DECOLORIZATION OF THE AZO DYE DRIMAREN BLUE X3LR BY THE CRUDE FILTRATE OF FUNALIA TROGII

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ABSTRACT

An extracellular Drimaren Blue X3LR decolorizing enzymatic activity was found in the crude filtrate of *Funalia trogii* grown by solid-state fermentation (SSF) using wheat bran and soya bean waste. Decolorization of the azo dye Drimaren Blue X3LR by the crude filtrate and partially purified enzyme of *Funalia trogii* were investigated and compared. Partially purified enzyme was characterized both by the effects of oxido-reductase inhibitors on decolorization yield and SDS-PAGE. In the absence of additional redox mediator, maximum decolorization ratios of 81.33% and 77.4 % were observed for Drimaren Blue X3LR at a reaction time of 2 min. using crude filtrate and partially purified laccase respectively. Decolorization yield was found to be higher with crude enzyme preparations. An initial colorless and then later orange band was obtained by the activity staining process with Drimaren Blue X3LR and laccase substrate (guaicol), respectively after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of this band was estimated as about 65 kDa by SDS-PAGE. $Na_2S_2O_5$ inhibited laccase and dye decolorizing enzyme activities but a significant peroxidase activity inhibition was not observed. Since the reaction was catalysed in the absence of H_2O_2 as co-substrate, it might be concluded that this enzyme is a laccase.

KEYWORDS: Decolorization, Drimaren Blue X3LR, *Funalia trogii*, Laccase, Solid-state Fermentation (SSF), SDS-PAGE

INTRODUCTION

The control of water pollution has become of increasing importance in recent years. The release of dyes into the environment constitutes only a small proportion of water pollution, but dyes are visible in small quantities due to their brilliance. Tightening government legislation is forcing textile industries to treat their waste effluent to an increasingly high standard. Although these dyes are not in themselves toxic, after release into the aquatic environment they may be converted into potentially carcinogenic amines [1, 2] that impact on the ecosystem downstream from the mill.

Colored industrial effluents from the paper and dying industries represent major environmental problems. The white rot fungi, which degrade lignin biopolymers by a range of extracellular enzymes, have been used to degrade and detoxify polyaromatic hydrocarbons, polychlorinated biphenyls and certain dyes [3, 4]. Fungal phenol oxidases such as lignin peroxidase and laccase may detoxify azo links from phenol-azo dyes and release molecular nitrogen [5, 6]. Laccase (02:p-diphenol oxido-reductase, EC.1.10.3.2) belongs to a small group of enzymes called the large blue copper proteins or blue copper oxidases. This group uses oxygen as an electron acceptor to remove hydrogen from phenolic hydroxy groups [7]. Mazmancı et al. (2002) investigated the decolorization of a dye Methylene Blue by a white rot fungus Coriolus versicolor [8]. A strain of Pycnoporus cinnabarinus decolorized industrial wastewater and the industrial vinyl sulfonyl dye Remazol Brillant Blue R The effect was attributed to a laccase in P. cinnabarinus [9, 10].

The aim of this work is to demonstrate that the decolorization of the Drimaren Blue X3LR dye (metal complex azo dye) was decolorized by laccase present in the strain of Funalia trogii.

MATERIALS AND METHODS

Organism. Funalia trogii was obtained from Environmental Biotechnology Laboratory of Environmental Engineering Department, University of Mersin, Turkey. The fungus was maintained on Potato Dextrose Agar (PDA, Merck) slants and incubated at 30°C for 5 days and stored at +4°C.

Chemicals. Drimaren Blue X3LR (commercial) was obtained from textile industries. All other chemicals used were obtained from Merck except Guaicol (analytical grade), which was obtained from Sigma.

Enzyme Production. The composition of solid-state fermentation (SSF) medium used for enzyme production was as following (g/kg): Wheat bran 900 and soybean residue 100. Medium was humidified with a 0.1 M pH 6.0 sodium phosphate buffers with a ratio 60 % (v/w). SSF medium in 1L Erlenmeyer flasks, was autoclaved (120°C and 1.2 atm.) for 60 minutes. SSF medium was inoculated by fungal stock cultures grown on Potato Dextrose Agar and was incubated for 10 days at 30°C, which is optimum temperature for maximum enzyme production (data not shown).

Enzyme Assay. After incubation, the cultures grown on SSF medium were dehumidified in incubator (Sanyo MIR 152) for 24 hours at 40°C. The powdered culture medium was kept in potassium phosphate (0.1M, pH 6.0) buffer (1g/10mL) for 15 minutes. Suspended solids were removed by centrifugation at 12 000 g (Hettich Micro 22R) for 15 minutes and then filtered from filter paper (Whatman, No; 1). The crude filtrate obtained was used in enzyme assays (laccase and HRP), decolorization, purification and characterization studies.

Laccase (02:p-diphenol oxido-reductase, EC.1.10.3.2) activity was determined by monitoring the absorbance at 465 nm with a 160-A UV/VIS spectrophotometer (Shimadzu A-160). Guaicol was used as substrate. Quinone formation by the oxidation of guaicol was determined at this wavelength. Relative HRP activity was also determined by the same method

except with the addition of 1.5 µL H_2O_2 into the medium Relative laccase and peroxidase activities wers expressed in colorimetric units. 1 CU (colorimetric unit) was defined as the enzyme activity that causes 0.1unit/h increase in optical density of the incubation mixture [11].

Protein estimation. Protein was determined by a modified Lowry method [12] with bovine serum albumin as a standard protein.

Enzyme purification. The culture filtrate obtained was saturated with $(NH_4)_2SO_4$ to a final concentration of 80%-satd. After 60 min. with gentle stirring, the filtrate was centrifuged for 15 min. at 6000 rpm. The pellet obtained was resuspended in 0.1 M potassium phosphate buffer, pH 6. The enzyme was then applied to a sephadex-G50 column and eluted with 0.1 M potassium phosphate buffer, pH 6. Fractions of 50 drop (approximately 2.4 mL) were collected. Fractions with laccase activity were combined and concentrated by ultrafiltration with Minicon Mini Plus Concentrator (Millipore, 10 000 NMWL). Laccase, peroxidase activities and total protein amount were measured at each purification step.

Decolorization assay. All decolorization assays by the crude filtrate and partially purified enzyme of Funalia trogii were performed at pH 3.0 (adjusted by 0.1M sodium tartarate buffer) and at 50°C, the optimum pH and temperature for decolorization (data not shown), at different dye concentrations (20-100). Drimaren Blue X3LR decolorization by the purified enzyme of Funalia trogii was assayed by measuring the decrease in A_{615} with a model 160-A UV/VIS spectrophotometer (including kinetic spectral measurement, Shimadzu A-160). 10 µL crude filtrate and 1990 µL dye solution were used in all analysis. Changing in the absorbance was detected during two minutes incubation period by spectrophotometric measurements, which were done at every 10 seconds; absorbance change is given as % change of colour intensity.

Inhibitor Effects. Two different inhibitors were used and their inhibition effects were investigated. 10 µL of different concentrations of sodium metabisulphide, $Na_2S_2O_5$, (1000-16.125 □M) and sodium azide, NaN_3, (2000-0.15625 □M) were supplied in the reaction medium containing 10 µL purified enzyme and 1980 µL dye solution. Changing in the absorbance was detected during two minutes incubation period by spectrophotometric measurements, which were done at every 10 seconds. Laccase and peroxidase activities were measured for each inhibitor concentrations.

Gel Electrophoresis and Staining. SDS-PAGE, to determine the molecular weight of the partially purified enzyme, was performed with a 10 % polyacrylamide containing 0.1 % sodium dodecyl sulphate according to the method of Laemmli [13]. The protein bands were visualized by Commassie Blue G-250 (Merck) and compared with Standard Molecular Weight markers (shown on Fig.4.)

Activity staining. For the activity staining of the decolorizing activity, the SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40 % for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. The renaturation of the enzyme proteins was carried out by placing the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM □-mercaptoethanol and 1 mM EDTA at 4□C. The gel was then transferred onto a glass plate, sealed in film, and incubated at 37□C for 4-5 h [14]. The gel was stained with Drimaren Blue X3LR and guaicol. Any clear and colored zones indicated the presence of the decolorizing activity of the enzyme.

RESULTS AND DISCUSSION

Enzyme purification. Purification steps were applied to obtain laccase preparations with higher specific activity. The procedure developed for laccase isolation and purification enabled us to obtain an enzymatic prepration purified 12.4 fold. The results are summarized in Table 1. Data shows that laccase and peroxidase couldn't be seperated by this procedure. So, inhibitor effects on laccase, peroxidase and dye decolorizing enzyme were studied in order to determine which of these enzymes participated to decolorization.

Table 1. Purification of laccase from *Funalia trogii*

	Laccase act. (CU)	Peroxidase act. (CU)	Total Protein (mg/mL)	Specific Activity (CU/mg)	Purification (fold)
Crude Filtrate	7,75	6,18	4,847	1,6	1
% 80 NH2SO4 precipitate	11,56	11,7	7,11	1,63	1,02
sephadex G-50	11,46	11,1	0,89	12,86	8.048,04
Konsantre (x3,5)	37,1	23,1	1,86	19,94	12,47

Decolorization of Drimaren Blue X3LR by Crude Filtrate and Partially Purified Enzyme of Funalia trogii:

In the absence of additional redox mediator, decolorization ratios of 81.33% and 77.4 % were observed for Drimaren Blue X3LR at a reaction time of 2 min. using crude filtrate and partially purified laccase respectively (Fig 1). Decolorization yield was found to be higher with crude enzyme preparations. This is most probably because of the redox mediator like molecules found in the crude filtrate, effective at decolorizing Drimaren Blue X3LR; some of which were lost by purification steps causes a decrease in decolorization ratio. Some other researchers reported the importance of redox mediators on decolorization (15, 16).

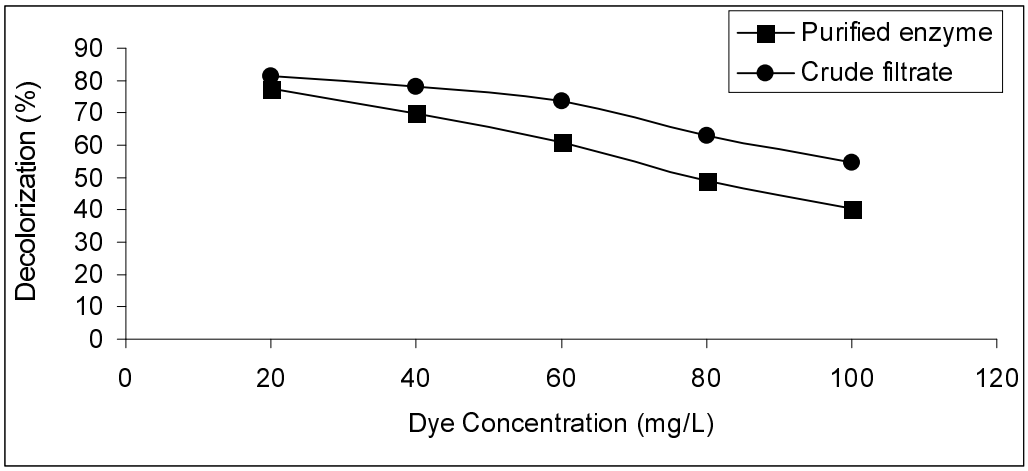

Figure 1. Decolorization (%) of Drimaren Blue X3LR by purified enzyme and crude filtrate

It was shown that dye decolorising enzymes such as LiP and MnP require the presence of H_2O_2 and/or VA. Colour removal started just with the addition of H_2O_2 and increased with higher H_2O_2 concentrations [17, 18, 19]. However, in this present study, it was found that Drimaren Blue X3LR decolorizing enzyme did not require H_2O_2.

Inhibitor Effects. Application of commonly used laccase inhibitors $Na_2S_2O_5$ and NaN_3 led to know the effect of laccase on decolorization. Decolorization and the laccase activities decreased parallel to each other by increasing inhibitor concentrations. NaN_3 inhibited both laccase and peroxidase activities and decolorization activity (Fig. 2). Such a result doesn't show us the relationship between decolorization and laccase activity. But provides us such a data that NaN_3 is an inhibitor for both laccase and peroxidase enzymes. $Na_2S_2O_5$ inhibited laccase and dye decolorizing enzyme activities but a significant peroxidase activity inhibition was not observed (Fig. 3). This result emphasizes the very important role of laccase on Drimaren Blue X3LR decolorization. In addition, the inhibition of Drimaren blue X3LR decolorizing enzyme by $Na_2S_2O_5$ and NaN_3 which are known to inhibits enzymatic reactions requiring oxygen (20, 21) shows that, this enzyme is an oxydoreductase group enzyme.

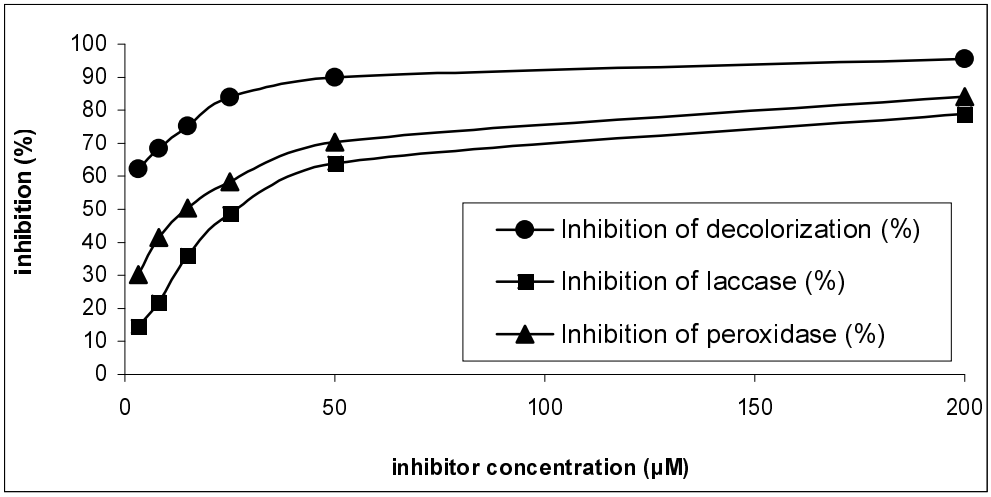

Figure 2. ***Inhibitory Effect of NaN_3 on Drimaren Blue X3LR oxygenase, Laccase and Peroxidase Enzymes.***

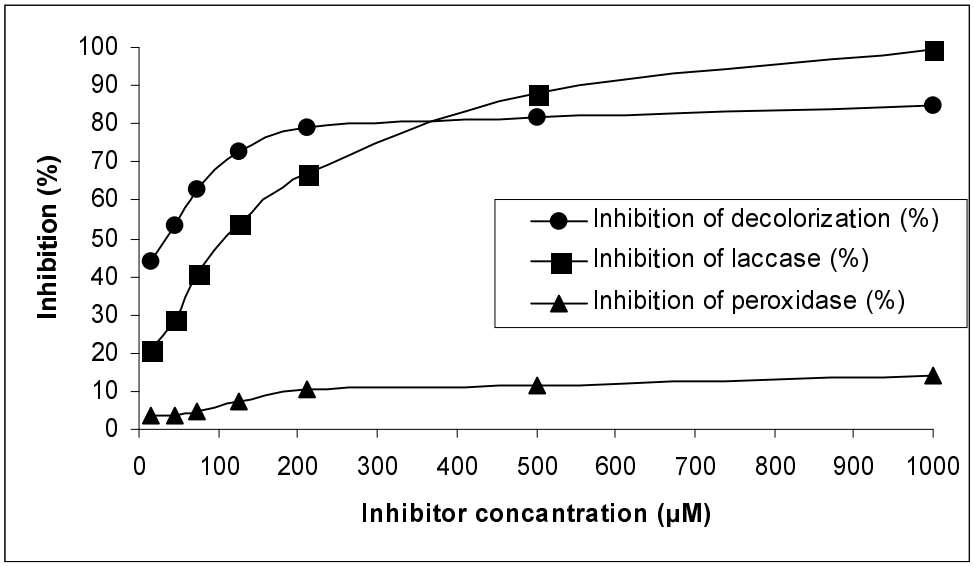

Figure 3. Inhibitory Effect of $Na_2S_2O_5$ on Drimaren Blue X3LR oxygenase, Laccase and Peroxidase Enzymes. Gel Electrophoresis.

The molecular weight of the enzyme was determined by SDS-PAGE with the molecular weight marker. In order to determine the molecular weight of the decolorizing enzyme band, the activity staining was done with Drimaren Blue X3LR after the gel was re-natured. After sufficient staining (30 min), a single non-colored zone appeared on the band (Lane B), which was calculated to be about 65 kDa. After staining with guaicol, this non-colored zone turned into orange-color (Lane C, Figure. 4). Hublik and Schinnera (2000) determined the molecular mass of laccase purified from *Pleorotus ostreatus* 66.8 kDa [22]. Höfer and Schlosser (1999) purified the laccase enzyme of *Trametes versicolor* having a single band with a molecular mass of approximately 68 kDa [23]. The degradation of the disazo dye Chicago Sky Blue 6B by a purified laccase from *Pycnoporus cinnabarinus* showed a band having a molecular size of 63 kDa determined by SDS-PAGE [24]. These results are in accordance with our findings.

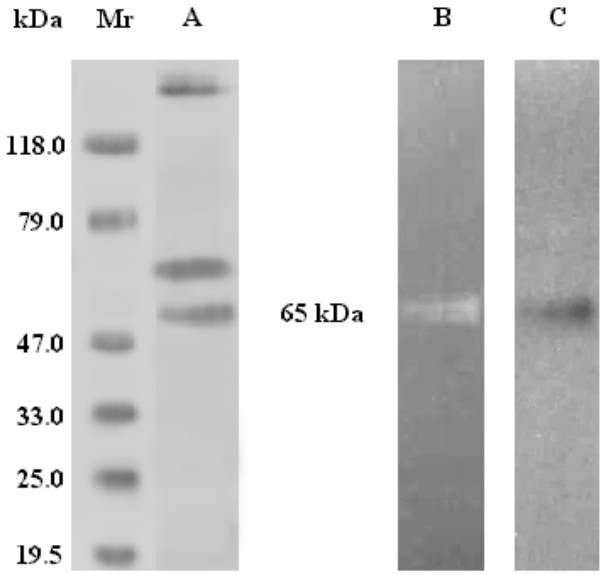

***Figure 4**. Separation (Lane Mr and A) and activity staining (Lane B and C) of the crude filtrate of Funalia trogii. Lane Mr standard molecular weight markers (β -galactosidase, 118.0 kDa; Bovine serum albumin, 79.0 kDa; Ovalbumin, 47.0 kDa; Carbonic anhydrase, 33.0 kDa; β- lactoglobulin, 25.0 kDa; Lysozyme, 19.5 kDa).*

CONCLUSIONS

A Drimaren Blue X3LR decolorizing enzyme was found synthesized during solid-state fermentation of wheat bran and soybean waste by *Funalia trogii* Decolorization was seen in the absence of H_2O_2. The molecular weight of enzyme protein was calculated to be about 65 kDa by SDS-PAGE. Development of orange color was detected after activity staining with laccase substrate guaicol. Inhibition of decolorization activity by oxydoreductase inhibitors, NaN_3 and Na_2S_2 indicated that Drimaren Blue X3LR decolorizing enzyme is an oxydoreductase group enzyme. These results indicate that this enzyme

might be a laccase. 81.33% and 77.4 % decolorization of Drimaren Blue X3LR by the same amounts of crude and partially purified enzymes respectively indicated that, some of the redox like molecules may lost by purification processes. This data has a great importance for industrial applications as eliminates purification processes and the need for additional redox mediators. Preparing a commercial laccase that is inexpensive means that one of the major barriers that limits the use of enzymes in wastewater treatment can be overcome. Scale up of such enzymatic processes is the subject of further study.

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