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Purification and Characterization of Laccase from *Ceriporiopsis subvermispora*

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Laccase from *Ceriporiopsis subvermispora* was purified and partially characterized using a combination of ammonium sulfate precipitation, DEAE-cellulose ion exchange chromatography and Sephadex G-100 molecular sieve column chromatography. The results demonstrated that the maximum laccase output from *C. subvermispora* fermentation reached 3900 U/L. The specific activity of the laccase increased from 10.28 U/mg in a crude enzyme solution to 245.27 U/mg after isolation and purification, with a purification factor of 23.86 and a yield of 24.40 %. The molecular mass of laccase was 63 kDa and the Michaelis-Menten constant value was 23.3 μmol/L. The optimal temperature for enzyme activity was 50 °C. The stabilization pH range was 4-5; within a pH 4-5 range, the relative activity was higher than 70 %.

Keywords: *Ceriporiopsis subvermispora*, Laccase, Purification, Laccase activity, Characterization.

INTRODUCTION

Lignin is a natural organic high polymer extremely abundant in nature. It is widely present in plant cell walls and constitutes the supporting skeleton of plants together with cellulose and hemicellulose. The structure of lignin consists of phenyl propane units linked in many ways, such as a carbon-carbon bonds and an alkyl ether bond, in an amorphous three-dimensional network. The difficulty of degradation of lignin, related to its complex structure, is the bottleneck for the effective utilization of biomass resources[1]. In the paper-making process using bamboo and wood, chemical pulping to remove lignin is still the main method utilized by pulp mills[2]. This process consumes large amounts of chemicals and produces toxic wastewater, causing serious pollution to the environment. In the field of cellulosic ethanol manufacturing, pretreatment of biomass feedstock is typically required to break the crystal structure of lignin and its wrapping of cellulose and hemicellulose; this is to increase the effective surface area of contact for enzymatic hydrolysis and to eliminate harmful toxic substances during fermentation, so as to promote biomass degradation[3].

White-rot fungi have an extremely high ability to degrade lignin and are the only microbes known to degrade lignin to CO_2 and H_2O in pure culture. The lignin-degrading enzymes secreted from white-rot fungi mainly include lignin peroxidase, manganese peroxidase and laccase[4,5].

Compared with lignin peroxidase and manganese peroxidase, laccase has better prospects for application for several reasons. First, lignin peroxidase and manganese peroxidase are secondary metabolites produced strictly under carbon- and nitrogen-limiting conditions. The presence of carbon and nitrogen nutrients in industrial wastewater limits their secretion from cells[6]. Second, lignin peroxidase and manganese peroxidase require a large amount of H_2O_2 as an auxiliary agent in the degradation of organic pollutants, which is difficult to achieve in real-world scenarios[7]. Third, laccase, with an oxidation-reduction potential of 780 mV, is able to directly reduce molecular oxygen to water and to catalyze the oxidation of organic pollutants even without H_2O_2 or other secondary metabolites[8]. Therefore, laccase degradation of lignin and many structurally similar environmental pollutants has drawn more and more attention in recent years. It has especially important research value and application prospects in aromatic compound conversion[9] and industrial wastewater treatment[10].

With the continuous development of technology in biochemical studies, researchers have further investigated and analyzed laccase using a variety of methods to reveal its protein molecular mass range, isoelectric point, optimal reaction temperature, optimal pH value for enzyme activity, thermal stability, pH stability, polysaccharide content, catalytic reaction kinetic constant and substrate specificity[11,12]. Studies found that fungal laccases have some similar properties, such as protein structure and pH stability, but laccases from different

strains, as well as laccase isoenzymes from the same strain, have large differences in enzymatic properties[13,14]. Therefore, in order to provide optimal conditions for catalysis by laccase, the strain type and its enzyme production conditions must be fully investigated and its specific enzymatic properties must be analyzed. Crude enzyme produced by liquid fermentation is distributed in fermentation broth, which exists in the form of a complex mixture containing many inorganic salts, polysaccharides and other protein molecules; this is not conducive to an in-depth and accurate study of laccase. Therefore, to investigate the structure, function and enzymatic properties of fungal laccase, it is a prerequisite to obtain complete laccase protein of high purity and activity from fungal fermentation broth using modern isolation and purification methods.

Ceriporiopsis subvermispora (*Basidiomycete*, *Polyporales*, *Ceriporiopsis*) produces high yields of laccase and manganese peroxidase[15,16]. A mutant strain, NL4, with a high yield of laccase and high genetic stability was bred in our laboratory through mutagenic treatment of *C. subvermispora* using the nitrogen ion mutagenesis technique at an implantation dose of 80×10^{14} ions/cm^2 at 30 keV. NL4 demonstrated a stable and high yield of laccase after 4 d of fermentation and its laccase yield reached 3900 U/L on 6 d, which is 4.79 times that of the original strain. Thus, it is worthy of in-depth studies. In this study, a combination of ammonium sulfate precipitation, dialysis and chromatography techniques was used to separate and purify laccase from *C. subvermispora*. The enzymatic properties of the purified laccase were then analyzed.

EXPERIMENTAL

Ceriporiopsis subvermispora NL4 was selectively breed in our laboratory previously and stored in potato dextrose agar (PDA) slant medium.

The components of the slope seed medium included 20 % potato juice, 2 % glucose and 2 % agar; the pH of the solution was neutral. The components of the enzyme production medium included wheat bran 3 %, peptone 0.2 %, beef extract 0.3 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, KH_2PO_4 0.3 % and VB1 20 mg/L; the pH value of the solution was natural. The medium was placed into 250 mL flasks (100 mL per flask) for further use. The components of the trace elements mixture included $MgSO_4 \cdot 7H_2O$ 3 g/L, NaCl 1 g/L, $FeSO_4 \cdot 7H_2O$ 0.1 g/L, glycine 0.5 g/L, $CoSO_4$ 0.1 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L, $ZnSO_4 \cdot 7H_2O$ 0.1 g/L, $CuSO_4 \cdot 5H_2O$ 0.1 g/L, VB1 0.003 g/L, H_3BO_3 0.01 g/L, $MnSO_4 \cdot H_2O$ 0.1 g/L and KA1 $(SO_4) \cdot 12H_2O$ 0.01 g/L; this solution was prepared with distilled water. Sterilization was carried out by autoclaving at 121 °C for 0.5 h.

Separation and purification of laccase

Preparation of crude enzyme solution: For slant cultures, the preserved strain was inoculated on slant medium and incubated at 28 °C for 10 d, then stored at 4 °C for future use. Next, a liquid seed culture technique was performed. The mycelium was scratched on each slant in 30 mL of sterile water, dispersed in an oscillator and 5 mL was inoculated in a 250 mL flask with 100 mL of seed culture medium. The mixture was shaken at150 rpm at 28 °C for 4 d. The submerged culture technique was carried out. Ten per cent of the cultured seed was inoculated into 100 mL of fermentation media and shaken at 150 rpm at 28 °C, for 8 d. The fermentation broth was filtered through four layers of gauze, placed in plastic containers at -20 °C for 24 h and then heated to 20 °C. At this point, the solution contained polysaccharides that separated as a flocculent precipitate. The enzyme solution was then centrifuged at 6000 rpm for 20 min. The supernatant was the crude laccase enzyme solution.

Ammonium sulfate precipitation: The crude enzyme solution was fractionally precipitated with the addition of ammonium sulfate at 30, 40, 50, 60, 70 and 80 % saturation and stored at 4 °C overnight. The precipitate was collected by centrifugation at 12000 rpm for 15 min. The supernatant was then discarded and the pellets were dissolved in 25 mL of 10 mmol/L PBS buffer (pH 6).

Dialysis: The precipitated enzyme solution was placed in a dialysis bag and dialyzed against 10 mmol/L PBS buffer (pH 6) for 24 h. The buffer was replaced three times during the dialysis.

DEAE-cellulose ion exchange chromatography: The dialyzed enzyme solution was loaded onto a DEAE-cellulose ion exchange column (2 × 45 cm) which was pre-equilibrated with PBS. The solution was then gradient-eluted with a linear gradient of PBS and phosphate buffer containing sodium chloride (0.5 mo1/L) at a flow rate of 0.4 mL/min. The fractions were collected in one tube per 10 min. Each fraction was subjected to 280 nm absorbance detection and laccase activity assay.

Sephadex G-100 molecular sieve column chromatography: Fractions with high enzymatic activity were pooled and dialyzed against 0.03 mo1/L PBS buffer; the buffer was replaced 3 times during dialysis. The dialysate was loaded onto a pre-equilibrated chromatography column (2 × 60 cm), eluted with eluent (pH 6) at a flow rate of 0.4 mL/min and the eluates were collected in aliquots of 3 mL per tube. The eluate fractions were assayed for laccase activity and 280 nm absorbance. Fractions with high enzymatic activity were pooled, placed in a dialysis bag, concentrated using the vacuum freeze-drying method and stored at 4 °C for future use.

Laccase activity assay: 2,2'-Azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used as the enzyme substrate for the laccase activity assay. The 3 mL reaction system consisted of 0.5 mL of ABTS (1 mmo1/L), 2 mL of citric acid sodium citrate buffer (0.04 mo1/L, pH 5) and 0.5 mL of crude enzyme solution. The reaction mixture was incubated in a 25 °C water bath for 5 min and the change of absorbance at 420 nm was determined using a spectrophotometer for 3 min. Reaction mixture without the initiation factor was used as the blank sample. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute at 25 °C.

Determination of protein content: Protein content was determined using the coomassie brilliant blue method; bovine serum albumin was used as the standard.

Determination of the enzymatic properties of laccase

Determination of laccase molecular weight: The molecular mass of the purified sample was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were run in a 5 % stacking gel at 80 V for

0.5 h, then in a 12 % separating gel at 120 V for 3 h. The molecular mass of the laccase was estimated by relative mobility after gel staining, bleaching and image scanning.

Effects of temperature on laccase activity: The enzymatic activity of the reaction system was determined after incubation at different temperatures. The relative enzymatic activity was calculated with the highest enzymatic activity as 100 %.

Thermal stability of laccase: The laccase solution was incubated in a water bath at different temperatures (30, 40, 50 and 60 °C) for 0.5 h and then cooled to room temperature. The enzymatic activity was determined and the remaining enzymatic activity was calculated with the enzymatic activity of the un-incubated enzyme solution as 100 %.

Effects of pH on laccase activity: Substrate preparation and enzyme solution dilution were carried out using citric acid-sodium citrate buffers of varying pH. Enzymatic activities were determined and relative enzymatic activities were calculated using the value of the highest enzymatic activity as the denominator.

pH stability of laccase: Citric acid-sodium citrate buffers of varying pH were added to the laccase solution and incubated at room temperature for 1 h; afterward, the enzymatic activity was determined. The remaining enzymatic activity was calculated with the laccase activity of the untreated enzyme as 100 %.

Determination of Michaelis-Menten constant value (K_m) of laccase: Enzymatic activities were determined after reacting with different concentrations of the substrate (ABTS). The K_m value was obtained through Lineweaver-Burk plotting.

RESULTS AND DISCUSSION

Ammonium sulfate precipitation of the laccase fermentation broth supernatant revealed that when the concentration of ammonium sulfate was 70 %, laccase activity recovery was 88 %. After that, enzyme activity recovery began to decline with the continuous increase of ammonium sulfate concentration (Fig. 1). Through ammonium sulfate precipitation, impurities in the crude fermentation broth were removed and the laccase solution was concentrated. Subsequently, salt and other small molecules were removed by dialysis.

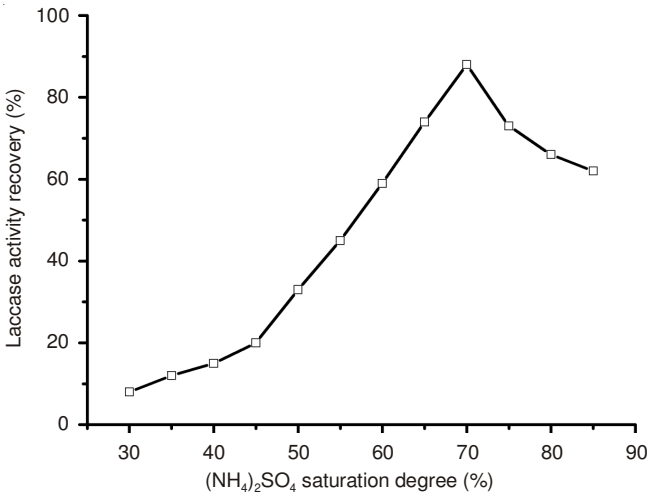

Fig. 1. Relationship of laccase activity recovery with $(NH_4)_2SO_4$ saturation degree

DEAE-cellulose ion exchange chromatography: After the crude laccase solution was loaded onto the DEAE-cellulose ion exchange column, the fractions with high laccase activity were collected and pooled (Fig. 2). Studies have shown that different proteins carry different numbers of charges and thus have different binding strengths with DEAE-cellulose. Accordingly, proteins can be separated and purified.

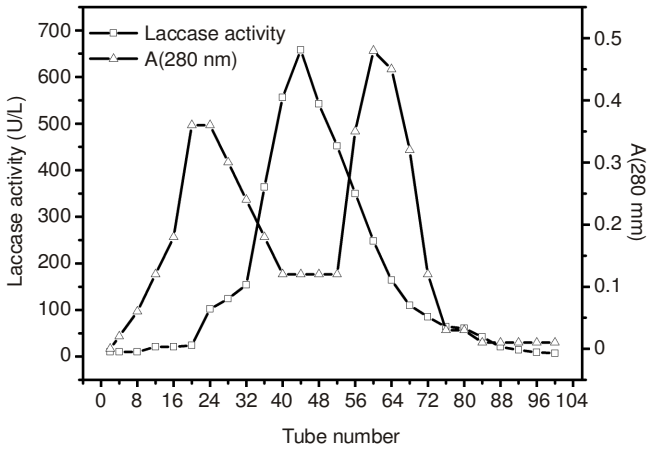

Fig. 2. DEAE-52 cellulose column chromatography outflow map of laccase

Sephadex G-100 molecular sieve column chromatography: The laccase samples pooled from DEAE-cellulose ion exchange chromatography were loaded on a Sephadex G-100 molecular sieve column and eluted with buffer. The chromatography results are shown in Fig. 3. A significant protein peak and a single activity peak were identified.

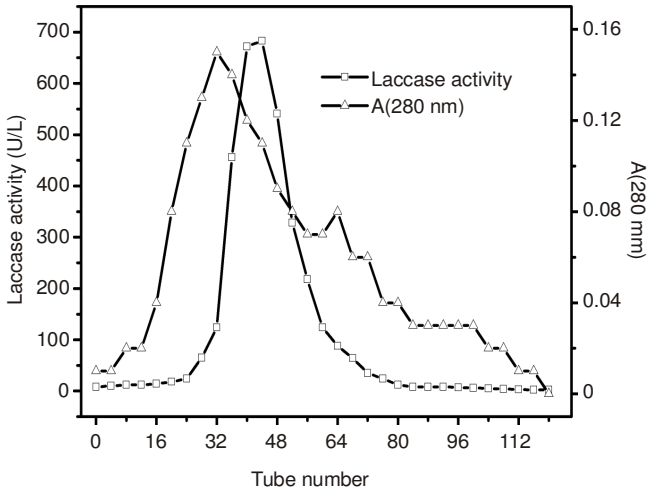

Fig. 3. Sephadex G-100 molecular sieve column chromatography outflow map of laccase

Purification results: The enzymatic activity and protein content of the fractions from the above steps were measured. The specific activity, recovery and purification factor of each of the steps were calculated and the results are shown in Table-1. Through 70 % ammonium sulfate precipitation, DEAE-cellulose ion exchange chromatography and Sephadex G-100 molecular sieve column chromatography, the specific activity increased from 10.28 U/mg in the crude enzyme solution to 245.27 U/mg. The purification fold was 23.86 and the recovery rate was 24.40 %.

TABLE-1
PURIFICATION RESULTS OF LACCASE

Purification procedure	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme solution	975.00	94.81	10.28	100.00	1.00
70 % ammonium sulfate precipitation	659.49	16.41	40.19	67.64	3.91
Dialysis	648.12	14.83	43.70	66.47	4.25
DEAE-cellulose	382.83	2.13	179.73	39.26	17.48
Sephadex G-100	237.90	0.97	245.27	24.40	23.86

Enzymatic properties

SDS-PAGE analysis: Purified laccase was visualized as a single protein band after undergoing appropriate concentration, SDA-PAGE and staining. This indicated that the laccase was electrophoretically pure. The molecular mass of the laccase was estimated to be 63 kDa by R_f-Log Mr mapping, according to the R_f of protein marker (Fig. 4).

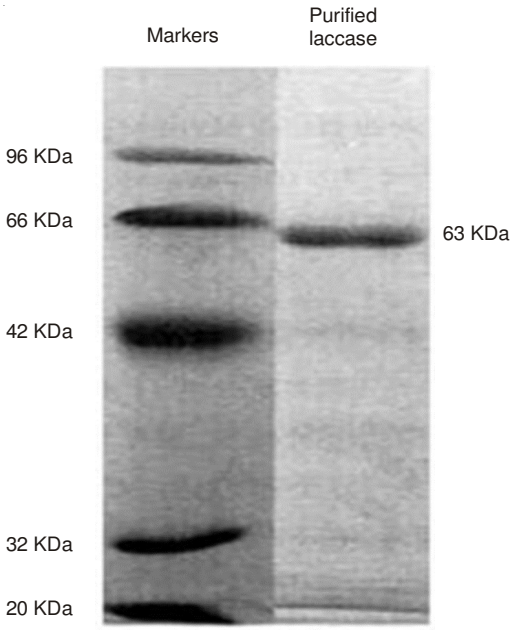

Fig. 4. SDS-PAGE of laccase after purification

Effect of temperature on enzymatic activity and stability of laccase: Optimal temperature and thermal stability are important properties of enzymes. At different temperatures, enzyme reaction rates vary greatly and tolerance of an enzyme to temperature is also related to its structure. With ABTS as the substrate, laccase activity was measured and the results are shown in Figs. 5 and 6.

In general, with the increase in reaction temperature, enzymatic activity will gradually increase. The curve trend in Fig. 5 reveals that, within the range of 20-50 °C, the reaction rate of laccase with ABTS as substrate gradually increased and reached the maximum at 50 °C. When the temperature was above 50 °C, the enzyme activity decreased rapidly and was almost undetectable when the temperature was above 75 °C. This indicated that high-temperature led to partial denaturation of laccase. Fig. 6 shows laccase activity after incubation at different temperatures over a 2 h span. After incubation at 30 °C for 2 h, approximately 80 % of the enzymatic activity remained. After incubation at 60 °C for 2 h,

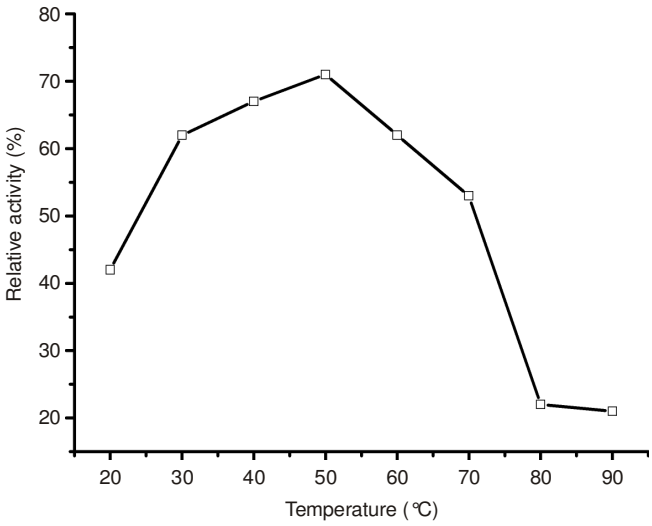

Fig. 5. Effects of temperature on laccase activity

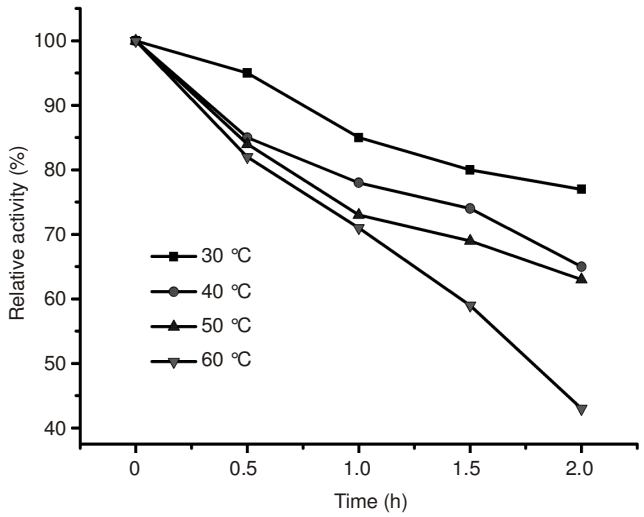

Fig. 6. Effects of temperature on laccase stability

only 40 % of the enzymatic activity remained. According to the results of the comprehensive analysis, when the temperature was above 50 °C, the relative activity decreased significantly, but the thermal stability was poor. Therefore, in this study, 30 °C was found to be the optimal reaction temperature of laccase. Therefore, in practical applications, the operating temperature should be determined by taking account of both the optimal reaction temperature and thermal stability requirement of laccase and thus can be selected within a range slightly lower than the optimal reaction temperature of laccase.

Effects of pH on laccase activity and stability: Enzymatic activity is closely related to environmental pH and each enzyme has a unique optimal pH range. Using ABTS as the substrate, at 30 °C and different pH levels, laccase activity

and stability were investigated. The results are shown in Figs. 7 and 8.

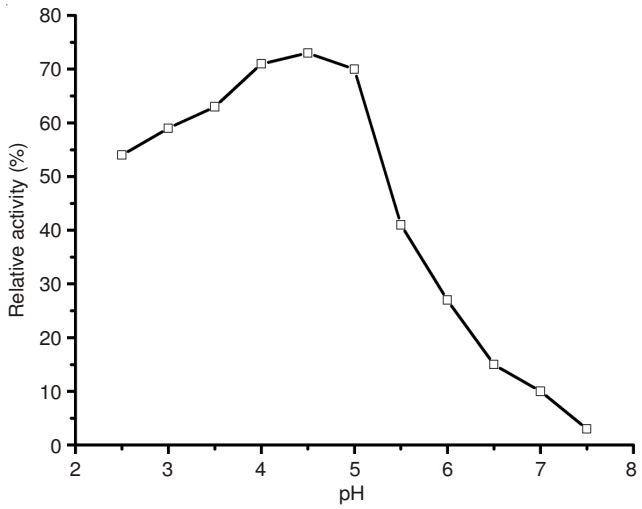

Fig. 7. Effects of pH on laccase activity

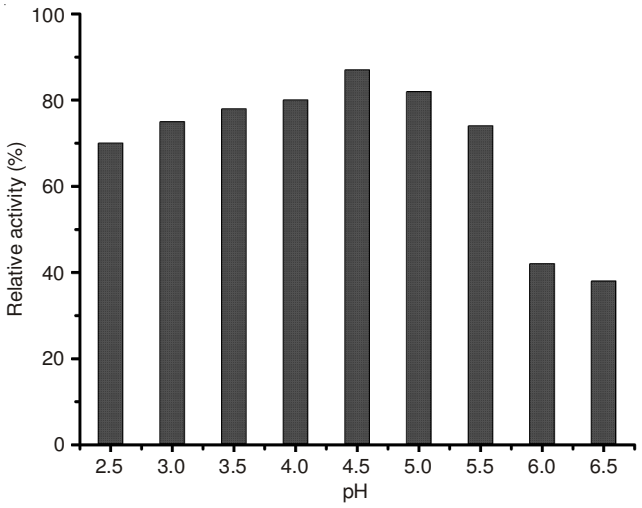

Fig. 8. Effects of pH on laccase stability

Fig. 7 revealed that laccase activity was significantly affected by pH. Within the pH range 2-4, the enzymatic activity increased slightly, indicating that the acidic environment inhibited part of the laccase enzymatic activity. Within the pH range 4-5, the enzymatic activity remained almost unchanged, indicating that this was the most appropriate pH range for laccase. When the pH was above 5, the enzymatic activity decreased rapidly and was almost undetectable in neutral and alkaline environments. According to Fig. 8, at a pH range of 4-5, laccase had good stability, but when the pH was greater than 5.5, the laccase relative activity was low. This indicated that an alkaline environment was not beneficial for preservation of laccase activity.

Determination of Michaelis-Menten constant value (K_m) of laccase: Laccase reaction rates were determined at 28 °C and a pH of 5 using varying concentrations of ABTS as the reaction substrate[4]. The K_m value was estimated to be 23.3 µmol/L using Lineweaver-Burk plotting with the reciprocals of the substrate concentration as the abscissa axis and the reciprocals of the reaction rate as the vertical axis (Fig. 9).

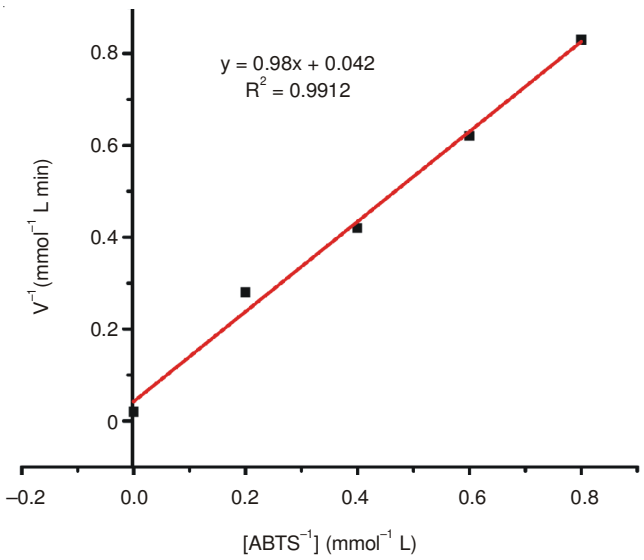

Fig. 9. Linewear-Burk plots of laccase from *C. subvermispora*

Conclusion

The highest yield of laccase from *C. subvermispora* fermentation was 3900 U/L. The specific activity of laccase increased to 245.27 U/mg after purification by combining 70 % ammonium sulfate precipitation, DEAE-cellulose ion exchange chromatography and Sephadex G-100 molecular sieve column chromatography.

The molecular mass of the homogeneous laccase was estimated to be 63 kDa by SDA-PAGE.

Analysis of its enzymatic properties revealed that it had typical characteristics of fungal laccase: a K_m value of 23.3 µmol/L; the optimal temperature and pH range for laccase activity was 50 °C and 4-5, respectively; and within a pH 4-5 range, the laccase activity was stable.

Compared to plant laccase and laccase produced from other wood-rotting fungi[17,18], the laccase from *C. subvermispora* demonstrated a good affinity to the substrate and thus has good application prospects in lignin degradation, organic dye decolorization and industrial wastewater treatment, *etc*.

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REFERENCES

1. L. Levin, L. Papinutti and F. Forchiassin, *Bioresour. Technol.*, **94**, 169 (2004).
2. H. Bermek, K. Li and K.L. Eriksson, *Bioresour. Technol.*, **85**, 249 (2002).
3. C.E. Wyman, B.E. Dale, R.T. Elander, M. Holtzapple, M.R. Ladisch and Y.Y. Lee, *Bioresour. Technol.*, **96**, 1959 (2005).

4. D.S. Arora and P.K. Gill, *Bioresour. Technol*., **77**, 89 (2001).
5. M. Fenice, G. Giovannozzisermanni, F. Federici and A. Dannibale, *J. Biotechnol*., **100**, 77 (2003).
6. P. Baborová, M. Möder, P. Baldrian, K. Cajthamlová and T. Cajthaml, *Res. Microbiol*., **157**, 248 (2006).
7. K. Fackler, C. Gradinger, B. Hinterstoisser, K. Messner and M. Schwanninger, *Enzyme Microb. Technol*., **39**, 1476 (2006).
8. K. Murugesan, I.-H. Nam, Y.-M. Kim and Y.-S. Chang, *Microb. Technol*., **40**, 1662 (2007).
9. E. Abadulla, T. Tzanov, S. Costa, K.-H. Robra, A. Cavaco-Paulo and G.M. Gubitz, *Appl. Environ. Microbiol*., **66**, 3357 (2000).
10. G. Songulashvili, V. Elisashvili, S.P. Wasser, E. Nevo and Y. Hadar, *Enzyme Microb. Technol*., **41**, 57 (2007).
11. K.M. Park and S.S. Park, *J. Microbiol. Biotechnol*., **18**, 670 (2008).
12. S. Vikineswary, N. Abdullah, M. Renuvathani, M. Sekaran, A. Pandey and E. Jones, *Bioresour. Technol*., **97**, 171 (2006).
13. D. Litthauer, M.J. van Vuuren, A. van Tonder and F.W. Wolfaardt, *Enzyme Microb. Technol*., **40**, 563 (2007).
14. S. Sadhasivam, S. Savitha, K. Swaminathan and F.-H. Lin, *Process Biochem*., **43**, 736 (2008).
15. A. Ferraz, A.M. Cordova and A. Machuca, *Enzyme Microb. Technol*., **32**, 59 (2003).
16. K. Yaghoubi, M. Pazouki and S.A. Shojaosadati, *Bioresour. Technol*., **99**, 4321 (2008).
17. S.R. Couto and J.L. Toca-Herrera, *Biotechnol. Adv*., **25**, 558 (2007).
18. V. Elisashvili and E. Kachlishvili, *J. Biotechnol*., **144**, 37 (2009).