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Decolorization and biodegradation of remazol brilliant blue R by bilirubin oxidase

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The dye-decolorizing potential of bilirubin oxidase (BOX) was demonstrated for an anthraquinone dye, remazol brilliant blue R (RBBR). The dye was decolorized 40% within 4 h by the BOX alone, whereas it was more efficient in the presence of 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), showing 91.5% decolorization within 25 min. The effects of operational parameters on decolorization were examined. The results showed that the decolorization efficiency decreased with increasing RBBR concentration, and a marked inhibition effect was exhibited when the dye concentrations were above 100 mg l^{-1}. The optimum temperature for enzymatic decolorization was 40 °C. BOX showed efficient decolorization of the dye with a wide pH range of 5–8.5. The maximum decolorization activity occurred at pH 8 with ABTS and at pH 5 without ABTS. Analysis of RBBR ultraviolet and visible (UV–VIS) spectra after BOX treatment indicated that the decolorization of RBBR was due to biodegradation. Our results suggested that ABTS can serve as an electron mediator to facilitate the oxidation of RBBR, and the BOX–ABTS mediator-involved dye decolorization mechanism was similar to that of laccase. Operation over a wide range of pH and efficient decolorization suggested that the BOX can be used to decolorize synthetic dyes from effluents, especially for anthraquinonic dyes.

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[**Key words:** Bilirubin oxidase; Remazol brilliant blue R; Decolorization; Biodegradation; Laccase]

Synthetic dyes are extensively used in the textile, cosmetic, printing, drug, and food-processing industries. It is reported that between 10 and 20% of about 0.7 million tons of dyestuffs being manufactured and used in dyeing processes may be found in wastewater each year (1). The total world colorant production is estimated to be 8 kT per year, and the textile wastewaters have been one of the main sources of severe pollution problems worldwide (2). Many dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, and water. The release of dye effluents into the environment is undesirable, not only because of their visual effect, but also because many dyes are made from known carcinogens, and their breakdown products are toxic and/or mutagenic to life (3).

To avoid the dangerous accumulation of dyes in the environment, many techniques have been used to remove dyes from wastewater. Traditional wastewater treatment technologies have been proven to be markedly ineffective for handling the wastewater of synthetic textile dyes because of the chemical stability of these pollutants (3, 4). In addition, those chemical or physical methods usually involve complicated procedures or are uneconomical. Therefore, there is a great need to develop an economic and effective way of dealing with textile dyeing waste in the face of ever increasing production activities. As a feasible alternative, biological processes have received increasing interest due to their low-cost and eco-friendly nature. In these processes, enzymatic processes present some interesting properties such as low energy requirements, easy process control, operation over a wide range of conditions, and a minimal environmental impact (5, 6).

Among enzymes, laccases (benzenediol oxygen oxidoreductase; EC 1.10.3.2), which belong to a family of multicopper containing oxidases and are widely distributed in nature, have a generally low substrate specificity and are able to transform a large number of phenolic and non-phenolic aromatic compounds with the concomitant reduction of oxygen to water (7). Laccases have been studied for many years due to their potential use as biocatalysts in pulp and paper bleaching (8). In addition, laccases are also considered an attractive alternative in the development of an effective technology for the biotreatment of dyestuffs. However, most classical laccases have an acidic pH optimum, and few have a near neutral pH optimum, which is the main limitation in their practical application to the decolorization of basic effluents (9). In the literature, much information is available on the use of laccases in textile effluent, but there is little information on the decolorization ability of other multicopper oxidases like bilirubin oxidase.

Bilirubin oxidase (BOX) (EC 1.3.3.5, from *Myrothecium verrucaria*) catalyzes the oxidation of bilirubin into non-colored corresponding substances at pH 7.8 in vitro (10). It has a molecular mass of 63 kDa and is a multicopper oxidase containing type 1, type 2, and type 3 coppers (in the ratio 1:1:2), similar to laccase (11, 12). There is also strong sequence homology between bilirubin oxidase and laccase (12). It has been reported that BOX can catalyze the oxidation of some typical laccase substrates such as ABTS and syringaldazine and exhibit a high catalytic activity near neutral pH (13). BOX has been used to determine

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the total bilirubin in serum in the field of clinical pathology for the diagnosis and treatment of jaundice and hyperbilirubinemia (14).

Our previous studies showed that *Myrothecium* sp.IMER1 can decolorize RBBR, Congo Red, and Indigo Carmine, and its BOX plays a major role in decolorization (15, 16), but the effects of environmental parameters on the decolorization of dyestuffs by BOX are unknown. Since dyestuffs and their derivatives are becoming a class of concerning water pollutant and are accumulating in the environment, there is a strong need to determine the optimal conditions for rapid degradation of dyes. RBBR is a typical anthraquinone dye used in the textile industry and represents an important class of toxic and recalcitrant organopollutants (15). Therefore, RBBR was selected for carrying out enzymatic decolorization and biodegradation studies.

With this view, we have undertaken a detailed study on the decolorization and biodegradation of RBBR and focused our attention on the effects of temperature, pH, dye concentration, and especially whether a mediator like ABTS, a substrate of BOX and laccase, can enhance the decolorization efficiency of RBBR.

MATERIALS AND METHODS

Dyes and chemicals Remazol brilliant blue R (RBBR, reactive anthraquinone, C. I.G1200) and ABTS were purchased from Sigma (USA). All other reagents and chemicals were of the highest purity available and of an analytical grade.

Enzyme preparation BOX preparation was carried out as described earlier (15). *Myrothecium* sp.IMER1 was cultivated in potato dextrose broth (PDB). A culture broth of 2 l at the maximum BOX activity was first filtered to remove fungal pellets and then centrifuged at 8000 $\times g$ for 15 min. The supernatant was frozen and then defrosted filtered to precipitate polysaccharides. The proteins were precipitated from the clear supernatant with ammonium sulphate (80% saturation). The precipitate was dialyzed for 24 h against 10 mM phosphate buffer (pH 7, buffer A), then filtered through a Millipore® membrane (0.22 μm). The filtrate was applied to a DEAE-Sepharose Fast Flow column that was equilibrated with buffer A. The bound proteins were eluted using buffer A with a linear gradient of NaCl (0–0.6 M). Major BOX fractions were selected, pooled together, concentrated, dialyzed, and lyophilized. The lyophilized extracts were dissolved in 2 ml of buffer A and then loaded onto a Sephadex G-100 column (2.5 cm × 60 cm), which was equilibrated with buffer A. The proteins were subsequently eluted with buffer A at a flow rate of 0.7 ml/min. BOX-positive fractions corresponding to the major peak were collected and used for subsequent decolorization.

Enzyme assay The BOX activity was assayed as follows: 2.0 ml of 30 μM bilirubin dissolved in 0.2 M Tris-H_2SO_4 buffer (pH 8.4) was added to 0.2 ml of the enzyme solution, followed by incubation at 37 °C. Measurement of the absorbance decrease of bilirubin was carried out at 440 nm with a spectrophotometer. 1 U was defined as the amount of enzyme that oxidized 1 μmol bilirubin/min (12).

Enzymatic dye decolorization The reaction mixture contained different dye concentrations and 10 U ml^{-1} BOX in a total volume of 4 ml in a citrate-phosphate buffer (pH 7). The reaction mixture was incubated at 28 °C in the dark, and dye decolorization was measured by monitoring the decrease in the absorbance maximum (RBBR 595 nm) in a UV–VIS spectrophotometer. In parallel, control samples were maintained with heat inactivated enzyme. The decolorization efficiency (P) was calculated according to the following formula: $P\ (A_1 - A_2)/A_1 \times 100\%$. Where A_1 represented the absorbance of the control, A_2 represented the absorbance of the reaction mixture, and P was the decolorization efficiency.

Effect of different parameters on dye decolorization The effect of pH on the enzymatic decolorization was monitored with a dye concentration of 80 mg l^{-1} at different pH ranging from 3 to 9. The pH of the reaction mixture was adjusted by citrate-phosphate (pH 3–8) and Tris–HCl (pH 8–9) buffers. To find out the effect of temperature on the enzymatic decolorization, the reaction mixture was incubated at different temperatures ranging between 20 and 60 °C with a 10 °C increment. In order to check the effect of enzyme quantity on decolorization, the decolorization reaction was started with different quantities of enzyme (4, 8, 12, 16, and 20 U ml^{-1}). The effect of the dye concentration was tested using 60, 80, 100, 180, and 300 mg l^{-1} concentrations. The assays were done twice in triplicate, the experimental error was below 10%, and the results shown are the means ± the standard deviation of less than 5%.

UV–VIS spectrophotometric analysis Decolorization and biodegradation were monitored by a UV–VIS spectroscopic analysis. The absorbance spectra of RBBR, before and after decolorization, were scanned by a Varian CARY50 UV–visible spectrophotometer (USA), and the changes in its absorbance spectrum (200–800 nm) were recorded.

RESULTS

Dye decolorization by BOX with and without ABTS Approximately 9.5% and 40% decolorization of RBBR by BOX alone were achieved within 25 min and 4 h, respectively (Fig. 1). In addition, about 98% decolorization was obtained within 24 h (data not shown). The result showed that BOX can decolorize RBBR effectively. However, the addition of 10 μM ABTS enhanced decolorization nearly 10-fold, and the maximum decolorization of 91.5% was observed within 25 min (Fig. 1). When RBBR alone and an RBBR with ABTS mixture were incubated without the enzyme, no decolorization was observed during 4 h (Fig. 1), which indicated that RBBR is stable in aqueous solution and ABTS itself does not decolorize the dye at all. Decolorization was therefore attributed to biodegradation by the enzyme.

Effect of initial dye concentration and BOX concentration The effect of the dye concentration on decolorization was tested with different initial dye concentrations from 60 to 300 mg l^{-1} with a constant amount of enzyme (10 U ml^{-1}) at 28 °C. The results revealed that the decolorization efficiency decreased with the increasing concentration of dye. The enzyme was able to decolorize RBBR (below 100 mg l^{-1}) in the range of 38–56% during the 4 h incubation (Fig. 2A). RBBR concentrations above 100 mg l^{-1} exhibited a marked inhibition effect. On the other hand, with the increase in dye concentration, the specific degradation was found to be effective up to a dye concentration of 80 mg l^{-1}. The specific degradation rapidly dropped at 100 mg l^{-1} and was almost constant over 180 mg l^{-1}. The behavior observed here indicated that BOX oxidized substrates in which the rate of substrate oxidation increased with the substrate concentration until saturation. When the BOX concentration was 10 U ml^{-1}, 80 mg l^{-1} may be presumed to be the cut-off concentration of the dye for the optimum decolorization.

The effect of the enzyme concentration on RBBR decolorization was studied by increasing the BOX activity from 4 U ml^{-1} to 20 U ml^{-1}. It can be seen in Fig. 2B that the decolorization efficiency linearly increased below 16 U ml^{-1} and became almost constant over 16 U ml^{-1}. On the other hand, the specific degradation increased with an increase in BOX concentration and became maximal at 12 U ml^{-1} and then it decreased. A subsequent increase in the BOX concentration up to 20 U ml^{-1} might have yielded a significantly low impact on the BOX dye decolorization reaction and resulted in a gradual decrease in the specific degradation. The results showed that the enzyme concentration of 12 U ml^{-1} was found to be the optimum concentration for maximum dye decolorization at the specified experimental conditions.

Effect of temperature The temperature effect was studied in the range of 20–60 °C. The decolorization efficiency increased (12–20%) at temperatures from 20 to 40 °C during a 2-h incubation. The

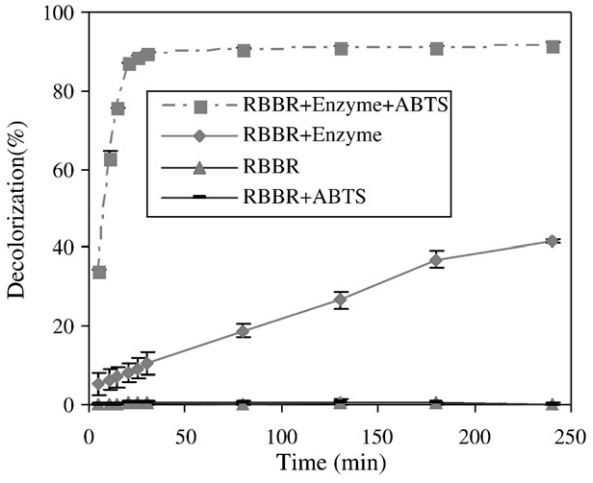

FIG. 1. Decolorization of RBBR by BOX (10 U ml^{-1}) with and without ABTS (10 μM) at 28 °C; pH = 7, 80 mg l^{-1} RBBR.

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