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Purification and characterization of laccase from the white-rot fungus *Pleurotus pulmonarius* VAST02.42

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Abstract. Due to capability to use a wide variety of phenolic and non-phenolic substrates, laccase (EC 1.10.3.2) has potential applications in the food, pharmaceutical, and environmental industries. In the present study, a laccase (*Pleu*Lac) from the white-rot fungus *Pleurotus pulmonarius* VAST02.42 that was newly isolated from Muong Phang special-use forest, was purified and studied on physical-chemical properties. *Pleu*Lac was successfully purified to apparent homogeneity (specific activity of 28 $U.mg^{-1}$) by using the anion-exchange chromatography and size-exclusion chromatography (Superdex G-100). A total activity of 532 U was acquired with a recovery of 8.3 % and purity of 9.6-fold. The molecular weight (Mw) of *Pleu*Lac was determined to be 35 kDa by SDS-PAGE electrophoresis. This enzyme exhibited an optimal activity at 40 ºC and pH of 5.5; it is stable at 25 ºC after incubation of 120 min and pH of 5.0. This purified and characterized laccase will serve for further study on catalyzing the oxidation of essential oils to value-added products in combination with peroxidase.

Keywords: Laccase, *Pleurotus pulmonarius*, white-rot fungus, Muong Phang, biocatalytic oxidation.

Classification numbers: 1.1.5, 1.4.3, 3.1.2.

1. INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, E.C.1.10.3.2) are multinuclear copper-containing enzymes that catalyze the oxidation of various phenolic and inorganic compounds with the concomitant reduction of oxygen to water. The Laccases are widely distributed in nature; however, the majority of laccases are often found in white-rot fungi [1, 2]. In fungi, laccases play a variety of physiological roles in their life cycle, such as lignin degradation, detoxification, pathogenesis, and morphogenesis [3]. The fungal laccases are highly promising for use in diverse industrial sectors due to their elevated redox potential and expanded substrate range. For instance, laccase from white-rot fungi *Podoscypha elegans* plays a role in decolorizing azo dyes [4]. Several fungal species producing laccase enzymes, which are

supposed to play an important role in biodegradation of lignin, are also known [5, 6]. The laccase is an essential enzyme in wastewater treatment, capable of replacing chemical compounds for degrading paper waste. A 5-fold decrease in lignin concentration was observed after adding laccase to wastewater compared to untreated wastewater. This is crucial because the presence of lignin and its derivatives in pulp effluent enhances environmental pollution [7]. Furthermore, the combination of laccase and peroxidase was also important for designing the allylic oxidation of α-pinene into value-added products such as biological pesticides [8, 9]. Taken together, the laccase has a great potential in industrial applications and it is predicted that the demand for laccase will increase in the future.

In the present study, a laccase produced by the white-rot fungus *Pleurotus pulmonarius* (VAST02.42) was successfully purified and characterized, i.e., molecular mass (M_W), the effect of pH, temperature, metal ions and kinetic constants. The appropriate knowledge about the structural and functional properties of *P. pulmonarius* VAST02.42 laccases could further help in the elucidation of the physiological function of this enzyme.

2. MATERIALS AND METHODS

2.1. Materials

Fungal strain and enzyme production

The *Pleurotus pulmonarius* VAST02.42 was newly isolated in the Muong Phang primeval forest ($27^0$27'N, $103^0$09'E) in Dien Bien, Viet Nam. This fungal species was chosen as a high laccase producer in a previously screening strategy of thirty ascomycetes and basidiomycetes for the lignocellulolytic enzymes (data not shown). The fungal isolate was grown on the plates of potato dextrose agar (PDA) at 23 ^{o}C and stored at the Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology (INPC-VAST). For the production of laccase, the fungal strain was grown in sterile plastic bags, each containing 2 % (w/v) of rice straw, yeast extract 2 g.L^{-1}, peptone 2 g.L^{-1}, and trace elements ($MgSO_4$ 0.2 g. L^{-1}, KH_2PO_4 0.5 g. L^{-1}, $(NH_4)_2SO_4$ 1.5 g. L^{-1}, and $CaCl_2$ 0.05 g. L^{-1}). The crude proteins were extracted with distilled water after ten days of fungal growth. The mycelium was removed by centrifugation (15,000 × g for 10 minutes) and filtration using a GF6 filter (Whatman PLC, UK). The clear supernatant was then harvested using an ultrafiltration system (30 kDa cut-off, Germany) at 11 °C.

2.2. Molecular identification and phylogenetic analysis

The fungal isolate was identified based on the sequence of the internal transcribed spacer (ITS1-5.8S-ITS2) region of the ribosomal gene repeat unit. The genomic DNA was isolated using Plant/Fungi DNA isolation Kit (Norgenbiotek, Canada) and purified using the GeneJET genomic DNA purification kit (Thermo Scientific, USA), following the manufacturer's instructions. The genomic DNA amplification was achieved by using a polymerase chain reaction (PCR) with a forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and a reverse primer ITS4 (5'-TCCTCCGCTTATTGATATTGC-3') [10]. For the reaction, a typical 25 µL PCR included: 12.5 µL of PCR Master mix kit (2X) (Themo Fisher, MA, USA), 3 µL DNA, 10 µM of each primer and nuclease-free water. The thermal cycling conditions were used: 95 °C for 3 min, 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C; and a 10 min extension at 72 °C. The PCR products were separated in 1.5 % agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. The sequences obtained were analyzed in the ABI Prism 3100 genetic analyzer (Applied Biosystems, MA, USA) and compared with GenBank databases using NCBI BLAST tool [11].

Phylogenetic analysis

Phylogenetic trees were constructed by using the MEGA® v6.0 software (Pennsylvania, PA, USA) [12]. The alignment was performed utilizing the Clustal W, and the evolutionary history was reconstructed with the maximum likehood method (ML) [13]. ML trees were constructed using 1000 bootstrap replicates. The sequence obtained from the fungus VAST02.42 was compared with available sequences on Genbank using the BLAST tool. *Psathyrella pygmaea* MG734744 was considered an outgroup species. TreeView software (S&N Genealogy, United Kingdom) was used to edit evolutionary tree images [14].

2.3. Enzyme activity assay

Laccase activity was determined spectrophotometrically with ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) as the main substrate at 436 nm (molar extinction coefficient, E_{max} = 29300 M^{-1} cm^{-1}). The assay mixture contained 0.5 mM ABTS and 0.1 M sodium acetate buffer at pH 3.0. The reaction was initiated by adding an appropriate amount of Laccase enzyme, and incubation at 30 ºC for 2 min. One unit of enzyme activity (IU) is defined as the amount of enzyme that releases 1 µmol of oxidized product per minute [15].

2.4. Enzyme purification

Protein purification was performed using fast protein liquid chromatography (FPLC) with an ÄKTA Pure system from GE Healthcare. Initially, proteins were added to a HiTrap™ DEAE cellulose column that had been equilibrated with buffer A (20 mM CH_3COONa, pH 5.5) at a flow rate of 2 mL/min. The adsorbed protein was washed with the same buffer and eluted by NaCl gradient (0 ~ 1.5 M) at a flow rate of 2 mL/min. Next, the activity fractions were applied to Sephadex G-100 gel filtration column equilibrated with buffer B (10 mM sodium acetate, pH 5.5, 50 mM NaCl) at a flow rate of 1 mL/min. Finally, elution on HiTrapTM Q XL was performed with a 100 mM CH_3COONa buffer (pH 5.5) starting from 0-1.0 M of NaCl at a flow rate of 0.3 mL/min. The collected portions containing laccase activity were pooled and ultra-filtrated (10 kDa cut-off) in the CH_3COONa buffer, pH 5.5 and preserved at -80 ºC for future studies.

2.5. Enzyme characterization

The purified enzyme's MW was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a separating gel of 12 % acrylamide. The enzyme sample was added to SDS gel-loading buffer (1 % SDS, 10 % glycerol, 10 mM Tris-Cl, pH 6.8, 1 mM ethylene diamine tetra acetic acid (EDTA), 2-mercaptoethanol, and bromophenol blue to serve as a tracking dye) and denatured at 95 °C for 5 min. Staining of the protein bands was done using Coomassie Brilliant Blue R-250, followed by fixing and destaining. The M_w of the laccase was determined by calibration against broad range M_w markers (Cell Signaling), which contained the proteins MBP-β-galactosidase (175 kDa), MBP-paramyosin (80 kDa), MBP-CBD (58 kDa), CBD-Mxe Intein-2CBD (46 kDa), CBD-Mxe Intein (30 kDa), CBD-BmFKBP13 (25 kDa), and lysozyme (17 kDa) (CBD-chitin binding domain, MBP-maltose-binding protein).

Effects of pH

The optimal pH of the purified laccase was measured using citrate-phosphate buffer adjusted to different pH levels in the range of 4.5 ~ 7.5 at 25 °C. To determine the pH stability, the purified laccase was incubated at various pH values (5.0 ~ 7.0) at optimal temperature. After 1 hour-intervals of incubation, aliquots of the sample were taken, and the remaining laccase activity was measured as described above.

Effects of temperature

The effect of different temperatures on laccase activity was determined at 30 - 60 °C as described above in the enzyme activities. Thermal stability was estimated by incubating the purified laccase at 25, 40, and 70 °C and measuring activity at selected times up to 120 min, at an interval time of 20 min. The catalytic activity at 30 ^{0}C before incubation was considered to be 100 %.

Metal ions effects

To elucidate the effects of different metal ions on the activity of purified laccase, 1 mM (final concentrations) of Ca^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, and Hg^{2+} was added into the reaction mixture separately, at optimal temperature for 1 hour. The activity was determined using ABTS as a substrate. All experiments were performed in triplicate.

2.6. Enzyme kinetics

The Michaelis-Menten constant (K_m) and catalytic rate constant (k_{cat}) were determined by measuring the activity of laccase for the most common substrates (ABTS) [15]. The K_m and k_{cat} parameters were calculated by applying the Lineweaver-Burk plot. Substrate concentrations were varied from 40 to 600 mM at 40 °C for 2 min.

3. RESULTS AND DISCUSSIONS

3.1. Identification of the isolated fungal strain

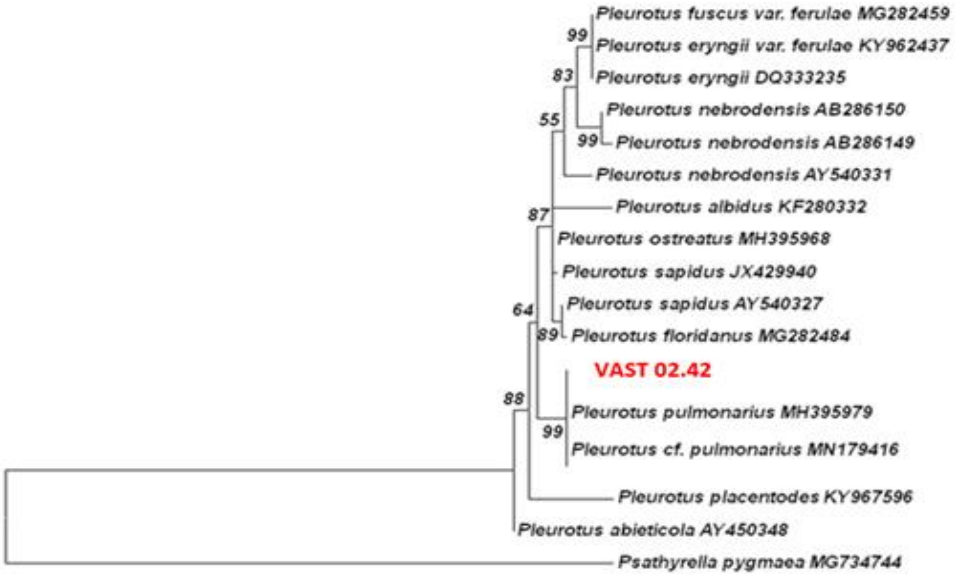

Figure 1. The phylogenetic tree of the fungal strain VAST02.42 was constructed using the rDNA-ITS sequence based on the Maximum Likelihood (ML) method. *Psathyrella pygmaea* MG734744 was used as the outgroup taxon.

Identification of the fungal isolates was conducted through partial gene sequencing as described in Section 2.2. A sequence of 703 nucleotides in length was obtained and then compared with those available in online database by applying the NCBI BLAST tool and

submitted to GenBank under accession number: OR770109. The results showed 100 % similarities with the sequences of *Pleurotus pulmonarius* MH395979 species. Based on this genetic identification, the strain VAST02.42 was authenticated as *Pleurotus pulmonarius (Pleurotaceae*, Basidiomycota). Phylogenetic trees of fungi (ITS region) are shown in Figure 1; the bootstrap values are used to estimate the confidence of the branches in a phylogenetic tree.

3.2. Purification of laccase from *P. pulmonarius* VAST02.42

After cultivation of *P. pulmonarius* (strain VAST02.42) that grown on solid-state culture with rice straw as a lignocellulose-rich substrate, a sufficient amount of crude protein with laccase was obtained for further purification steps. As a result, a total laccase activity up to 6432 U could be reached at the first step of purification. Accordingly, the specific activity of *Ple*Lac was increased 5.1-fold from 2.9 $U.mg^{-1}$ in crude extract to 14.7 U.mg-1 after separation by the anion exchange column DEAE-cellulose (Table 1).

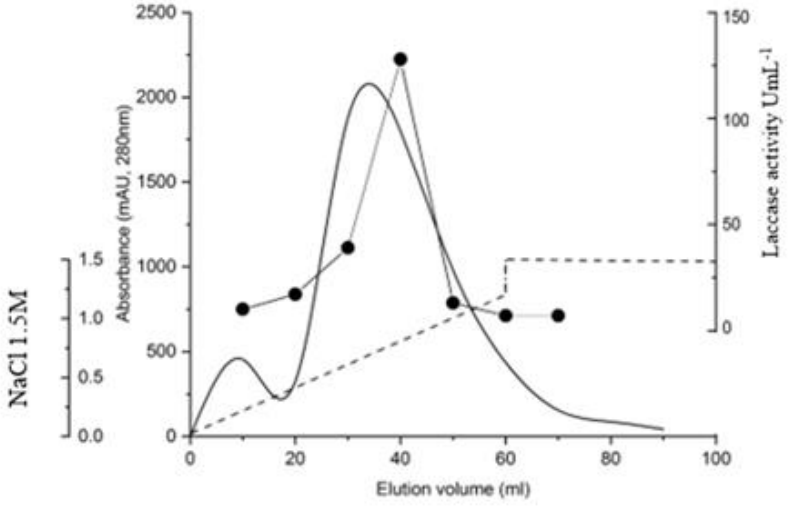

Figure 2. FPLC elution profile of the final purification steps of *Ple*Lac (HiTrap™ Q XL column); absorbance at 280 nm (solid line), *Ple*Lac activity (black circles), and NaCl gradient (dashed line).

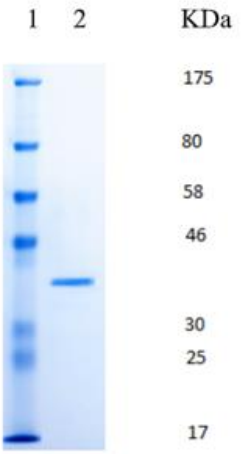

Figure 3. Protein bands on SDS-PAGE gel after laccase activity-based purification (Lane 1: marker, lane 2: pure protein).

Table 1. Summary of *Ple*Lac purification from *Pleurotus pulmonarius*.

Purification steps	**Protein (mg)**	**Total laccase activity (U)**	**Specific activity (U mg^{-1})**	**Yield (%)**	**Purification (fold)**
Culture fluid	2243	6432	2.9	100	1.0
DEAE Cellulose	205	3011	14.7	46.8	5.1
Sephadex G-100	54	889	16.5	13.8	5.7
HiTrap^{TM} Q XL	19	532	28	8.3	9.6

Afterwards, fractions with laccase activity were pooled with a total activity of 3.011 U and subsequently applied to gel exclusion chromatography with Sephadex G-100 resin. The final purification step was performed on a HiTrap™ Q XL column with elution using a NaCl gradient of 1.5 mM. A peak of laccase activity was collected that corresponded to the protein spectrum shoulder at λ = 280 nm (Figure 2). The laccase-active protein (designated as *Ple*Lac) was successfully purified, appearing as a single protein band on a 12 % SDS-PAGE gel with a molecular mass (Mw) of 35 kDa (Figure 3). The total activity of *Ple*Lac was calculated to be 532 U, corresponding to a 9.6-fold purification and an 8.3 % recovery (Table 1).

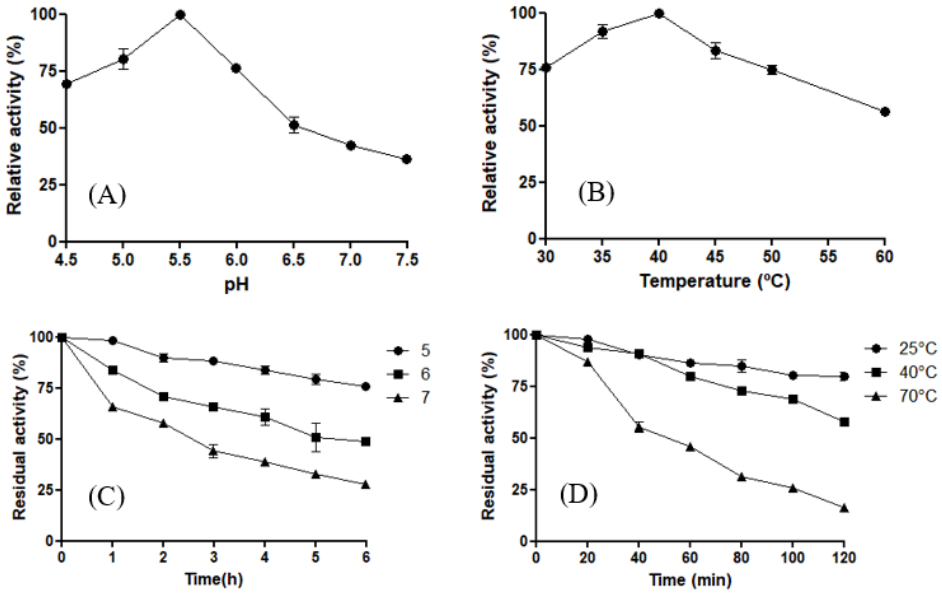

Figure 4. Influence of pH and temperature on *Ple*Lac activity.
Optimum pH (A), optimum temperature (B), pH stability (C), and thermal stability (D).

The effect of pH and temperature on *Ple*Lac activity was determined using ABTS as a substrate. The optimal pH of *Ple*Lac was determined to be between pH 4.5 ~ 7.5. The highest activity was observed at pH 5.5 in citrate-phosphate buffer and lost its activity at pH 7.5 (Figure 4A). The optimal pH range of *Ple*Lac was different from other Lacs. Most laccases from fungi showed higher activity at the pH range of 3.0 to 4.0, such as that from *Pleurotus ostreatus* exhibited its optimum activity at pH 3.0 [16] or optimal pH of *Pleurotus sajor-caju* laccase was determined at pH 3.2 [17]. Whilst, the purified *Ple*Lac exhibited its pH-optimum in the range of

weak-acid and neutral pHs. The stability of *Ple*Lac was determined by measuring residual activity after incubation of the enzyme at various pH 5.0 ~ 7.0 for each hour. The *Ple*Lac maintained its activity at pH 5.0 for 3 h and was gradually denatured at pH 6.0. At the higher pH of 7.0, *Ple*Lac's half-life was determined to be about 3-h incubation and decreased its activity rapidly after longer incubation (Figure 4C).

The optimal temperature of *Ple*Lac was measured between 30 - 60 ºC (Figure 4B). The *Ple*Lac exhibited optimum activity at 40 ºC. This enzyme showed thermostability at moderate temperatures (e.g., 25 - 40 ºC), while the activity down rapidly at high temperatures (Figure 4D). Thus, the enzyme lost its activity up to 80 % at 70 ºC during 120 min of incubation. These results show that the *Ple*Lac from *P. pulmonarius* was not stable at high temperatures. This properties of *Ple*Lac were similar to other laccases, e.g., that from *Lentinus squarrosulus* with thermostability at 30 ºC [15].

3.4. Effect of metal ions on the *Ple*Lac activity

The effect of various metal ions at 1 mM was determined with the main substrate ABTS at 40 °C. As the result, Cu^{2+} did not seem to affect the *Ple*Lac activity since its activity was conserved equivalent to that of the negative control (100.8 %). Copper plays an important role in the active site of laccase, which contains conserved amino acid motifs responsible for binding to copper atom, thus it could be required for this enzyme production, i.e., by fungal- or microbial fermentation. However, the purified laccase (mature protein enzyme) was used in the current study, so this metal ion may not absolutely be necessary for enzyme activity. Enzyme activity even slightly decreased in the presence of Na^{+}, K^{+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} and remarkably inhibited by Hg^{2+} and Fe^{3+} with its relative activity of 61.22 % and 65.11 % respectively compared to maximum activity (Table 2). Consistent with previous studies, Mainak showed a few metal ions acted as the inhibitor of the enzyme in 1 mM concentration, i.e., Hg^{2+}, Fe^{3+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} [15].

Table 2. Influence of metal ions on *Ple*Lac activity.

Metal ions	Relative activity (%)
None	100
Na^{+}	89.78 ± 1.12
K^{+}	90.35 ± 1.27
Cu^{2+}	100.82 ± 0.83
Mn^{2+}	93.33 ± 1.34
Ca^{2+}	84.32 ± 1.98
Hg^{2+}	61.22 ± 1.11
Mg^{2+}	89.76 ± 0.84
Fe^{3+}	65.11 ± 0.92

3.5. Enzyme kinetics

Kinetic constants of purified *Ple*Lac were studied with ABTS as substrate. The reaction rates were varying at substrate concentration from 40 mM to 600 mM. The results showed Michaelis-Menten constants (K_m) and the catalytic rate (k_{cat}) values of the purified *Ple*Lac were 41 mM and 334 s, respectively, which lead to a catalytic efficiency (k_{cat}/K_m) of 8.1 $mM^{-1}.s^{-1}$ (Table 3). The kinetic data in the K_m values using ABTS as the substrates have been reported

previously in numerous studies [15, 18, 19]. The *P. pulmonarius* laccase showed a K_m value to be similar to those of *Pleurotus ostreatus* HP-1 (46.51 mM) [18]. However, the K_m value of *Ple*Lac was 574-fold and 138-fold higher than that obtained for *Lentinus squarrosulus* and *Mycena purpureofusca*, respectively [15, 19].

Table 3. Kinetic parameters of *Ple*Lac in comparison with others.

Organism	K_m (mM)	k_{cat} (s)	k_{cat}/K_m ($mM^{-1}. s^{-1}$)	Reference
P. pulmonarius VAST02.42	41	334	8.1	This study
Lentinus squarrosulus MR13	0.0714	303	4243	[15]
P. ostreatus HP-1	46.51	244.32	5.25	[18]
Mycena purpureofusca	0.296	405	1368	[19]

4. CONCLUSIONS

The anion exchange and gel filtration column chromatography technique were used to purify laccase enzyme from *P. pulmonarius* VAST02.42 up to 9.6-fold. The molecular weight on SDS-PAGE of *Ple*Lac was 35 kDa. Biochemical properties of *Ple*Lac showed that the optimal pH was 5.5 and stability at the neutral pH range. Interestingly, most laccases from fungi showed higher activity at the pH range of 3.0. Whilst, the purified *Ple*Lac exhibited its pH-optimum in the range of neutral pHs. The optimal temperature was 40 ºC for *Ple*Lac and the activity was maintained at a moderate temperature (25 ºC). The values of kinetic parameters K_m and k_{cat} of the purified *Ple*Lac were 41 mM and 334 s, respectively. The enzyme's activity seems unaffected by Cu^{2+} but was decreased by Na^+, K^+, Mn^{2+}, Ca^{2+}, and Mg^{2+}. In conclusion, our findings confirmed that *P. pulmonarius* VAST02.42 excretes Laccase. The Laccase obtained from *P. pulmonarius* VAST02.42 differed from those produced by other fungi in terms of optimal pH, optimal temperature, and the effect of metal ions. These results provide fundamental information for using this enzyme in industrial applications and for further study on its application in catalyzing the oxidation of essential oils (e.g., α-pinene) to create value-added products in combination with peroxidase.

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Credit authorship contribution statement. Do Huu Nghi designed the research. Dang Thu Quynh, Le Viet Hoang, Le Mai Huong, and Do Huu Nghi performed the research, analysis. Do Huu Nghi and Dang Thu Quynh prepared and revised the paper.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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