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Full Length Article

Laccase Production by *Pycnoporus sanguineus* Grown under Liquid State Culture and its Potential in Remazol Brilliant Blue R Decolorization

Rakrudee Sarnthima[1*] and Saranyu Khammuang[1*]
[1]*Protein and Enzyme Technology Research Unit and Center of Excellence for Innovation in Chemistry, Department of Chemistry, Faculty of Science, Mahasarakham University, Maha Sarakham 44150, Thailand*
[*]For correspondence: rsarnthima@yahoo.com; skhammuang@yahoo.com

Abstract

In this research, laccase production by *Pycnoporus sanguineus* was investigated under liquid state fermentation. The fungus was cultured in glucose yeast peptone (GYP) liquid medium in the absence and presence of certain laccase inducers including 1.0 mM of gallic acid, copper(II) sulfate, ferulic acid, 2,5-xylidine and ethanol. The best laccase inducer was copper(II) sulfate and followed with ferulic acid, whereas the rest could not induce laccase activity by this specie. Copper(II) sulfate at low concentration tended to induce more laccase activity and the static culture condition was better than shaking at 140 rpm. Manganese independent peroxidase (MIP) activity was detected in lower level than laccase. In the presence of copper(II) sulfate, fungal biomass tended to decrease except in the case of 3.0 mM $CuSO_4$ in which instead slightly increased in biomass. Native polyacrylamide electrophoresis (native PAGE) accompanies with activity staining and coomassie staining suggested that the laccase and MIP activities might be of the same protein. Protein pattern of $CuSO_4$ induced was slightly different in various concentrations. The crude enzyme could decolorize RBBR approximately 60% within 24 h at 32°C. © 2013 Friends Science Publishers

Keywords: Anthraquinone; Fermentation; Inducer; Ligninolytic enzyme; *Pycnoporus* sp.

Introduction

White rot fungi are important microorganisms for lignin degrading enzymes producer. Those enzymes mainly comprise lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and laccase. Lignin degrading enzymes play an important role in lignin hydrolyzing, which carbon and nitrogen sources were then used by the fungi. Some white rot fungi contain all three enzymes however; others contain two enzymes which may be lignin peroxidase and manganese peroxidase or manganese peroxidase and laccase (Giardina *et al.*, 2000). Peroxidases need hydrogen peroxide for their activity but laccases do not need therefore laccases is probably more convenient for applications.

Laccases (E.C. 1.10.3.2, *p*-diphenol:dioxygen oxidoreductases) are multi-copper oxidases that able to oxidize a variety of phenolic compounds, with simultaneously reduction of molecular oxygen to water. These enzymes have a mononuclear copper site containing one type-1 Cu (blue Cu) and a tri-nuclear copper site containing one type-2 Cu and two type-3 Cu. Substrates are oxidized near the mononuclear site and the electrons are transferred to the tri-nuclear site, where the molecular oxygen is reduced. These enzymes are secreted in multiple isoforms depending on the fungal species and the environmental conditions (Baldrian, 2006; Rosario Freixo *et al.*, 2012). Some isoforms are constitutively expressed while other can be inducible. It was known that laccase gene transcription is regulated by metal ions, various aromatic compounds related to lignin or lignin derivatives, nitrogen and carbon sources (Piscitelli *et al.*, 2011).

Laccases could be used in a wide range of applications, such as in the pulp and paper industry, in the food and textile industries, in soil bioremediation, in synthetic chemistry, food and cosmetic industries (Gomaa, 2005; Singh Arora and Kumar Sharma, 2010). High amounts of laccase enzyme, then, are required thus the development of simple and efficient way for laccase production is of always interest. One way to get enzyme in large quantities, the fungus cultures in liquid medium is necessary to study because many enzymes with biotechnological applications have been successfully cultured in a liquid process.

Laccase production in solid substrate fermentation of selected agro-residues by *Pycnoporus sanguineus* had reported (Vikineswary *et al.*, 2006). The lignin-degrading enzymes by the fungus *Pycnoporus* sp. in Thailand have been rarely reported. Thongkred and co-workers (Thongkred *et al.*, 2011) have reported in *P. coccineus* and *P. sanguineus* and its former crude enzyme were able to oxidize polycyclic aromatic hydrocarbons (PAHs). In this

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research we aimed to study laccase production by *P. sanguineus* in liquid culture. The influence of certain types of inducers; copper(II)sulfate, 2,5-xylidine and ethanol on growth and enzyme production by this fungus were investigated and evaluated the potential of synthetic dye decolorization.

Materials and Methods

Fungus and Media Preparation

White rot fungus, *Pycnoporous sanguineus* was taken from Forestry Research and Development Unit, Forestry Department. It was maintained in the PDA at 4°C, and sub-cultured every 1-2 months. Fungal culture medium (30 mL) was prepared according to the formula glucose yeast peptone (GYP) prepared in an Erlenmeyer flask-sized 125 mL, ratio of nutrients ($g•L^{-1}$) as follows: glucose 10 g, yeast extract 5 g, peptone 5 g, $MgSO_4•7\ H_2O$ 1 g adjusted to pH 5.5 before sterilization.

Culture Conditions for Laccase Production

Five selected laccase inducers including 2,5-xylidine, copper(II) sulfate, gallic acid, ferulic acid and ethanol (1.0 mM) were added to the liquid medium on day-4 of fungal cultures grown at room temperature (~28±3°C) by shaking at 140 rpm speed. By completely randomize for at least three flasks, the activities of lignin degrading enzymes were monitored in the culture medium every second days for 2 weeks. The fungus was also grown in liquid medium of the above conditions under non-shaking condition. Concentration of copper(II) sulfate in the cultures was studied at various concentrations ranging from 0-3.0 mM.

Protein Determination and Ligninolytic Enzymes Activity Assay

Protein in solution was analyzed by Bradford (1976) using reaction with Bio-Rad protein assay reagent and measuring absorbance at 595 nm by spectrophotometer, using bovine serum albumin as standard protein. Analyze the activity of laccase using ABTS as substrate, according to previous reports (Khammaung and Sarnthima, 2007), and 1 U is the amount of enzyme required to oxidize substrate to produce 1 µmole per minute at the given conditions (in 50 mM sodium acetate buffer, pH 4.5 at 30°C). The light absorption coefficient (ε_{420}) of free radicals, $ABTS^{\cdot+}$, which equals 3.6 x 10^4 $M^{-1}•cm^{-1}$. Lignin peroxidase (LiP) activity was measured using veratryl alcohol as substrate in the presence of H_2O_2, according to the method of Collins and co-workers (1996). Activity of manganese peroxidase (MnP) and manganese-independent peroxidase (MnIP) were analyzed according to the modified method of Ngo and Lenhoff (1980) as described previously (Sarnthima and Khammuang, 2008).

Analysis of Reducing Sugar and Fungal Biomass

Observations on the growth of fungus was monitored by follow the use up of glucose (reducing sugar) in culture liquid with dinitrosalicylic acid method (Ghose, 1987), using glucose as standard. At the final day of culture, biomass of each fungal culture was separated by filtering on filter papers, Whatman paper No. 1, washed with distilled water many times. Biomass on filter paper was dried at a temperature ~60°C until constant weight. Biomass of each culture would subtract the weight of the filter paper.

SDS-PAGE

Ten micrograms of crude protein from the 14-days culture of *P. sanguineus* after water exchanged via dialysis were separated by Native-PAGE technique to observe activity zymogram and protein pattern. Electrophoresis was run at 100 volt/gel and after running the gels were washed with distilled water and incubated with a solution of buffered sodium acetate buffer, pH 4.5 containing substrate of laccase, ABTS or syringaldazine. The substrates of peroxidase (DMAB/MBTH/EDTA/H_2O_2) were also incubated to detect peroxidase activity. Reactions were done at 32°C until the color of the reaction products of each substrate appeared. Protein pattern was observed by staining a gel with coomassie brilliant blue R-250.

Dye Decolorization Study

The crude enzyme of the 14-days culture of *P. sanguineus* of (0.2 $U•mL^{-1}$) were used to decolorize synthetic anthraquinone dye, Remazol brilliant blue R (RBBR) in (0.1 M) sodium acetate buffer, pH 4.5 at 32°C. RBBR concentration was monitored at the maximum absorption wavelength (λ_{max}592) for 24 h using the spectrophotometer. Results were reported as decolorization percentage of the dye.

Statistical Analysis

Design of the experiments was completely randomized with three replications. The values reported are means ± standard deviations. Data were analyzed variance and significant differences among means by one-way ANOVA using SPSS (Version 17.0, SPSS Inc., 2008, Chicago, USA). Significant differences of $P < 0.05$ were declared.

Results

Effect of Laccase Inducers on Laccase Production

This work investigated ligninolytic enzyme production by *P. sanguineus* grown in liquid state medium of GYP in the presence of 5 types laccase inducers including copper(II) sulfate, 2,5-xylidine, gallic acid, ferulic acid and ethyl alcohol. The result showed that $CuSO_4$ could induce laccase activity to the highest approximately 1.6 $U•mL^{-1}$ at day-8

culture and followed with ferulic acid around 0.4 U•mL^{-1} at day-12 culture, whereas other compounds could not induce as shown in Fig. 1a.

Protein amount was detected in the culture medium after inducer addition at day-4. In each case, protein concentration was around 0.2 mg•mL^{-1} except in the case of ferulic acid which yielded protein concentration around 0.5 mg•mL^{-1} as shown in Fig. 1b. Consideration to the reducing sugar taken for growth, *P. sanguineus* grown in the presence of ferulic acid, more rapidly decreased in reducing sugar and completely used up within day-8 of culture (Fig. 1c) accompanying with protein concentration sharply increased (Fig. 1b).

Among tested, $CuSO_4$ could be the best choice for laccase inducer of *P. sanguineus* and yielded laccase with specific activity of about 6.4 U•mg^{-1} protein at day-14 of culture as shown in Fig. 2.

Effect of Shaking Condition on Ligninolytic Enzymes Production

When culturing *P. sanguineus* in GYP medium containing (1.0 mM) $CuSO_4$ under shaking comparison to non-shaking conditions at room temperature. Monitor a decrease of reducing sugar, secreted proteins and enzymes periodically, the results showed that when cultured the fungus in non-shaking condition, laccase production was clearly higher than those of shaking one (Fig. 3a) with the highest laccase activity approximately 5.3 U•mL^{-1} at day-14. Protein content was increased slowly (Fig. 3b) and reducing sugar consumption decreased more slowly than those of shaking. The amount of reducing sugar increased by the subsequent addition of $CuSO_4$, which is measured by an increase at day-6 and day-8 before it was gradually decreased and has been used up at day-14 of culture (Fig. 3c).

The results of these experiments also showed that the fungus *P. sanguineus* grown in the presence of 1.0 mM $CuSO_4$, laccase production could be induced. Non-shaking conditions could induce laccase at very high specific activity, up to about 19 U•mg^{-1} proteins, whereas when cultured in shaking condition the specific activity obtained only about six times lower (3.2 U•mg^{-1} proteins), as shown in Fig. 4.

Effect of Copper(II) Sulfate Concentration

The effect of the $CuSO_4$ concentration on ligninolytic enzymes production by *P. sanguineus* grown in liquid medium in the absence of the inducer, no laccase activity could be detected throughout 14 days of culture. The addition of inducer, laccase activity could be found in all concentrations tested. And lower $CuSO_4$ concentration, laccases are likely to be induced than those of higher concentration, except at $CuSO_4$ 3.0 mM seems to induce the production of laccase slower but gave higher laccase activity as shown in Fig. 5(a). The activity of other ligninolytic enzymes, the activity of manganese-

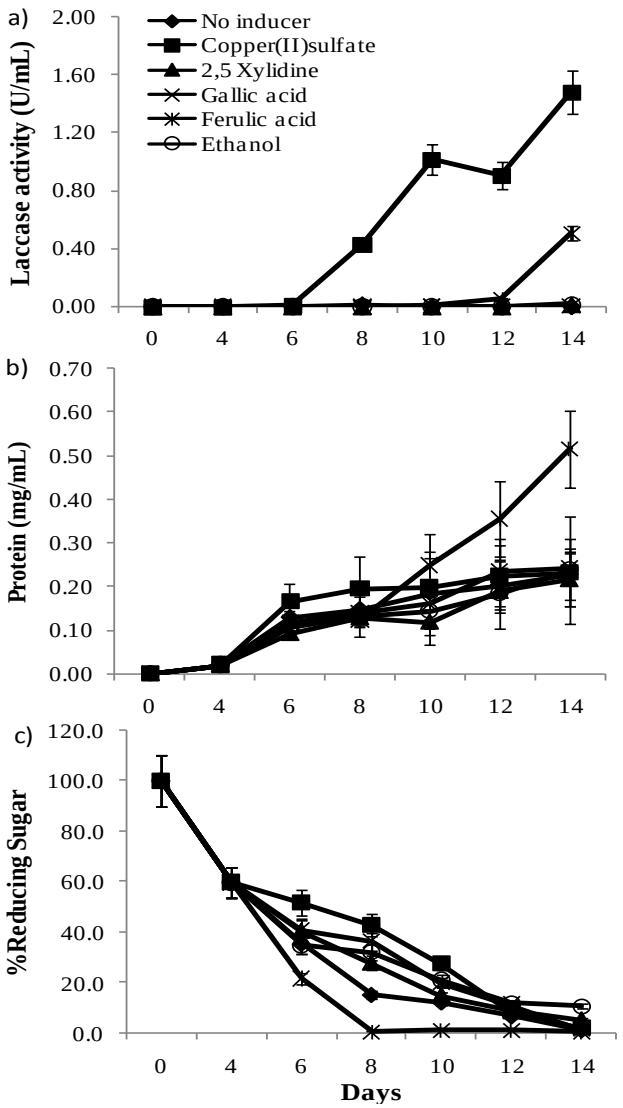

Fig. 1: Laccase activity (a) protein concentration (b) and residual reducing sugar percentage (c) of *P. sanguineus* cultured in GYP medium at shaking condition of 140 rpm at room temperature. Error bars indicate standard deviations (n=3)

independent peroxidase (MIP) can be detected in the similar pattern of laccase activities but in the lower level as shown in Fig. 5(b).

When protein secretion was in consideration, *P. sanguineus* grown in the presence of 0.05–1.0 mM $CuSO_4$ showed similar protein appearance and intensity. Copper(II) sulfate-induced conditions, except that the concentration of 3.0 mM $CuSO_4$, gave a little bit higher protein content than those of no inducer as shown in Fig. 5(c). When considering the use of glucose as a carbon source for growth, the addition of $CuSO_4$ at low concentrations (0.05-0.2 mM) slowly decreased the percentage of reducing sugar and gradually decreased and used up at about day-12 of culture. However, in conditions of higher $CuSO_4$

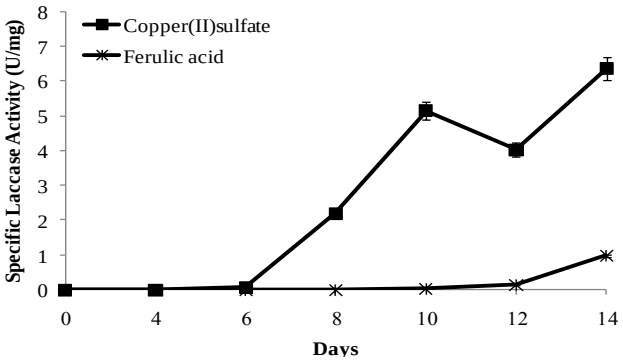

Fig. 2: Specific laccase activity of *P. sanguineus* cultured in GYP medium in the presence of various laccase inducers at room temperature under shaking condition at 140 rpm. Error bars indicate standard deviations (n=3)

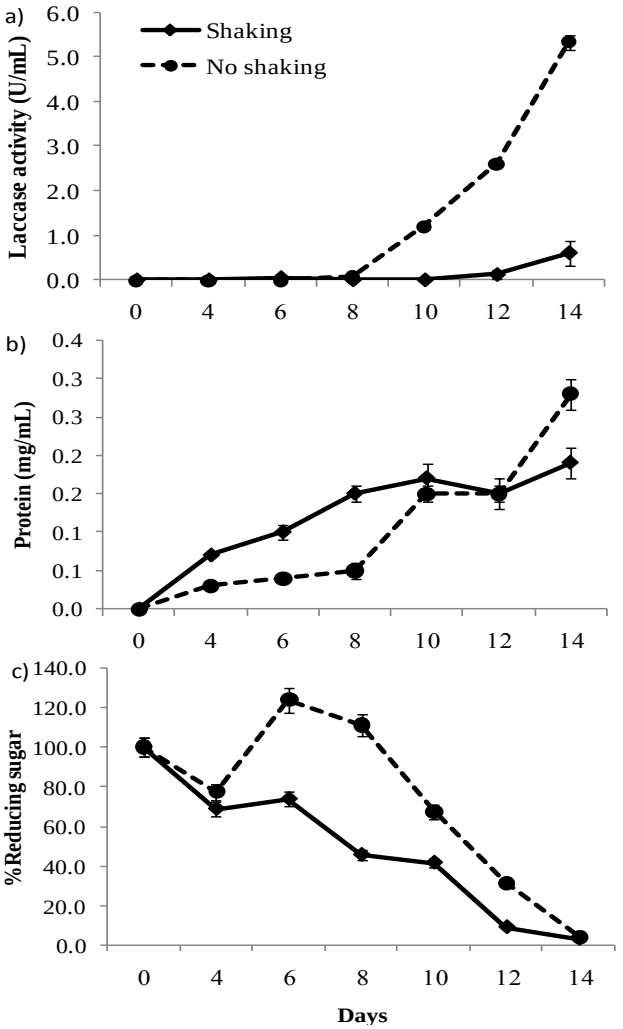

Fig. 3: Laccase activity (a) protein concentration (b) and residual reducing sugar percentage (c) of *P. sanguineus* cultured in GYP medium in the presence of 1.0 mM $CuSO_4$ under non-shaking condition at room temperature. Error bars indicate standard deviations (n=3)

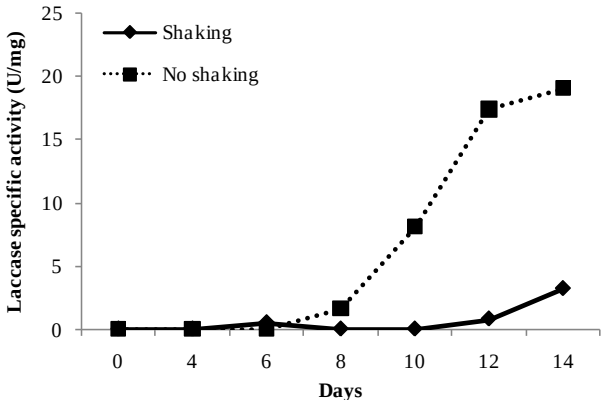

Fig. 4: Specific laccase activity of *P. sanguineus* cultured in GYP medium in the presence of 1 mM $CuSO_4$ at room temperature under non-shaking and shaking condition at 140 rpm. Error bars indicate standard deviations (n=3)

concentration (1.0 and 3.0 mM), the amount of reducing sugar increased after the addition of inducer on day-4. The addition of 3.0 mM $CuSO_4$ detected a higher amount of reducing sugar about 170% at day-6 and then gradually decreased until day-10 before sharply decreased to about 20% at the last day of culture. The addition of 1.0 mM $CuSO_4$ increased the amount of reducing sugar in day-6 (~100%) and gradually decreased until it disappeared on day-12, as shown in Fig. 5(d).

When culturing *P. sanguineus* for 14 days, biomass was harvested, oven-dried and weighed. The result showed that the concentration of $CuSO_4$ inducer also affect fungal biomass. In the addition of $CuSO_4$ are likely to decrease fungal biomass. Except at high concentration of 3.0 mM $CuSO_4$, that found fungal biomass increased significantly compared to the condition of 1.0 mM $CuSO_4$ as shown in Fig. 6.

Compared laccase production to the biomass, *P. sanguineus* fungus was cultured in an environment where the concentration of $CuSO_4$ of 0.05-1.0 mM the proportion of laccase to biomass did not significantly different. While the condition added 3.0 mM $CuSO_4$, even if it has the same enzyme activity levels with the other concentrations, however, this case has a higher biomass. As a result, the ratio of laccase production above the biomass decreased significantly. The culture with the $CuSO_4$ concentration of 0.05, 0.2, 1.0 mM, the laccase activity towards biomass is higher than 1,000 $U \cdot g^{-1}$ d.wt. biomass. However, in the addition of $CuSO_4$ 3.0 mM gave laccase to biomass equal to about 700 $U \cdot g^{-1}$ d.wt. biomass as shown in Fig. 7.

Gel Electrophoresis of Crude Enzyme of *Pycnoporus sanguineus*

When examination the pattern of proteins secreted by *P. sanguineus* cultured in GYP medium supplemented with $CuSO_4$ at various concentrations using native PAGE stained with substrates of laccase and manganese peroxidase.

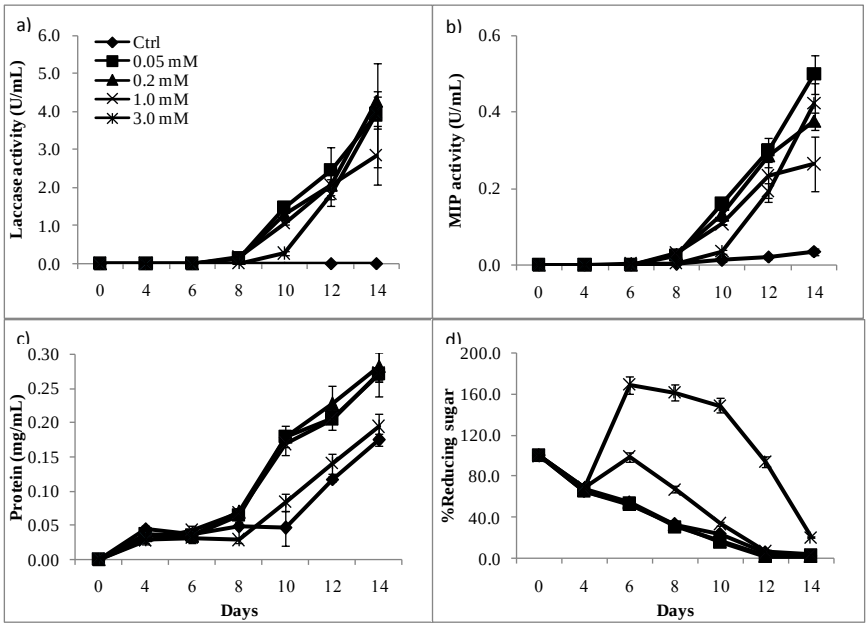

Fig. 5: Laccase activity (a) manganese peroxidase activity, MIP (b) protein concentration (c) and residual reducing sugar percentage (d) of *P. sanguineus* cultured in GYP medium in the presence of 0-3.0 mM $CuSO_4$ under non-shaking condition at room temperature. Error bars indicate standard deviations (n=3)

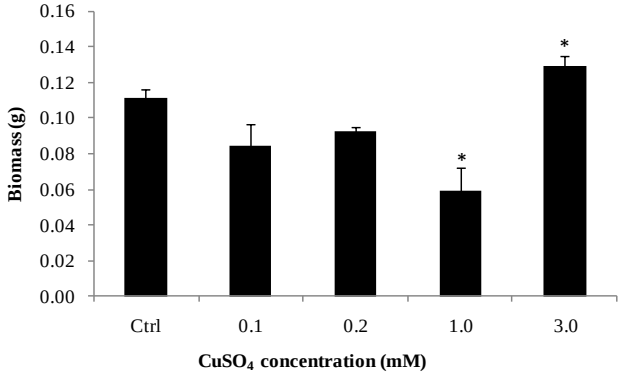

Fig. 6: Biomass of *P. sanguineus* cultured in GYP medium in the presence of various concentrations of $CuSO_4$ under non-shaking condition at room temperature after 14 days. Error bars indicate standard deviations (n=3). * indicates significant different ($p < 0.05$)

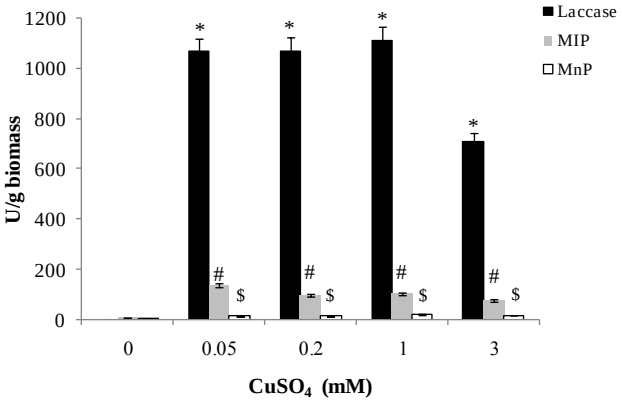

Fig. 7: Laccase production by *P. sanguineus* cultured in GYP medium in the absence and presence of various concentrations of $CuSO_4$ under non-shaking condition at room temperature for 14 days. Error bars indicate standard deviations (n=3). Similar symbols indicate significant different compare to control, no $CuSO_4$ inducer ($p < 0.05$)

The results showed that, when stained with the laccase substrate ABTS and syringaldazine, $CuSO_4$ induced in each concentration, appeared one dark green and one light green bands with less electrophoretic mobility (lane 1-3), except in case where the addition of 3.0 mM $CuSO_4$ no higher MW protein band observed (lane 4) as shown in Fig. 8(a). When the gel was stained with another laccase substrate, syringaldazine, only one pink band appeared at the position that match the dark green color as shown in Fig. 8(b). Also, Fig. 8(c) showed the blue-violet band of the gel stained with substrates of manganese peroxidase. It is found that this band position matches the color of green and pink bands as well.

The gel was stained for protein pattern with a coomassie brilliant blue R-250. The pattern of crude enzyme of this specie was similar and there are more than five protein major bands that move with difference electrophoretic mobility. By the presence of higher $CuSO_4$ concentrations (1.0 and 3.0 mM), some of protein bands were missing especially in case of 3.0 mM $CuSO_4$ (lane 3, 4). However, the protein is expected to have the activity of ligninolytic enzymes, found at the same position in all $CuSO_4$ induced conditions as shown in Fig. 8(d). The crude lininolytic enzymes from this fungal specie could be

decolorized RBBR to about 60% within 24 h experiment as shown in Fig. 9.

Discussion

In this study, we found that very low laccase was produced and excreted in normal culture by *P. sanguineus*. It was clearly inducible in the presence of certain inducers. Copper ions are known to be a good laccase inducer for many laccases producing fungi including *Phanerochaete chrysosporium* (Gnanamani *et al.,* 2006), *Pleurotus ostreatus* (Hou *et al.,* 2004), etc. Ferulic acid was also reported as an effective laccase inducer in culture of *Pycnoporus cinnabarinus* (Herpoel *et al.,* 2000). Even though, xylidine was reported to be the best inducer of laccase activity for *Trametes versicolor* (Rodrı´guez Couto *et al*., 2002; Revankar and Lele, 2006a, b; Saraiva *et al.,* 2012), our results showed no laccase induction by this substance (xylidine). Ethanol vapors addition as inducer of laccase produced by *P. cinnabarinus* ss3, which plays a regulatory role on both gene-expression and protease-activity levels (Meza *et al.,* 2005; 2007). However, even though ethanol was presented as a promising inducer for laccase production by *P. sanguineus* (Valeriano *et al*., 2009), our work showed no laccase induction observed by ethanol. Which might be due to low concentration of ethanol used (only 46 mg•L^{-1} ethanol used in our study whereas 20 g•L^{-1} were used in their study).

In contrast to our knowledge that laccases are oxidases requiring molecular oxygen as electron acceptor for complete catalysis reaction, and shaking condition is favor for oxygen dissolution. Most laccase produced microorganisms were experimented at agitation state. However, we found that higher laccase activity induction by *P. sanguineus* when cultured in liquid medium under non-shaking condition. This phenomenon was also observed in laccase production by another white rot, *Lentinus polychrous* in our laboratory (unpublished data). Laccase activity in *Bjerkandera adusta* observed only in nutrient rich medium (NRM) static condition, whereas the highest level of Aryl alcohol oxidase (AAO) was observed under shaking condition (Tripathi *et al.,* 2011). Moreover, in this same study laccase production was clearly higher detected under static condition by *Lentinus squarrosulus* in nutrient poor medium (NPM) than shaking condition. This indicates that culture condition have important influence effect on laccases and other enzymes production differently in each strain.

According to copper inducer, different concentrations have been reported, some as low as µM range (Janusz *et al.,* 2006), while as high as 30 mM concentration (Gnanamani *et al.,* 2006). In this study, we reported the concentration between 0.05-3.0 mM $CuSO_4$ with laccase activity around 2.85–4.29 U•mL^{-1}. Unlike in the case of Cu^{2+} induced laccase in *Lentinus polychrous*, which is the enzyme excretion was lowered when 3.0 mM $CuSO_4$ presented in

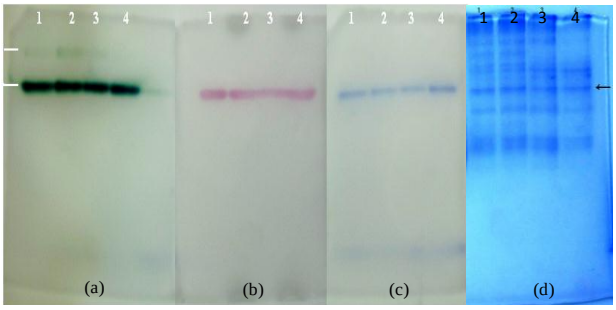

Fig. 8: Native-PAGE of crude enzyme produced by *P. sanguineus* stained with laccase, ABTS (a); syringaldazine (b); manganese peroxidase substrates, DMAB/ MBTH/EDTA/H_2O_2 (c) and coomassie brilliant blue R-250 stain (d). Lane 1-4; crude enzyme of 0.05, 0.2, 1.0 and 3.0 mM $CuSO_4$, respectively

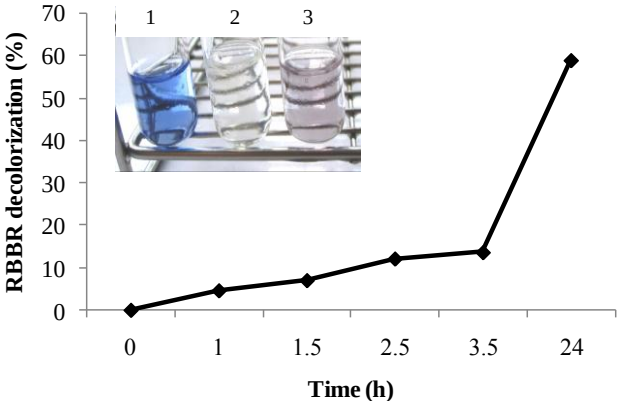

Fig. 9: Fig. 9: RBBR decolorization activity of crude enzyme produced by *P. sanguineus* (0.2 U•mL^{-1}) in 0.1 M sodium acetate buffer, pH 4.5 at 32ºC; inset represents decolorization of the dye after 24 h treatment; 1: dye; 2: enzyme; 3: reaction

culture medium, in this fungus the laccase activity is still higher induced. Species variation, experiments at higher inducer concentration than 3.0 mM should be further study. We also found that even the laccase activity was induced at the same levels as of lower inducer concentration, but higher biomass has been observed. Fonseca *et al.* (2010) reported that with 1 mM Cu^{2+}, *P. sanguineus* biomass underwent a dramatic growth inhibition and marked delay in growth with 0.5 mM Cu^{2+}. The relationship between laccase activity and biomass production is of interests to understand.

Two laccase isoforms (Lac I, II) obtained from *P. sanguineus* cultured in liquid cultures containing 0.05 g•L^{-1} 2,5-xylidine as inducer (Garcia *et al.,* 2006). Only a single laccase could be observed for the *P. sanguineus* cultured in diluted molasses (Litthauer *et al.,* 2007). Three novel laccase isozyme genes, lacA, lacB, and lacC, have been identified from Trametes sp. AH28-2. Lower Cu^{2+}

concentration than 0.5 mM can induce LacA and a novel laccase (LacC) and the LacC will disappear when Cu^{2+} concentration is increased up to 1−2 mM (Xiao *et al.*, 2006). Copper ion and certain metal ions affected laccase isozymes production, LacI and LacII were also obtained in barley bran submerged cultures of *T. versicolor* (Lorenzo *et al*., 2006). Our results indicate there might be more than one isoform of laccase from this fungal strain.

The ability of the purified laccase from *P. sanguineus* to decolorize RBBR was demonstrated effectively without adding any redox mediators (Lu *et al.*, 2007) which more than 80% decolorization within 10 min at 40°C with 2-5 $U{\bullet}L^{-1}$ at pH 3.0. In our work, low efficiency RBBR decolorization might be due to using the crude enzyme with low amount of laccase activity (only about 0.2 $U{\bullet}L^{-1}$ used) and dye decolorizing conditions have not yet optimized.

In conclusion, copper(II) sulfate at low concentrations (0.05-1.0 mM) could induce the production of laccases by this fungus, *P. sanguineus* when cultured in GYP medium. The non-shaking condition yielded a higher laccase production than that of shaking. Proteins secreted by *P. sanguineus* fungus showed similar patterns of $CuSO_4$ at various concentrations and proteins that contain both laccase and MIP activities in the same bands. This crude enzyme showed potential for wastewater treatment containing RBBR. Although copper ions could improve laccase production as noted in this present study, further studies are still needed to find the way to improve the production enough for industrial uses such as molecular cloning and over expression.

Acknowledgements

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