Journal of
Environmental and Applied Bioresearch
Published online Oct 01, 2015 (http://www.scienceresearchlibrary.com)

ISSN: 2319 8745
Vol. 03, No. 04, pp. 192-198

Research Article

Open Access

PRODUCTION CHARACTERIZATION AND DE-COLORIZATION OF ANTHRAQUINONE DYE BY LACCASE FROM ISOLATED *GANODERMA LUCIDUM* UPAG08

Khanchai Danmek[1*], Somchart Thana[2] and Choke Sorachakula[3]

[1]Program of Biotechnology, School of Agriculture and Natural Resources, University of Phayao, Phayao 56000, Thailand
[2]Program of Animal Science, School of Agriculture and Natural Resources, University of Phayao, Phayao 56000, Thailand
[3]Development Division, Royal Project Foundation, Chiang Mai 56000, Thailand
*Corresponding author's email: khanchai.da@up.ac.th

Received: July 11, 2015 / Accepted : July 27, 2015
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Abstract

The production of laccase by Tropical Mushroom *Ganoderma lucidum* UPAG08 isolated in Thailand by Semi-synthethic (SS) medium cultivation under static culture condition at 30 ^{O}C was investigated. Response Surface Methodology (RSM) was used for production and of laccase. Laccase yield (mU/ml) was significantly ($p \leq 0.05$) increased when incubation day and number of mycelia agar plug increased while the number of supporter had no significant influence on laccase production by *G. lucidum* UPAG08. The model showed the maximum laccase yield appeared at 16.36 days and 21 pieces of mycelia agar plug and 20 pieces of growth support matrix. When both sorbitol and casein were used as the sole carbon and nitrogen source respectively, the laccase activity was 306.90±9.12 mU/ml which was 2.13 fold greater than the control medium containing glucose, yeast extract and peptone (143.99±15.20). The enzyme retained more than 50% of residual activity, even after 48 hr of incubations in pH between 4.5 to 6.5. The kinetic parameters *Km* and *V*max indicate that 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) is a preferred specific substrate for laccase activity when compared to 2, 6-Dimethylphenol (2, 6-DMP). The reaction of crude laccase (20 mU) supplemented with ABTS showed capacity to de-colorized Remazol Brilliant Blue R dye (RBBR) in 51.14±6.53%. The results suggest that crude laccase from *G. lucidum* UPAG08 have potential use as biological pretreatment of anthraquinone dye of RBBR.

Keywords: *Ganoderma lucidum*, laccase, laccase characterization, de-colorization, RBBR

Introduction

White rot basidiomycetous fungi are well known for their role of lignin degradation in nature. They produce ligninolytic enzymes including laccase (E.C. 1.10.3.2), lignin peroxidase (E.C. 1.11.1.14), and Manganese peroxidase (E.C. 1.11.1.13) (Stajic et al., 2004). These enzymes are also involved in the degradation of phenolic compound and commercial dyes (Camarero et al., 2005, Soares et al., 2001, Wang et al., 2011). Laccase (benzenediol: oxygen oxidoreductases) is a multi-copper blue oxidase that couples the four electron reduction of oxygen and the oxidation of a phenolic substrate by coupling it to the reduction of oxygen to water. Laccase is an important group of enzymes and are commonly used in bioremediation protocols of both environmental phenolic and non-phenolic compounds. They also have been reported to decolorize several synthetic dyes (Wang et al., 2011). The redox potential of laccase is rather low or less than those of nonphenolic substrates which would be expected to limit their role in lignin degradation (Bourbonnais et al., 1997).

Among several applications, laccase has been widely used in biotechnological processes including bio-pulping, textile production and degradation of synthetic dyes (Howard et al., 2003). Several white rot fungi have been recognized for their ability to produce ligninolytic enzymes including *Trametes* sp. (Zang et al., 2006), *Pycnoporus sanguineus* (Low et al., 2009), and *Ganoderma lucidum* (Punnapayak et al., 2009). *G. lucidum* was found to be ligninolytic basidiomycete which ability to produce high level of laccase (Bonnen et al., 1994). However, the great biodiversity of mushroom showed different pattern of three ligninolytic enzymes including lignin peroxidase, manganese peroxidase, and laccase. Besides, some ligninolytic basidiomycetes had capacity to produce laccase with low to high activity and used to degrade or decolorize phenolic dyes which also differences in their ability of dye de-colorization (Zille et al., 2005). Screening new white rot fungus producing high level of laccase is a focus of much attention. Extracellular laccase is constitutively produced in small amounts but their production can be stimulated by the presence of a wide variety of inducing substrates (Arora and Gill, 2011, Ding et al., 2012). Several reports showed the ligninolytic basidiomycetes enzyme production are highly regulated by nutrients such as carbon sources, nitrogen sources, and presence of inducers and supporters (Gnanamania et al., 2006, Revankar and Lele, 2006). In addition, several factors are carried out to enhance laccase activity by optimizing the nutritional requirements and the environmental conditions for submerged cultivation (Mikiashvili et al., 2006). This study reported for the production of laccase from tropical mushroom *G. lucidum* UPAG08. Based on the promising prospect of using laccase for bioremediation applications, laccase was examined the kinetic parameters of pH optimal, stability, *Km*, and *Vmax*. Finally, an application of de-colorization of RBBR dye *in vitro* by crude laccase was also studied.

Materials and Methods

Fungal culture and culture medium

A tropical mushroom of *G. lucidum* UPAG08 was used for the investigation. It was originally collected from deciduous dipterocarp forest, Phayao Province, Northern region of Thailand

in 2013. The preliminary testing of laccase activity by plate screening on Malt extracted Agar (MEA) supplemented with Remazol Brilliant Blue R (RBBR) dye. The result showed that this fungus gave positive testing of laccase activity with zone of clearance. The culture was transferred and preserved in Potato Dextrose Agar (PDA) slants at 4^{O}C in the culture collection of the Biotechnology program, School of Agriculture and Natural Resources, University of Phayao, Thailand.

Semi-synthetic medium (SS) (Danmek et al., 2014) was modified and used in this studied. The medium consists of 2.0% (w/v) glucose, 0.2% (w/v) peptone, 0.1% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 0.01% (w/v) $CaCl_2$, 0.2% (v/v) Tween 80 and 0.5 ml of trace elements in stock solution consists of 0.0005 % (w/v) $ZnSO_4.7H_2O$, 0.0005% (w/v) $FeSO_4.7H_2O$, 0.002% (w/v) $CuSO_4.7H_2O$, and 0.005% (w/v) $MnSO_4.H_2O$. The medium pH was adjusted to 6.0. Anthraquinone dye **(**Remazol Brilliant Blue R; RBBR) was used for the *in vitro* testing and 2, 6-Dimethylphenol (2, 6-DMP) and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were used as redox mediators for determination of laccase activity. These reagents were purchased from a Sigma-Aldrich company.

Production of laccase

The mycelium of *G. lucidum* UPAG08 was inoculated on PDA plates and incubated at 30 ^{O}C for 7 days in the dark. Twenty pieces of 0.5 cm diameter (Ø) agar plug acquired from the growing margins of fungal mycelia were inoculated into 250 ml Erlenmeyer flask containing 100 ml of SS medium supplementary with 20 pieces of 1.0 cm^3 growth support matrix of sponge (Scotch Brite, 3M Spain, S.A.) and incubated under static culture condition at 30 ^{O}C for 15 day. At the end of experiment, fungal mycelia were removed from the enzyme solution by filtration through a Whatman No. 1 filter paper.

Statistical optimization production for laccase by Response Surface Methodology (RSM)

The experimental design and statistical analysis were performed according to the response surface analysis method using Minitab15 software. Central composite experimental design (CCD) (Box and Wilson, 1951) with quadratic model was employed to study the combined effect of three independent variables namely incubation day (χ_1, days), number of mycelia agar plug (χ_2, pieces) and number of 1 cm^2 growth support matrix (Scotch Brite, 3M Spain, S.A.) (χ_3, pieces). The dependent variables (γ) measured was laccase activity (γ; mU/ml). This dependent variable was expressed individually as a function of the independent variables known as response function. In CCD, the range and the levels of the variables investigated in this study are given in the Table 1. A 2^3-factorial CCD, with six axial points and six replications at the center points (n_o = 6) leading to a total number of 20 experiments was employed (Table 1) for the optimization of the condition of laccase production. The second degree polynomials (Eq. 1), was calculated to estimate the response of the dependent variable. The variance for each factor assessed was partitioned into linear, quadratic and interactive components and were represented using the second order polynomial function as follows:

$$\gamma = \beta_0+\beta_1\chi_1+\beta_2\chi_2+\beta_3\chi_3+\beta_{11}\chi_1^2+\beta_{22}\chi_2^2+\beta_{33}\chi_3^2+\beta_{12}\chi_1\chi_2+\beta_{13}\chi_1\chi_3+\beta_{23}\chi_2\chi_3 \quad \text{(Eq. 1)}$$

Where γ is the predicted response, χ_1, χ_2, χ_3 are independent variables, β_0 is the offset term, and β_1, β_2, β_3 are interaction terms. The significance of all terms in the polynomial functions were assessed statistically using *F*-value at a probability (*P*) of 0.01 or 0.05. The regression coefficients were then used to generate contour maps from the regression model. The two-dimensional (2D) plots were generated by keeping one variable constant at the center point and varying the other variables within the experimental range. The three factors which influence highly the enzyme production are incubation day, number of mycelia agar plug, and number of 1 cm^2 growth support matrix are considered for optimization in enzyme production. All experiments were made in triplicate. The encoded values of the initial incubation day, number of mycelia agar plug, and number of 1 cm^2 growth support matrix, as well as the mean values of the triplicate results obtained in the twenty performed assays executed in terms of laccase activity in the final production have been recorded (Table 1)

Table 1. Central composite design matrix code values for laccase activity

Run no.	Incubation day (days) (χ_1)	No. of mycelia agar plug (pieces) (χ_2)	No. of 1 cm^2 growth support matrix (pieces) (χ_3)
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Effect of carbon, nitrogen and growth support matrix on laccase activity

Carbon sources

Six groups of carbon source were explained and showed as monosaccharides (glucose, fructose, arabinose, and xylose), disaccharides (sucrose, and cellobiose), polysaccharide (molass), sugar alcohols (sorbitol, and inositol), lipid carbons (tributyrin, and glycerol), and other carbon sources (sodium acetate, sodium citrate, sodium tartrate, sodium succinate, and malic acid). To prepare modified SS medium, glucose was replaced by 0.1 M final concentration of each carbon sources. Cultures were incubated under static culture condition at 30 ^{O}C.

Nitrogen sources

Inorganic nitrogen sources including ammonium sulfate $(NH_4)_2SO_4$, sodium nitrate ($NaNO_3$), and sodium nitrite ($NaNO_2$) and organic nitrogen sources including peptone, yeast extract, casein, urea, and corn steep liquor were used in this study. In the experiment, each nitrogen sources with a final concentration of 0.3% (w/v) replaced peptone and yeast extract in SS medium (control). Cultures were incubated under static culture condition at 30 ^{O}C.

Growth support matrix

Rice straw, coconut husk, and eucalyptus were used in this study as natural growth support matrix. All samples were prepared by cutting 1.0 cm^3 pieces of materials, dried in hot air oven at 60 °C, and then each used as growth support matrix. A control commercial was used (Scotch Brite, 3M Spain, S.A.). To prepare modified SS medium, 20 pieces of each materials were added into 250 ml Erlenmeyer flask with 100 ml medium.

Determination of Laccase activity and protein

Laccase activity was determined at room temp (28-30^{O}C) by spectrophotometrically monitoring the reaction absorbance at 469 nm during 1.0 min. One (1) ml reaction mixtures consisting of 1.0 mM 2,6-DMP (ε_{469} = 49,600 $M^{-1}cm^{-1}$; referred to product concentration) in 50 mM sodium citrate buffer pH 5.0 and enzyme solution. One unit of laccase expressed as international unit (IU) was defined as the amount of enzyme which increased the absorbance at 465 nm by 1 μmol of product concentration per minute.

pH optimum and stability

Laccase activity was investigated at three different kinds of buffer with pH values ranging between 3.0 to 8.0 including 0.1 M sodium acetate buffer pH 3.0 to 5.0, 0.1 M sodium citrate buffer pH 4.0 to 6.0, and 0.1 M sodium phosphate buffer pH 6.0 to 8.0. Before determination, enzyme was incubated in condition for 60 min without substrate at room temperature. The effect of the pH on laccase stability was examined by incubating enzymes in 0.1 M buffers pH values ranging between 3.0 to 8.0 at 30 °C for 48 hr. After incubation, each reaction was determined residual laccase activity.

Effect of substrate specificity; determination of *Km* and *Vmax*

Two types of substrate which of 2, 6-DMP (ε_{469} at 469 nm = 49,000 $M^{-1}cm^{-1}$) and ABTS (ε_{420} = 27,500 $M^{-1}cm^{-1}$) were also chosen to test the substrate specificity of laccase. The Michalis-Menten for *Km* and *Vmax* values were determined by varying substrate concentrations ranging from 0 to 20 mM, in the same manner of laccase determination.

De-colorization of anthaquinone dye of RBBR

Anthaquinone dye of RBBR was used, and prepared by dissolving in 0.1 M sodium citrate buffer pH 5.0 in the total volume of 5.0 ml. The capacity of laccase for de-colorization of RBBR dye was accessed by reacting mixture containing the final concentration of 0.01 % (w/v) dye solution, with or without 0.1 mM mediator of ABTS, and laccase followed with incubated at 30^{o}C in the dark under static condition for 24 hr. The oxidation activity was determined by monitoring the decreasing in absorbance on a spectrophotometer using the wavelength range between 550 to 750 nm. The data values were calculated percentage of dye de-colorization using following equation; % Dye de-colorization = ((A_0-A_t/$A0$)x100)) when A_0 = initial concentration of chromogenic substance at absorbance maximum and A_t = observed concentration of chromogenic substance at absorbance maximum.

RESULTS AND DISCUSSION

Production of laccase

The white rot fungus *G. lucidum* UPAG08 is efficient natural laccase producer, one of lignin degrading enzymes that involved in the degradation of lignin and also in several substances including chromogenic dyes, and polycyclic aromatic hydrocarbons (PAHs) (Zille et al., 2005, Punnapayak et al., 2009). Fungus has been qualitative screened on MEA supplementary with RBBR dye indicator. A positive result was characterized by de-colorization which arose from oxidation of dark green color of RBBR dye leading to visible clear zone on agar plates. Qualitative testing, the isolated *G. lucidum* UPAG08 was able to decolorize RBBR in solid media of MEA. The result indicated and was found to be the first step of laccase screening same the experiment of Okino and collaborators (Okino et al., 2001). Quantitative screen was conducted in media to inform the ability in laccase production from fungi. Laccase activities ranged from 18.64±3.25 to 86.59±7.26 mU/ml were achieved from MEB and SS medium, respectively.

In the cultures of SS medium with growth support matrix of commercial sponge, laccase activity first appeared on the 9th day (9.23±3.34 mU/ml) and then, the activity increased with highest level on the 15th day of incubation. Crude laccase activity obtained from the cultures of SS medium was 4.65 fold higher than those obtained in the cultures of MEB. Similar results were described by Ullrich and collaborators with *Agaricus blazei* (Ullrich et al., 2005). Laccase production was influenced by the presence of growth support matrix. At the same culturing under static culture of 100 ml of SS medium in 250 ml Erlenmeyer flasks without growth support matrix, laccase activity was 36.03±6.58 mU/ml but with growth support matrix of sponge was 79.06±7.74 mU/ml. The results showed *G. lucidum* UPAG08 needs growth support matrix for the aerial mycelia formation and laccase production. Similar results were described by Neifar and collaborators (Neifar *et al*., 2010). Moreover, some growth support matrix included coconut husk served simultaneously as a preferred support matrix for laccase production from *Pycnoporus sanguineus* (Karim and Annuar, 2009, Low et al., 2009).

Statistical optimization production for laccase by Response Surface Methodology (RSM)

Statistical optimization for laccase production was achieved by the Response Surface Methodology (RSM). The coefficient of determination (R^2) estimated was 0.9592, indicating of the variability in the response could be explained by the model (Table 2 and 3). The model reveals that incubation day (χ_1) had a significant effect (P<0.01) on $\gamma_{\text{Laccase activity}}$ as it had the largest coefficient followed by number of mycelia agar plug (χ_2). Positive coefficient of χ_1 and χ_2 indicated a linear effect to increase $\gamma_{\text{Laccase activity}}$. However, quadratic terms (χ_1^2 and χ_2^2) had negative effects, along with interaction term ($\chi_1\chi_2$) that decrease $\gamma_{\text{Laccase activity}}$. The number of 1 cm^2 supporter (χ_3), and its other terms (χ_3^2, $\chi_1\chi_3$, and $\chi_2\chi_3$) had no significant influence on laccase production by *G. Lucidum* UPAG08. The result of the regression analysis of a full second-order polynomial model for optimization of laccase production was indicated in Figure 1. In addition, judging from the regression coefficients and only considering the significant terms, we obtain the polynomial model for laccase yield and showed as Eq. 2. and Figure 2. described as the 2D contour plot of the combined effects of incubation day and number of mycelia agar plug on the laccase production, respectively. It was obvious that the laccase yield (mU/ml) was significantly increased when incubation day and number of mycelia agar plug increased. The model predicted that the maximum laccase yield of 100.63 mU/ml appeared at 16.36 incubation days and 20 pieces of mycelia agar plug. Verification of the calculated optimum of the model (Eq. 2) for laccase production was done and representing this maximum point and yielding laccase of 115.96± 5.53 mU/ml. The excellent correlation between predicted and measured values of these experiments justifies the validity of the response model and the existence of an optimum point.

$$\gamma_{\text{Laccase activity}} = -631.709+80.972\chi_1+9.129\chi_2-2.269\chi_1^2-0.069\chi_2^2-0.428\chi_1\chi_2 \quad \text{(Eq. 2)}$$

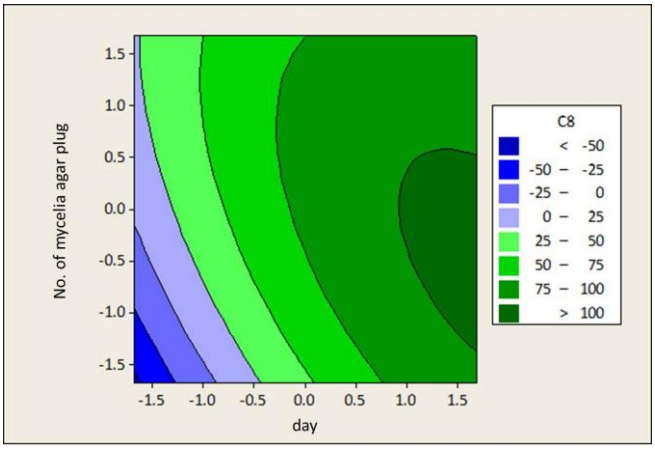

Figure 1 Contour plot (2D) of the combined effects between incubation day and mycelia agar plug on laccase activity by G. lucidum UPAG08. Fixed level: growth support matrix of sponge =0

Table 2. Analysis of variance for the experimental results of the central composite design

Regression	SS	df	MS	*F*-value	Sig.	R^2	adj. R^2
Linear	13976.964	3	4658.988	22.172	0.000[a]	0.8061	0.7697
Residual	3362.008	16	210.125				
Quadratic	16631.889	9	1847.988	26.135	0.000[a]	0.9592	0.9225
Residual	707.083	10	70.708				
Total	17338.972	19					

[a] significant at 1% level

Table 3. Results of regression analysis of a full second-order polynomial model for optimization of laccase production of *G. lucidum* UPAG08

Factor	Coefficients estimated	*t* - value	*p*-value
Intercept	79.822	23.296	0.000[a]
χ_1	30.193	13.281	0.000[a]
χ_2	8.585	3.776	0.004[a]
χ_3	6.172	2.715	0.422
$\chi_1\chi_1$	-9.067	-4.097	0.002[a]
$\chi_2\chi_2$	-6.852	-3.096	0.011[a]
$\chi_3\chi_3$	3.409	1.541	0.154
$\chi_1\chi_2$	-8.566	-2.884	0.016[b]
$\chi_1\chi_3$	1.703	0.574	0.579
$\chi_2\chi_3$	0.206	0.069	0.946

[a] significant at 1% level
[b] significant at 5% level

Carbon sources

Different carbon sources that were tested for laccase production. *G. lucidum* UPAG08 showed high activities of laccase on several carbon sources except sucrose, inositol, tributyrin, acetate, citrate, malate, succinate and tartarate (Figure 2). The aerial mycelia formation and highest laccase activity (mU/ml) was obtained when sorbitol (245.07±5.21) followed by xylose (240.37±9.92), molass (187.84±36.17), glycerol (122.81±7.32), glucose (112.52±4.51), cellobiose (97.45±8.99) and fructose (82.98±3.40) was used as carbon sources respectively (Figure 2). The SS medium with sorbitol stimulated the highest laccase activity of *G. lucidum* UPAG08, which was 2.18 fold greater than SS medium with glucose. Revankar and Lele (2006) observed the effect of carbon sources on laccase production by *Coriolus versicolor* MTCC 138 and showed that glucose was rapidly utilized for growth but less effect for laccase production. However, xylose stimulated laccase activity more than glucose which was in contrast for *Trametes* sp. AH28-2 (Zhang et al., 2006). In addition, the effect of fructose on laccase activity of *G. lucidum* UPAG08 was not similar as those reported for the production of laccase from *P. sanguineus* (Eugenio et al., 2009). Supporting this result, the use of fructose instead of glucose resulted in an increase the specific activity of laccase from *P. sanguineus* and *Basidiomycetes* strain CECT 20197 (Mansur et al., 1997). Sorbitol differed from the other sugar carbon sources in which it was sugar alcohol and was not a cyclic compound (Elisashvili et al., 2002, Saravanakumar et al., 2010, Stajic et al., 2004). Accordingly, the high level of laccase activity in *Pleurotus* sp. and *P. ostreatus* was observed in cultivation on the sugar alcohol of mannitol. However, medium containing mannitol decreased laccase activity of *T. pubescens* MB89 when compared with the control using glucose. In addition, *T. hirsuta* produced a maximal laccase activity in the presence of glycerol (Rodriguez et al., 2006). Summarize of this results, laccase production has been found to be highly dependent on the *Taxa* of fungi, nutritive medium composition especially type and culture condition.

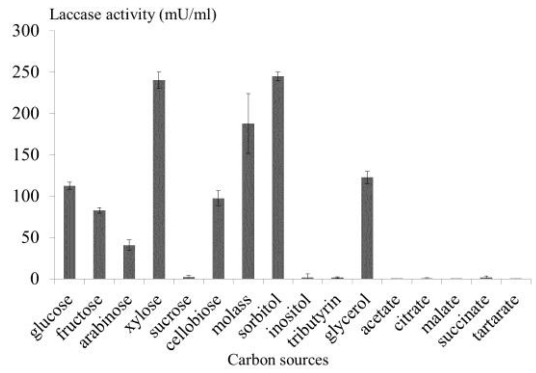

Figure 2. Effect of carbon sources on the laccase activity

Nitrogen sources

Two types of nitrogen source of inorganic and organic were examined. Overall investigation, significant ($p \leq 0.05$) highest laccase activity (mU/ml) was obtained when casein (306.90±9.12) followed by corn steep liquor (181.47±4.45) and yeast extract (141.57±12.12) was used as the sole nitrogen source when compared with the control medium containing both peptone and yeast extract (143.99±15.20) (Figure 3). On the other hand, the effect of peptone on laccase activity of *G. lucidum* UPAG08 did not similar as those capacities of *T. pubescens* MB89 (Songulashvili et al., 2007, Strong, 2011). Moreover, peptone resulted in an increase the specific activity of laccase more than yeast extract and ammonium ion by *G. lucidum* 447 and *T. pubescens* MB89 (Songulashvili et al., 2007, Strong, 2011). Supporting these results, organic nitrogen sources are more effective with a view to obtaining high laccase activity included from *C. versicolor* MTCC 138, *P. ostreatus* and *P. sanguineus* and could not be replaced by simpler nitrogen substitutes (Mishra and Kumar, 2007, Revenkar and Lele, 2006).

Both inorganic nitrogen sources tested, especially $NaNO_3$, and $NaNO_2$ did not stimulate both laccase and aerial mycelia formation by *G. lucidum* UPAG08. Results of *P. eryngii* and *P. ostreatus* showed the low level of laccase activity in the medium containing KNO_3 (Stajic et al., 2006). However, some white rot fungus included *G. lucidum* 447 showed the maximal

values of laccase activity in culture containing KNO_3 as the sole nitrogen source (Songulashvili et al., 2007). They suggested that the positive effect of KNO_3 might be due to the prevention of culture medium acidification. Accordingly in this study, replacing the nitrogen source of SS medium by 0.3% w/v of casein resulted in significantly ($p \leq 0.05$) increased laccase production approximately 2.13 folds when compared the control medium containing yeast extract and peptone. Organic nitrogen sources including both casein, and peptone appeared to be the superior nitrogen sources whereas ammonium and nitrate ion appeared to be inferior nitrogen sources for laccase production.

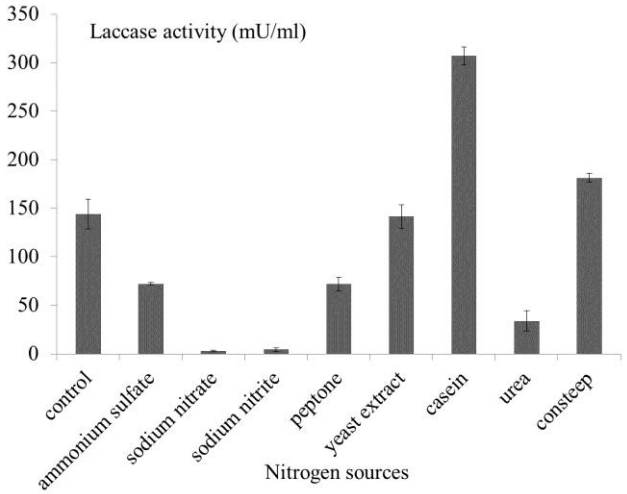

Figure 3. Effect of nitrogen sources on the laccase activity

Growth support matrix

Medium containing pieces of growth support matrix of commercial sponge, rice straw, coconut husk, and eucalyptus supported aerial mycelia formation. Rice straw and eucalyptus gave laccase activity (mU/ml) of 154.16±18.13 and 155.87±15.05 significant ($p \leq 0.05$) same as the control (143.99.18±15.02) while coconut husk stimulated significant highest levels of laccase at 261.25±25.58 (Figure 4). Karim and Annuar (2009) summarized that coconut husk is a natural inducer for the production of laccase and one of the potential candidate can served simultaneously as a support matrix for fungal growth. Comparison of the result with those previously reported by *P. sanguineus*, indicated that high laccase levels was obtained when the natural supporters included coconut husk and grey sponge were used (Low et al., 2009). Rosales and collaborators (Rosales et al., 2002) had similar results, natural growth support matrix of potato peeling induced a higher laccase activity than synthetic support matrix of sponge or even lack of support matrix In tests with white rot fungus of *Tamete hirsuta*. The increase in yield resulting by the used of growth support matrix might results from natural nutrients and inducers released into the medium the breaking down of cellulose, hemicellulose, and lignin (Low et al., 2009). According to the results obtained in the present work, coconut husk have an enormous potential as supports for laccase production by *G. lucidum* UPAG08.

The kinetic parameters of laccase

There are several reported that different buffer system influenced on laccase activity included citric acid-sodium phosphate buffer (Han et al., 2006) sodium citrate buffer (Zhang et al., 2006), sodium succinate buffer (Simonic et al., 2010), sodium acetate buffer (Zille et al., 2005) and sodium tartrate buffer (Dhouib et al., 2005, Eggert et al., 1996). The highest laccase activity of 42.25±3.57 mU/ml was achieved in sodium citrate buffer pH 5.0 (significant, $p>0.05$).

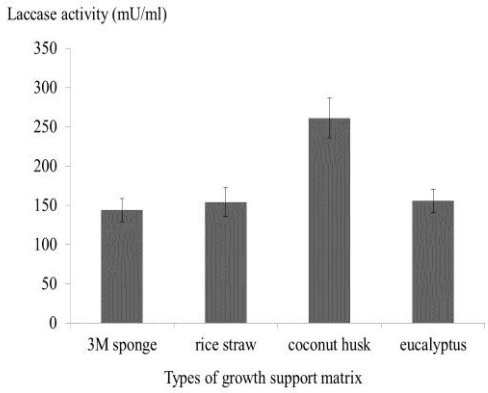

Figure 4. Effect of growth support matrix on the laccase activity

For pH stability, the enzyme showed high relative activity retained after 48 hr of incubation in several buffer systems pH between 4.5 to 6.5 (Figure 5). The white rot fungus of *G. lucidum* UPAG08 crude laccase acitivity showed the relative activity similar curve as compared with*T. versicolor* 951022 and *Ceriporiopsis subvermispora* (Han et al., 2005). In high acidic and basic condition under pH 3.5 or over pH 8.0, the activity of laccase was inactivated. The resulting might be related to the enzyme was terminated at acidic condition or binding of the hydroxide ion with the specific site at basic condition. Liu and collaborators supported this results and showed laccase from *P. ostreatus* strain 10969 would found to be inactivated enzyme when pH was increased to 8.0 or decreased to 3.0 (Liu et al., 2009).

The kinetic parameters (*Km* and *V*max) were investigated and ABTS is considered a specific substrate for laccase more than 2, 6-DMP. *Km* values were found to be 0.08 mM and 0.64 mM when each ABTS and 2, 6-DMP was used as the substrate respectively. *V*max value was obtained with ABTS (0.12 $\mu mol.min^{-1}.ml^{-1}$) when compared with 2, 6-DMP (0.07 $\mu mol.min^{-1}.ml^{-1}$). The parameter results were observed similarity as laccase from *C. rigida* (Saparrat et al., 2002) and *G. lucidum* (Jeon et al., 2008). On the other hand, *T. hirsute* IMA2002 showed 2, 6-DMP as specific substrate for laccase activity more than ABTS (Almansa et al., 2004). For this observation, selection of the appropriate storage solvent (sodium citrate) and specificity with substrate of ABTS seems to be crucial for laccase in order to maintain high activity of laccase for both storage and biodegradation pretreatment process.

De-colorization of anthaquinone dye of RBBR

The oxidation capacity was associated with crude laccase (20mU) from *G. lucidum* UPAG08 which was able to decolorize dark blue chromogenic dye of RBBR in 48 hr. Results were observed with a decrease of maximum absorbance of the visible peak between 550-750 nm. Laccase without ABTS mediator was not decolorized the color of RBBR dye while laccase with ABTS mediator showed high capacity of 51.14±6.53% dye de-colorization after 48 hr of reaction at $30^{O}C$ when observed at the visible peak of ABTS absorption (595 nm) (Figure 6). Supporting these results, *G. lucidum* UPAG08 crude laccase needs mediator and showed high efficiency in decolorizing RBBR simiarility as the result from *P. cinnabarinus* and *T. villosa* (Camarero et al., 2005).

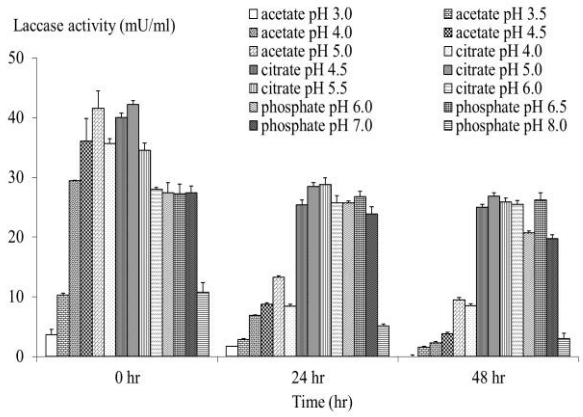

Figure 5. Effect of pH and stability on the laccase activity

Moreover, the addition of a redox mediator was necessary for dyes de-colorization by a commercial laccase (Soares et al., 2001, Wang et al., 2011). The results suggest that crude laccase from *G. lucidum* UPAG08 have potential use as biological pretreatment of anthaquinone dye of Remazol Brilliant Blue R (RBBR).

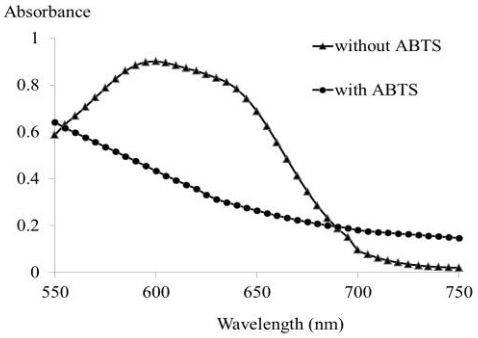

Figure 6. Visible absorbance peak of laccase to de-colorize RBBR within 48 hr

CONCLUSIONS

Many researchers have found that laccase production were dependent on the composition of media nutrients included carbon, nitrogen and other supplemented sources. Like other *Ganoderma* species, *G. lucidum* UPAG08 produced high laccase activity when cultivation under static culture condition at 30 ^{O}C. The hightest laccase activity was observed in SS medium containing carbon source of sorbitol, nitrogen source of casein, 20 pieces of mycelia agar plug and 21 pieces of growth support matrix (coconut husk) and incubated in 16.36 days.

It is apparent from the present studies that the laccase from *G. lucidum* UPAG08 might be used in several industries because the enzyme has a wide range of temperature and pH stabilities and can de-colorize anthaquinone dye of Remazol Brilliant Blue R (RBBR) which was generally used in textile industries.

ACKNOWLEDGEMENTS

This research was partially supported by budget research grants ID 255803023 of 255801012 from National Research Council of Thailand (2014) and the Unit Excellence of Biomass Utilization for Beef Cattle Production, University of Phayao (2015). Additionally we thank Mr. Kawintra Khangkhan, Mr. Anut Khattisa and Mr. Tosawat Anorach, member of Faculty of Agriculture and Natural Resources, University of Phayao for technical advice and assistance.

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