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Research Article

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Enhanced production of an acid-tolerant laccase by cultivation of *Armillariella tabescens*

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ABSTRACT

The production of laccase from Armillariella tabescens by submerged fermentation was studied. Various agro industrial residues were tested to determine the most suitable solid substrate for laccase production. Wheat bran led to the highest levels of activity (6.1 U/mL) and was selected as substrate in this study. Peptone was the best nitrogen source for laccase production by this fungus. Ferulic acid, guaiacol as well as copper sulphate proved to be the effective inducers for laccase production, resulting in increases of activities higher than 50% as compared to control value. In addition, the effects of pH and temperature on enzyme activity were investigated. The optimum temperature and pH for the crude laccase were 45 °C and 1.5, respectively, when in citrate buffer, with 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. Interestingly, the crude laccase is highly stable under acidic conditions, with pH values ranging from 1.5 to 5.0.

Key words: Laccase production, *Armillariella tabescens*, Submerged fermentation, Acid stability

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INTRODUCTION

The eco-friendly laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) that are copper containing eznymes can catalyze the oxidation of a wide range of aromatic compounds. The oxidation reaction occurs via the one electron oxidation mechanism coupled with the reduction of molecular oxygen to water [1]. Because of their relatively broad substrate specificity and versatility, it has been demonstrated that laccases possess potential in dustrial and environment applications including decolorization of synthetic dyes, pulp bleaching in the paper industry, detoxification of industrial effluents, bioremediation of soil, as catalysts for organic synthesis, as tools for medical diagnostics, and even as ingredients in cosmetics [2]. Moreover, a laccase from *Lepiota ventriosospora* was latterly found to display inhibitory activity against HIV-1 reverse transcriptase [3]. Laccases are produced by higher plants, insects, bacteria and fungi [4]. Most of the known laccases are from fungi[5, 6, 7]. *Armillariella tabescens* belonging to white-rot wood decay fungi is a non-toxic, edible fungus with curative properties in the treatment of cholecystitis, hepatitis, appendicitis and otitis media, and has received much attention from researchers during the last decade [8,9].

In previous study, polysaccharides from *A. tabescens* have been found to possess dispelling effects of alcohol in mice [10], and it was discovered that this fungus is capable of producing extracellular laccase by fermentation. Cultivation of *A. tabescens* can not only harvest the mycelium which could use as material of Chinese fork medicine but also produce laccase. Herein we describe the determination of suitable operating conditions to enhance production of laccase from *A. tabescens* by submerged fermentation. In addition, the effect of temperature and pH on laccase activity and the stability of the crude enzyme were investigated.

EXPERIMENTAL SECTION

Microorganism and inoculum

The strain *A. tabescens* GIM5.4 was supplied by the Guangdong culture collection center (Guangzhou, China). It was maintained at 4ºC on potato dextrose agar (PDA) slants and subcultured every 2 months. The culture medium for inoculum contained per liter: 20 g glucose, 15 g peptone, 5 g $(NH_4)_2SO_4$, 5 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, and 20 g cornmeal, at pH 6.0. Erlenmeyer flasks (500 mL) with sterile culture medium (100 mL) were inoculated with one agar plugs (diameter, 10 mm) and incubated for 6 days at 160 r/min and 25 °C. Glass beads (15 g) were added and the flasks were shaken at 200 r/min for 30 min. The resulting suspension was used as inoculum.

Medium and cultural conditions for submerged fermentation

Various agro industrial residues such as soybean meal, wheat bran, wheat straw, rice straw, rice bran and corn bran were obtained from the local market. The particle size was 0.25-0.85 mm. Generally, the basal culture medium contained per liter: glucose 5 g; peptone 10 g; $(NH_4)_2SO_4$ 1 g; KH_2PO_4 5 g; $MgSO_4 \cdot 7H_2O$ 1 g; $CuSO_4$ 0.2 g; $CaCl_2 \cdot 2H_2O$ 0.2 g; vitamin B_1 0.1 g; pH 6.0. Basal culture medium (50 mL) and agro industrial residue (0.5 g) were added into Erlenmeyer flasks (250 mL) and autoclaved at 121 °C for 30 min. Then inoculum (5 mL) was added. The Erlenmeyer flasks were incubated for 6 days at 150 r/min and 25ºC.

Enzyme assay and biomass measurement

The culture supernatants were centrifuged (4 °C, 8000 × *g*, 10 min). The laccase activity was determined spectrophotometrically with ABTS as the substrate and oxidation monitored at 420 nm[11]. The laccase reaction mixture contained 1 mL of enzyme, appropriately diluted with citrate buffer (0.1 M, pH 4.5), and 0.5 mM ABTS (3 mL). The reaction was monitored by measuring the increase in absorption at 420 nm for 5 min, at 45 °C. One laccase activity unit was defined as the amount of enzyme that oxidized 1 mmol of ABTS per minute. Activities are expressed in U per milliliter of crude enzyme extract (U/mL). Biomass was estimated by determining the dry weight of the mycelium after drying to constant weight at 80 °C.

Effect of temperature and pH on laccase activity and stability

The optimum temperature of the crude enzyme was studied over a range of 20–60 °C. To determine the thermal stability, the enzyme was kept at different temperatures (4–60 °C) for 1 h in citrate buffer (0.1 M, pH 4.5) and the residual laccase activity was measured as described above. To investigate the effect of pH, the activity was tested at pH values ranging from 1.0 to 6.0. The pH stability of the enzyme was assessed by pre-incubation at 4 °C for 1 h.

RESULTS AND DISCUSSION

Screening of substrate for laccase production

Several agro industrial residues were screened for laccase production by cultivation of the fungus *A. tabescens*. As shown in Fig.1, wheat bran was the most suitable substrate for laccase production. Corn bran and soybean meal were also considered as promising substrates while low laccase activities were observed when wheat straw and rice bran were used as substrates. This suggests that the nutrient components in wheat bran are more propitious for enzyme production of *A. tabescens*. Thus, wheat bran was selected as substrate in this study.

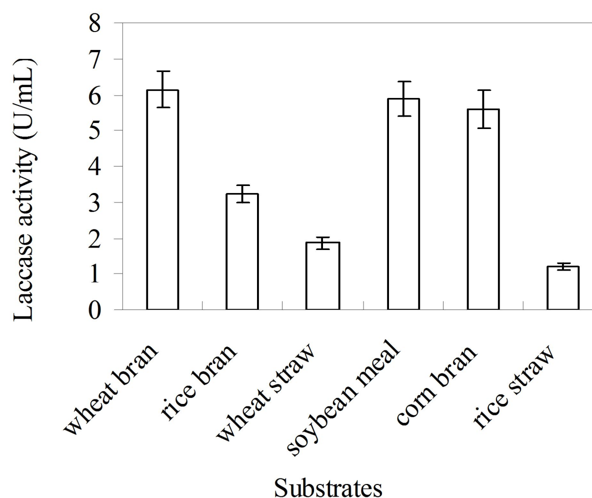

Fig.1 Laccase production by cultivation of *A. tabescens* using different substrates

Experiments were performed in triplicate. Each value represents the mean ± standard deviation.

Effect nitrogen source on laccase production

Nitrogen source is an important factor influencing the efficiency of fungi to produce laccase. Many researches have reported the effect of different carbon and nitrogen sources on laccase production by various fungi [12, 13, 14]. Therefore, effects of different nitrogen sources on laccase production were investigated. Fig.2 shows that there was a positive correlation between laccase production and biomass. The results indicate that the organic nitrogen sources such as peptone, yeast extract, tryptone were more suitable than the inorganic source. Similar results were obtained in production of laccase by *Streptomyces psammoticus* [15]. Among the organic sources tested peptone was the best for laccase production and growth of *A. tabescens.* Peptone was included as the nitrogen source in the basal media that was used for all the experiments. Replacement of peptone with other nitrogen sources led to reduce laccase yield, which indicated that peptone is the suitable nitrogen source for laccase production by *A. tabescens.*

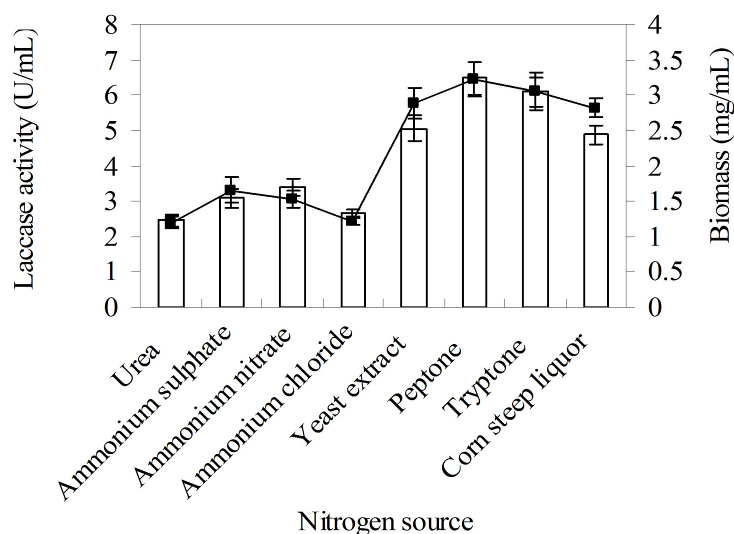

Fig.2 Effect of nitrogen source on laccase production and biomass

Amount of nitrogen source added in the culture medium was according to the concentration of nitrogen (1.5 g/L). Experiments were performed in triplicate. Each value represents the mean ± standard deviation. Symbols: laccase activity (□), biomass (■).

Effect of inducers on laccase production

Aromatic and phenolic compounds have been widely employed to enhance laccase production by different organisms and the nature of the compound that induces laccase activity differs greatly with the species [16, 17]. Several aromatic inducers and copper sulphate were used in study (Fig.3). The results show that among aromatic compounds ferulic acid (6.85 U /mL) and guaiacol (6.77 U/mL) evidently enhanced the level of laccase production by *A. tabescens*. However, inducing effect was not observed when the other aromatic compounds were used, even addition of phenol in the culture medium caused decline in laccase activity and biomass. Besides aromatic inducers, copper sulphate was also proven to be an effective inducer. The result confirmed copper ions being capable of inducing laccase synthesis [18].

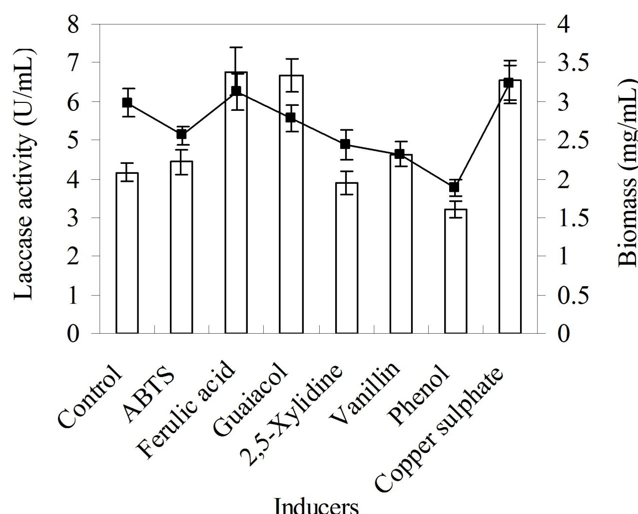

Fig.3 Effect of inducers on laccase productionand biomass

All the inducers were added at 1mM concentration after 24 h of cultivation. Experiments were performed in triplicate. Each value represents the mean ± standard deviation. Symbols: laccase activity (□), biomass (■).

Effect of temperature on laccase activity and stability

The effect of temperature on laccase activity was investigated at temperatures between 20 and 60 °C. The optimum temperature for the crude laccase was 45 °C (Fig.4). To examine thermal stability, the enzyme was incubated for 1 h at different temperatures. At 45 °C, the laccase retained approximately 50% of its initial activity. However, it was almost completely inactive at temperatures above 60 °C.

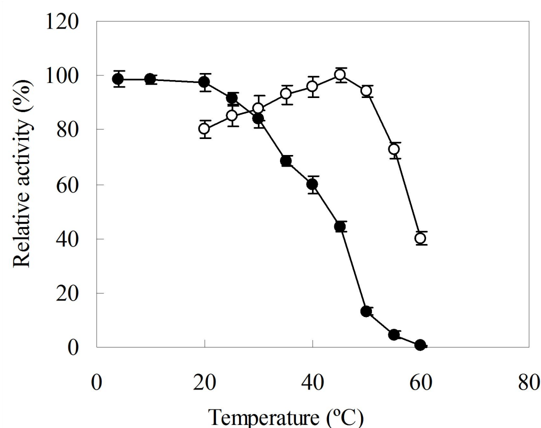

Fig. 4: Effects of temperature on the activity and stability of the crude laccase

Laccase activity was measured in the presence of ABTS dissolved in 0.1 M citrate buffer at pH 4.5. The laccase was kept at different temperatures (4–60 °C) for 1 h to determine the stability. Initial activity at 45 °C was assumed to be 100%. Experiments were performed in triplicate. Each value represents the mean ± standard deviation. Symbols: laccase activity (○), stability (●).

Effect of pH on laccase activity and stability

The effect of pH on laccase activity and stability was tested over a pH range of 1−6 using HCl-KCl buffer for pH 1−1.5 and citrate buffer for pH 1.5−6.0. The crude laccase was active within a narrow pH range of 1.5−2.5 (Fig.5). The optimal pH value was determined to be 1.5 in the citrate buffer, only 75 % relative activity was observed at pH 1.5 in the HCl-KCl buffer. This result indicates that the type of buffer may influence laccase activity. When the pH was increased to 4.0, the relative activity decreased to less than 50%. Almost no activity was observed when the pH was above 6.0. Overall, the pH stability profile indicated that the enzyme was unstable at pH values above 6, but reasonably stable over pH values from 1.5 to 5, maintaining 80% of its original activity after incubation for 1h (Fig. 5). Most laccases from fungi such as *Coltricia perennis* [19], *Paraconiothyrium variabile* [6] and *Scytalidium thermophilum* [20] have an optimal pH range of 3−6 and have good stability in near neutral conditions. Laccases from *Trametes pubescens* and *Podospora anserine* were found to have good stability under alkaline conditions [21,

22]. Hence, the stability of the laccase from *A.tabescens* in acidic conditions is rare among fungal laccases.

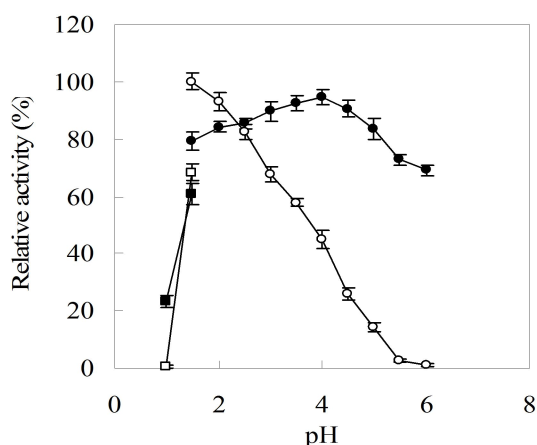

Fig. 5: Effects of pH on the activity and stability of the crude laccase

The laccase activity (○, □) was determined in HCl-KCl aqueous solution (□, ■) at pH 1–1.5 and citrate buffer (○, ●) at pH 1.5–6.0. Initial activity at pH 1.5 in citrate buffer was assumed to be 100%. To determine the pH stability (●, ■), the laccase was pre-incubated at different values of pH for 1 h at 4 °C. Laccase activity at the start of the incubation period was assumed to represent 100% stability. Experiments were performed in triplicate. Each value represents the mean ± standard deviation.

CONCLUSION

Laccase production from a medicinal fungus *A. tabescens* by submerged fermentation has been demonstrated. The most suitable substrate and nitrogen source were wheat bran and peptone, respectively. In order to further enhance the production of laccase, various inducers including aromatic compounds and copper sulphate were tested. Ferulic acid, guaiacol and copper sulphate proved to be effective inducers, leading to increases in laccase activities higher than 50%, as compared to control level. The effect of temperature and pH on the laccase activity was investigated. The optimum temperature and pH were found to be 45 °C and 1.5, respectively in a citrate buffer system. Interestingly, the laccase exhibited acid-tolerant activity and this unusual property may have potential industrial applications.

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