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Eichhornia crassipes: Agro-waster for a novel thermostable laccase production by *Pycnoporus sanguineus* SYBC-L1

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The aim of this study was to explore the utilization of an intractable waster of *Eichhornia crassipes* in laccase production by *Pycnoporus sanguineus* SYBC-L1. *E. crassipes* as the sole carbon and nitrogen source was confirmed to produce laccase (7.26 U/g dry substrate). The fermentation medium for the maximum enzyme production was optimized and the laccase was then purified and characterized. The optimized culture medium was 25.1% *E. crassipes*, 13.9% sawdust, 1.5 mM $CuSO_4$, 40 μM gallic acid, 65% moisture content and initial pH 6.0. A maximum laccase activity of 32.02 U/g dry substrate was detected at 9th day, which was 4.5-fold compared to the initial medium. The molecular mass of the purified Lac-S was 58.4 kDa. The optimum activity of Lac-S for DMP was at pH 3.0 and 70°C. Lac-S showed not only high catalytic activities at low temperature, but also good stabilities toward pH and temperature. The residual catalytic activities of Lac-S were 30%, 40% and 50% at 0°C, 10°C and 20°C, and the half-lives at 50°C, 60°C and 70°C were 21.7, 9.7 and 1.5 h, respectively. The results provide a significant basis for *E. crassipes* further utilization and Lac-S specific application in harsh industry.

[**Key words:** *Eichhornia crassipe*; Laccase; Solid-state fermentation; Thermostability; *Pycnoporus sanguineus*]

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multicopper oxidase that is found in plants, insects, bacteria and fungi, but the major producer is white-rot fungi, such as *Trametes pubescens* (1), *Pleurotus ostreatus* (2), *Pycnoporus sanguineus* (3). Among them, the genus *Pycnoporus* is one of the most promising laccase producer (4,5). Laccase catalyzes a broad range of organic and inorganic substrates, including mono-phenols, di-phenols, poly-phenols, amino-phenols, methoxy-phenols, aromatic amines. Since its low substrate specificity and high catalytic efficiency, laccase is exploited for a variety of industrial and environmental applications, including bio-pulping, bio-bleaching, bio-detoxication, bioremediation, lignin modification effluent treatment (6,7). To achieve the laccase bio-catalytic functions in industrial applications, the laccase fermentation with high production and low cost is essential and critical. In recent years, in order to reduce the costs, there is a significant trend to use of agro-industrial residues to produce high value-added products (e.g., enzymes, saccharides, bio-fuels). For laccase production, the most suitable fermentation materials are lignocellulosic wastes. These wastes contain cellulose, hemicellulose and lignin (8), and which can been metabolized by white-rot fungi because of their abilities on the lignocellulose degradation (9,10). The degradation is mainly caused by the extracellular enzyme system secreted by the microorganisms, and for lignin degradation, the ligninase is composed of at least three enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. In particular, the most widely studied enzyme is laccase because of its special catalytic property, which can oxidize the substrates in the absence of H_2O_2 (11). Furthermore, the lignin, cellulose and hemicellulose used in laccase production act not only as the substrate, but also as the inducer. For this reason, many agro-industrial lignocellulosic materials such as wheat straw, olive leaves, coconut flesh had been used in laccase production (2,3,12). In this study, *Eichhornia crassipes* has been selected to produce laccase.

E. crassipes, commonly known as water hyacinth, is an abundant lignocellulosic resource in the world. It is a perennial free-floating aquatic plant, which is native to the Amazon Basin of South America. In the past two centuries, it was introduced into many parts of the world as an ornamental garden pond plant, since then it had led to an overall worldwide distribution and production (13). In China, *E. crassipes* was first introduced from South America as a good fodder plant in 1901, thereafter, they grew rapidly and increased annually, however, only a few was utilized, and most was discharged as wastes, which had caused a series of environmental problems. Nowadays, it is becoming more and more difficult to eradicate, and the pollution control costs are becoming higher and higher (14), so it is meaningful for *E. crassipes* to find some other new applications.

In laccase production, there are many important factors, such as the suitable substrates, culture conditions and fermentation

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patterns. Most researches on laccase production have been performed in liquid culture pattern, however, this fermentation technique cannot reflect the natural living conditions, especially for ligninolytic fungi. The solid-state fermentation (SSF) is defined as the fermentation process carrying out on a solid substrate with the insert substrate or the natural substrate as the solid support (15). The fungi grew under SSF condition can been close to their natural habits. Therefore, when dealing with filamentous fungi, SSF offers advantages over liquid cultivation. SSF has emerged as a potential technology for the production of microbial products by fungi.

In the previous study, the laccase production had been studied in submerged fermentation by *P. sanguineus* SYBC-L1 (16). This paper was explored the laccase production in SSF. The aim of this present study was to investigate the potential capability of *E. crassipes* as the raw material on laccase production by *P. sanguineus* SYBC-L1. In addition, this study also focused on screening low-cost raw materials for using as the solid substrates without adding any other costly chemicals. Further, the culture parameters for laccase production were optimized and the laccase was purified and characterized.

MATERIALS AND METHODS

Microorganism The strain used in this study was *P. sanguineus* SYBC-L1 (16). The fungus was incubated on a potato dextrose agar (PDA) plate at 30°C for 5 days. The spores were collected from the surface of culture plate and then inoculated into the PDA liquid medium. The medium was then incubated at 30°C under 200 rpm for 24 h.

***Eichhornia crassipes* preparation and laccase production** *E. crassipes* was collected from Taihu basin in Jiangsu Province. All the parts of the plant were dried and broken into filaments, and then they were used as the substrate for laccase production in SSF.

The solid medium consisted of 10 g *E. crassipes* and 30 ml distilled water was made for determining whether laccase could product by using *E. crassipes* as the sole substrate.

SSF cultivation was carried out under solid-state conditions in 250 ml Erlenmeyer flasks. Spore culture fluids (4 ml) were spread aseptically on the surface of the substrate in each flask and the cultures were incubated at 30°C for 9 days.

Screening of solid support-substrate In order to obtain the support-substrate for laccase production, eight agro-industrial wasters of steel wool, nylon sponge, sawdust, bagasse, banana skin, rice straw, wheat straw and sorghum stalk were studied. Prior to use, steel wool and nylon sponge were pre-treated by boiling for 10 min and washing thoroughly with distilled water, while the others were dried and cut into 2 cm pieces.

The medium for support-substrate screening was consisted of 10 g *E. crassipes*, 5 g support-substrate and 30 ml distilled water. The screening medium was prepared in 250 ml flasks and then autoclaved at 121°C for 20 min. The fermentation was performed for 9 days at 30°C. The control experiments were carried out without support-substrate supplementation.

Optimization the concentration of medium composition The optimal contents of *E. crassipes* and sawdust on the solid culture medium for laccase production by *P. sanguineus* SYBC-L1 were studied. The tests were performed on the basal solid culture medium in 250 ml flasks, in which the contents of *E. crassipes* and sawdust were 10 g and 5 g respectively. The effects of different concentrations of the solid sources were evaluated respectively by replacement of the corresponding composition with the following concentrations: *E. crassipes* (10.0%, 15.0%, 20.0%, 25.0%, 30.0% and 35.0%, *w/w*) and the support-substrate of sawdust (5.0%, 7.5%, 10.0%, 12.5%, 15.0% and 17.5%, *w/w*).

The effects of inducers for laccase production were also investigated in this paper. Copper sulfate ($CuSO_4$) and gallic acid were used in this test. $CuSO_4$ (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mM) and gallic acid (0.0, 10.0, 20.0, 40.0, 60.0 and 80.0 μM) were varied to study their influences on laccase production. The SSF was carried out at 30°C in 250 ml flasks with a solid medium containing 10 g *E. crassipes*, 5 g sawdust and 30 ml sterile water.

Effect of incubation conditions The SSF incubation conditions for laccase production were investigated on the solid medium as follows: the initial moisture content (55%, 60%, 65%, 70%, 75% and 80%, *w/w*), incubation temperature (25°C, 28°C, 30°C, 32°C, 35°C and 37°C) and initial pH (2.0–8.0).

The cultivation medium of *E. crassipes* and sawdust was prepared at their optimal contents and the SSF was incubated at 30°C for 9 days. All the experiments were performed respectively at least triplicates.

Optimization of laccase production by response surface methodology The optimization of laccase production was performed by using statistical design in two steps. Plackett-Burman design (PBD) was employed to select the most significant parameters for laccase production by *P. sanguineus* SYBC-L1. The effects of six independent quantitative variables, including initial pH (X_1), gallic acid (X_2), copper sulfate (X_3), sawdust (X_4), initial moisture content (X_5) and *E. crassipes* (X_6), were studied. Each variable was tested at two levels (+1 and −1) and center point (0), respectively. The detail of the design is shown in Table S1. The central composite rotatable design (CCRD) with two variables was used further for enhancing the laccase production by *P. sanguineus* SYBC-L1. The low, middle and high levels of the two variables were denoted as −1.41421, −1, 0, +1 and +1.41421, respectively. The range and the level of each factor are given in Table S2. According to this design, the relationships of variables were determined by fitting a second-order polynomial equation to data obtained from the 12 runs.

$$Y = a_0 + a_1X_4 + a_2X_6 + a_{12}X_4X_6 + a_{11}X_4^2 + a_{22}X_6^2 \quad (1)$$

where Y was the predicted laccase production, X_4 was sawdust, X_6 was *E. crassipes*, α_0 was the constant coefficient, α_1 and α_2 were the linear coefficients, α_{12} was the interaction coefficient, α_{11} and α_{22} were the quadratic coefficients.

Design-Expert software (version 8.0, Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental designs and regression analysis. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). All the experiments were carried out in triplicates.

Laccase extraction For laccase activity assay, 10 g of the fermented matter was firstly macerated with 90 ml of citrate/phosphate buffer (20 mM, pH 6.0) for 2 h at room temperature, the crude laccase was then extracted by centrifugation at 8000 ×*g* for 10 min and the supernatant was collected and used for enzyme assay.

Laccase assay Laccase activity was determined by oxidation of 2,6-dimethoxyphenol (DMP, $\varepsilon_{470\ nm} = 49{,}600\ M^{-1}\ cm^{-1}$) (17). The reaction mixture contained 100 μl laccase fluid, 0.5 ml DMP (10 mM) and 1.9 ml citrate/phosphate buffer (100 mM, pH 3.0). Enzyme assay was carried out by monitoring the increase in absorbance of the reaction product at 470 nm. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μM of DMP per minute.

Protein estimation Protein concentration was calculated based on the previously described protocol (18) using bovine serum albumin as the standard.

Laccase purification The laccase was purified by three steps including salt-out with $(NH_4)_2SO_4$, ion-exchange chromatography with DEAE-cellulose 52 and gel filtration chromatography with Sephadex G-100.

The crude laccase was obtained by soaking the fermented matter with citrate/phosphate buffer. The soaking supernatant was then salt fractionated with $(NH_4)_2SO_4$ of 80% saturation overnight. The protein precipitate was dissolved in a small volume of citrate/phosphate buffer (20 mM, pH 6.0) and then dialyzed against the same buffer until without $(NH_4)_2SO_4$ detection. The dialyzed protein solution was applied to the DEAE-cellulose 52 column (1.6 × 30 cm) pre-equilibrated with citrate/phosphate buffer (20 mM, pH 6.0). The column was eluted with a step gradient of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl at a flow rate of 1 ml per minute. The active fractions were pooled, dialyzed, concentrated and loaded onto the Sephadex G-100 column (1.6 × 60 cm). The column was then washed with the same citrate/phosphate buffer. The laccase-active rich fractions were pooled, concentrated and stored at −20°C.

Gel electrophoresis of the purified Lac-S The molecular mass of purified laccase (Lac-S) was detected with sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). The assay was carried out according to the protocol described by Laemmli (19) with 5% (*w/v*) stacking gel and 12% (*w/v*) separating gel. The gel was stained with Coomassie brilliant blue R-250 for 1 h at room temperature. The molecular mass of Lac-S was determined by comparing with the standard protein markers (TaKaRa biotechnology Co., Ltd., Dalian, China).

Effect of pH on the purified Lac-S The optimal pH value of Lac-S was measured by examining the enzyme activities at a pH range of 2.0–8.0 using DMP as the substrate. To determine the stabilities at different pH values, Lac-S was equilibrated respectively with 100 mM citrate/phosphate buffer (pH 4.0 and 6.0) or 100 mM Tris–HCl buffer (pH 8.0 and 10.0) for different time (0–70 h) at 30°C.

Effect of temperature on the purified Lac-S In the optimum temperature assay, Lac-S was determined at a temperature range from 0°C to 100°C with DMP as substrate. The thermostability of Lac-S was studied by incubating in citrate/phosphate buffer (100 mM, pH 6.0) at a temperature range of 40–70°C for different time (0–5 h). The residual laccase activity was measured with time under standard assay condition. The enzymatic inactivation constant (k) of Lac-S at different temperature was determined by the following equation $dR/dt = kR$, where R is residual activity (%) at t (h). The half-life ($t_{1/2}$) at different temperature was calculated as $t_{1/2} = \ln 2/k$.

RESULTS AND DISCUSSION

***Eichhornia crassipes* for laccase production in SSF** In the products fermentation, the most important factor to take into account is medium components, especially, the availability and cost of

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