

Decolorization of rhodamine B and congo red by partial purified laccase from *Lentinus polychrous* Lév

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Abstract

This study reports decolorization of Rhodamine B and Congo red by a partially purified laccase from *Lentinus polychrous* Lév. in the presence or absence of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) as a redox mediator. The decolorization study showed that about 90% of Rhodamine B could be decolorized within 52 h, whereas Congo red could be decolorized more than 75% within 3 h. The most effective concentration of the redox mediator was 0.10 mM ABTS. The optimum pH for Rhodamine B decolorization was at pH 4.0-5.0 and optimum temperature was at 50 °C. The optimum pH for Congo red decolorization was at pH 6.0-7.0, but increasing temperature had less of an effect. The results and availability of the fungus indicated that laccase from *L. polychrous* Lév. has high potential for the treatment of waste dyes.

Keywords: Congo red, decolorization, laccase, *Lentinus polychrous* Lév., rhodamine B

Introduction

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) are part of a large group of enzymes that catalyze the one-electron oxidation of a vast number of phenol and non-phenol substrates. Molecular oxygen then serves as the terminal electron acceptor and becomes reduced to two molecules of water (Baldrian 2006). Laccases are found in eukaryotes and prokaryotes including fungi, plants, insects and bacteria (Mayer and Staples 2002). Laccases from fungi have been predominantly studied; where they are

believed to have a major role in lignin degradation. Their ability to use compounds, with irregular structures such as lignin, as a substrate means laccases have a broad spectrum of substrates. The broad substrate specificity means these enzymes have a wide range of applications, ranging from effluent decolorization and detoxification (Eduardo et al. 2003) to pulp bleaching (Widsten and Kandelbauer 2008), and removal of phenolics from wines (Minussi et al. 2002). There is currently much interest in the removal of colors from waste waters, synthetic dyes are extensively used in industrial dyeing and printing processes, and are released as industrial effluents. Decolorization of these dyes by chemical or physical (adsorption and precipitation methods, chemical degradation or photodegradation) is financially and often methodologically demanding (Eichlerova et al. 2005). However, enzyme-based dye decolorization methods have an advantage as there is minimal impact on the ecosystem, as well as low energy requirements (Michniewicz et al. 2008). The ability to oxidize substrates by laccases can be enhanced by the addition of redox mediators. The basis of the laccase-mediator concept is the use of low-molecular-weight compound, which once oxidized by the enzyme to a stable radical, act as a redox mediator, oxidizing other compounds that in principle are not substrates of laccase (Camarero et al. 2008).

Rhodamine B (Basic violet 10, C.I. 45170) is a fluorescent cationic dye which the dye type becomes more important in textile dyeing because of its more rigid structure than other organic dyes. Due to its cationic structure, it can apply for anionic fabrics which contain negative charges such as polyester fibers, wool, silk, and acrylic fibers. The dye is brilliant and most fluorescent among other synthetic dyes. Congo red is a diazo dye based on benzidine ([1,1'-biphenyl]-1,4'-diamines) which are known to be carcinogenic (Zollinger 2003). The development of waste water treatment biotechnology has been increasing interested due to its eco-friendly benefits. A recent study on the decolorization of Rhodamine B dye by the laccase mediator system (LMS) has shown that 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was the best redox mediator, giving the highest Rhodamine B decolorization by laccase from *Trametes versicolor* (Khammuang and Sarnthima 2009). The aim of this study was to evaluate the decolorization of synthetic dyes Rhodamine B (fluorone dye) and Congo red (diazo dye) by laccase produced by *L. polychrous* Lév. in the presence and absence of ABTS as a redox mediator.

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Materials and methods

Enzyme production and purification

Lentinus polychrous Lév. was collected from Rujira Mushroom Farm in Ka La Sin province (Northeast, Thailand), and was maintained at 4 °C on potato dextrose agar (PDA) plates with periodic subculture. The fungus mycelia plugs from active growing culture on a PDA plate (5-7 days) were cultivated for a month under solid-state fermentation on rice bran and rice husk (2:1 by weight) prepared according to the method of Sarnthima et al. (2009).

The fungal crude enzyme was extracted from solid culture using distilled water (extraction ratio of culture medium: distilled water, 1:3 g mL⁻¹) and stirred using a magnetic stirrer for 45 minute then filtrated through a gauze, centrifuged at 6,000 rpm for 10 min at 4 °C. Protein precipitation was performed on the supernatant obtained with ammonium sulfate ((NH₄)₂SO₄) at 0-40% and 40-85% saturation. The pellet was re-suspended in 0.1 M sodium acetate buffer (pH 4.5) and 40-85% suspended solution was applied to a Sephacryl S-300 column (GE Healthcare) pre-equilibrated with 0.15 M NaCl in 20 mM sodium acetate buffer (pH 4.5) with a flow rate of 0.3 mL min⁻¹. Proteins were eluted with the same buffer and the amount of protein was determined by measuring absorbance at 280 nm. The fractions containing laccase activity (tube no. 43-60) were pooled, dialyzed against 20 mM Tris-HCl buffer, pH 7.5 and then loaded on a DEAE-cellulose (Sigma) column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) with a flow rate of 3.0 mL min⁻¹. After washing away the unabsorbed proteins with the same buffer (about 10 column volumes), the absorbed proteins were eluted with a linear gradient of 0-0.7 M NaCl in the starting buffer. Fractions containing laccase activity (fractions 137-155) were pooled.

The purity of the enzyme in each step was checked by SDS-PAGE according to the method of Laemmli (1970) with a 12% separating gel and a 4% stacking gel. The gel was run at a constant volt (100 volts/gel) and protein bands were visualized using silver stain. The native-PAGE was also performed according to Ornstein (1964). After completion of the run, the gel was removed from the glass plates and soaked with 0.1 M sodium acetate buffer (pH 4.5) containing ABTS or syringaldazine for laccase activity detection and a combination of 3-methyl-2-benzothiazolinone hydrazone (MBTH), 3-(dimethylamino) benzoic acid (DMAB), H₂O₂ and Mn²⁺ for Mn-peroxidase activity visualization. The gel was incubated at 32 °C until a color band appeared.

Enzyme assay and proteins determination

Laccase activity was assayed as previously described (Sarnthima et al. 2009) using 10 mM ABTS as a substrate in 0.1 M sodium acetate buffer pH 4.5. The reaction was incubated at 32 °C for 10 minutes, stopped with 50% (w/v) trichloroacetic acid (TCA) and the absorbance was measured at 420 nm ($\epsilon = 36000 \text{ M}^{-1}\text{cm}^{-1}$). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 μmole of substrate per minute.

Protein concentration was determined by the Bradford (1976) method using Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as a standard protein.

Dye decolorization

Two synthetic dyes, Rhodamine B (Fluka) and Congo red (CARO ERBA) were studied for decolorization. Firstly, the mediator ABTS was tested at various concentrations (0, 6.25, 12.5, 25, 50 and 100 μM). Reaction mixtures contained 0.1 M sodium acetate buffer (pH 4.5) and 10 μM Rhodamine B or 50 μM Congo red in the absence

or presence of the mediator. Otherwise state, reactions were started by the addition of the enzyme solution (0.02 U mL⁻¹ reaction mixtures) and were incubated at 32 °C. After the reactions the absorbance of the tested dyes was measured at 555 and 500 nm, respectively.

The effect of pH on decolorization of both dyes was studied using a 0.1 M citrate-phosphate buffer (pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0). Due to color change in the Congo red when added to the buffer pH two different λ_{max} 500 nm or 484 nm were measured for the reactions in pH range of 3.0 to 4.5 and 5.0 to 8.0, respectively. Temperature effect on enzymatic decolorization of both dyes was also investigated at various temperatures (30, 35, 40, 45, 50, 55 and 60 °C) at the optimum pH for 3 h. The absorption spectra were scanned from 260-800 nm using a UV-Vis Spectrophotometer (hp 8453, Hewlett Packard).

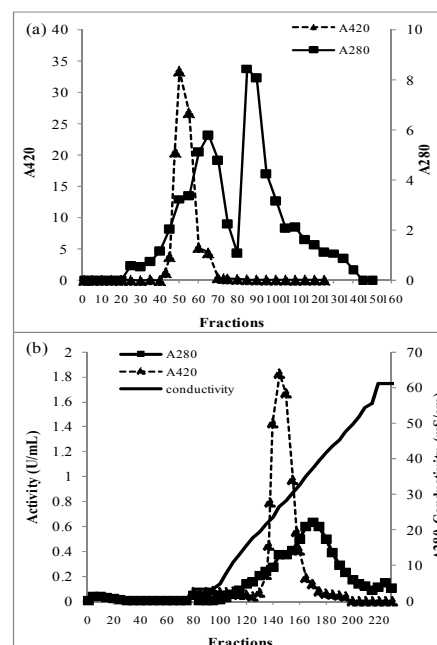


Figure 1: The Sephacryl S-300 gel filtration profile of *Lentinus polychrous* Lév. laccase purification (a) and the DEAE-cellulose column profile (b).

Results and discussion

Purification study of laccase

Laccase was partially purified from the crude extract of a solid state culture of *L. polychrous* Lév., by salt fractionation, Sephacryl S-300 and DEAE-cellulose columns respectively. After the two chromatography steps, the enzyme was purified only about 5.3-fold, with an overall yield of 5.6% and a laccase specific activity of 14.3 U mg⁻¹. The elution profile from the gel filtration chromatography on a Sephacryl S-300 showed the laccase activity as a single peak (fractions 43-60) as shown in Fig. 1a. The pooled fractions after dialysis were subjected to an ion-exchange chromatography on a DEAE-cellulose column, which also showed the laccase activity as a single peak (fractions 137-155) (Fig. 1b). The purification of the enzyme in each step as summarized in Table 1. However, the final step provided only partially purified laccases (< 50% purity) with molecular mass around 45 kDa accompany with low-molecular-weight proteinaceous impurities according to

SDS-PAGE result (**Fig. 2a**). These laccase-enriched fractions showed three close bands on native-PAGE containing both laccase and peroxidase activities (**Fig. 2b**) which is similar to the band patterns previously reported (Sarnthima et al. 2009). According to this results, we suggested those three bands of protein might be laccase due to its ability to oxidize ABTS and syringaldazine in the absence of H_2O_2 . However, to confirm this more successful purification steps and N-terminal sequencing of each protein is needed.

Those proteins have a molecular mass around 45 kDa according to SDS-PAGE, which were lower than the most commonly reported fungal laccase, between 50-130 kDa (Morozova et al. 2007). The molecular mass of laccases from these results were in a similar range as the molecular masses of laccase from *Fomitella fraxinea* (47 kDa) which has only one isozyme (Park and Park 2008) and lower molecular mass compared to the laccase from *Perenniporia tephropora* (63 kDa) (Younes et al. 2007), *Phlebia radiata* (61 kDa) (Kaneko et al. 2009), *Pycnoporus sanguineus* laccase (61.4 kDa) as determined by SDS-PAGE (Lu et al. 2007). Another gel filtration technique might be suitable for removing low-molecular-weight contaminant proteins. Moreover, the %yield activity should be much more of a concern in the future.

Decolorization of synthetic dyes

The laccase substrate range can be extended to non-phenol compounds in the presence of a low-molecular-weight compound acting as a redox mediator. A recent study, reported decolorization of Rhodamine B dyes with laccase-mediator system (LMS) by laccase from *T. versicolor* (Khammuang and Sarnthima 2009). In this report, ABTS was chosen as a redox mediator for studying decolorization of Rhodamine B and Congo red by the partial purified laccase from *L. polychrous* Lév.. The results in this study showed that Rhodamine B and Congo red decolorization in the presence of ABTS was more efficient than in the absence of mediator.

The ability of *L. polychrous* laccase to decolorize Rhodamine B and Congo red was enhanced in the presence of ABTS and increased at increasing ABTS concentrations (**Fig. 3a**). Both dyes' decolorization reactions, ABTS were saturated at about 25 μ M. These results support that ABTS is a potential redox mediator for Rhodamine B and Congo red decolorization. The pH profile for decolorization of both dyes showed a peak of maximum activity in a pH range of 4.0-5.0 and 6.0-7.0 for Rhodamine B and Congo red, respectively (**Fig. 3b**). For temperature effect, Rhodamine B decolorization was maximal at 50 $^{\circ}$ C, whereas with Congo red decolorization there were slight increases in decolorization percentages at higher temperatures (**Fig. 3c**). This unusual observation might be due to the relatively high decolorization of Congo red which was reached within a short time before the thermal denaturation of the enzymes.

The effect of dye concentration was studied for Rhodamine B (5-40 μ M) and Congo red (25-200 μ M) using the same amount of laccase activity (0.02 U mL⁻¹ reaction mixture). The results shown in **Fig. 4a-b**, indicate that the partially purified laccase from *L. polychrous* Lév. decolorized Rhodamine B to 56% within 3 h at 50 $^{\circ}$ C, at a dye concentration equal to or less than 20 μ M (**Fig. 4a**). However, Congo red could be decolorized up to 75% within 3 h at 30 $^{\circ}$ C, at a dye concentration equal to or less than 50 μ M (**Fig. 4b**). The absorption spectra of enzymatic-catalyzed Congo red decreased over time (**Fig. 5a**), whereas Rhodamine B reaction spectra shifted to shorter wavelengths around 540 nm (**Fig. 5b**). Previously, we

have reported the optimum conditions of Rhodamine B decolorization by *T. versicolor* were at pH of 4.0-5.0 (sodium acetate buffer), temperature of 35 $^{\circ}$ C with the proportion of dye and enzyme being 0.01 mM Rhodamine B and 0.02 U mL⁻¹ laccase activity in the presence of 0.1 mM ABTS (Khammuang and Sarnthima 2009). The laccase from *L. polychrous* Lév. in this study catalyzes Rhodamine B decolorization at a similar optimal pH condition, but with a lower decolorization rate.

Table 1 Purification table of *Lentinus polychrous* Lév

Purification step	volume (mL)	Lcc.activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purity Fold
Crude extract	400.0	1.03	412.0	0.38	150.4	2.7	100.0	1.0
0-40% (NH ₄) ₂ SO ₄	100.0	0.42	42.4	1.74	174.2	0.2	10.3	0.1
40-85% (NH ₄) ₂ SO ₄	10.0	30.62	306.2	2.03	20.3	15.1	74.3	5.5
Sephacryl S-300	25.0	2.84	71.1	0.59	14.7	4.8	17.3	1.8
DEAE cellulose	73.0	0.31	22.9	0.02	1.6	14.0	5.6	5.1

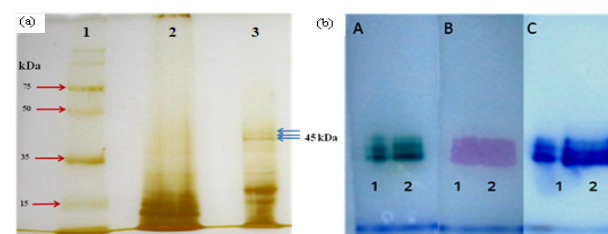


Figure 2: SDS-PAGE (12%) of the partially purified laccase from *Lentinus polychrous* Lév. stained with silver stain (a), lane 1: marker; lane 2: crude enzyme; lane 3: partially purified laccase and Native-PAGE (12 %) (b) Native-PAGE (12%) with activity staining of laccase substrates ABTS (b-A), Syringaldazine (b-B) and Mn-peroxidase substrates (b-C). lane 1: crude laccase and lane 2: partially purified laccase.

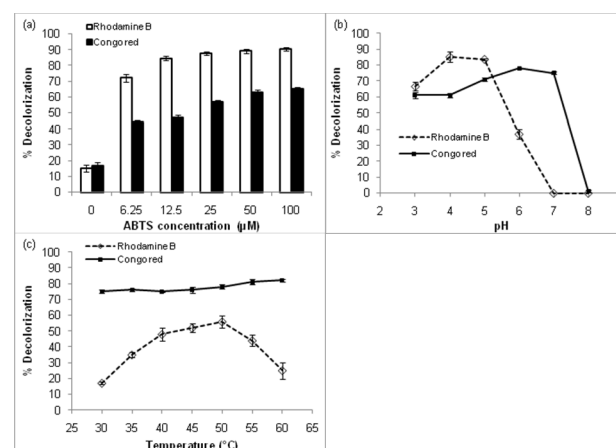


Figure 3: Effect of ABTS concentration on decolorization of Rhodamine B and Congo red (a), effect of buffer pH on decolorization of Rhodamine B and Congo red (b) and effect of temperature on Rhodamine B (c) by the partially purified laccase from *Lentinus polychrous* Lév.. Data were mean values \pm SD.

Partially purified laccase from the compost of this fungus also had an optimum pH of 4.0 for decolorization of Remazol brilliant blue royal (RBBR) (Khammuang and Sarnthima 2007). However, Younes et al. (2007) showed that laccase from *P. tephropora* was

Younes et al. (2007) showed that laccase from *P. tephropora* was able to decolorize the fluorone dye Pink Bengal without 1-hydroxybenzotriazole (HBT) to a higher percentage (49%) than with the redox mediator (35%) at pH 4.0 with 50 mg L⁻¹ of dye and 0.8 U mL⁻¹ laccase activity. They suggested that Pink Bengal decolorization was not improved by the laccase and HBT, while there was very little decolorization of the Janus green (azo dye) by this laccase under both sets of conditions (17% without HBT and 22% with HBT). In contrast, RBBR (anthraquinone dye) could be decolorized up to 91% without HBT (Younes et al. 2007). These results indicated that high decolorization efficiency depended much more on the type of dye rather than mediator.

Moreover, decolorization of the azo dyes Congo red, Orange G and Amido black 10B from dye industry effluent by *Thelephora* sp. were examined by Selvam et al. (2003). They found that the fungus mycelia was able to decolorize only 33.3% of 50 µM Orange G within 9 days, whereas 97.1% of 50 µM Congo red was removed within 8 h and 98.8% of 25 µM Amido black 10B within 24 h. When the azo dyes were treated with the enzyme solution at the same dye concentrations, it was found that a maximum decolorization of 19% for Orange G and 15% for Amido black 10B were achieved by laccase (15 U mL⁻¹ reaction mixture), whereas 12% Congo red was decolorized by 5 U mL⁻¹ reaction mixture. The results in our work showed higher efficiency due to a higher Congo red decolorization percentage (> 70% within 3 h) and a lower amount of laccase activity used (0.02 U mL⁻¹ reaction mixture). Moreover, these results contrast with results of laccase from *P. radiata* strain BP-11-2, which did not decolorize Congo red, Brilliant green, Rhodamine 6G or Methyl orange (Kaneko et al. 2009). Laccases from different sources have different optimum conditions for each substrate or dye type. This work also indicated that xanthenes dye, Rhodamine B is more difficult to decolourize than azo dye, Congo red.

The absorption spectra of Congo red showed a decrease in the absorbance at its λ_{max} according to treatment times, whereas Rhodamine B showed shifted spectrum at its λ_{max} to a shorter wavelength around 540 nm. The result is quite similar to the spectrum of decolorization products by laccase from *T. versicolor* previously report (Khammuang and Sarntima 2009), which showed shorter wavelength at 540 and 500 nm. It is also possible that the decolorization process of Rhodamine B by the enzyme from *L. polychrous* Lév. might be similar to that of *T. versicolor* laccase, while the reaction mechanism might be similar to a microwave-assisted photocatalytic system by an N-de-ethylation and/or a cleavage of conjugated chromospheres structure as proposed by He et al. (2009). Eggert et al. (1997) have demonstrated that the two *Pycnoporus cinnabarinus* laccase-less strains could not metabolize ¹⁴C-ring-labeled DHP to ¹⁴CO₂, addition of purified laccase to the mutants increased ¹⁴CO₂ evolution to give rates comparable to the wildtype. These results indicate clearly that the enzyme is absolutely essential for lignin degradation in *P. cinnabarinus*. Therefore in the laccase-catalyze synthetic dye decolorization, the products somehow transparent or change the colour depended on its structural stability after losing one electron (and proton). In our experiment, *L. polychrous* laccase catalyzed decolorization of Rhodamine B reduced in colour intensity at its λ_{max} , whereas in Congo red reaction, a completely clear solution obtained. However, to elucidate the catalysis mechanism of this laccase, characterization of each product is required. Moreover, a full characterization of the purified enzyme and kinetics study of synthetic dyes decolorization by the laccase-enriched fractions enough to perform.

Conclusion

In conclusion, the results showed that the partial purified laccase from *L. polychrous* Lév. was able to decolorize Congo red better than Rhodamine B in the presence or absence of ABTS as a redox mediator. This reasonably high decolorization efficiency makes this fungal enzyme of potential interest for treatment of real effluents containing these dyes. Further purification for full characterizations and kinetics studies are underway in our research group. As the fungus is a common in Thailand, enzymes obtained from it would be much more practical than those obtained from external sources.

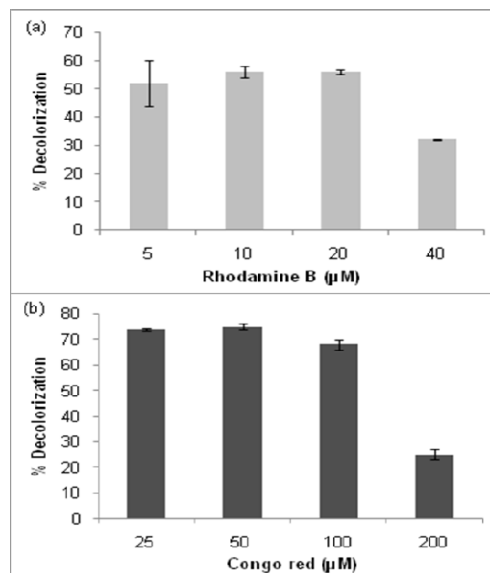


Figure 4: Effect of Rhodamine B concentration (a) and Congo red concentration (b) on decolorization activity by partially purified laccase from *Lentinus polychrous* Lév. Data were mean values \pm SD.

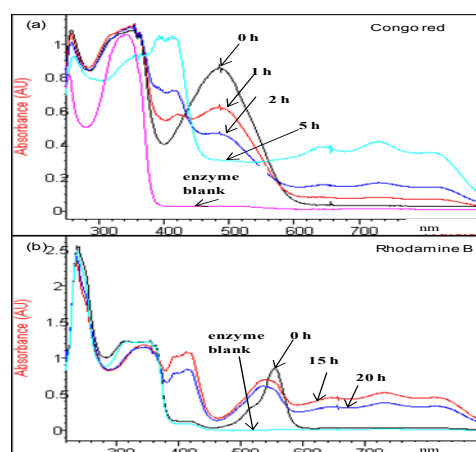


Figure 5: UV-vis spectra recorded during the *Lentinus polychrous* Lév. laccase catalyzed decolorization of Rhodamine B (a) and Congo red (b).

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