Isolation, identification of a laccase-producing fungal strain and enzymatic properties of the laccase

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Abstract

A new type of thermostable laccase was isolated from *Paraphoma* sp. GZS18, and its partial enzymatic properties were determined. A strain GZS18 of laccase with high yield was screened from forest soil and identified as *Paraphoma* sp. GZS18 through morphological characteristics and ITS sequence analysis. The laccase of *Paraphoma* sp. GZS18 (Lac-P) was obtained through cation–anion exchange chromatography, gel filtration chromatography, and other purification processes. The testing result shows that Lac-P is a single protein of 75 kDa, and the 11 amino acid sequences in the N-terminal are AX^aVSVASREMT (X^a was the non-standard protein). The optimum temperature and optimum pH of lac-P activity are substrate-independent. The temperature is in the range of 50–70 °C, and pH has high catalytic efficiency in the acidic range. Lac-P has good stability in the temperature and pH. The half time at 70–60 °C is 1.5 and 4 h, respectively. At pH 6–9 and room temperature, there is more than 80% activity 24 h later. Lac-P is tolerant of most metal ions and low concentrations of inhibitors but is inhibited by Hg²⁺, Fe²⁺ and NaN₃. The laccase from *Paraphoma* sp. GZS18 at high temperature and pH 6–9, with strong stability, has better industrial application characteristics.

Keywords Characterization · Laccase · *Paraphoma* sp. · Purification

Introduction

Laccases (*p*-diphenol: oxygenoxidoreductases; EC1.10.3.2), the biggest sub-group of blue multi-copper oxidases, directly utilize the redox ability of cupric ions, catalyze the oxidation of several aromatic compounds, forming water and oxygen molecules (Yang et al. 2015a; Campos et al. 2016). The active center of laccase contains a special structure composed of four cupric ions. These four cupric ions include one type I, one type II, and two type III, which exhibit different magnetic and spectroscopic properties (Farver et al. 2011; Du et al. 2012). Laccase, with extensive substrate specificity, can catalyze and oxidize multiple organic and inorganic compounds, such as phenol and its derivatives, arylamine

and its derivatives, pigments, and lignin (Tinoco et al. 2011). Laccase, as a multifunctional enzyme, is widely applied to various industries and areas, including environmental remediation. With advancements in technology, scholars have developed new application fields of laccase (Prins et al. 2015; Du et al. 2015).

Laccase is widely distributed in plants, fungi, and some bacteria (Elisashvili et al. 2010). Fungi of different species exhibit laccase activity and have been used to separate and purify several types of laccases (Yan et al. 2015). All white-rot fungi can produce various amounts of laccase. In recent years, researchers have discovered that new species in ascomycetes, basidiomycetes, and deuteromycetes can produce laccase (Liu et al. 2016). For example, *Paecilomyces* sp. WSH-L07 produced maximum laccase output by 2 mM copper and methylene blue as inducers (Liu et al. 2009). The species from *Phoma* genus have been proven to produce laccase. Some papers (Junghanns et al. 2008; Hofmann and Schlosser 2016) reported that *Phoma* sp. UHH 5-1-03 could produce approximately 1117 U/L laccase after being cultivated in complex culture medium. Fungal laccase is widely used, and generally has an optimal reaction temperature of

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45 °C; in particular, laccase from *Trametes versicolor* can effectively degrade multiple synthetic dyes and has an optimal reaction temperature of 40–50 °C, limiting its industrial application (Levasseur et al. 2009; Ebrahimpour et al. 2011). Therefore, researchers must develop new strains with heat resistance, and the laccase that can resist acid and alkali, chelating agents, and some toxic compounds.

In this research, a novel thermotolerant strain (GZS18) of *Paraphoma* sp. producing laccase and the characteristics of laccase produced are investigated for the first time. After screening, separation, and identification, a novel strain (GZS18) of *Paraphoma* sp. was obtained, laccase produced after re-separation and purification, and characteristics of purified laccase were studied.

Materials and methods

Chemicals, microorganism, media, and culture conditions

2,6-Dimethoxyphenol (DMP), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), guaiacol, syringaldazine (SGZ), Acid Blue 129, Remazol Brilliant Blue R, Methyl Blue, Toluidine Blue and Reactive Black 5 were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade and obtained from Sinopharm Chemical Reagent (Shanghai, China).

Microorganisms (include strain GZS18) were isolated from various sources, such as bamboo samples and rotten wood, and maintained on PDA slants (potato extract 200 g/L, glucose 20 g/L, agar 20 g/L) at 34 °C. The *Paraphoma* sp. strain GZS18 was preserved by the Institute of Biochemistry and Nutrition in Guizhou University, China. Fungal inocula were prepared by growing the strains on a rotary shaker at 150 rpm and 34 °C in 250 mL flasks containing 100 mL of PDA medium. After 7 days of cultivation, the prepared inocula (5 mL) were then transferred into a 250-mL flask that contained 50 mL of PDA media. The cultivation was carried out at 34 °C on a rotary shaker incubator at 150 rpm for 5 days. In all experiments, multiple flasks were run at the same time, and at least three flasks were used.

Strain identification

The strain was identified following the methods reported (Liang et al. 2011; Liu 2013).

Determining laccase activity

Laccase (EC 1.10.3.2) activity was determined by monitoring the absorbance change at 420 nm related to the rate of oxidation of 1 mM ABTS to its cation radical in 25 mM

Na-acetate buffer (pH 3.8) at room temperature (Du et al. 2012). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol ABTS per minute. All experiments were performed at least twice using 3–5 replicates. The data presented correspond to mean values, the standard deviation being lower than 15%.

Purification of laccase

The fermentation broth of *Paraphoma* sp. strain GZS18 was filtered with four-layered gauze. The filtrate was then poured into a 7-kDa dialysis bag. It was dialyzed for 4 h with deionized water four times the fermentation broth volume. The fungal solution obtained after four dialyses was crude laccase sample. DEAE–Cellulose chromatographic column (5 cm × 20 cm) was balanced with 10 mmol/L Tris–HCl (pH 6.9). The crude laccase sample was centrifuged at 10,000 r/min. The supernatant was taken and then adjusted to 10 mmol/L (pH 6.9) with 1 mol/L Tris–HCl (pH 6.9). The sample was loaded and then eluted stepwise, with 10 mmol/L Tris–HCl (pH 6.9) and NaCl of different concentration gradients, at a flow rate 4 mL/min. The eluent was collected, combined with active components, and effectively dialyzed. Lac1 was obtained as a result. CM–Cellulose chromatographic column (2.5 cm × 20 cm) was balanced with 10 mmol/L HAc–NaAc (pH 4.2) buffer solution. Lac1 was adjusted to 10 mmol/L (pH 4.2) with 1 mol/L HAc–NaAc (pH 4.2). The sample was loaded. It was then eluted stepwise, with 10 mmol/L HAc–NaAc (pH 4.2) and NaCl of different concentration gradients, at a flow rate of 4 mL/min. The eluent was collected, combined with active components and adequately dialyzed. Lac2 was obtained as a result. SP–Sepharose chromatographic column (1.0 cm × 15 cm) was balanced with 10 mmol/L HAc–NaAc (pH 4.0) buffer solution. Lac2 was adjusted to 10 mmol/L (pH 4.0) with 1 mol/L HAc–NaAc (pH 4.0). The sample was loaded. Elution was then performed with 10 mmol/L HAc–NaAc (pH 4.2). Linear gradient elution was also performed using 10 mmol/L HAc–NaAc (pH 4.0) and NaCl of different concentration gradients at a flow rate of 3 mL/min. The eluent was collected and combined with active components. Lac3 was obtained, as a result. Superdex-75 gel filtration chromatographic column was balanced with 0.15 mol/L NH₄HCO₃ (pH 8.5). Lac3 was dissolved with 0.15 mol/L NH₄HCO₃ (pH 8.5) and centrifuged at 12,000 r/min for 10 min. The supernatant was taken. The sample was loaded and then eluted with 0.15 mol/L NH₄HCO₃ (pH 8.5) buffer solution at a flow rate of 0.5 mL/min. It was combined with active components and then freeze dried. Laccase from *Paraphoma* sp. strain GZS18 was obtained (Park et al. 2015; Yang et al. 2015b).

Biochemical characterization of purified laccase

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) purity detection of fungal laccase: separation gel concentration was 12% and spacer gel concentration was 5.0%. After electrophoresis, Coomassie Brilliant Blue R-250 staining fluid was added until it covered the gel surface. A gel imager was used to capture images of the electrophoretic result. The glycosylation of the laccase was determined by treating the denatured enzyme (20 µg) with 2 U of endo-glycosidase H (Glyko, USA) at 37 °C for 3 h. N-terminal sequencing of the purified laccase was determined by Shanghai GeneCore Biotechnologies (Shanghai, China) using an Applied Biosystems Protein Sequencer PROCISE™ 494. The optimal temperature and pH, thermal and pH stabilities, and effects of metal ions and inhibitors were determined using ABTS, DMP, SGZ, and guaiacol as the substrate (Lee et al. 2012). Thermal stability was measured after incubating the purified laccase at temperatures ranging from 0 to 80 °C for 1 h, and then the residual activity was determined every 30 min, the initial laccase activity was assigned as 100%. The optimal temperature was determined from 20 to 80 °C. The optimal pH and pH stability were determined in 0.1 M citrate buffer (pH 2.5–5.0), 0.1 M phosphate buffer (pH 6.0–8.0), and 0.1 M glycine–NaOH buffer (pH 9.0–10.0). The stability at pH 2.5–10.0 was tested after incubating the enzyme for 24 h at 30 °C. The highest value of laccase activity was assigned as 100%. The effects of the metal ions Fe^{2+} , Cu^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Ba^{2+} , Ca^{2+} , K^{+} , Hg^{2+} and Ag^{+} at 0–10 mM were investigated. The effects of typical laccase inhibitors (EGTA, NaN_3 , SDS, DTT, and sodium thioglycolate) at 0–10 mM on laccase activity were also observed by incorporating them to the assay mixture before determining residual activity. Laccase activity was determined after incubation at 30 °C for 10 min. Laccase activity without metal ions and inhibitors was assigned as 100%.

Results and discussion

Characteristics and identification of strain GZS18

ABTS in the culture medium turns green when a strain capable of producing laccase was used. More than 30 strains with obvious oxidation capacity were obtained after screening and separation on a PDA plate containing ABTS. The strain GZS18 possessed a higher production. It had the highest laccase activity 1867.78 ± 159 U/L among all the tested strains.

The strain GZS18 was described and identified according to Aveskamp's method (2010). The ITS region of the GZS18 strain was amplified, sequenced, and submitted to GenBank (Accession No. KT 205556). A Blast search in GenBank was performed using the strain GZS18 sequence

as the query. Close matches that showed maximal sequence identities of 95% included *Paraphoma* spp. and other related species. A novel laccase-producing strain was isolated and identified as *Paraphoma* sp. strain GZS18 according to the morphological characteristics and the comparison of DNA gene sequences.

Molecular mass and N-terminal amino acid sequence of laccase

Crude laccase obtained from the 108-h cultures was subsequently purified. After three sequential steps, laccase produced by *Paraphoma* sp. GZS18 was purified using alt-out with ammonium sulfate, hydrophobic interaction, and ion-exchange chromatographies. *Paraphoma* sp. GZS18 contains several laccase genes in its genome. Figure 1 indicated that two laccase isozymes were present in the crude culture. However, it remains possible that the two different bands represent the same enzyme, with the difference in charge/molecular weight the result of post-translational modification. After sequential purification steps, the laccase was purified 7.3-fold from its initial culture broth with a final yield of 44.5%, and the specific activity of the purified enzyme was measured to be a protein of 297.5 U/mg (Table 1). The purified laccase exhibited a single band both on SDS–PAGE (Fig. 1), suggesting a homogenous form. The purified laccase from the *Paraphoma* sp. GZS18 was named as Lac-P. After calculation, a molecular weight of approximately 75 kDa Lac-P was obtained (Fig. 1). The molecular weights of the typical fungal laccases were 60–90 kDa (Park and Park 2014). However, reports describing the molecular mass of purified laccase at 190 (Edens et al. 1999) and

Fig. 1 SDS–PAGE of Lac-P (M–Marker, 1–purified laccase; 2–crude laccase from fermentation broth)

Table 1 A summary of the purification steps of laccase from *Paraphoma* sp. GZS18

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	139.3	5681	40.8	1	100
Cation exchange	15.8	3567	225.8	5.5	62.8
Superdex-75	8.5	2529	297.5	7.3	44.5

51 kDa exist (Neifar et al. 2010). The laccase was found to be a glycoprotein with an estimated carbohydrate content of 14%. The carbohydrate content in the range of 10–20% of laccase included the report of other basidiomycetes laccases, *Trametes versicolor*, *Coriolus hirsutus*, and *Cladosporium cladosporioides* (Du et al. 2015; Halaburgia et al. 2011).

The N-terminal amino acid sequence of the *Paraphoma* sp. GZS18 laccase was measured. The sequence was AX^aVSVASREMT (X^a was the non-standard protein), which was 45.5% similar to amino acid sequence at N-terminal of *Phoma* sp. Laccase (Junghanns et al. 2009), 36.4% similar to *Pycnoporus sanguineus* (Iracheta-Cárdenas et al. 2016) and less similar to the N-terminal amino acid sequence of other species, such as *Paraconiothyrium variable* (Forootanfar et al. 2011) and *Cyathus bulleri* (Garg et al. 2012). The analysis on the amino acid sequence at the N-terminal of laccase indicated that significant differences existed between different species.

Effects of temperature on laccase activity and stability

Four substrates commonly used for laccase activity testing (ABTS, DMP, SGZ, and guaiacol) were chosen to study the optimal reaction temperature needed for laccase catalysis of different substrates and temperature stability. Figure 2a showed that the optimal temperature of Lac-P was substrate dependent, that is, catalysis of different substrates required different optimal temperatures. When DMP, SGZ, and ABTS were used as substrates, the optimal temperature of Lac-P was 60 °C. However, catalysis of guaiacol required an optimal temperature 70 °C, which was slightly higher. A sharp decrease in laccase activity at a high temperature is probably related to two reasons: laccase structure changes and less dissolved oxygen in the laccase activity evaluation system at a high temperature (substrate catalysis by laccase directly requires participation of oxygen) (Yang et al. 2013; Liu et al. 2017).

Temperature stability of Lac-P was studied, with ABTS as the substrate (Fig. 2b). Lac-P seemed stable when the

Fig. 2 Effects of temperature on the activity (a) and stability (b) of the purified laccase

temperature was below 65 °C and it became unstable when the temperature exceeded 75 °C. When the temperature was maintained at 85 °C for 5 min, Lac-P basically lost its activity. The half-life of pure enzyme Lac-P was 1.5 h at 70 °C and 4 h at 60 °C. The half-life of Lac-P at room temperature could last for as long as 2 weeks (no supporting data are given). The stability of pure enzyme at 70 °C was slightly lower than that of crude enzyme, which was probably because small organic molecules secreted by some fungi in crude enzyme solution protected laccase stability. In pure enzyme solution, these small molecules disappeared; so Lac-P was not protected, and its stability naturally reduced. In addition, the primary structure of laccase also influenced temperature stability. Autore et al. (2009) analyzed amino acid sequence of multiple fungal laccases with excellent heat thermostability and found that 16 amino acids extending from the C-terminal might maintain laccase stability and that revision or site-specific mutagenesis of amino acid at the C-terminal causes significant changes of laccase activity and stability. However, the optimal reaction temperatures of most fungal laccase were between 40 and 60 °C (Solé et al. 2012). The temperature stability test of Lac-P shows that its activity basically remained unchanged when the temperature was kept at 30–65 °C for 60 min, that its relative activity was 50% when the temperature was kept at 75 °C for 20 min, and that it already lost its activity when the temperature was kept

at 85 °C for 10 min. Therefore, Lac-P showed excellent heat resistance, which may be due to the high optimal growth temperature of the *Paraphoma* sp. GZS18 strain, the fungal source of Lac-P.

Effects of pH on laccase activity and stability

Laccase's optimal pH was also substrate dependent, but it demonstrated the highest catalytic efficiency within the acidic range (Fig. 3). The optimal pH for Lac-P catalyzing ABTS and SGZ was 3.0, 4.0, and 4.5 for the substrates DMP and guaiacol. For the different substrates, Lac-P only showed high conversion efficiency within a narrow acid pH range. Beyond pH 5.0, enzyme activity rapidly dropped to 45.0% of the maximum activity. Lac-P is suitable in more acidic conditions. When pH exceeded 6.0, Lac-P was basically unable to oxidize these substrates. The pH value of the reaction system might result in different catalytic efficiencies by influencing the combination state of enzyme and different substrates.

In this research, the temperature was kept at 25 °C and pH was kept at 6–9 for 24 h and laccase still had more than 80% residual activity. In particular, it still had a 95% residual activity when the temperature was kept at 25 °C and pH was kept at 6.0. However, slightly less desirable results were achieved within the pH range of 3–5. Many fungal laccases had this characteristic, namely, an acidic optimal pH but

a high stability under neutral condition or alkaline condition (Sadhasivam et al. 2008). For example, optimal pH of *Lentinus* sp. laccase was 2.5, but it was stable within the pH range of 6–10 (Hsu et al. 2012). The laccase of marine fungus *Cerrena* sp. H7 has an optimal catalytic pH of 3.0 but is most stable at pH 7–10 (Yang et al. 2014). The specific mechanism involved was unknown. In addition, based on different reports, the researchers found that all the three iso-enzymes of basidiomycetes *Steccherinum ochraceum* strain 1833 remain stable within the pH range of 2–6 (Chernykh et al. 2008).

Effect of metal ions and inhibitors on the enzyme activity

The influence of inorganic metallic salt and typical laccase inhibitors on Lac-P activity is shown in Table 2. Mn^{2+} activated Lac-P in a concentration-dependent manner (0.1–10 mM). Among the metal ions tested, Hg^{2+} and Ag^{+} had greater influence on laccase activity and demonstrate higher inhibiting effect. Hg^{2+} had extremely intense inhibiting effect on Lac-P, and it also had intense inhibiting effect on other fungal laccases, such as *P. ostreatus*; however, it had a mild inhibiting effect on certain fungal laccases (Liu 2013). Ca^{2+} , Zn^{2+} , Mg^{2+} , K^{+} and Ba^{2+} exhibited activation of Lac-P up to a certain level, beyond which it exhibited an inhibition of laccase activity. Fe^{2+} and Cu^{2+} activated Lac-P in a concentration-dependent manner up to 1 mM. Copper is an important element for laccase activity. Cupric ions existing in the solution have an activation effect on most laccases and inhibiting effect on certain laccases. Research has shown that Cu^{2+} (even at low concentration, such as 0.1 mM) demonstrate activation effect on Lac-P, too (Du et al. 2015; Othman et al. 2014). Chernykh et al. (2008) found that cupric ions had the activation effect on laccase at low concentration, and they had the inhibition effect on laccase at high concentration. In general, Lac-P was influenced by type and concentration of metal ions and had certain tolerability towards most metal ions.

NaN_3 had a considerable inhibiting effect on Lac-P. Lac-P activity was intensely inhibited by 1 mmol/L NaN_3 . When 0.1 mmol/L NaN_3 existed in the reaction system, laccase activity was almost completely inhibited. This intense inhibiting effect might be due to the combination of NaN_3 and II and III cupric ion sites in laccase protein, which inhibited electron transport (Ausec et al. 2015). In the other inhibitors investigated, low-concentration inhibitors had less influence on enzyme activity while high-concentration inhibitors had significant influence on enzyme activity. When 1 mmol/L SDS was added into the reaction system, residual enzyme activity of laccase was 52%. Lac-P had lower sensitivity to 1 mmol/L DTT, approximately < 18% enzyme activity

Fig. 3 Effects of pH on the activity (a) and stability (b) of the purified laccase

Table 2 Effect of metal ions and inhibitors on purified laccase activity

Effectors	Relative activity (%)						
	Concentration (mM)						
	0	0.1	0.5	1	3	5	10
Ag ⁺	100	40 ± 2.2	24 ± 1.2	14 ± 0.2	9 ± 0.2	0	0
Hg ²⁺	100	36 ± 1.2	14 ± 0.2	0	^a	^a	^a
Mn ²⁺	100	111 ± 1.2	131 ± 1.4	191 ± 2.2	231 ± 3.2	376 ± 2.3	392 ± 3.5
NaN ₃	100	20 ± 1.2	5 ± 0.6	0	0	0	0
SDS	100	103 ± 3.4	78 ± 1.4	52 ± 2.4	6 ± 0.5	0	0
Sodium thio-glycolate	100	83 ± 7.1	53 ± 3.7	13 ± 1.5	3 ± 0.7	0	0
DTT	100	172 ± 3.7	136 ± 4.7	82 ± 2.4	23 ± 1.5	9 ± 0.7	0
EGTA	100	113 ± 4.2	94 ± 1.4	78 ± 4.8	33 ± 2.9	5 ± 0.5	0

Salts of Fe²⁺, K⁺, Ca²⁺, Cu²⁺, Mg²⁺, Zn²⁺ and Ba²⁺ showed little or no effect on laccase activity

^aSalts of Fe²⁺, Cu²⁺, and Hg²⁺ at concentrations beyond 1 mM interfered with the color development during enzyme activity assay and hence the effect could not be measured

was inhibited. SDS, NaN₃, sodium thioglycolate, DTT and EGTA of 10 mmol/L all intensely inhibited Lac-P activity.

Conclusions

Many laccases have been separated and studied, but the present work is the first to report on the *Paraphoma* sp. laccase and its characteristics. A newly isolated *Paraphoma* sp. was separated from PDA culture medium containing ABTS. Purified extracellular laccase of *Paraphoma* sp. GZS18 was obtained after separation, and its characteristics were investigated under different of pH values, temperatures, ions, and inhibitors. The molecular weight of the laccase was approximately 75 kDa. Laccase activity remained stable below 60 °C and then destabilized beyond 70 °C. The optimal pH was between 3.0 and 5.0, but the enzyme remained stable under neutral and alkaline conditions. The laccase was tolerant of most metal ions, except for Ag⁺ and Hg²⁺, and Cu²⁺ promoted laccase activity to some degree. Low-concentration SDS, DTT, and EGTA slightly influenced the laccase activity. Overall, the *Paraphoma* sp. GZS18 laccase possesses a promising industrial application potential.

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Compliance with ethical standards

Conflict of interest The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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