1330.1	483	[25]
Lacc3		genemark.4314_g	541	-
Lacc4		fgenesh1_pm.C_scaffold_6000411	522	-
Lacc5		estExt_fgenesh1_pm.C_110124	628	-
Lacc6	POXA1b	estExt_Genewise1Plus.C_61245	534	[31]
Lacc7		fgenesh1_pm.C_scaffold_6000308	513	-
Lacc8		genemark.8511_g	534	-
Lacc9	POX1, LAC1	estExt_fgenesh1_pm.C_60392	529	[27]
Lacc10	POX2, POXC	fgenesh3_kg.6_#_42_#_gi	533	[28, 29]
Lacc11		e_gw1.6.1130.1	557	-
Lacc12		estExt_Genewise1.C_110409	515	-

[a]Laccase gene names were taken from Castanera *et al.* [24].

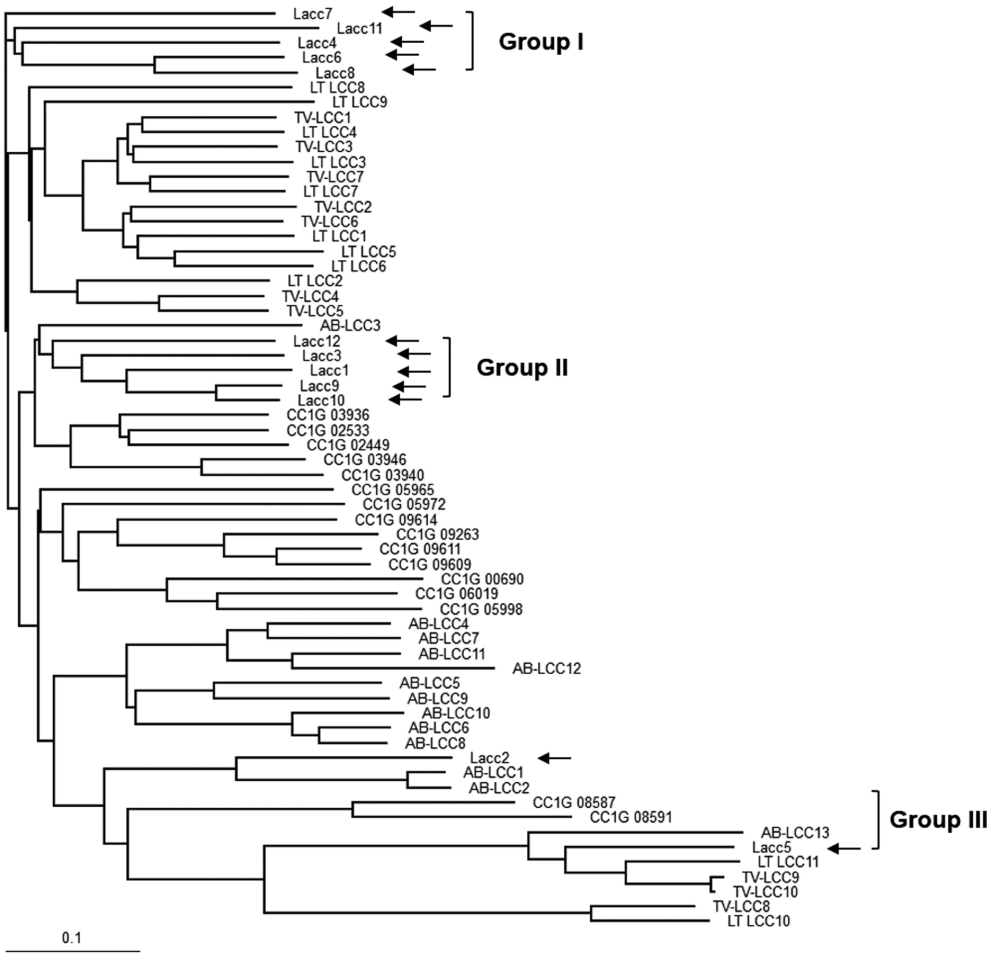

Fig. 1. Phylogenetic analysis of laccases from *Pleurotus ostreatus*. Laccase protein sequences were obtained from the Comparative Fungal Genomics Platform. AB, *Agaricus bisporus*; CC, *Coprinopsis cinerea*; LT, *Lentinula tigrinus*.

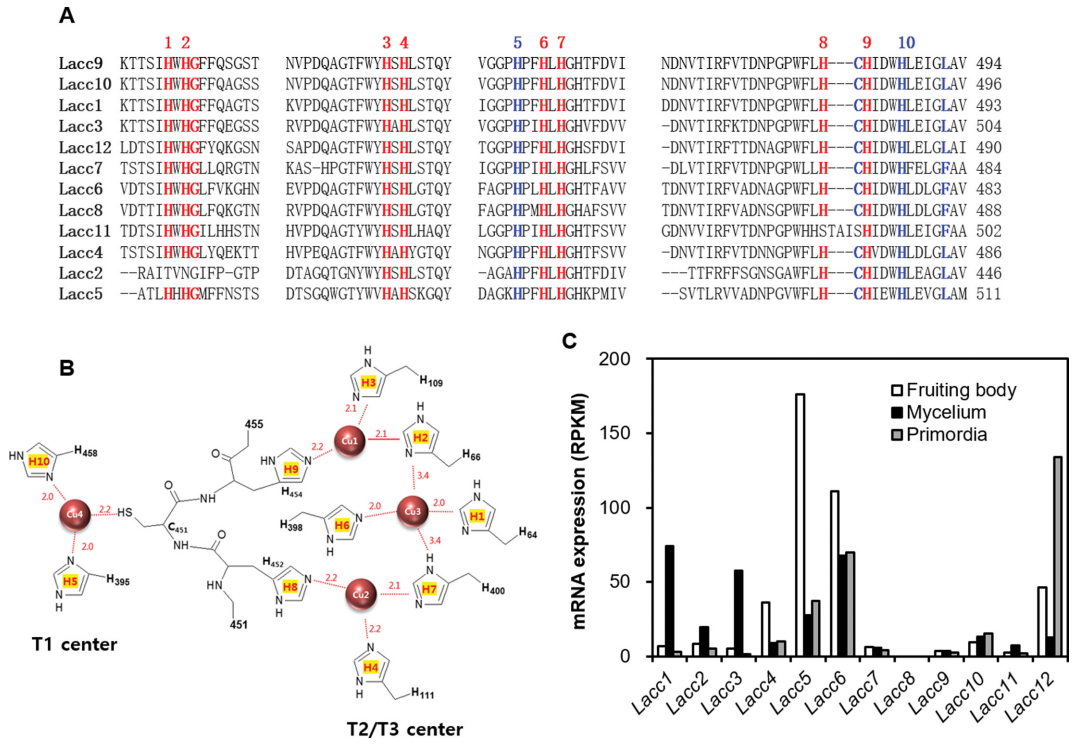

Fig. 2. Conserved sequence regions and laccase expression in *Pleurotus ostreatus*. A, Conserved sequence regions; the numbers above the sequence indicate conserved histidine residues; B, Arrangement of histidine residues in the catalytically important T1 and T2/T3 centers; coordinate bonds between the amino acid residues and copper ions in the laccase structure were redrawn using the laccase structure of *Trametes versicolor* (PDB code: 1GYC); C, RNA-Seq analysis of laccase genes in the mycelia, primordia, and fruiting bodies of *P. ostreatus*. RPKM, Reads Per Kilobase per Million mapped reads.

2A). Ten histidine residues in the conserved sequence signatures form coordinate bonds with four copper ions, thereby constituting the enzyme active site, which makes them essential for laccase activity (Fig. 2B). Interestingly, the conserved HXHG sequence was not found in *Lacc2*, and *Lacc11* did not contain the copper binding cysteine residue in the T1 center (Fig. 2A).

Transcriptional regulation of the laccase genes during different developmental stages was investigated by transcriptome analysis of the mycelium, primordium, and fruiting body (Fig. 2C). Among the twelve genes investigated, *Lacc5* was highly expressed in the fruiting body, whereas *Lacc1* and *Lacc3* were expressed in the mycelium. *Lacc12* was specifically expressed in the primordium. None of these genes have been extensively studied to date. Notably, *Lacc6* was highly expressed at all stages. *Lacc6* encodes a laccase protein similar to the blue laccase (GeneBank No. CAA06291) from *P. ostreatus* [31]. *Lacc2* and *Lacc7~Lacc11* were not significantly expressed under these conditions.

Heterologous expression of laccase genes in *Pichia pastoris*. Data from the transcriptome analysis indicated that *Lacc5* and *Lacc12* were the main laccases active during fruiting body maturation and primordiation stages, respectively, whereas *Lacc2*, *Lacc8*, and *Lacc11* were not expressed at any stage (Fig. 2C); in addition, *Lacc2* and *Lacc11* contained unusual active site arrangements (Fig. 2A). *Lacc6*, which was ubiquitously expressed, is the most studied laccase in *P. ostreatus* [31]. Moreover, these laccases were scattered among the three phylogenetic groups: *Lacc4*, *Lacc6*, and *Lacc11* in group I; *Lacc12* in group II; and *Lacc2* and *Lacc5* in group III. Therefore, theses laccases were selected for further characterization. We constructed laccase expression vectors by cloning the six synthesized laccase genes adjacent to the AOX1 promoter in the *Pichia* expression vector pPICHOLI-2. Next, culture conditions for the production of laccase protein in *P. pastoris* GS115, using *Lacc6* as a model gene, were investigated. In this system, methanol serves as the carbon source for yeast cell growth, as well as the inducer for gene expression. However, the methanol concentration needed to be optimized, as methanol exerts toxic effects on the cells on reaching a critical concentration in the culture medium.

In order to overproduce mushroom laccase in *P. pastoris* harboring pPICHOLI-*Lacc6*, the effects of methanol feeding frequency, in terms of cell growth and extracellular laccase activity, were investigated in batch culture. When 1% methanol was fed every 6 hr, both cell growth and production of the laccase protein decreased due to methanol toxicity (Fig. 3A). Cell growth was maintained at high levels at a feeding interval of 12~24 hr, whereas extracellular laccase production decreased significantly when methanol was fed every 24 hr.

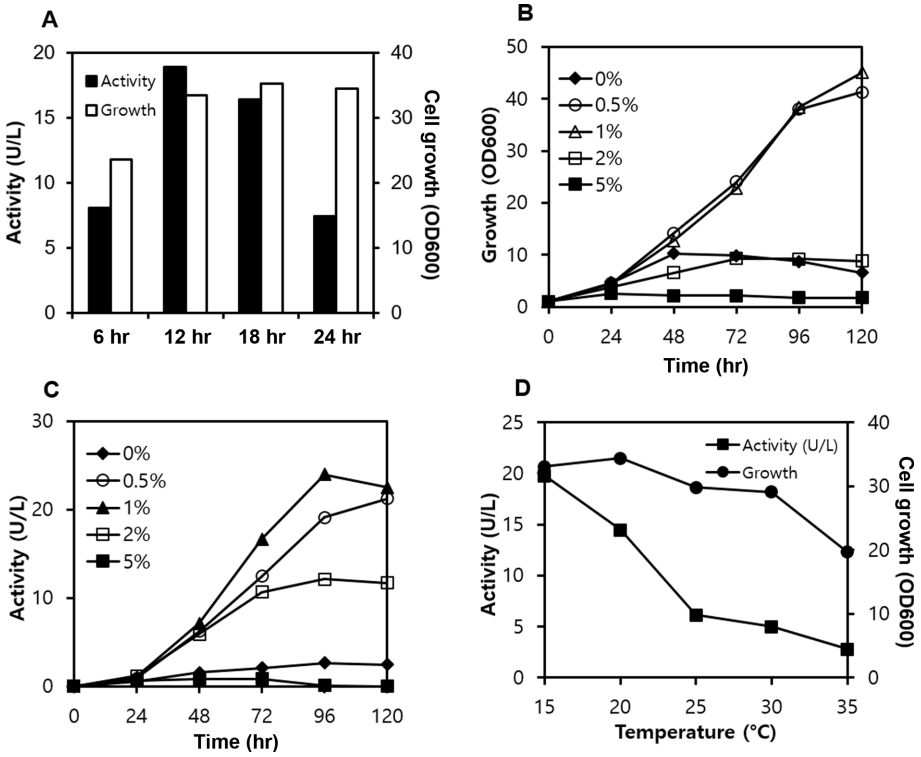

Fig. 3. Optimization of culture conditions for expression of laccase genes in *Pichia pastoris*. A, Optimal methanol feeding interval; methanol (1%) was fed intermittently at the time intervals indicated; B, C, Optimal methanol concentration for cell growth and laccase production; D, Effect of culture temperature on cell growth and production of extracellular laccase protein; yeast cells were grown for 72 hr at specific temperatures with intermittent addition of 1% methanol every 12 hr.

Extracellular laccase activity and cell growth reached maximal levels when methanol was fed at 12-hr intervals. Next, the effects of methanol concentration were examined by feeding methanol at different concentrations every 12 hr. Cell growth and laccase activity approached maximum levels after 120 hr of incubation (Fig. 3B and 3C). Methanol was found to inhibit both cell growth and laccase production at concentration of ≥ 2%. The optimal concentration ranged from 0.5% to 1% (Fig. 3B and 3C).

Heterologous proteins are generally expressed at 30°C in *P. pastoris* while numerous studies have shown that temperature is one of the important variables in the *Pichia* expression system [32-34]. Accordingly, the effects of culture temperature on enzyme production were examined in the present study. Yeast cells grew to optical densities of 30~35 at 600 nm (OD600) at temperatures ranging from 15°C to 30°C (Fig. 3D), with longer lag times at 15°C (data not shown). Cell growth was significantly inhibited at temperatures higher than 35°C. Extracellular laccase activity in the culture broth was also temperature-dependent; laccase activity decreased with increasing culture temperature (Fig. 3D). Cells grown at 20°C produced more laccase during earlier stages of the culture; however, the laccase productivity was overtaken by cells grown at 15°C when the culture time exceeded 48 hr (data not shown). Laccase activity remained low at temperatures higher than 25°C (Fig. 3D). The requirement for low temperatures may be attributed to the instability of laccase protein at elevated temperatures.

Biochemical characterization of *P. ostreatus* laccase enzymes. *P. ostreatus* laccase enzymes were produced using optimized culture conditions (15°C, and 1% methanol fed at 12-hr intervals) in *P. pastoris*. *Lacc6*, *Lacc8*, and *Lacc12* were observed as major protein bands, whereas *Lacc2*, *Lacc5*, and *Lacc11* were not detected or detected as smaller and minor protein bands (Fig. 4A). Notably, *Lacc2* and *Lacc11* encode defective proteins that lack HXHG and contain incomplete HCH signatures, respectively (Fig. 2A). Laccase activity was assessed by decolorization of malachite green, as described in our previous report [4]. As shown in Fig. 4B, *Lacc6* and *Lacc12* induced complete decolorization of malachite green, as indicated by the disappearance of the maximum absorbance peak at 622 nm, in a time-dependent manner (Fig. 4C). *Lacc2*, *Lacc5*, *Lacc8*, and *Lacc11* did not decolorize malachite green (Fig. 4B). Despite its high concentration, *Lacc8* was non-functional (Fig. 4B). Additionally, the *Lacc8* gene was not expressed during any stage of mushroom development (Fig. 2C), suggesting that *Lacc8*, which is closely related to *Lacc6* (Fig. 1), appears to be in the degenerate state at the level of both transcription and translation.

We next investigated the biochemical characteristics of

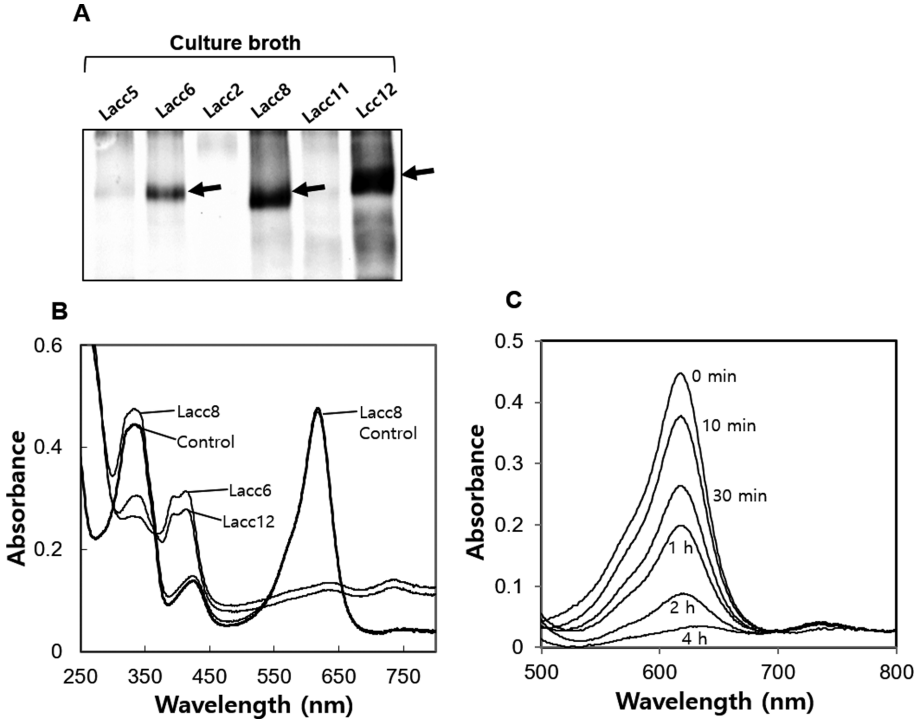

Fig. 4. Extracellular production of laccase protein by *Pichia pastoris* harboring laccase genes. A, SDS-PAGE analysis of culture supernatant; arrows indicate the electrophoretic position corresponding to laccase protein; B, Decolorization of malachite green by culture supernatant containing laccase proteins; C, Time-dependent decolorization of malachite green by *Lacc12*.

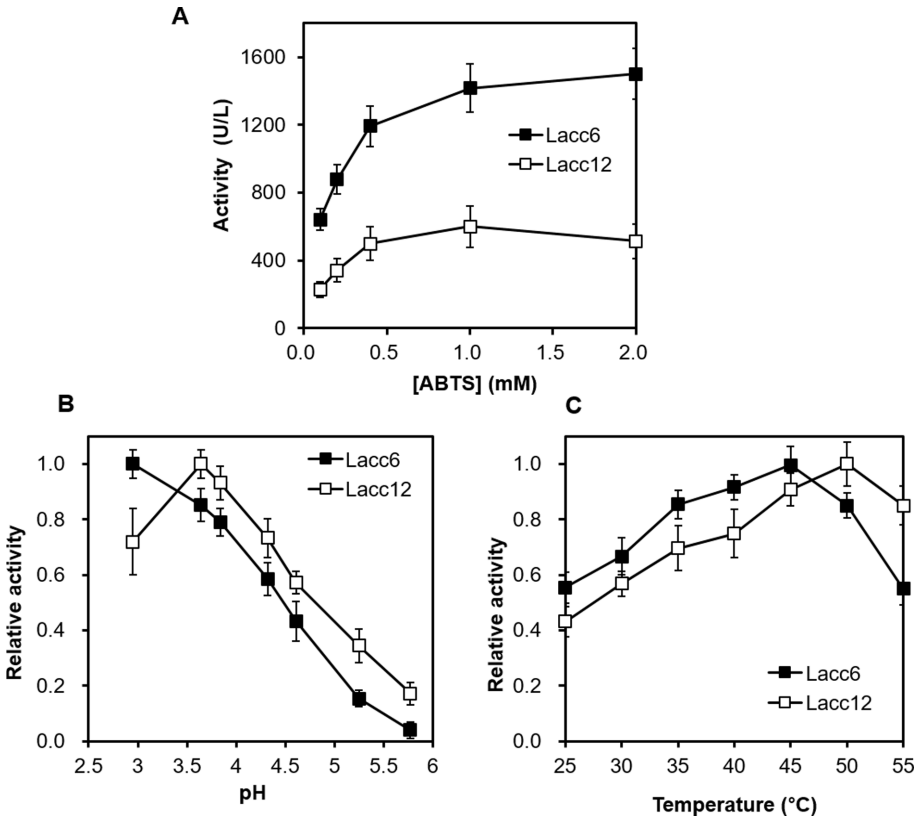

Fig. 5. Biochemical characteristics of *Lacc6* and *Lacc12*. A, Activity vs. substrate concentration profile; B, Effect of pH on laccase activity; C, Effect of temperature on laccase activity. ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

two functional laccase enzymes, *Lacc6* and *Lacc12*. The oxidation activity of laccase was measured using various concentrations of ABTS. The activity increased proportionally to ABTS concentration, and became saturated at above 2 mM, showing maximum activities of 1,560 units/L and 600 units/L for *Lacc6* and *Lacc12*, respectively, and K_m values of 0.152 mM and 0.316 mM for *Lacc6* and *Lacc12*, respectively (Fig. 5A). V_{max}/K_m, which represents the enzyme catalytic activity at low substrate concentration, was 10,263 units/L/mM and 1,898 units/L/mM for *Lacc6* and *Lacc12*, respectively. These findings indicate that *Lacc6* is 5.4-fold more active than *Lacc12* at low substrate concentration. Both enzymes were highly active at acidic pH. Notably, *Lacc6* showed lower pH optimum than *Lacc12* with optimum pH values of < 3.0 and 3.5 for *Lacc6* and *Lacc12*, respectively (Fig. 5B). The effects of temperature on laccase activity were also slightly different. Specifically, *Lacc12* showed a 5°C higher temperature optimum than *Lacc6*; however, both enzymes were essentially thermophilic, with optimum temperatures of 45°C and 50°C for *Lacc6* and *Lacc12*, respectively (Fig. 5C).

DISCUSSION

The genome of *P. ostreatus* contains 12 laccase genes clustered into three major groups (Fig. 1). *Lacc2* and *Lacc10* have been shown to be overexpressed in the mycelia of *P. ostreatus* in submerged culture when induced by wheat straw extract in a chemically defined medium [24]. However, the present study showed that expression of the two laccases was negligible in sawdust medium at all developmental stages (Fig. 2C), indicating that *P. ostreatus* expresses different laccase enzymes depending on the medium conditions. *Lacc1* and *Lacc3* were specific to the mycelial stage in solid medium (Fig. 2C). *Lacc6* was highly expressed in all developmental stages whereas *Lacc5* and *Lacc12* were specific for fruiting bodies and primordia, respectively (Fig. 2C). *Lacc6* was characterized as a blue laccase under the name POXA1b [31].

A comparative study of the enzymatic characteristics of the primordium-specific laccase *Lacc12* and the constitutive laccase *Lacc6* showed that the ABTS oxidation activity of *Lacc12* was 40% that of *Lacc6*. Moreover, the optimal pH and temperature for *Lacc12* activity were 0.5 pH units and 5°C higher than those for *Lacc6* (Fig. 5B and 5C). These findings may reflect the physiological conditions during the primordiation stage. In *L. edodes*, laccase activity in the sawdust substrate decreased dramatically at the primordial stage, along with a sharp increase in cellulase activity [35]. These findings suggest that laccase activity is necessary for the vegetative growth of mycelia in the sawdust substrate and potentially for lignin degradation. Degradation of lignin or production of lignin-derived compounds may induce primordiation. Upon primordiation, laccase activity in the substrate is no longer required. Instead, activity within primordia is necessary for the completion of primordiation, with cellulases of the mycelia beneath the primordia digesting the cellulosic substrate to provide a source of carbon and energy. *Volvariella volvacea* shows a high level of expression of a laccase gene, *lac1*, as well as high laccase activity, particularly during the primordial stage [36]. Taken together, these findings indicate that different laccases function at various stages of development as well as in lignin degradation.

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