e-ISSN:2320-3528
p-ISSN:2347-2286

Research & Reviews: Journal of Microbiology and Biotechnology

Cloning and Expression Analysis of Vv-lac3, A Novel Functional Laccase Gene Involved in Stipe Elongation, Based on the Genomic Sequence of *Volvariella volvacea*

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Research Article

Received date: 23/06/2015
Accepted date: 01/08/2015
Published date: 07/08/2015

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Keywords: *Volvariella volvacea,* Laccase, Fruiting body formation, Enzyme.

ABSTRACT

Fungal laccases always play important roles in various physiological and developmental processes. Here, we cloned a laccase gene, and named *Vv-lac3*, cDNA from the *Volvariella volvacea*, and expressed the cDNA in *Pichia pastoris*. The *Vv-lac3* cDNA consisted of 1599 bp containing an open reading frame (ORF) encoding a 532 amino acids. The analysis of the deduced amino acids revealed that *Vv-lac3* possessed a 19-amino acids signal peptide at the N-terminal end and a 513-amino acid mature protein. The *Vv-lac3* contained four conserved copper-binding sites that which is the typical structure of fungal laccases. Phylogenetic analysis showed that *Vv-lac3* had a high degree of identity with other basidiomycete laccases. Native-PAGE and SDS-PAGE analysis demonstrated that the product of *Vv-lac3* cDNA from *P. pastoris* was a functional laccase with a molecular mass of 65 kDa. The expression of the *Vv-lac3* gene in *V. volvacea* increased during button stage to the elongation stage; it reached peaked in the elongation stage, and then decreased in the maturation stage, which suggests that this gene plays a regulatory role in stipe elongation in *V. volvacea*.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) belong to a family of blue multicopper oxidases that catalyze the oxidation of a variety of aromatic substrates along with the reduction of molecular oxygen to water [1]. Laccases have attracted extensive attention due to their functional diversity; for example, they are involved in lignin biodegradation, plant pathogenesis, and stress defense [2]. The multigene family of laccases is a common feature in fungi [1]. The first example of the multigene family of laccases was described in *Agaricus bisporus* [3]. Since, seventeen laccases have been characterized from *Coprinopsis cinerea* and, eleven laccases from *Laccaria bicolor* [4] and *Flammulina velutipes* have been characterized [5].

Volvariella volvacea (Bull.: Fr. Sing.) is an economically important edible mushroom; it is a straw-degrading basidiomycete that has been cultivated extensively in the southern provinces of China for several centuries. The previous reports have led to the suggestion that *V. volvacea* is unable to degrade lignin because none of the lignin-degrading enzymes (lignin peroxidase, Mn-dependent peroxidase and laccase activity) were detected in this species [6]. However, recently, the genome sequencing of *V. volvacea* has been completed [7,8], and a total of eleven laccase genes have been detected. Laccases are multi-functional enzymes, and they may play other putative roles in addition to fruiting body formation [9]. To clarify the function of *V. volvacea* laccases and reveal why *V. volvacea* is unable to degrade the lignin during the earlier stages in the colonization of the rice straw substrate, we sought to learn more about the laccase genes from *V. volvacea*.

In this study, we cloned a *Vv-lac3* gene from *V. volvacea* and successfully expressed the *Vv-lac3* cDNA in *Pichia pastoris*. We have also determined the effect of pH on the activity of this laccase activity. In addition, the expression of this gene was investigated during the fruit body development of *V. volvacea*, suggesting that the expression of *Vv-lac3* was important for stipe elongation.

MATERIALS AND METHODS

Strains and vectors: The heterokaryon H1521 strain of *V. volvacea* was obtained from the Mycological Research Center of

Fujian Agriculture and Forestry University, Fuzhou, China, and deposited in the Agricultural Culture Collection of China (accession no. ACCC52633).

Escherichia coli DH5α (TIANGEN, China) was used as the host for the cloning procedures. PZeroBack/Blunt Vector (Takara, Japan) was used to subclone the cDNA fragment for sequencing. The pPIC9K plasmid with an alpha-factor signal peptide and *Pichia pastoris* GS115 (Mut^+ His^-) were purchased from Invitrogen (USA).

Isolation of total RNA: *V. volvacea* strain H1521 was cultured on rice saw compost [10]. Samples at different development stages were harvested according to the method of Tao et al [11] and frozen in liquid nitrogen.

Total RNA was isolated from samples using an E.Z.N.A.™ Plant RNA Kit (OMEGA, USA) according to the manufacturer's instructions. The first strand of cDNA was synthesized using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen, China). All cDNA was stored at -20°C for the subsequent experiments.

Transcription pattern analysis of laccase genes in *V. volvacea* with DGE data: The mRNA extracted from stipes of four developmental stages of the fruiting body was submitted to BGI (Shenzhen, China) for construction and sequencing of digital gene expression (DGE) libraries. The construction and sequencing of the DGE tag libraries were as described by Tao et al [11]. The DGE data were deposited in the NCBI's GEO database under accession number: GSE43297.

Then, the DGE data were used for transcription pattern analysis of the laccase genes in *V. volvacea*. Briefly, the expression levels of these genes were measured based on the number of tags mapped solely to the laccase genes, which were then normalized to TPM (number of tags per million clean tags) [12].

Furthermore, qRT-PCR was performed to verify the expression of *Vv-lac3* analysed using the DGE data. SYBR Premix Ex TaqTM *II* (Tli RNaseH Plus) (Takara, Japan) was used in this study. A total reaction volume of 25 μL was prepared according to the manufacturer's protocols. The qRT-PCR programmer was as follows: initial denaturation 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s. The primers for *Vv-lac3* and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) genes [11,13], used as an internal standard, were designed with Primer Premier 5.0. And then, we used the $2^{-\Delta\Delta Ct}$ method for qRT-PCR data analysis [14]. All experiments were conducted in triplicate.

Cloning of Vv-lac3 cDNA, construction of expression vector and transformation: To clone the *Vv-lac3 cDNA*, PCR was performed with primers lac3OF and lac3OR, and the cDNA from the button stage as a template. The PCR temperature program was initiated at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 4 min, and a final extension at 72°C for 10 min. The reaction mixture for PCR was as follows: 2 μl of cDNA, 2.5 μl of dNTP Mixture, 2.5 μl of Pfu buffer (with $MgSO_4$), 0.5 μl of Pfu DNA Polymerase, 1 μl each of forward and reverse primers, and 15.5 μl of ddH_2O. The PCR products were cloned into pZeroBack/Blunt Vector (Tiangen, China) for sequencing (Sangon Biotech, China).

The ORF of *Vv-lac3* without the native signal peptide sequence was flanked by Avr*II* and Not*I* restriction sites at the 5'- and 3'-ends respectively, with PCR using lac3-F-Avr*II* and lac3-R-Not*I* primers. The fragments were subcloned into a pZeroBack/Blunt Vector followed by digestion with the restriction enzymes Avr*II* and Not*I* restriction enzymes and then ligation into the corresponding sites of the Pichia pastoris expression vector pPIC9K and the validated recombinant plasmid was named pPIC9k-*Vv-lac3* .

Both the recombinant plasmids pPIC9K-*Vv-lac3* and pPIC9k without *Vv-lac3* , which were used to prepare negative control strains, were linearized using the restriction enzyme Sac*I* and transformed into *P. pastoris* GS115 by electroporation (Invitrogen). The transformants were selected on MD agar plates (1.34% yeast nitrogen base [YNB], 4×10^{-5} biotin, 2% dextrose) at 28°C, after which His^+ transformants were screened using direct PCR with the lac3-F-Avr*II* and lac3-R-Not*I* primers.

Expression, purification and analysis of heterologous *Vv-lac3*: The His^+ transformants were transferred to BMGY agar plates (2% Tryptone, 1% yeast extract, 1.34% YNB, 4×10^{-5} biotin, and 1% glycerinum) at 28°C for 2 days. BMM agar plates (1.34% YNB, 4×10^{-5} biotin, 0.5% (v/v) methanol, 0.1 mM $CuSO_4$ and 0.2 mM ABTS [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid),100 mM of potassium phosphate, pH 6.0] [15] were applied to screen the transformants that were secreting *Vv-lac3* according to the presence of a green zone around the His^+ transformants colonies.

The *Vv-lac3* transformants were inoculated in 100 ml BMG (100 mM of potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} biotin and 1% glycerol) at 28°C and 150 rpm until the OD600 value reached 10. Then, the *P. pastoris* cells were collected by centrifugation at 1500 g for 5 minutes, and re suspended with 50 ml BMM (containing 0.3 mM $CuSO_4$ and 0.8% alanine) at 28°C and 150 rpm [15]. Methanol was added daily to a final concentration of 0.5 % (v/v) and 1 ml of culture was taken daily from the flask. Supernatants were collected by centrifugation prior to the measurement of laccase activity according to a previously described method [16]. We defined one unit of laccase activity as the amount of laccase that catalyzed the oxidation of 1 μmol of ABTS min-1 [17]. In addition, Native-PAGE was conducted with 8 ml of 12% (w/v) separating gel and 2 ml of 5% (w/v) stacking gels to identify laccase. After electrophoresis, protein band with laccase activity was stained with 1 mM ABTS in 0.1 M acetate buffer (pH 5) [16].

After performing of the experiments mentioned above, supernatants with the highest laccase activity were harvested by centrifugation at 10,000 g for 10 min and concentrated to a volume of 5 ml using PEG4000. The concentrate solution was applied to a Sephadex G-15 column (10 × 300 mm) pre-equilibrated with 0.05 M phosphate buffer (pH 6.8). It was eluted using the same buffer and the eluted protein was detected by 1 mM ABTS in 0.1 M acetate buffer (pH 5), after which the eluted proteins were applied to a DEAE-cellulose column (10 × 300 mm, DE52) pre-equilibrated with 0.05M phosphate buffer (pH 6.8). After washed with 500 ml of the same buffer, the unbound proteins were removed from the column. Subsequently, the bound laccase was eluted with sodium chloride solution in gradient concentration (from 0.05M to 0.45M). Eluted proteins were pooled, concentrated to 2 ml using PEG4000, and stored at -20°C for further investigation.

e-ISSN:2320-3528
p-ISSN:2347-2286

The purified protein was investigated using 10% w/v sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After which, the protein band was stained with Coomassie Brilliant Blue R-250 at room temperature for 2 h. Protein molecular weight markers (Takara, Japan) were used to estimate the molecular weight of heterologous *Vv-lac3* . In addition, the optimum pH of the purified *Vv-lac3* was assayed by monitoring the oxidation of ABTS at 420 nm (ε_{420}=3.6 × 104/mol/cm). The reaction mixture containing an appropriate amount of purified *Vv-lac3* and 1 mM of ABTS in McIlvaine buffer at different pH ranges (pH 2.5 to pH 7). The activity of purified *Vv-lac3* under optimal pH was defined as 100%.

Protein sequences analysis and phylogenetic tree construction: The basic physical and chemical characteristics of *Vv-lac3* were analyzed using ExPASy Protpara (http://www.expasy.ch/tools/protparam.html) [18], whereas the signal peptide was predicted by signalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP) [19]. The analysis of the amino acid conservation domains was performed using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) [20]. NetNGlyc 1.0 Server (http://genome.cbs.dtu.dk/cgi-bin/webface2.fcgi) [21] was applied to the analysis of the N-glycosylation sites.

To generate the phylogenetic tree, the amino acid sequences of the 18 typical laccases from other fungi were obtained from NCBI according to the approach used by Valderrama [22]. Then, these sequences and the predicted laccase amino acid sequences of laccase encoded by *Vv-lac3* gene were aligned with ClustalX 1.83 [23]. A neighbor-joining tree was constructed using a bootstrap method (the number of bootstrap relationships was 1,000) using MEGA 5.1 software [11,24,25].

RESULTS

Laccase genes in the *V. volvacea* genome and their transcription patterns: Based on the homology search results using local BLAST analysis by comparing the sequence of six laccase genes from *V. volvacea* V14 downloaded from NCBI to the genome of strain PYd21 (GenBank: ANCH00000000.1), 11 putative laccase genes were found in PYd21 genome. Among them, six laccase amino acid sequences were most closely matched with the previously cloned laccase genes in *V.volvacea*, namely, *lac1* (AY249052.1) [10], *lac2* (AY338483.1) [26], *lac3* (AY338484.1), *lac4* (AY338486.1), *lac5* (AY338485.1), and *lac6* (AY338487.1). Thus, we named these genes following the convention established by Chen. In addition, Bao et al [7] published the multigene family of laccases in *V.volvacea* strain V23. They named the laccase genes in V23 using another rule (order on the scaffolds). We aligned the laccase genes loci between PYd21 and V23; 11 laccase genes exhibited similar loci in both genomes **(Figure 1)**, which indicated that this laccase loci structure in *V. volvacea* could be conservative. Furthermore, all of these genes from the PYd21 genome exhibited high homology with the sequences retrieved from *V. volvacea* V23 (Table S2. Identity ≥ 98%, E-value=0).

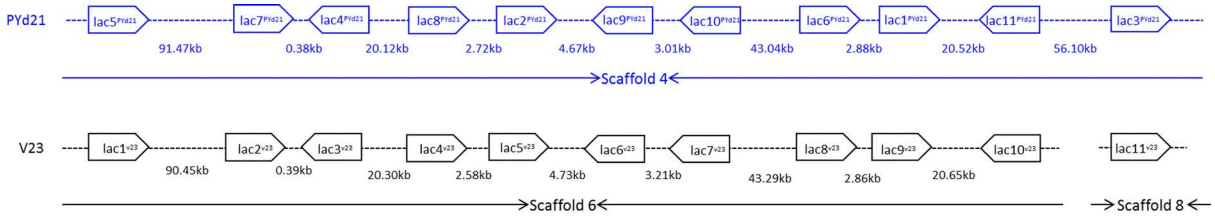

Figure 1. Distribution of laccase genes in the genome of *V. volvacea* strain PYd21 and V23. Arrow-shaped boxes indicate the laccase genes, whereas the direction of arrows shows the direction for each gene. The numerical values below the line indicate the distances (bp) between pairs of laccase genes. Blue one indicates laccase genes from the PYd21; black one indicates laccase genes from V23.

Tags were mapped to every laccase gene for each developmental stage and normalized to TPM. Differences in the expression levels of these genes were detected when compared to different developmental stages **(Table 1)**. All of the laccase genes, except *lac7, lac8* and *lac10*, were expressed in at least one stage. Among these genes, *Vv-lac3* exhibited the highest expression levels in every sample, and it also showed an interesting expression pattern, thus *Vv-lac3* was chosen for further study.

Table 1. Differential expression levels of the laccase genes from PYd21 at different developmental stages (from button stage to maturation stage). BU: Button stage, day 10 after inoculation on rice saw compost; EG: Egg stage, day 13 after inoculation; EL: Elongation stage, day 13.5 after inoculation; MA: Maturation stage, day 14 after inoculation.

	lac1	lac2	lac3	lac4	lac5	lac6	lac7	lac8	lac9	lac10	lac11
BU	0	1.96	8.73	4.99	0.36	0	0	0	0.18	0	0.89
EG	0.33	0.33	71.09	14.55	3.34	0.5	0	0	1.5	0	1.34
EL	0	0	92.2	9.88	0	0.67	0	0	1.17	0	0.5
MA	0	1.17	27.14	11.23	0	1.01	0	0	0.5	0	0

Low transcript levels of *Vv-lac3* analyzed based on the DGE data were detected in the button stage. The expression of *Vv-lac3* then increased dramatically at the egg stage and reached peaked at the elongation stage. The expression level of *Vv-lac3* decreased at the mature stage. These results were further confirmed by qRT-PCR **(Figure 2)**. The transcription pattern of *Vv-lac3* strongly suggested that this gene may play an important regulatory role in the elongation stage.

The structure of *Vv-lac3* gene: Based on *V. volvacea* whole genome sequencing data from strain PYd21, primers (lac3-OF and lac3-OR) were designed to clone *Vv-lac3* cDNA. The full-length cDNA of *Vv-lac3* consisted of 1599 bp. Comparison of the *Vv-lac3* genomic DNA sequence and cDNA sequence using DNAMAN (version 5.2.2) revealed that the coding region was interrupted by 13 introns **(Figure 3)**. The size of 13 introns ranged from 50 to 80 bp, and all of the splice junctions of the introns conformed to the GT-AG rule [27].

e-ISSN:2320-3528
p-ISSN:2347-2286

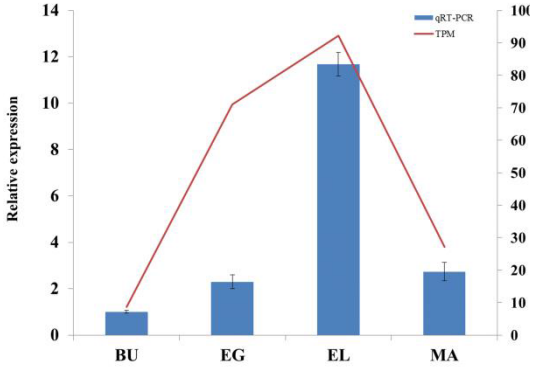

Figure 2. Comparison of vv-lac3 expression using qRT-PCR and DGE. TPM (Transcripts per Million clean tags): a standardized indicator, pointing out number of transcript copies in every 1 million clean tags. BU: Button stage, day 10 after inoculation on rice saw compost; EG: Egg stage, day 13 after inoculation; EL: Elongation stage, day 13.5 after inoculation; MA: Maturation stage, day 14 after inoculation.

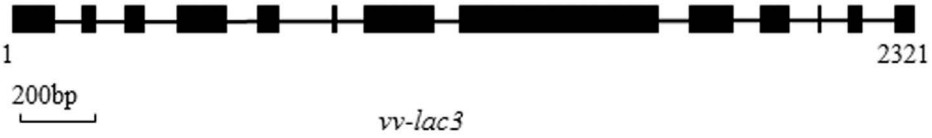

Figure 3. Gene model of vv-lac3, whereby black boxes indicate exons and transverse lines indicate introns.

Characterization of *Vv-lac3* protein and phylogenetic tree: The cDNA of *Vv-lac3* encoded 532 amino acids with a putative signal peptide of 19 amino acids and a mature protein of 496 amino acids. Analysis of *Vv-lac3* using ExPASy Protparam revealed that theoretical isoelectric point (pI) of deduced amino acids was 4.62. An InterProScan search indicated that the deduced protein contains three multicopper oxidase domains (type 1, IPR001117; type 2, IPR011706; type 3, IPR011707). Three N-glycosylation sites (Asn-Xaa-Thr/Ser, in which Xaa is not Pro), at positions 89, 114, and 451 of the deduced amino acids, were found using NetNGlyc 1.0 Server, suggesting that laccase3 from *V. volvacea* is a glycoprotein. The alignment of the deduced amino acid sequence indicated four copper-binding sites (L1-L4), including 10 conserved histidine residues and 1 cysteine residue, were found in this protein **(Figure 4)**. The presence of the conserved histidine residues and cysteine residue located in the four copper-binding sites suggested that *Vv-lac3* belongs to the family of typical laccases.

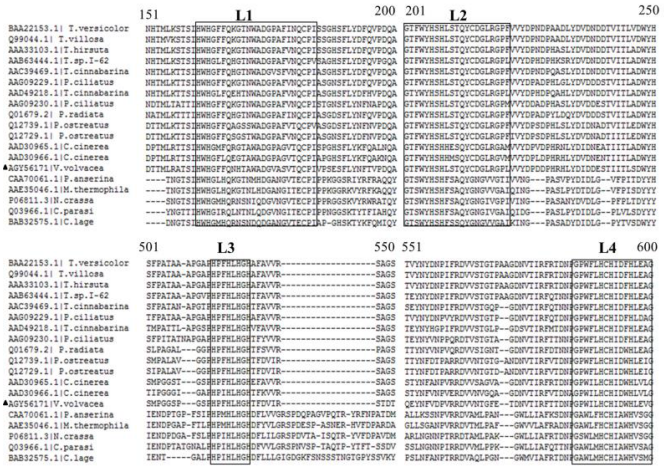

Figure 4. Alignment of the deduced amino acid sequence of *Vv-Lac3* with homologous laccases using Clustal X (1.8). Four black boxes indicate four fungal laccase signature sequences (L1-L4), including 10 conserved histidine residues and 1 cysteine residue. ▲i*V. volvacea Vv-Lac3* protein.

Phylogenetic analysis demonstrated that all of the laccases we collected from Basidiomycota or Ascomycota divisions formed independent clades, which is taxonomically consistent. Moreover, the *Vv-lac3* clustered with *Lac2* and *Lac3* from C. cinerea **(Figure 5)**, which have been verified as real laccases. In addition, the BLASTP results revealed that the deduced protein product of *Vv-lac3* showed high identity with other fungi laccases, such as, *C. cinerea* (AAD30965.1, 65%), *L. bicolor* (XP_001874989, 61%), *Coprinus comatus* (AFD097049.1, 63%), *Stropharia aeruginosa* (AFE48786.2, 62%), *Cyathus bulleri* (ABW75771.2, 63%), *C. cinerea* (AAD30966.1, 65%), and *F. velutipes* (AIW01083.1, 61%).

Heterologous expression of *Vv-lac3* in *P. pastoris*: The *Vv-lac3* cDNA, without the native signal sequence, was inserted the downstream of the α-factor secretion signal of the *P. pastoris* expression vector pPIC9K. The pPIC9K-Vv-lac3 and pPIC9K sequences were digested with Sac I. Then, the pPIC9K-*Vv-lac3* and pPIC9K were transformed into *P. pastoris* GS115 and screened by BMM plates. The positive transformants containing pPIC9K-*Vv-lac3* produced green zones around their colonies, whereas those containing pPIC9K did not display any color changes **(Figure 6)**. The colony displaying the deepest green zones on the BMM plates, which might be the most efficient *Vv-lac3* producing colony, was selected for the subsequent experiment.

e-ISSN:2320-3528
p-ISSN:2347-2286

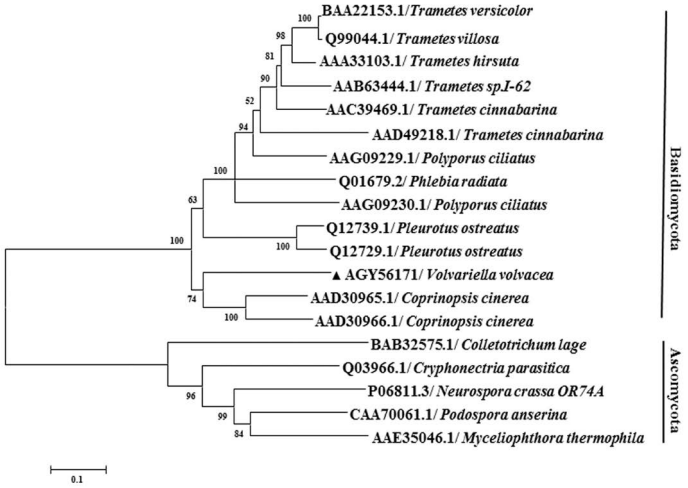

Figure 5. Neighbor-joining tree of *V. volvacea Vv-Lac3* protein sequences with amino acid sequences of laccases identified in other fungi. The tree was constructed with Poisson correction using MEGA 5.1 and, bootstrap values (1,000 replications) higher than 50% are indicated for the nodes. ▲*V. volvacea Vv-Lac3* protein.

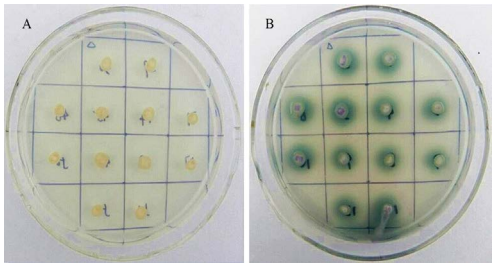

Figure 6. Screening of positive transformants with BMM medium. A: pPIC9K acting as negative control, B: pPIC9K-*vv-lac3*.

The laccase activity peaked after 21 days of cultivation (296.83 U/L), and heterologous *Vv-lac3* was purified and detected by Native-PAGE and SDS-PAGE, both of which displayed a single band **(Figure 7)**. The molecular weight of purified *Vv-lac3* was approximately 65kD, which is consistent with the features of the previously characterized fungal laccases (60 to 80 kDa) [2]. Similar to many fungal laccases whose optimum pH range is 3.0-4.5 for ABTS [28], the optimum pH of *Vv-lac3* is 4.5 **(Figure 8)**. When the pH exceeded 4.5, the activity declined and was almost completely depleted at pH 7.0.

DISCUSSION

Laccases were first discovered in the Japanese lacquer tree *Rhus vernicifera* (reviewed by Thurston) [2]. Subsequently, laccases have also been identified in other plants [29,30], in various fungi [31-34], in bacteria [35-38], and in insects [39]. Laccases carry out a diverse array of biological roles. In plants, laccases are involved in lignin biosynthesis [40]. In bacteria, laccases appear to participate in the protection of the spores against stress factors such as hydrogen peroxide or UV radiation [37]. In fungi, laccases are also involved in the biosynthesis of pigments [41], lignin degradation [42], conidiation and sporulation [43].

In addition to the roles in lignin degradation and plant pathogenesis, fungal laccases participate in lignin bioconversion in *A. bisporus* and *L. edodes* [44,45], the development of fruiting bodies in *V. volvacea* [26], gill browning after fruit body harvest in *L. edodes* [46], and stipe elongation in *F. velutipes* [5]. In this study, the transcription of 11 laccase genes was first analyzed together during the progress of stipe elongation in *V. volvacea*. Among these genes, *Vv-lac3* exhibited the highest expression levels in every sample, thus *Vv-lac3* cDNA was cloned and expressed in *P. pastoris*. The analysis of the deduced protein showed that the predicted protein sequence of *Vv-lac3* carried four conserved sequence regions L1-L4, which are useful for identifying laccases [47]. The high sequence identity with laccases from *C. cinerea, L. bicolor,* and *F. velutipes* suggested that the protein encoded by the *Vv-lac3* gene was a laccase. The phylogenetic tree provided further evidence for that *Vv-lac3* is a *sensu stricto* laccase gene. In addition, the presence of green zones around their colonies and the results analyzed using Native-PAGE and SDS-PAGE proved that the *Vv-lac3* gene encoded a functional laccase.

The optimal pH of Vv-lac3 was 4.5, and the activity of laccase was almost depleted at pH 7.0, which was similar to the activity of CcLCC6I whose optimal pH is 3 and the activity of CcLCC6I is completely depleted between pH 6.0 to 7.6 [48]. However, the subculture of vegetative mycelia was performed on the rice straw compost (pH 8.0) [49]. The difference in pH between the laccase activity and *V. volvacea* growth could account for the inability to degrade the lignin and, hence, the inability to draw nutrition from lignified growth substrates and, in turn, leading to the relatively poor mushroom yields even on wastes with small quantities of lignin such as rice straw compost.

The stipe of *V. volvacea* is very small in the button stage, and stretches in the egg stage, and then extends to almost full length in the elongation stage [50]. The expression level of the *Vv-lac3* gene was in consistent with the change of stipe tissues during the development of the *V. volvacea* fruit body. The transcription pattern implied that the *Vv-lac3* gene may play an important regulatory

role in the stipe elongation. Knock out experiments, which are a useful for studying the function of genes, should be performed to further confirm the regulatory role of *Vv-lac3* in stipe elongation. However, it is still a challenge for us to knock genes out in *V. volvacea* [51]. Transcription pattern analysis of genes during the development of fruit body could provide the basis for elucidating the different parameters of the stipe elongation of the fruit body in *V. volvacea* given that stipe elongation is considered to be the cause of rupture of the universal veil and, in turn, the reduction of commodity value [52-54]. In summary, our preliminary results may have benefits in the promotion of *V. volvacea* cultivation.

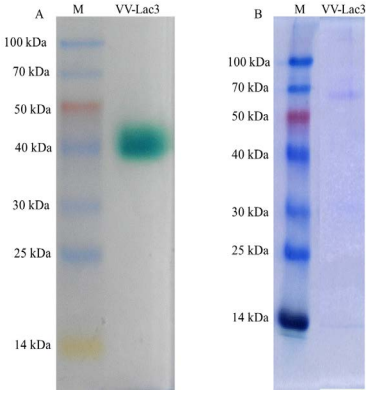

Figure 7. Native-PAGE and SDS-PAGE of purified *Vv-Lac3* secreted by *P. pastoris*. M: protein marker, *Vv-Lac3*: laccase expressed in *P. pastoris* GS115. A: Native-PAGE of purified *Vv-Lac3* secreted by *P. pastoris*; B: SDS-PAGE detection of purified *Vv-Lac3* during the process of electrophoresis, the mobility of proteins depends not only on the size of proteins, but also on the charge of proteins in protein analysis using native PAGE. While SDS-PAGE overestimates the molecular masses. Thus, the band sizes are different in the two types of gel.

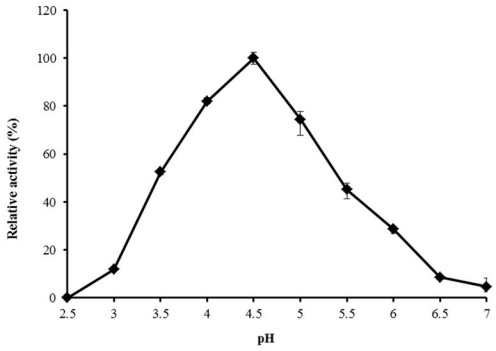

Figure 8. The optimal pH of *Vv-Lac3*. Optimal pH of purified *Vv-Lac3* was measured at a pH range from pH 2.5 to pH 7.0 at 32 °C with ABTS as substrate. The activity of purified *Vv-Lac3* under optimal pH is defined as 100%. Values represent the mean ± standard deviation of three separate measurements.

ACKNOWLEDGMENTS

This work was supported by grants from the National Key Basic Research Program of China (2014CB138302), and the China Agriculture Research System (CARS24). The authors thank the Fujian Edible Fungi Engineering Technology Research Center and the National Fungi Breeding Center (Fujian Division) for providing the experimental facilities.

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