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Laccase from Spent Mushroom Compost of *Lentinus polychrous* Lev. and its Potential for Remazol Brilliant Blue R Decolourisation

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Abstract: Laccases from spent *Lentinus polychrous* Lev. mushroom compost were extracted, purified and characterized including evaluation of its potential in synthetic dye decolourisation. The enzyme was partial purified by ammonium sulfate fractionation, chromatography on DEAE-cellulose and Superdex 200 HR columns, respectively. The final step purification provided an 18.5% yield and a purification of 48.4 fold. The results from SDS-PAGE indicated that the laccase had a molecular weight of about 32 kDa. The enzyme's pH optimum was pH 3.0 when using 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) as substrate and the optimum temperature was 55°C. The decolourisation of a model dye, Remazol Brilliant Blue R (RBBR), by the partial purified enzyme was investigated. The results showed that decolourisation ability was highest at about 66% within 3.5 h at pH 4.0.

Key words: Characterization, laccase, *Lentinus polychrous* Lev., purification, RBBR, decolourisation

INTRODUCTION

Laccases (EC 1.10.3.2; benzenediol:oxygen oxidoreductases) are multi-copper oxidases that catalyze the one electron oxidation of several aromatic substrates with the simultaneous reduction of dioxygen to two molecules of water (Piontek *et al*., 2002). It is a major enzyme in fungal ligninolytic systems involved in lignin degradation. The substrate ranges of these enzymes are fairly broad including polyphenols, aromatic amines, polycyclic aromatic hydrocarbons and synthetic dyes (Solomon *et al*., 1996). Moreover, other non-phenolic compounds and radical mediators such as N-hydroxybenzothiazone and 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) are also reported as substrates. Because of its catalytic properties and broad substrate specificity, laccase has great potential applications including pulp delignification (Bajpai, 1999; Wong *et al*., 2000; Record *et al*., 2002), dye decolourisation, waste water and contaminated soil treatment (Collins *et al*., 1996; Campos *et al*., 2001; Soares *et al*., 2001; Ahn *et al*., 2002; Wesenberg *et al*., 2003; Dias *et al*., 2004; Palmieri *et al*., 2005; Mechichi *et al*., 2006), as well as uses in organic synthesis (Mustafa *et al*., 2005; Ncanana and Burton, 2007).

In commercial cultivation, mushrooms are grown on various ligninolytic substrates. After harvesting, a considerable amount of residual compost is discarded as a by-product. This residue may therefore become a potential source of ligninolytic enzymes (Ball and Jacksohn, 1995; Trejo-Hernandez *et al*., 2001; Law *et al*., 2003). *Lentinus polychrous* Lev., a nationwide distributed white-rot basidiomycete, is one of the commercial mushrooms of interest in Thailand. Our Laboratory suggests that the mushroom might be a good producer of laccases and other ligninolytic enzymes including Mn peroxidase and Mn-independent peroxidases (unpublished data). These fungal extracellular enzymes might be very useful for a wide range of applications in the near future. The objective of this study was to examine the laccase activity in spent compost of *L. polychrous* Lev. The research describe here the purification procedure, some characterization and the potential use of the enzyme in environmental applications, especially the enzymatic decolourisation of synthetic recalcitrant dyes.

MATERIALS AND METHODS

Fungal strain and preparation of crude enzymes: White rot fungus, *L. polychrous* Lev. was grown under conditions of solid-state fermentation of a commercial mushroom growth conditions for about 9 months (during August, 2005-April, 2006) at a local farm in Maha Sarakham province, Northeastern, Thailand. Preparation

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and purification as well as some characterization were performed at Protein and Enzyme Technology Research Unit, Faculty of Science, Mahasarakham University. Crude laccases were prepared by extraction with 0.1 M sodium acetate buffer, pH 4.5 under magnetic stirring for 30 min at room temperature (1 g solid substrate/5 mL buffer) and filtration through sheet cloth. The filtrate was then centrifuged at 3,000 rpm for 20 min.

Laccase activity assay: The routine assay for laccase was based on the oxidation of 2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) (Sigma). A modified method of Shin and Lee (2000) was used. Briefly, 50 μL of the enzyme solution was incubated in 940 μL of 0.1 M sodium acetate buffer (pH 4.5) containing 10 μL of 10 mM ABTS at 30°C for 10 min. The reaction mixture was stopped by adding 50 μL of 50% (w/v) Trichloroacetic acid (TCA). Oxidation of ABTS was monitored by spectrophotometer at 420 nm ($\varepsilon = 3.6\times10^4\ M^{-1}\ cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol/min of substrate under assay conditions.

Enzyme purification: The crude laccases were fractionated by $(NH_4)_2SO_4$ in the range of 50-85% saturation. The filtrate was centrifuged at 12,000 x g for 30 min. The precipitate was re-suspended in small volume of 20 mM Tris-HCl buffer (pH 6.5) and dialyzed against large volume of 20 mM Tris-HCl buffer (pH 6.5) using dialysis tubing with a molecular weight cut off of about 4,000 Da. The crude enzymes were then passed through a column (1.6×7.0 cm) of DEAE-cellulose (Whatman) equilibrated with 20 mM Tris-HCl buffer (pH 6.5). Unadsorbed proteins were eluted with the starting buffer, while adsorbed proteins eluted with a linear gradient of 0-0.7 M NaCl in the starting buffer. Laccase activity fractions were pooled, concentrated using Amicon Ultra-15 (10,000 MWCO) and centrifuged at 5,000 rpm, for 20 min at 25°C. The concentrated DEAE fractions were next subjected to gel filtration chromatography on Superdex 200 HR (Amersham Biosciences) column (1.6×65 cm) equilibrated with 0.15 M NaCl in 0.1 M sodium acetate buffer (pH 4.5) and eluted at a flow rate of 0.5 mL min^{-1}. Fractions contained laccase activity were pooled, concentrated and kept at 4°C.

Protein determination: Protein concentrations were determined according to Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad) and Bovine Serum Albumin (BSA) was used as the standard.

Enzyme characterization: Native and denaturating PAGE were used for purity analysis. Laccase activities were detected by incubating the gel at 30°C in 0.1 M sodium acetate buffer (pH 4.5) containing 1 mM ABTS. The SDS-PAGE was carried out in accordance with the procedure of Laemmli (1970), using a 15% resolving gel and a 5% stacking gel. After electrophoresis, the protein bands were visualized by coomassie brilliant blue R 250 staining. The molecular weights of proteins were estimated according to Rainbow Molecular Weight Marker (high range) (Amersham Biosciences).

Laccase activity was measured at 30°C in the pH range 2.0-8.0 (0.1 M McIlvain buffer). The laccase activity was also determined at various temperatures between 30 and 80°C at the standard assay pH 4.5.

Decolourisation of Remazol Brilliant Blue R (RBBR): Dye decolourisation capability of the partial purified laccase from *L. polychrous* Lev. was monitored with 5 mg L^{-1} dye concentration at different pH values ranging from 2.0 to 8.0 (0.1 M McIlvain buffer) at 30°C and reaction was started with the enzyme 0.05 U mL^{-1}. The reaction mixtures were monitored by following the decrease in absorbance of RBBR (λmax; 592 nm) using a JENWAY 6400 Spectrophotometer (LABQUIB, England). Experiments were monitored immediately after enzyme adding and after that every 30 min incubation. Dye decolourisation was expressed in terms of percentage calculated according to the equation.

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

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

Enzyme extraction and purification: The spent mushroom compost after *L. polychrous* harvest was evaluated as a source of one lininolytic enzymes named laccase. Because of this natural substrate, extracellular laccase may play different roles for mushroom growth. As expected, some amounts of laccases were found in the residual compost. An extract prepared from 0.2 kg residual compost in 1 L of buffer solution contained laccase activities 27.0 U equivalent to specific compost activities of 0.135 U g^{-1} of dry residual compost or 0.225 U mg^{-1} proteins. The crude enzymes were fractionated with $(NH_4)_2SO_4$. This precipitation step gave the laccase enzyme with a 3 fold purification, but about a half of activity was lost. The precipitate fraction was then purified on a DEAE-cellulose column. The laccases were found to bind to the

Table 1: Purification of laccase from *Lentinus polychrous* Lev. (200.0 g)

Purification steps	Vol. (mL)	Act. (U mL^{-1})	Total Act. (U)	Prot. (mg mL^{-1})	Total prot. (mg)	Specific Act. (U mg^{-1})	Purification (fold)	Yields (%)
Crude enzyme	1,000.00	0.027	27.00	0.120	120.000	0.225	1.00	100.0
50-85% ppt	34.00	0.414	14.08	0.605	20.570	0.684	3.04	52.2
DEAE_conc.[a]	3.80	2.230	8.47	0.608	2.310	3.667	16.30	31.4
GF_conc.[b]	1.10	4.650	5.02	0.419	0.460	10.890	48.41	18.6

[a]: DEAE pooled fractions and [b]: Gel filtration pooled fractions after concentrated by ultrafiltration, respectively

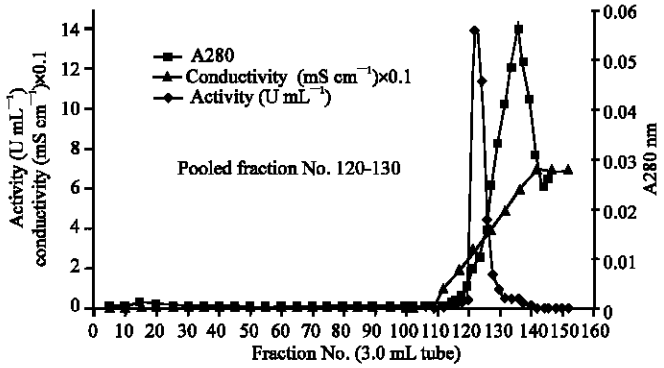

Fig. 1: An anion exchange chromatography of 50-85% saturation of *Lentinus polychrous* Lev. on DEAE-cellulose. Elution of adsorbed proteins was carried out with a linear gradient of 0-0.7 M NaCl in 20 mM Tris-HCl buffer (pH 6.5), flow rate 30 mL h^{-1}

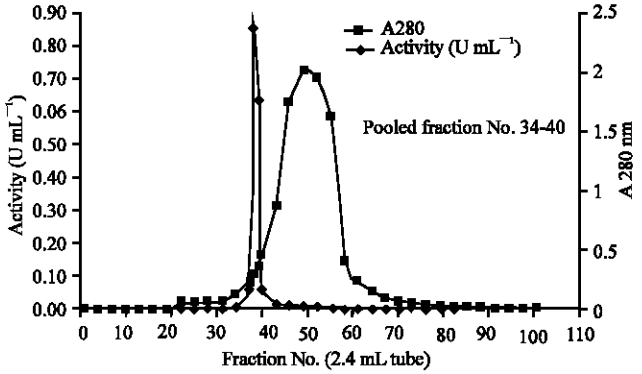

Fig. 2: Gel filtration profile on Superdex 200 HR of DEAE fraction. Eluent: 0.15 M NaCl in 0.1 M sodium acetate buffer (pH 4.5). Flow rate: 30 mL h^{-1}. Fraction size: 2.4 mL

anion-exchange media and were eluted out during ionic strength of 0.1 to 0.3 M NaCl in the starting buffer (Fig. 1). From this step, the laccase was purified 16.3 fold in a yield of 31.4% (Table 1). The activity peak from the Superdex 200 HR column was collected at an elution fraction between 34 and 40 (2.4 mL each). Peak activity not perfectly coincided with peak protein concentration (Fig. 2). The active fractions had the characteristic blue-green color after reacted with substrate ABTS, these were pooled and concentrated. Comparison of total dry matter of the final preparation with total protein obtained by the methods of Bradford (1976) indicates high purity of laccase was obtained with the specific activity of the final preparation, 10.89 U mg^{-1}, 18.5% yield and a purification of 48.4 fold (Table 1).

Enzyme characterization: Purity of the enzyme protein was confirmed by PAGE-electrophoresis both native and denaturing conditions. SDS-PAGE showed one major band with a molecular weight of about 32 kDa (Fig. 3a) by coomassie stained. The laccase activity staining in non-denaturing PAGE was detected in a pattern of bands exactly corresponding to those detected by protein staining (Fig. 3b). The laccase of *L. polychrous* Lev. was

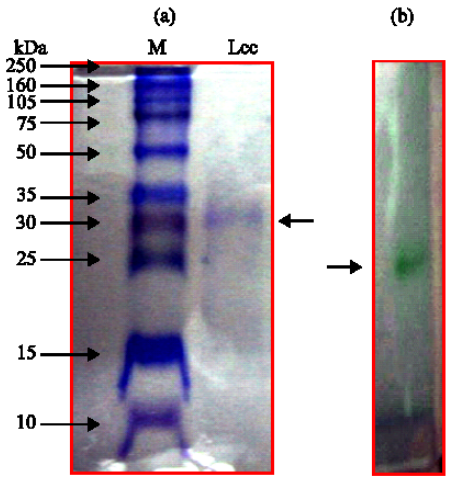

Fig. 3: Electrophoresis of purified laccase from *Lentinus polychrous* Lev. (a) SDS-PAGE. Left lane: molecular mass markers, Right lane: partial purified laccase (b) Native-PAGE, stain with ABTS

Table 2: Effect of pH on laccase activity: 0.1 M McIlvain buffer was used for assay at 30°C

pH	Relative activity (%)
2.0	61.3
3.0	100.0
4.0	76.4
5.0	51.3
6.0	39.7
7.0	16.0
8.0	1.9

Data are the values obtained in duplicate assays

Table 3: Effect of temperature on laccase activity: 0.1 M sodium acetate buffer (pH 4.5) was used for assay

Temperature (°C)	Relative activity (%)
30	63.8
40	67.0
45	72.0
50	83.0
55	100.0
60	86.7
65	80.0
70	36.0
80	9.9

Data are the values obtained in duplicate assays

assayed at different pH values using ABTS substrate to determine the effect of pH on laccase activity. The pH profile showed optimum activity at pH 3.0 and the laccase was inactive at pH 8.0 (Table 2). The optimum temperature for its activity was 55°C (Table 3). The activity of the laccase slightly increases as the ambient temperature is raised from 30 to 55°C. The increase in enzyme activity was about 40%. The enzyme was susceptible to thermal denaturation at 80°C and almost all enzyme activity disappeared after 10 min (less than 10% activity remained).

Table 4: Percentage Decolourisation of RBBR after treated with the partial purified laccase from *Lentinus polychrous* Lev. at 30°C

	Decolourisation of RBBR[b] (%)				
pH[a]	30 min	60 min	90 min	150 min	210 min
2.0	9.6	11.6	14.0	16.6	18.3
4.0	30.0	44.4	57.5	62.3	65.8
6.0	11.4	16.4	19.5	24.0	25.8
8.0	0.0	0.4	0.8	1.2	1.9

[a]: 0.1 M McIlvain buffer; [b]: final dye concentration of 5 mg L^{-1} and the enzyme activity 0.05 U mL^{-1}. Data are the values obtained in triplicate assays

RBBR decolourisation by the purified laccase: Table 4 shows the percentage decolourisation of the model anthraquinone dye, RBBR by the partial purified laccase of *L. polychrous* Lev. Among the four tested pH values, the decolourisation was affected by pH to a great extent. From our experiments, RBBR was most decolorized 65.8% within 3.5 h at pH 4.0, below or above which the decolourisation percentage decreased remarkably.

DISCUSSION

Spent mushroom compost of *L. polychrous* Lev. contains ligninolytic enzyme, laccase, which might be of use in various applications. In this study, we reported the purification and some characterization of laccase from *L. polychrous* Lev. in order to define its enzymatic properties and compare it to other fungal laccases. The laccase was purified to about >90% homogeneity by $(NH_4)_2SO_4$ fractionation (50-85% saturation), DEAE-cellulose anion exchange chromatography column and gel filtration on Superdex 200 HR, respectively. The laccase was adsorbed on DEAE-cellulose column and eluted out with about 0.1-0.3 M NaCl in the buffer. The laccase has a molecular weight about 32 kDa. The molecular mass agrees with the mass of the laccase-like stilbene oxidase produced by *Botrytis cinerea* Pers.: Fr. (Pezet, 1998) and appears to be lower than that of other reported laccases from white rot basidiomycetes which are in the range of 55-65 kDa (Eggert *et al*., 1996; Munoz *et al*., 1997; Cambria *et al*., 2000; Shin and Lee, 2000; Garzillo *et al*., 2001). The enzyme has an optimum pH at pH 3.0 and a temperature optimum at 55°C. The optimum pH when ABTS as substrate was similar to that of the laccase from *Pleurotus ostreatus* (Palmieri *et al*., 1997, 2003), *Rigidoporus lignosus* (Cambria *et al*., 2000). Its optimum temperature was also similar to that of the laccase-like stilbene oxidase produced by *Botrytis cinerea* Pers.:Fr. (Pezet, 1998) and those of laccases from Pleurotus spp. (Palmieri *et al*., 1997, 2003). The enzyme shows a good percentage in decolourisation of RBBR at pH 4.0 and 30°C (65.8% within 3.5 h). The effect of pH on RBBR decolourisation from the present study is 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). On the other hand, optimal decolourization rates occurred at pH 5.0 when the dye solution was treated with laccase from *Trametes trogii* (Mechichi *et al*., 2006). The RBBR decolourisation ability of this laccase might be more improved if we can use in higher amount or purer enzyme. The enzyme amount in each experiment was progressive lower than other reports used. Therefore, large scale preparation of the enzyme is still required. Recently, a small molecular weight redox mediator was found to be effective at decolorizing of several synthetic recalcitrant dyes. For example, Soares *et al.* (2001) reported that decolourization of RBBR by laccase from *Aspergillus* microorganism (Novo Nordisk) showed a sharp increase in the presence of either 1-hydroxybenzotriazole (HOBT) or Violuric Acid (VA), whereas no decolourisation took place when the laccase alone was used. The characteristics of this laccase; acidic pH of catalytic activity and fairly high temperature optimum and its potential in synthetic dyes decolourisation, suggest that it should be further purified and fully characterized including native molecular weight, pI, substrate specificity, kinetics and stability. 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.

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