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ORIGINAL PAPER

Production of laccase by a newly isolated deuteromycete fungus *Pestalotiopsis* sp. and its decolorization of azo dye

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Abstract The effect of various carbon and nitrogen sources on the production of laccase by newly isolated deuteromycete *Pestalotiopsis* sp. was tested under liquid-state fermentation. Twenty grams per liter of glucose and 10 g l^{-1} ammonium tartrate were found to be the optimized concentrations of carbon and nitrogen sources, respectively. The influence of different inducers and inhibitors on the laccase production was also examined. Adding the Cu up to optimum concentration of 2.0 mM in medium (include 20 g l^{-1} glucose and 10 g l^{-1} ammonium tartrate), the highest laccase activity of 32.7 ± 1.7 U ml^{-1} was achieved. Cu had to be supplemented after 2 days of growth for its maximal effect, an addition after 6 days of growth, during which laccase activity was dominantly formed, resulted in distinctly reduced laccase activity. In addition, Direct Fast Blue B2RL can be effectively decolorized by crude laccase, the decolorization percentage of which was 88.0 ± 3.2% at pH 4.0 within 12 h. The results suggest that *Pestalotiopsis* sp. is a high potential producer of the industrially important enzyme laccase.

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Introduction

Laccase (benzenediol:oxygen oxidoreductases; EC 1.10. 3.2) is multi-copper oxidases widely distributed among plants, insects, and fungi [17, 33]. It has been found in different genera of ascomycetes [12], some deuteromycetes [18, 29], and most basidiomycetes [17, 33]. Recently, laccase has also been reported to be widespread in bacteria [2]. Fungal laccases are believed to be involved in the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation [33].

Laccase catalyses the oxidation of both phenolic and non-phenolic compounds [8] and is able to mineralize a wide range of synthetic dyes [1, 25]. It makes this biocatalyst suitable for application in several bioprocesses, including biopulping, biobleaching, bioremediation, biosensor, various food technological uses, and in the treatment of industrial wastewater [34]. The later is a field of increasing research due to a recent trend toward stricter government legislation concerning the release of contaminated effluents. Therefore, rather large amounts of crude and purified laccases are required [17]. However, in several organisms, extracellular laccases are constitutively produced in small amounts [7]. Their production can be considerably enhanced by a wide variety of substances, including aromatic or phenolic compounds such as xylidine or guaiacol [7, 14, 23], or aqueous plant extracts [4]. Recently, an important effect of copper on laccase synthesis in *Trametes versicolor* and *Pleurotus ostreatus*

was reported [11, 27]. Laccase production can also be influenced by different carbon and nitrogen sources [20, 32].

In our previous work, we isolated a deuteromycete, *Pestalotiopsis* sp., from the organic layers of the forest floor in Zijin Mountain (32°5′N, 118°48′E), Nanjing, China in November 2004. And this fungus had demonstrated a strong laccase activity when it incubated in solid substrate of *Quercus variebilise* litter without any other inducers [19]. This is a continual study. The aim of this work is to examine the effect of various carbon and nitrogen sources, and different putative inducers and inhibitors on the production of laccase of this fungus under liquid conditions. Moreover, the in vitro decolorization ability of the crude enzyme obtained in the cultures was to be investigated.

Materials and methods

Effect of carbon and nitrogen sources on laccase production

The deuteromycete, *Pestalotiopsis* sp. was maintained, through periodic transfer, at 4°C on potato-dextrose agar (PDA) plates. Shaken-flask cultures of the organism were grown at 28 ± 2°C in a darkened atmosphere with continuous agitation (150 rpm) in Erlenmeyer flasks. Each flask (250 ml) was plugged with a cellulose stopper and contained 50 ml of basal mineral medium. The basal mineral medium used for experimental cultures included KH_2PO_3 3.0 g l^{-1}, $MgSO_4$ 1.5 g l^{-1}, VB_1 0.01 g l^{-1}, mineral solution 1 ml l^{-1}, and unless otherwise stated, 10 μmol l^{-1} $CuSO_4{\cdot}5H_2O$. Individual carbon and nitrogen source were also added to the basal mineral medium at the concentrations stated below. The mineral solution used included 1.5 g $CaCl_2{\cdot}2H_2O$, 0.3 g $ZnSO_4{\cdot}7H_2O$, 0.25 g $FeSO_4{\cdot}7H_2O$, 0.2 g $MnCl_2{\cdot}5H_2O$, 0.05 g $CoCl_2{\cdot}6H_2O$, 0.01 g H_3BO_3, 0.01 g $NaMoO_4{\cdot}2H_2O$ in a liter. The pH was adjusted to 5.6 with 0.25 M H_3PO_4, and the medium was then autoclaved at 121°C for 20 min. Each flasks was then incubated with three fungal discs (3-mm diameter), which had been incubated 7–9 days earlier on PDA plates at 27°C. Inoculated flasks were cultivated in a time course of 12 days.

Maltose, glucose, cellobiose, lactose, glycerol, and (α-cellulose were tested as carbon sources to indicate their effects on laccase production. They were added individually to the basal mineral medium (containing 10 g l^{-1} NH_4NO_3 as nitrogen source) at 10 g l^{-1}.

The effect of different glucose concentrations on the laccase activity was also examined. Gradient carbon-source concentrations, 5, 10, 20, 40 g l^{-1} of glucose, were tested in the basal mineral medium (also containing 10 g l^{-1} NH_4NO_3 as nitrogen source) to optimize the laccase production.

Beef extract, ammonium tartrate, yeast, ammonium nitrate, ammonium oxalate, ammonium chloride, ammonium sulfate, meat peptone, acrylamide, and urea were tested as nitrogen sources to examine their effects on laccase production. They were added individually to the basal mineral medium (containing 20 g l^{-1} glucose as carbon source) at a N-source concentration of 10 g l^{-1}.

Effect of laccase inducers and inhibitors

The laccase formation of the fungus was followed over 12 days under the incubation in a medium (including 10 g l^{-1} ammonium tartrate, 20 g l^{-1} glucose) with various putative laccase inducers or inhibitors. The culture condition was the same as described above. The putative inducers and inhibitors were well-described aromatic compounds (guaiacol and 2,5-xylidine), and several other reagents ($AgNO_3$, $CdSO_4$, CuSO4, $HgCl_2$, $MnSO_4$, $ZnSO_4$, NaN_3, KCN). Some of them are known to impose a state of oxidative stress on the mycelia. 2,5-Xylidine was dissolved in 50% ethanol as stock solution, and sterilized by filtration. The final concentration of ethanol in the growth medium was always less than 0.5%, and an equivalent amount of ethanol was added to control flasks (final, 0.35%) without extra inducers. Stock solutions of $AgNO_3$, $CdSO_4$, $CuSO_4{\cdot}5H_2O$, $HgCl_2$, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, guaiacol, NaN_3, and KCN were prepared in water. Copper ion was added to actively growing fungal cultures after 2 days of cultivation at a final concentration of 2 mM. The other inducers and inhibitors were also added after 2 days of incubation, but at a final concentration of 1 mM.

Gradient concentrations of copper (final 0.1, 0.5, 1.0, 1.5, 2.0 mM) were added to the basal mineral medium (included 10 g l^{-1} glucose and 10 g l^{-1} ammonium tartrate) at different time of the shaken-flask cultivations (day 0, day 2, day 4, day 6), respectively.

Analytical determination

Samples were harvested periodically, filtered through pre-weighed Whatman filter paper No. 1. The filtrate was then centrifuged at 10,000×*g* at 4°C, and the clear supernatant was collected for determining enzyme activity and carbon remaining. The residual fungal biomass collected on the tared filter paper was washed with distilled water and dried at 80°C to a constant

weight. Experiments were carried out in duplicate and samples were analyzed in triplicate. Data were expressed as mean ± standard deviation, and the difference shown in figures was performed using with one-way ANOVA.

Laccase activity was determined spectrophotometrically using the method of Niku-Paavola et al. [24], with ABTS as the substrate. The laccase reaction mixture (in a total volume of 3 ml) contained 0.5 ml crude enzyme and 1.8 ml succinic acid buffer (41.67 mM, pH 3.0) and 0.7 ml 20 mM ABTS. The reaction was monitored at room temperature (25°C) by measuring the change in A_{436} ($\varepsilon = 29.3\ mM^{-1}\ cm^{-1}$) for 2 min. One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute. Enzyme activity was expressed in U ml^{-1}.

Glucose concentration in the fermentation samples was determined enzymatically using a commercially available assay (Infinity Glucose Reagent, Sigma). Cellobiose and lactose concentrations were measured by employing purified cellobiose dehydrogenase using the method described by Baminger et al. [6]. Glycerol and maltose levels were determined using commercially available test kits (Boehringer Mannheim, Germany).

Azo dye (Direct Fast Blue B2RL) decolorization

Culture broth was collected at the maximum laccase activity, filtered, and centrifuged as above. The reaction mixture (final volume 10 ml) consisted of an aqueous solution of dye (final, 50 mg l^{-1}) and crude enzyme (final, 30 U ml^{-1}) in 20 mM sodium acetate buffer at pHs 3.0–5.0. The gradient pHs were prepared according to the book of "Experimental methods and technology of biochemistry" [35]. Since the dyes are photodegradable, the reaction was carried out in test tubes at room temperature in static dark condition.

The absorbance values (A) of the reaction mixtures were measured spectrophotometrically at the maximum wavelength of 550 nm. The residual dye concentrations were calculated by the equation $C_x = A_x C_0 / A_0 \times 100\%$ (C_0 the initial dye concentration; C_x the residual dye concentration; A_0 the absorbance value of the initial reaction mixture; A_x the absorbance value of the residual reaction mixture), and the dye decolorization was expressed as a percentage. A parallel control test containing the same amount of a heat-denatured laccase (heated in microwave for 15 min) was also performed.

In an attempt to investigate the role of laccase in dye decolorization by *Pestalotiopsis* sp., sodium azide (final, 0.01 mM) was added to each shaken-flask of 50 ml basal mineral medium (containing 10 g l^{-1} glucose and 10 g l^{-1} ammonium tartrate) after 2 days of incubation, when laccase activity was first detected. Flasks were cultivated in a time course of 12 days at 28 ± 2°C. Culture broth was collected at the maximum laccase activity, filtered, and centrifuged. Then the supernatant was tested to enzyme assay and dye decolorization. The procedures were done as described above.

The assays were done twice, and samples were analyzed in triplicate. Values shown are means ± standard deviation.

Chemicals

All solvents and chemicals were of analytical grade, unless otherwise stated. All enzyme activities, substrate content, and decolorization tests were assayed with a U-3000 spectrophotometer (Hitachi Ltd, Tokyo, Japan) in the Laboratory of Botanical Science, Nanjing University, China.

Results and discussion

Effect of carbon and nitrogen sources on laccase production

The strain of *Pestalotiopsis* sp. has recently been isolated [19]. In order to improve laccase production, a range of carbon sources added in the medium was examined. The results show that the laccase formation was strongly affected by the substrates (Fig. 1). In general, substrates that were utilized efficiently and rapidly by the organism usually stimulated a comparatively high level of laccase activity. Maltose and glucose caused the similar laccase activities. Maltose gave the highest activities of 1.8 ± 0.2 U ml^{-1} among the carbon sources tested (Fig. 1). Cellobiose and lactose showed lower activities than maltose and glucose. They were consumed more slowly than glucose and maltose (Fig. 1b). And when using these two as substrates, respectively, the maximum activity levels were obtained after a longer cultivation time (Fig. 1a). Both (α-cellulose and glycerol, which are poorly utilized for growth by the fungus, resulted in the lowest laccase levels.

Laccase activities obtained with glucose were comparable to those obtained with maltose. However, glucose is cheaper than maltose. Therefore, glucose was chosen to be the carbon resource in this work. The effect of different glucose concentrations on the laccase activity is shown in Fig. 2. The laccase activities increased with glucose concentrations (Fig. 2). Increasing the glucose concentration from 5 to 20 g l^{-1}

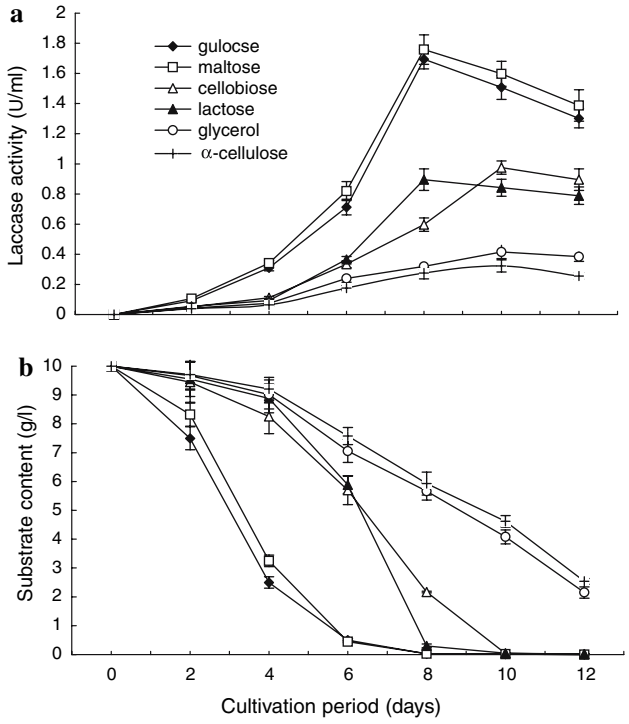

Fig. 1 Effect of various carbon sources (**a**) on laccase formation by *Pestalotiopsis* sp. and substrate consumption (**b**) when grown in basal mineral medium (containing 10 g l^{-1} single carbon source and 10 g l^{-1} NH_4NO_3). *Bars* represent standard deviation

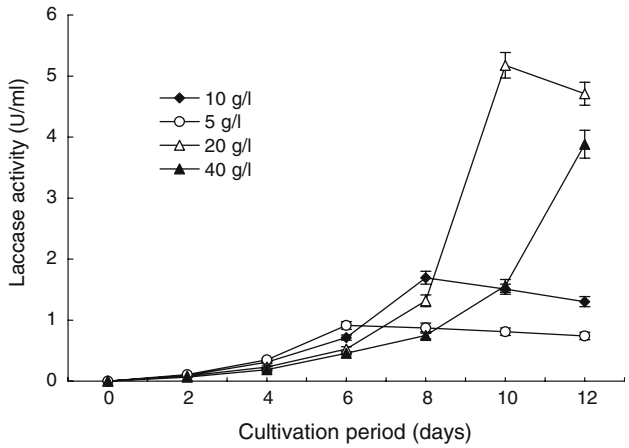

Fig. 2 Effect of varying concentrations of glucose (5–40 g l^{-1}) on laccase formation by *Pestalotiopsis* sp. when grown in basal mineral medium (containing 10 g l^{-1} NH_4NO_3). *Bars* represent standard deviation

resulted in a more than fivefold increase (0.9 ± 0.1 to 5.2 ± 0.2 U ml^{-1}) of laccase activity. A further increase to 40 g l^{-1} did not further enhance the laccase activity, but lower activities (3.9 ± 0.2 U ml^{-1}) were obtained. Glucose is often found to repress genes that are used in the metabolization of alternative carbon sources, and this was the case in the present study. This glucose repression is well known in fungi, and is thought to be an energy-saving response [30].

Another factor essential to efficient laccase production by fungi is the nitrogen source used for cultivation. Both its nature and the concentration employed have been reported to be of considerable importance [14, 17, 32]. The effect of different nitrogen sources on laccase production by *Pestalotiopsis* sp. when grown on 20 g l^{-1} glucose in basal medium was assayed (Fig. 3).

The highest enzyme production (14.2 ± 0.4 U ml^{-1}) was achieved when using ammonium tartrate (10 g l^{-1}) as the N-source. A further increase in the concentration of this nutrient (15 g l^{-1}) did not result in an increase in laccase formation. While decreasing the concentration of this nitrogen source led to reduced laccase activities (data not shown). The distinct effects of the different type of N-source on laccase formation are also noted in Fig. 3. The N-source could not be replaced by acrylamide. When using it as the sole nitrogen source, laccase activity was almost negligible.

Laccase production was found to be high in a medium containing ammonium tartrate (10 g l^{-1}) as the nitrogen source and glucose (20 g l^{-1}) as the carbon source in basal mineral medium. This confirms the reports about high N levels helpful to laccase formation of fungi [17]. In contrast, some authors [15, 28] reported that laccase activity increased under N limiting conditions.

Effect of putative laccase inducers and inhibitors

Results of these shaken-flask experiments are summarized in Fig. 4. Supplementing guaiacol and 2,5-xylidine

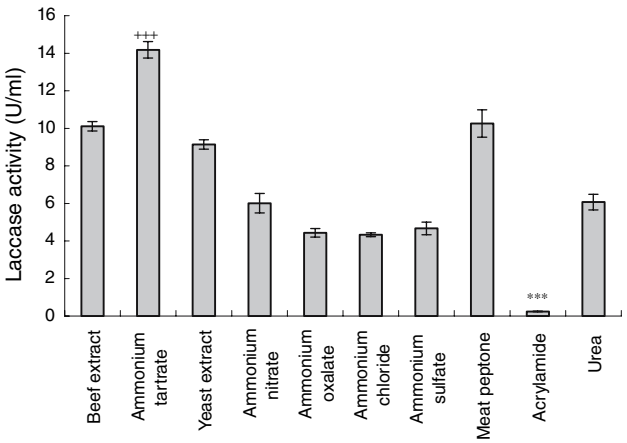

Fig. 3 Effect of different nitrogen sources on laccase formation by *Pestalotiopsis* sp. when grown in basal mineral medium (containing 20 g l^{-1} glucose and 10 g l^{-1} single nitrogen source). Cultivation period was 12 days. *Bars* represent standard deviation. Significantly higher activities than others for $^{+++}P < 0.001$. Significantly lower activities than others for $^{***}P < 0.001$

to the growth medium did not increase the laccase formation significantly compared to the controls (Fig. 4). This result appears to be quite different to the reports for other fungi [17, 34], in which the addition of aromatic compounds was routinely shown to boost laccase formation.

Interestingly, the addition of some of the metal ions resulted in markedly enhanced formation of extracellular laccase activity. The stimulatory effects of copper, which was much more pronounced ($P < 0.001$) than that of the aromatic inducers, and manganese ($P < 0.01$) were especially remarkable, achieving high levels of laccase activity with 32.7 ± 1.7 and 20.1 ± 1.1 U ml^{-1}, respectively. This is contrasted the other reagents which led to a significant decrease ($P < 0.001$) in laccase formation compared to the reference experiment. Ag^{+}, Cd^{2+}, Hg^{2+}, NaN_3, and KCN were highly toxic for the fungus in the concentration selected, as is evident from both the distinct decrease in laccase activity (Fig. 4) and the complete inhibition of its growth after their addition (Table 1).

As we know, most studies dealing with the production of laccase focused on the type, concentration, and the feeding time of inducers stimulating laccase formation [5, 20]. Since the addition of Cu to the growth medium stimulated laccase production by *Pestalotiopsis* sp. to the greatest extent, its effect on laccase formation was studied in more detail.

Figure 5 shows the results of different concentrations of Cu addition at different time of the shaken-flask cultivations. Increasing Cu in the basal mineral medium (contained 10 g l^{-1} glucose and 10 g l^{-1} ammonium tartrate) up to a critical concentration led to a considerable increase in laccase activity. And also, the start time of $CuSO_4$ addition distinctly influenced laccase formation (Fig. 5). When $CuSO_4$ was supplemented to the basal medium at the time of inoculation, no growth inhibition was observed in the low concentrations of Cu (<0.5 mM), while higher concentrations (1.0–2.0 mM) partially inhibited fungal growth (Table 2). Nevertheless, the highest enzyme activities were found in the cultures supplemented with high copper concentrations (Fig. 5). When $CuSO_4$ with the glucose in the medium of approximately 7.5 g l^{-1}, was added to the fungal cultures after 2 days of growth, high laccase activity occurred. Enzyme formation was found to be lower when $CuSO_4$ was added after 4 days

Table 1 Fungal biomass remaining after 12 days incubation in basal mineral medium (included 20 g l^{-1} glucose, 10 g l^{-1} ammonium tartrate) with different inducers and inhibitors (Cu, 2 mM final; others each 1 mM final added after 2 days of incubation)

Putative inducers and inhibitors	Mycelial dry weight (mg ml^{-1})
Control	3.3 ± 0.1
Guaiacol	2.9 ± 0.0
Xylidine	3.5 ± 0.2
Ag^{+}	0.4 ± 0.1
Cd^{2+}	0.5 ± 0.0
Cu^{2+}	2.3 ± 0.2
Hg^{2+}	0.3 ± 0.1
Mn^{2+}	3.6 ± 0.0
NaN_3	0.1 ± 0.1
KCN	0.1 ± 0.1

Control was 0.35% ethanol

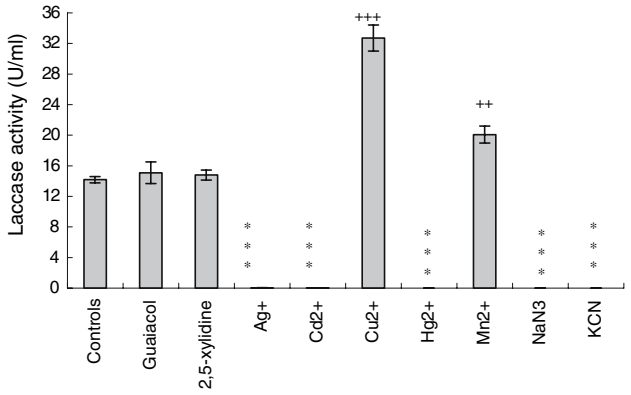

Fig. 4 Effect of different inducers and inhibitors (Cu, final 2 mM; others each final 1 mM added after 2 days of incubation) on laccase formation by *Pestalotiopsis* sp. when grown in basal mineral medium (included 20 g l^{-1} glucose, 10 g l^{-1} ammonium tartrate). Controls were done with the addition of 0.35% ethanol in above described basal mineral medium. Cultivation period was 12 days. *Bars* represent standard deviation. Significantly higher activities than controls for $^{+++}P < 0.001$, $^{++}P < 0.01$. Significantly lower activities than controls for $^{***}P < 0.001$

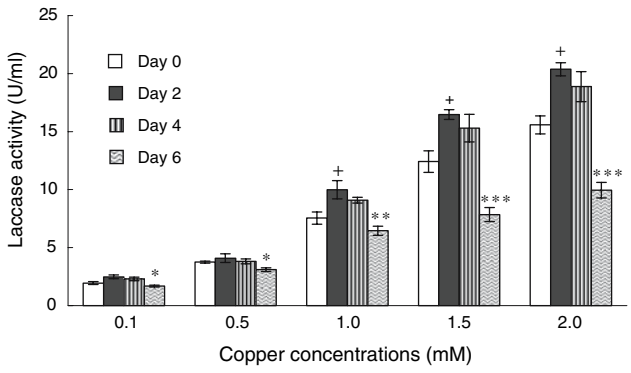

Fig. 5 Effect of various concentrations of $CuSO_4$ and varying times of its addition on laccase formation by *Pestalotiopsis* sp. in basal mineral medium (contained 10 g l^{-1} glucose and 10 g l^{-1} ammonium tartrate). *Day 0* addition of Cu before incubation, *day 2* addition after 2 days of incubation, *day 4* addition after 4 days of incubation, *day 6* addition after 6 days of incubation. Cultivation period was 12 days. *Bars* represent standard deviation. Significantly lower activities than others for $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significantly higher activities than others for $^{+}P < 0.05$

Table 2 Fungal biomass remaining after 12 days incubation in basal mineral medium (contained 10 g l^{-1} glucose and 10 g l^{-1} ammonium tartrate) with different concentrations of copper added on day 0

Treatment (mM)	Mycelial dry weight (mg ml^{-1})
0.1	3.3 ± 0.3
0.5	3.2 ± 0.4
1.0	2.5 ± 0.1
1.5	2.1 ± 0.3
2.0	1.8 ± 0.1

of growth under the glucose concentration in the medium with approximately 2.5 g l^{-1}. These results are in contrast to those observed when fungal cultures were amended with $CuSO_4$ after nearly complete consumption of glucose (6 days of growth). In this case, laccase production by *Pestalotiopsis* sp. decreased significantly for all concentrations of Cu tested (Fig. 5). It should be noted, however, that distinct laccase production only started when glucose was almost completely consumed from the culture medium, regardless of the time of Cu supplementation.

Copper is an essential micronutrient for most of living organisms [10], and copper requirements by microorganisms are usually satisfied in very low concentrations, ranging between 1 and 10 μM. Copper present in higher concentrations in its free, cupric form, is extremely toxic to microbial cells [21]. In this paper, the optimal copper concentration for the laccase formation was found to be 2.0 mM. This is much higher than the concentrations typically used in cultivation media (2–600 μM) for the production of laccase in wild-type or recombinant strains [11, 13, 27]. The reason that copper effectively stimulates laccase formation has not been clearly understood. In the cases of *T. versicolor*, *Ceriporiopsis subvermispora*, and *P. ostreatus*, the expression of laccase was regulated at the level of gene transcription by copper [27]. In the case of ascomycete *Podospora anserina*, in which laccase mRNA increased in response to copper and aromatic compounds, it was postulated that laccase acts as a defense mechanism against oxidative stress [16]. This protective function was partly attributed to the chelation of copper ions during synthesis of the laccase enzyme. Based on our studies, this putative protective mechanism seems not to affect *Pestalotiopsis* sp., since the formation of 32.7 ± 1.7 U laccase activity per milliliter, corresponding to a laccase concentration of approximately 0.65 μM, is only responsible for chelating 2.6 μM Cu. This is only about 0.2% of the 2.0 mM added during the cultivation. Furthermore, fungi possess other, more effective mechanisms of resistance against increased copper concentrations, such as intracellular complexing by metallothioneins and trapping of the metal by cell-wall components [10]. Apparently, the further investigation in such field associated with *Pestalotiopsis* sp. is necessary and would probably add to our understanding of the degradation process of the fungi.

Dye decolorization

Figure 6 shows the color remaining during the reaction of culture liquid of *Pestalotiopsis* sp. on Direct Fast Blue B2RL at various pHs over 16 h. As can be observed, the highest decolorization rate was obtained at pH 4.0. within 12 h, the decolorizaition percentage was as high as 88.0 ± 3.2%, followed by 85.4 ± 2.5% at pH 4.5. As in the case of the control, with heat-denatured laccase, they did not show any change in the maximum wavelength over the incubation time. This indicates that such dye decolorization is biological. Furthermore, the above results suggest that *Pestalotiopsis* sp. employed secreted acid-stable enzymes, which are useful in decolorizing dyes from acidic textile effluents, such as those resulting from wool and polyester processing.

In order to determine the role of laccase in dye decolorization, 0.01 mM NaN_3 (final concentration) was added to inhibit the laccase activity, followed the work of Anthony et al. [3]. Over a 12-day incubation, almost no laccase activity was demonstrated in the inhibitor-presented flasks, while biomass production was almost identical to control flasks (without the addition of sodium azide). The degradation of the former samplings was only 30.1 ± 1.0%, compared to 88.0 ± 3.2% of the latter samples within 12 h of reaction. The

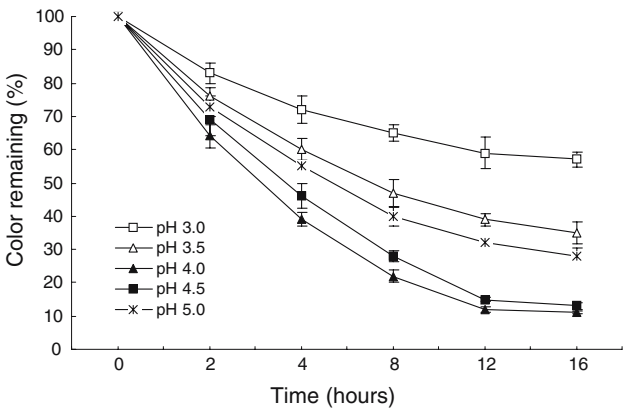

Fig. 6 Color remaining during the decolorization of Direct Fast Blue B2RL by culture liquid of *Pestalotiopsis* sp. at various pHs within a time course of 16 h. *Bars* represent standard deviation

findings suggest that laccase secreted by *Pestalotiopsis* sp. is mainly involved in the decolorization of textile dye Direct Fast Blue B2RL. However, given the absence of detectable levels of laccase enzyme, another process must account for the remaining color removal observed in samplings.

Conclusions

In conclusion, this study has explored how to increase the laccase formation of the newly isolated deuteromycete, *Pestalotiopsis* sp. We can obtain a high laccase yield on the basal mineral medium containing glucose ($20\ g\ l^{-1}$), ammonium tartrate ($10\ g\ l^{-1}$), and stimulating enzyme formation by the addition of Cu up to 2 mM. The maximum laccase production in our experiment was $32.7 \pm 1.7\ U\ ml^{-1}$, which was much higher than or quite comparable to those activities obtained from many white-rot fungi such as *T. versicolor* [22], *Lentinula edodes* [31], *Trametes modesta* [26], *P. ostreatus* [27], *C. subvermispora* [9], and so on. Furthermore, it is easier to culture and propagate than white-rot fungi. In addition, It has been shown that the crude laccase from the *Pestalotiopsis* sp. can be used for the decolorization of azo dye Direct Fast Blue B2RL, achieving decolorization as high as $88.0 \pm 3.2\%$ at pH 4.0 in 12 h. The use of the enzyme may conceivably be extended to other type textile dyes, suggesting its high potential to the removal of dyes from industrial effluents.

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References

1. Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco PA, Gubitz G (2000) Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol 66:3357–3362
2. Alexandre G, Zhulin IB (2000) Laccase are widespread in bacteria. Trends Biotechnol 18:41–42
3. Anthony V, Anissa LH, Durand SR (2004) Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. Int Biodeterior Biodegradation 53:65–70
4. Ardon O, Kerem Z, Hadar Y (1996) Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract. J Biotechnol 51:201–207
5. Arora DS, Gill PK (2001) Effects of various media and supplements on laccase production by some white rot fungi. Bioresource Technol 77:89–91
6. Baminger U, Subramaniam SS, Renganathan V, Haltrich D (2001) Purification and characterization of cellobiose dehydrogenase from the plant pathogen *Sclerotium* (*Athelia*) *rolfsii*. Appl Environ Microbiol 67:1766–1774
7. Bollag JM, Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48:849–854
8. Bourbonnails R, Paice MG (1997) Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of ABTS. Appl Environ Microbiol 36:823–827
9. Carmen R, Salas L, Vicuna R, Kirk T (1993) Extracellular enzyme production and synthetic lignin mineralization by *Ceriporiopsis subvermispora*. Appl Environ Microbiol 59:1792–1797
10. Cervantes C, Gutierrez-Corona F (1994) Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol Rev 14:121–137
11. Collins PJ, Dobson AD (1997) Regulation of laccase gene transcription in *Trametes versicolor*. Appl Environ Microbiol 63:3444–3450
12. Dekker RFH, Barbosa AM (2001) The effect of aeration and veratryl alcohol on the production of two laccases by the ascomycete *Botryosphaeria* sp. Enzyme Microb Technol 28:81–88
13. Dittmer JK, Patel NJ, Dhawale SW, Dhawale SS (1997) Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. FEMS Microbiol Lett 49:65–70
14. Eggert C, Temp U, Eriksson K-EL (1996) The ligninolytic system of the white-rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl Environ Microbiol 62:1151–1158
15. Eggert C, Temp U, Eriksson K-EL (1996) Laccase-producing white-rot fungus lacking lignin peroxidase, and manganese peroxidase. ACS Symp Ser 655:130–150
16. Fernandez-Larrea J, Stahl U (1996) Isolation and characterization of a laccase gene from *Podospora anserina*. Mol Gen Genet 252:539–551
17. Gianfreda L, Xu F, Bollag JM (1999) Laccases: a useful group of oxidoreductive enzymes. Bioremediation J 3:1–25
18. Gigi O, Marbach I, Mayer AM (1891) Properties of gallic acid-induced extracellular laccase activity of *Botrytis cinerea*. Phytochemistry 20:1211–1213
19. Hao J, Tian X, Song F, Zhang Z, Zhang P (2006) Involvement of lignocellulolytic enzymes in the decomposition of leaf litter in a subtropical forest. J Eukaryot Microbiol 53(3):193–198
20. Hou H, Zhou J, Wang J, Du C, Yan B (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochem 39:1415–1419
21. Labbé S, Thiele DJ (1999) Pipes and wiring: the regulation of copper uptake and distribution in yeast. Trends Microbiol 7:500–505
22. Lee I, Jung K, Lee C, Park Y (1999) Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. Biotechnol Lett 21:965–968
23. Mansur M, Suárez T, González AE (1998) Differential gene expression in the laccase gene family from basidiomycete I-62 (CECT 20197). Appl Environ Microbiol 64:771–774
24. Niku-Paavola ML, Raaska L, Itavaara M (1990) Detection of white-rot fungi by a non-toxic stain. Mycol Res 94:27–31
25. Nyanhongo GS, Gomes J, Gubitz GM, Zvauya R, Read JS, Steiner W (2002) Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*. Water Res 36:1449–1456
26. Nyanhongo G, Gomes J, Cubitz G, Zvauya R, Read J, Steiner W (2002) Production of laccase by a newly isolated strain of *Trametes modesta*. Bioresource Technol 84:259–263

27. Palmieri G, Giardina P, Bianco C, Fontanella B, Sannia G (2000) Copper induction of laccase isoenzymes in the lignin-olytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66:920–924
28. Pointing SB, Jones EBG, Vrijmoed LLP (2000) Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia 92:139–144
29. Robles A, Lucas R, Magdalena MC, Omar NB, Perez R, Antonio G (2002) Characterization of laccase activity produced by the hyphomycete *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH32. Enzyme Microb Technol 31:516–522
30. Ronne H (1995) Glucose repression in fungi. Trends Genet 11:12–17
31. Savoie J, Mata G, Billette C (1998) Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*. Appl Microbiol Biotechnol 49:589–593
32. Sethuraman A, Akin DE, Erriksson KE (1999) Production of ligninolytic enzymes and synthetic lignin mineralization by the bird's nest fungus *Cyathus stercoreus*. Appl Mcirobiol Biotechnol 52:689–697
33. Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26
34. Xu F (1999) Laccase. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation, vol 3. Wiley, New York, pp 1545–1554
35. Zhang L, Zhang T, Li L (1981) Experimental methods and technology of biochemistry. Higher Education Press, Beijing, pp 372–373