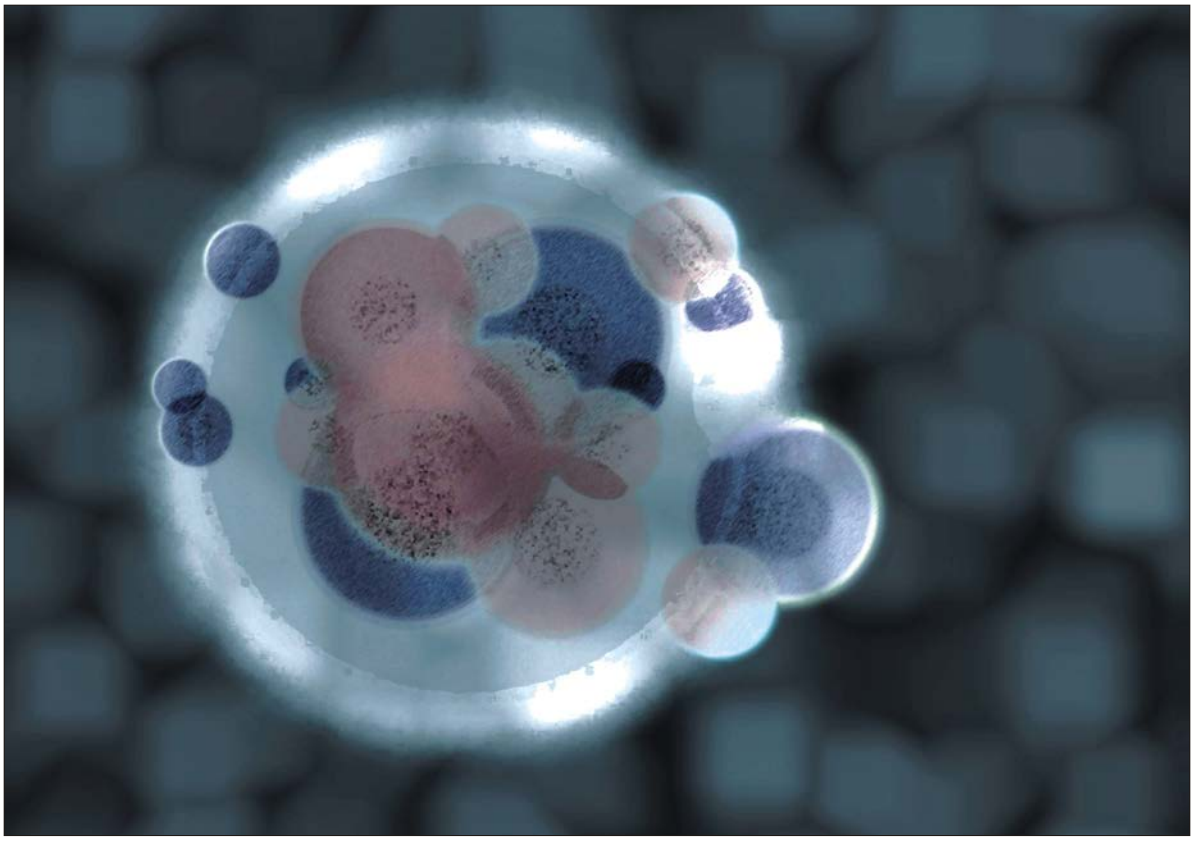

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Laccase Activity from Fresh Fruiting Bodies of *Ganoderma* sp. MK05: Purification and Remazol Brilliant Blue R Decolorization

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Abstract: The primary aim of this study was to screen for laccase activity from various mushroom fruiting bodies found in Maha Sarakham and nearby provinces of Northeast of Thailand. The enzyme activity was followed by a spectroscopic property of an oxidized product of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm. The results found that crude water extract of mushroom *Ganoderma* sp. MK05 gave the highest specific activity of laccase. Consequence aim was to partial purification of the crude water extract of the selected mushroom and to evaluate the dye decolorization activity. The crude enzyme was partial purified by ammonium sulfate fractionation in the range of 40-70% saturation and followed with DEAE-cellulose anion exchange column to 1.34 and 3.07 purification folds, respectively. Native gel electrophoresis result showed one protein band with laccase activity. The partial purified laccase from this mushroom showed a high efficient decolorization ability of anthraquinone dye, Remazol Brilliant Blue R with above 80% within 5 h at 30°C.

Key words: White rot fungi, ligninolytic enzyme, oxidoreductase, synthetic dye, anthraquinone dye, biotechnology

INTRODUCTION

Laccases (p-diphenol: O_2 oxidoreductase; EC 1.10.3.2) catalyze the oxidation of a wide range of inorganic and aromatic compounds, particularly phenols, while reducing molecular oxygen to water, are of great biotechnological interests. They found high potential applications in biobleaching of kraft pulp (Camarero *et al*., 2007; Gutierrez *et al*., 2007), recalcitrant synthetic dye decolorization (Camarero *et al*., 2005) and detoxification (Dodor *et al*., 2004; Wu *et al*., 2008), organic synthesis (Ncanana and Burton, 2007) etc. These enzymes are multi-copper oxidases which widely distributed among plants, fungi and bacteria.

Most reports laccases are from white rot fungi, especially basidiomycetous fungi such as *Pleurotus ostreatus* (Palmieri *et al*., 2005), *Trametes trogii* (Mechichi *et al*., 2006), *Hericium erinaceum* (Wang and Ng, 2004a), *Tricholoma giganteum* (Wang and Ng, 2004b), *Cantharellus cibarius* (Ng and Wang, 2004), *Trametes versicolor* and *Phanerochaete chrysosporium* (Fragoeiro and Magan, 2008), *Lentinula edodes* (Hong *et al*., 2008). Several reports describe laccases as extra cellular enzymes, whereas only a few has been reported as intra cellular enzymes as in the mushroom fruiting bodies. The laccases were isolated from fruiting bodies such as *Lentinula edodes* (Nagai *et al*., 2003), *Cantharellus cibarius* (Ng and Wang, 2004), *Hericium erinaceum* (Wang and Ng, 2004a), *Albatrella dispansus* (Wang and Ng, 2004c), *Ganoderma lucidum* (Wang and Ng, 2006a) and *Pleurotus eryngii* (Wang and Ng, 2006b). The enzymes-secreted and non-secreted forms even though comes from the same mushroom might not have the same characteristics and/or may have different physiological roles.

In Thailand, the mushroom diversity is one of the most valuable bio resources. Reports of laccases from mushroom fruiting bodies have only a few. Therefore, the main objective of this study was to report a screening of laccases from mushroom fruiting bodies found in Maha Sarakham and nearby provinces in Northeast of Thailand. The purification of selected mushroom has been set up. The potential application of partial purified laccase on synthetic dyes decolorization was also evaluated.

MATERIALS AND METHODS

Enzyme extraction: The mushroom fruiting bodies were collected from forest in Maha Sarakham and nearby provinces during June 2005 through September 2006. The fresh mushrooms were cleaned by washing with tab water and then blended with distilled water (10 mL distilled

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water per 1 g mushroom) using a blender. The liquid fraction was filtered through a double layer of cheese cloth to remove solid stuffs and then subjected to centrifugation at 3,000 rpm for 15 min to discard the fine precipitate. The supernatants (named crude water extracts) were then determined for laccase activity and protein concentrations.

Laccase activity assays and protein determination: Laccase activity was spectrophotometric assayed at 30°C of oxidized 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (blue green color cation radical) as previously described by Khammuang and Sarnthima (2007). Briefly, the assay mixture contained 0.1 mM ABTS and 0.1 M sodium acetate buffer (pH 4.5). Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). One unit of enzyme is the amount of enzyme required for catalyze 1 µmole substrate to product per minute at certain assay condition.

Protein concentration was determined by Bradford (1976) method using the Bio-Rad Protein Assay Reagent (Bio-Rad) and using bovine serum albumin (BSA) as a protein standard.

Partial purification and characterization: Crude extract of UM 01 (assigned name as *Ganoderma* sp. MK05), which showed highest laccase specificity, was subjected to purification processes. Firstly, to find the suitable salt saturation range for fractionation of laccase protein, the crude enzyme was fractionated in a sequence saturation of ammonium sulfate ($[NH_4]_2SO_4$) from 0-20, 20-40, 40-60, 60-80 and 80-100% saturations. The salt was gradually added into the crude enzyme until reaching a desired saturation with simultaneously gently stirred at 4°C for 30 min each. After centrifugation at 10,000 rpm, 30 min at 4°C, the precipitate was collected and the supernatant was further added salt to the higher salt saturations. The precipitation and centrifugation steps were repeated until finishing an 80-100% saturation step. Each precipitate fractions were re-suspended in a small volume of 0.1 M sodium acetate buffer (pH 4.5), salt removing via. dialysis before laccase activity assay and protein determination. The laccase specific activities in each salt fractionation were compared to select the most optimum range for fractionation laccase protein from the rest of crude enzyme.

For the rest of the crude enzyme, it was fractionated by ammonium sulfate to 40% saturation, stirred for 4 h. After centrifugation at 10,000 rpm, 15 min, the precipitate was discarded whereas the supernatant was further added salt to reach 70% saturation, stirred overnight. Otherwise state, all steps were performed at 4°C. The salt fractionated enzyme was subjected to centrifugation again (10,000 rpm, 30 min) to get a precipitate and then re-suspended in a small volume of 0.1 M sodium acetate buffer (pH 4.5) prior to enzyme activity assay and protein determination. After dialysis against starting buffer, 0.02 M sodium phosphate buffer (pH 7.4) to remove salt and lower ionic strength, the dialyzed fraction was loaded onto a DEAE-cellulose anion exchange column which pre-equilibrated with the starting buffer. The unbound proteins were washed out from the column by the washing buffer (0.02 M sodium phosphate buffer, pH 7.4), whereas the bound proteins were then eluted out by a linear gradient of 0-1.0 M NaCl in the starting buffer. Fractions contained laccase activities were pooled, concentrated by ultra filtration devices at a MWCO 10 kDa. Polyacrylamide gel electrophoresis (PAGE) of a discontinuous gel (12% separating gel, Tris-HCl pH 8.8 and 5% stacking gel, Tris-HCl pH 6.8) was performed to analyze the purity and enzyme activity pattern.

***In vitro* dye decolorzation:** Remazol brilliant blue R (RBBR), anthraquinone dye (chemical structure as shown in Fig. 1) was used as a synthetic dye model for evaluate the decolorization ability of laccases from the *Ganoderma* sp. MK05. The dye was purchased from Sigma, USA. To find the optimum pH on RBBR decolorization, the reactions were investigated at various pH conditions from acidic to basic. The reactions were experimented in 0.05 M of phosphoric buffer (pH 3.7), sodium acetate buffer (pH 4.5, 5.5) and Tris-HCl buffer (pH 7.0, 8.0). After that, the effect of dye concentration on decolorization ability was studied at various RBBR concentrations (4, 8, 12 and 24 mg L^{-1}) at its optimum pH by the partial purified laccase (0.05 U mL^{-1}). The enzymatic treatments were monitored a decrease in maximum absorbance of the dye at wavelength 592 nm. All experiments were performed duplicate at 30°C in an incubator water bath. Decolorization of RBBR by the partial purified laccase from *Ganoderma* sp. MK05 was expressed in terms of percentage calculated according to the Eq. 1.

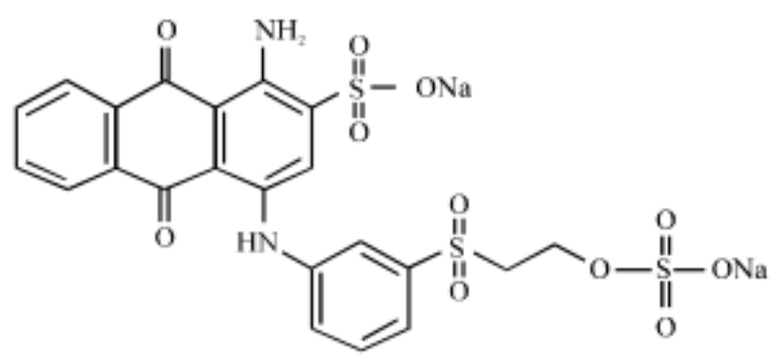

Fig. 1: Chemical structure of Remazol brilliant blue R

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

where, A_0 is an absorbance at 592 nm immediately measured after adding the enzyme solution and A_t is an absorbance at 592 nm after each time intervals.

RESULTS AND DISCUSSION

Laccase activities were screened from water crude extracts of 24 mushroom fruiting bodies. The crude enzyme of mushroom *Ganoderma* sp. MK05 (Fig. 2) showed the highest specific activity, thus the crude enzyme of this mushroom was subjected to purification processes. Most protein containing laccase activity was salting out around 40-60% $(NH_4)_2SO_4$ saturation (Table 1). Therefore, the rest of crude enzyme was subjected to further purify by $(NH_4)_2SO_4$ fractionation in a range of 40-70% saturation as described in partial purification and characterization. This precipitation step gave the laccase enzyme with a 1.34 fold purification, but about 75% of starting activity was lost (Table 2). The salt fractionate enzyme was desalted and then passed through a DEAE-cellulose anion exchange column. The enzymes were adsorbed on DEAE-cellulose in 0.02 M sodium phosphate buffer (pH 7.4) and were eluted out with 0.1-0.2 M NaCl in the starting buffer (Fig. 4). The adsorption of *Ganoderma* sp. MK05 laccase on DEAE-cellulose is in line with the report that laccase from mushroom *Hericium erinaceum* (Wang and Ng, 2004a), but in contrast to the inability of laccases from *Tricholoma giganteum* and *Cantharellus cibarius* to bind to DEAE-cellulose as reported by Wang and Ng (2004b) and Ng and Wang (2004).

The laccase active fractions (D0-2) were pooled, concentrated and desalted by ultrafiltration and dialysis techniques. The laccase was purified about 3.07 fold in a yield of 13.6% (Table 2). Native-PAGE of salt fractionation enzymes (Fig. 3b) and the last step purified enzyme showed one single band of activity stained by its substrate ABTS (Fig. 5b), but with at least three protein bands stained with coomassie brilliant blue R-250 (Fig. 3a, 5a). The results indicated that the enzyme was only partial purify and had only one laccase enzyme.

The effect of pH on dye decolorization was studied in 0.05 M of various buffer conditions (phosphoric buffer pH 3.7, sodium acetate buffer pH 4.5, 5.5, Tris-HCl buffer pH 7.0, 8.0). Among tested pH values, the decolorization of RBBR was affected by pH to a great extent. RBBR was most decolorized (80.8% within 5 h) at pH 5.5, below or above which the decolorization percentage decreased remarkably (Fig. 6a).

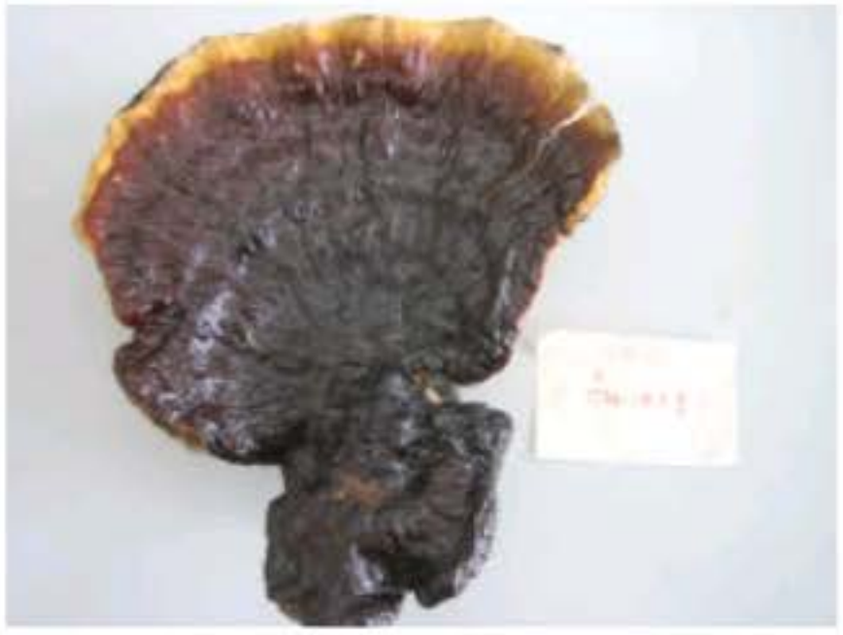

Fig. 2: *Ganoderma* sp. MK05 mushrooms collected from Maha Sarakham Province (Northeast of Thailand) used for partial purification of laccase enzyme. The crude enzyme extracted by distilled water had a laccase activity 2.472 U mL^{-1}, protein 2.76 mg mL^{-1} and with a specific activity of 0.897 U mg^{-1}

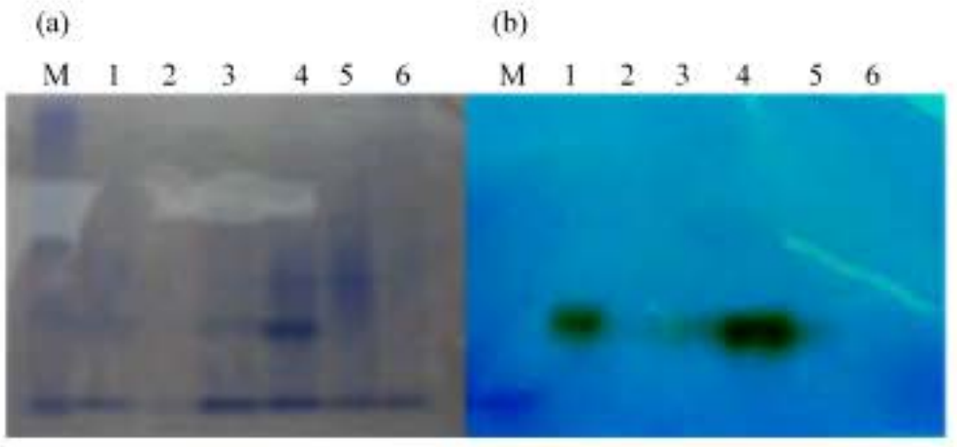

Fig. 3: Native-PAGE of various $(NH_4)_2SO_4$ fraction of *Ganoderma* sp. MK05. Stained with coomassie Blue R-250 (a) and a zymogram with ABTS substrate (b) M, protein marker; 1, crude enzyme; 2, 80-100% sat. (10 µg); 3, 60-80% sat. (10 µg); 4, 40-60% sat. (10 µg); 5, 20-40% sat. (10 µg); 6, 0-20% sat. (10 µg)

Table 1: Laccase activity of different ammonium sulfate precipitation of *Ganoderma* sp. MK05

Saturation (%)	Activity (U mL^{-1})	Proteins (mg mL^{-1})	Specific activity (U mg^{-1})	Fold
Crude enzyme	2.472	2.76	0.895	1.00
0-20	0.137	2.57	0.053	0.06
20-40	0.185	3.27	0.056	0.06
40-60	4.958	2.77	1.790	2.00
60-80	0.333	1.22	0.272	0.30
80-100	0.014	0.10	0.140	0.15

The effect of pH on RBBR decolorization from the present study was similar to those observations for many other fungal laccases. RBBR decolourisation by *P. ostreatus* laccases gave the highest capability at pH 4.0 (Palmieri *et al*., 2005) as similar as those obtained

Table 2: Purification table of laccase purification from fruiting body of *Ganoderma* sp. MK05 mushroom (52.99 g)

Step	Vol. (mL)	Activity (U mL^{-1})	Protein (mg mL^{-1})	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Fold	Yield (%)
Crude enzyme	368.00	0.268	0.35	98.624	128.80	0.766	1.00	100.00
40–70% sat. ppt	16.00	1.491	1.45	23.856	23.20	1.028	1.34	24.19
D0 pooled	1.54	0.487	0.25	0.750	0.38	1.948	2.54	0.76
D1 pooled	1.24	4.672	2.28	5.793	2.83	2.049	2.67	5.87
D2 pooled	2.68	5.005	2.13	13.413	5.71	2.349	3.07	13.60

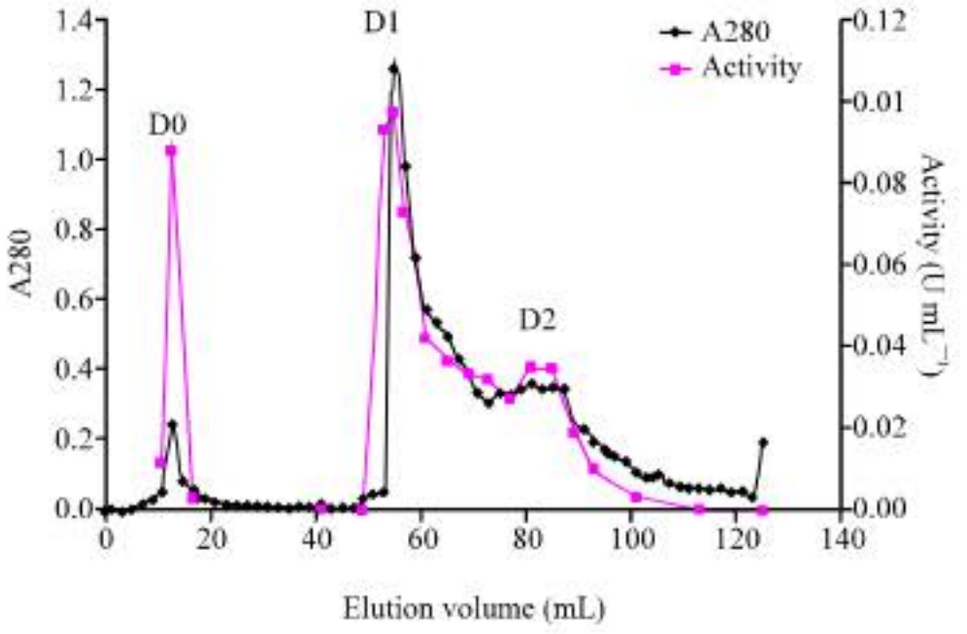

Fig. 4: DEAE-cellulose column chromatography profile of a 40-70% saturation $(NH_4)_2SO_4$ fraction of *Ganoderma* sp. MK05. Washing buffer: 0.02 M sodium phosphate buffer (pH 7.4); elution buffer: 0-1.0 M NaCl in 0.02 M sodium phosphate buffer (pH 7.4); flow rate: 0.5 mL min^{-1}

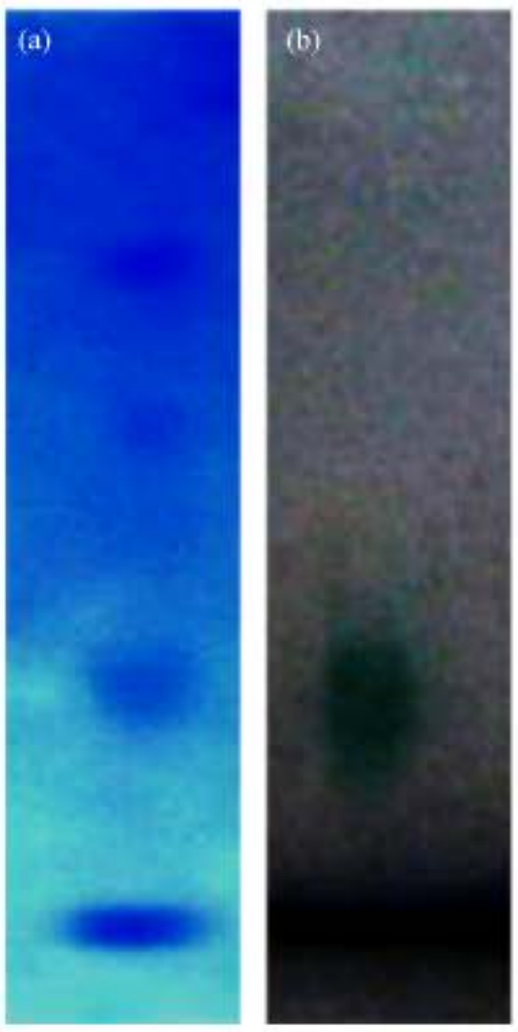

Fig. 5: Native-PAGE of the partial purified laccase from *Ganoderma* sp. MK05. (a) Stained with coomassie brilliant blue R-250 and (b) Stained with ABTS substrate

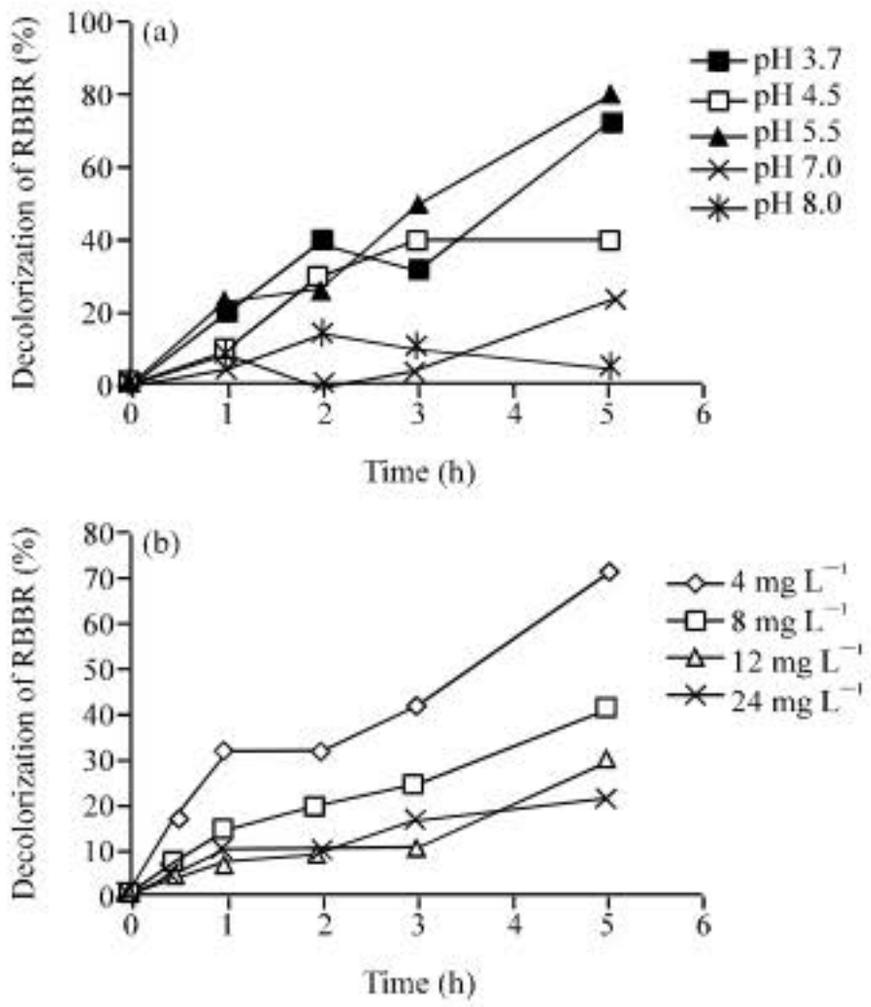

Fig. 6: RBBR decolorization percentage by the partial purified laccase from *Ganoderma* sp. MK05 at 30°C. The reactions contained the enzyme 0.05 U mL^{-1}, (a) Treated at various pH values (phosphoric buffer pH 3.7, sodium acetate buffer pH 4.5, 5.5, Tris -HCl buffer pH 7.0, 8.0). (b) Treated in 0.05 M sodium acetate buffer (pH 5.5) using various dye concentrations

by *Lentinus polychrous* Lev. from the earlier report (Khammuang and Sarnthima, 2007). On the other hand, optimal decolorization rates occurred at pH 5.0 when the dye solution was treated with laccase from *Trametes trogii* (Mechichi *et al*., 2006). The decolorization of RBBR was also effect by the proportion of enzyme and initial dye concentration (Fig. 6b). The potential in synthetic dyes decolorization of this laccase suggests that further purify and full characterization is needed. In addition, industrial and environmental applications should also be further investigated, together with mediators of interest, especially for enzymatic degradation of toxic recalcitrant dyes or aromatic pollutants. Liquid culture fermentation of the fungus and decolorization by the cultures are underway studying in our laboratory.

CONCLUSION

The crude water extract of fresh *Ganoderma* sp. MK05 fruiting body was partial purify by fractionation of 40-70% saturation $(NH_4)_2SO_4$ and followed by DEAE-cellulose anion-exchange chromatography. The laccase from fruiting body of this mushroom has one protein. The partial purified enzyme showed a high efficiency in RBBR decolorization with above 80% at optimum condition (pH 5.5) within 5 h at 30°C. Due to only a low amount of laccase activity (0.05 U mL^{-1}) used in the effective decolorization experiment, make this enzyme is of interest to further purification, characterization and application on decolorization in other groups of synthetic dyes.

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