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Decolorization of Remazol Briliant Blue R by Laccase from White Rot Fungus *Polyporus* sp. S133

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Abstract

The decolourization of the recalcitrant dye RBBR by the culture filtrate of *Polyporus* sp. S133 and its isolated laccase was investigated. The laccase alone decolorized RBBR. A small molecular weight redox mediator (HBT) was necessary to increase the decolorization. The purified laccase totally decolorized the dye of 200 mg l^{-1} initial concentration of RBBR when only 1.5 U ml^{-1} of laccase was used in the reaction mixture. The effects of different physicochemical parameters were tested and optimal decolorization rates occurred at pH 5 and at a temperature of 50 °C. The effect of surfactants on the decolourization of RBBR was tested with Tween 80, Tween 20, and Brij 35. It was demonstrated that Tween 80 was inhibiting substrate for the decolorization while Tween 80 and Brij 35 was no inhibiting effect for the decolorization. Provided that all of the condition is included, it is suggested that laccase may be suitable for the wastewater treatment of similar anthraquinone dyes.

Keywords: Decolorization; Laccase; Remazol Brilliant Blue R (RBBR); *Polyporus* sp. S133

Introduction

Synthetic dyes are environmental interest because of their widespread use and their potential for forming toxic aromatic amines. Synthetic dyes are extensively used in several industries including textile, paper, printing, cosmetics and pharmaceuticals (Marmion, 1991). There are many structural varieties, such as, acidic, basic, disperse, azo, diazo, anthraquinone based and metal complex dyes. On the basis of dyeing process, textile dyes are classified as reactive dyes, direct dyes, disperse dyes, acid dyes, basic dyes and vat dyes. It is estimated that 10–15% of the dyes are lost in the effluent during dyeing process (Zollinger, 1987). Many dyes are difficult to decolorize due to their complex structure and synthetic origin. Decolorization of textile dye effluent does not occur when treated aerobically by municipal sewage systems (Willmott *et al*., 1998). Brightly colored, water-soluble reactive and acid dyes are the most problematic, as they tend to pass through conventional treatment systems unaffected (Willmott *et al*., 1998). RBBR is one of the most important dyes in the textile industry. It is frequently used as starting material in the production of polymeric dyes. RBBR is an anthracene derivative and represents an important class of toxic and recalcitrant organopollutants.

White rot fungi have long been known to decolorize dyes (Glen and Gold, 1983). These versatile fungi can degrade not only a broad range of recalcitrant dyes but also the complex polymer (Field *et al*., 1993; Barr and Aust, 1994). The biodegradation ability of the white-rot fungi is assumed to be associated with the production of lignolytic

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enzymes such as lignin peroxidase and laccase (Ghodake *et al.*, 2008; Couto and Herrera, 2006). Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) is amulticopper oxidase, widely distributed among plants, fungi, and bacteria (Mayer and Staples, 2002; Claus, 2003). It catalyzes the oxidation of a broad range of organic and inorganic substrates, including diphenols, polyphenols, diamines, aromatic amines, and ascorbate by a one-electron transfer mechanism (Thurston, 1994).

There are extensive studies focused on laccase from fungi like *Daedalea quercina*, *Sclerotium rolfsii*, *Ganoderma lucidum*, *Trametes trogii*, and *Pycnoporus sanguineus* (Baldrian, 2004; Ryan *et al.*, 2003; Murugesan, 2007; Zouari-Mechichi *et al.*, 2006; Litthauer *et al.*, 2007). Laccases can be applied extensively in many fields, including waste detoxification and textile dye transformation due to their low substrate specificity (Couto and Herrera, 2006). Although some bacterial laccases have been characterized (McMahon *et al.*, 2007; Robinson *et al.*, 2001), little information is available concerning their substrate specificities towards dye decolorization. Large amounts of chemically different dyes are used for textile dyeing and a significant proportion of these dyes enter the environment as waste water. Not all these dyes could be degraded and/or removed with physical and chemical processes, and sometimes the degradation products are more toxic (Robinson *et al.*, 2001). Currently, one of the possible alternatives for treatment of textile effluents is the use of bacteria or their enzymes, which can oxidize a wide spectrum of synthetic dyes (Robinson *et al.*, 2001). In this paper, decolorization of the dye RBBR by the laccase from *Polyporus* sp. S133 was studied, as it is representative of an important class of recalcitrant anthraquinonetype dyes.

Materials and Methods

Chemicals

N-hydroxybenzotriazole (HBT) and the surfactants (Tween 80, Tween 20, and Brij 35) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). RBBR was provided by Sigma (St. Louis, USA). The structure of RBBR was showed in Figure 1.

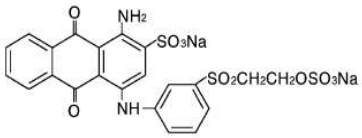

Figure 1.The chemical structures of Remazol Brilliant Blue R.

All other chemicals were purchased from Wako Pure Chemical Industry Co. Ltd. (Osaka, Japan) at the highest purity available.

Microorganism and culture condition

Polyporus sp. S133, a white rot fungus, was selected for this study for its ability to decolorize RBBR in nutrient medium and to produce a high titer of laccase. Composition of nutrient medium used for decolorization studies is (g/l): malt extract 20, glucose 20, and peptone 1. Pure culture is maintained on nutrient agar slants at 4°C by transferring culture once in a month.

Laccase production

Time course of laccase production by *Polyporus* sp. S133 was studied in 100 ml nutrient broth at 30°C at static condition. Laccase activity (as mentioned in following section) was measured in crude cell extract of *Polyporus* sp. S133 cells grown at different time intervals. For higher laccase production, 10% inoculum of 12 h grown *Polyporus* sp. S133 was inoculated in 3 l nutrient medium and incubated 12 h at 30°C. Cells were collected by centrifugation at 8000×*g* for 15min and suspended (150 mg/ml) in 50mM sodium phosphate buffer (pH 7.0) containing 5 mg ml^{-1} lysozyme. Cells

are further incubated at 37°C for 45 min in water bath and then disrupted by sonication. This cell free extract was solubilized in cholic acid (0.33 mg/mgprotein) on magnetic stirrer at 4°C for 30 min. The cell lysate obtained was centrifuged twice at 15,000×*g* for 30 min at 4°C and the clear supernatant used immediately or stored at 20°C until its use to purify laccase.

Purification of laccase

The supernatant containing laccase activity 0.04 U was heated at 60°C for 10 min and centrifuged at 8000×*g* for 20 min. The clear supernatant obtained after centrifugation was loaded on a DEAE cellulose fast flow column (15mm×120mm). The column was washed with the same buffer by two times of the column volume and the enzyme was eluted with a linear gradient of 0-1.0M NaCl. Fractions containing laccase activity were pooled and dialyzed against 1mM sodium phosphate buffer (pH 6.0). The dialyzed sample was concentrated (1–2 ml) by ultrafiltration and loaded on Biogel column (10mm×500mm) equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The protein elution was carried with the same buffer at 6 ml/h flow rate. Fractions containing laccase activity were pooled and stored at 20°C until use.

Protein determination and enzyme activity

The protein concentration of each fraction is monitored by absorbance at 280nm or Lowry methods with bovine serum albumin as a standard (Lowry *et al.*, 1951). Laccase activity was determined at 30°C by measuring increase in optical density at 420 nm in a reaction mixture of 2 ml containing 0.66mM ABTS in 0.1 M acetate buffer (pH 4.9) and 100µl enzyme (Hatvani and Mecs, 2001). One unit of enzyme activity was defined as a change in absorbance unit/min/ mgprotein

Decolorization of RBBR by purified laccase

Unless otherwise indicated all experiments were performed using 3 ml disposable cuvettes with 2 ml final reaction volume. The reaction mixture was composed of 100 mM acetate buffer pH 5, 200 mg/l RBBR and 1.5 U/ml laccase. The decolorization was monitored by scanning the spectrum between 400–800 nm using UV-VIS spectrophotometer. All experiments were performed in duplicate and incubated with shaking (120 rpm) for appropriate time. Controls were performed using heat inactivated enzymes after incubation at 100 °C for 10 min. Dye concentrations were calculated from the calibration curve prepared from the dye concentration and the measured absorbance at l_{max} (595 nm) (Rajkumar, 2007). The percentage of decolorization was calculated as follow.

$$\text{Decolorization (\%)} = \left(1 - \frac{C}{C_0}\right) \times 100$$

where C_0 is initial dye concentration and C is final dye concentration (Sayan, 2006).

Results and Discussion

Decolorization of RBBR by purified laccase

In order to assess the degrading ability of the purified laccase obtained from the above culture, the decolorization of RBBR was studied. Decolorization was performed in one step as well as by successive additions of dye to the reaction mixture. RBBR was incubated in pure laccase without HBT and the spectra of the reaction mixture were recorded each 20 min. Figure 2A shows the visible absorbance spectrum for RBBR after treatment with the purified *Polyporus* sp. S133 laccase. A maximal absorbance was seen at 595 nm and this peak decreased with time, which is associated with oxidation of the dye. Following 24 h and 48 h of incubation, the percentage of decolorization was 26 % and 60 %. The final overlay shows that almost complete decolorization

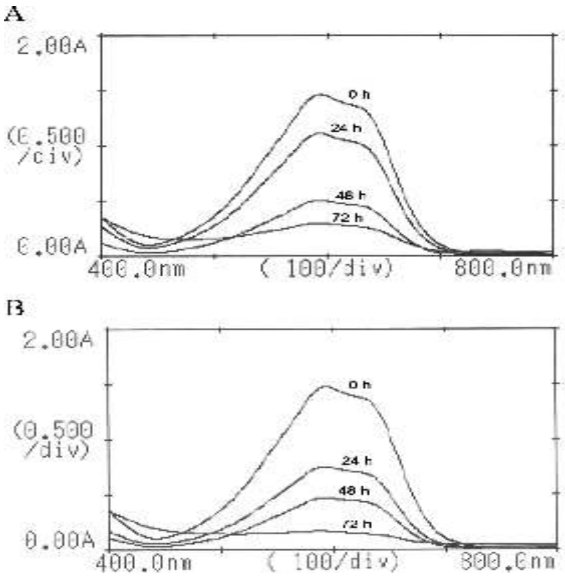

Figure 2B shows the visible absorbance spectrum for RBBR after treatment with the HBT, which contained 1.5 U/ml laccase. Following 24 h and 48 h of incubation, the percentage decolorization was 52% and 85%, respectively. Our results suggest that fungi that have laccase, and corresponding redox mediator, will be more readily detected using RBBR as the chromophoric substrate. This study with laccase purified from *Polyporus* sp. S133 showed that the laccase alone could decolorize RBBR. In the latter study, employing purified laccase with HBT increase 20% decolorization. This could be explained by the fact that anthraquinone dyes such as RBBR act as enzyme substrates that are directly oxidized by laccase involved by some small molecule like HBT as mediators (Soares *et al.*, 1991). The final overlay shows that complete decolorization occurred within 72 h. RBBR has been conveniently used to screen large numbers of fungi for lignolytic activity (Lonergan *et al.*, 1993). Eggert *et al.* (1996) showed that the lignolytic system of white rot fungi is unusual in that it lack both lignin peroxidase and manganese peroxidase. Also, a natural mediator for the versatile laccase has been identified in some white rot fungi (Eggert et *al.*, 1996). Fungal laccase in combination with variety of redox mediators and found that the redox potential of the laccases varied depending of the source of the laccase. This could conceivably dictate that need or nature of redox mediator for the degradation of a particular dye to occur (Li *et al.*, 1999).

Effect of dye and laccase concentration on RBBR decolorization

The effect of initial dye concentration was performed by increasing the RBBR concentration from 200 to 500 mg/l. As shown in Figure 3A, the laccase from *Polyporus* sp. S133 decolorized up to 90% of RBBR when the concentration used is below or equal to 300 mg/

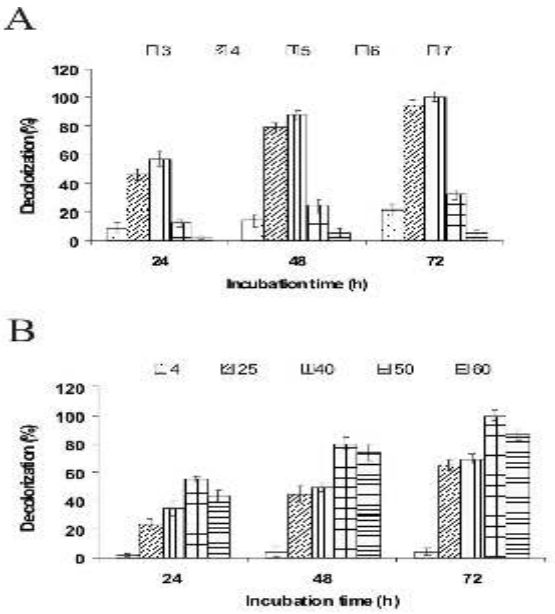

Figure 3. Effect of dye (A) and enzyme (B) concentration on the decolorization of RBBR by the laccase of *Polyporus* sp. S133

However, only 75% of colour removal was obtained when the dye concentrations were between 400 and 500 mg/l The behavior observed here is similar to that of laccase oxidation of substrates in which the rate of substrate oxidation increased with the substrate concentration until saturation.

Our results are in agreement with that of (Deveci *et al*., 2004), who demonstrated that the dye decolorization rate was optimal by increasing RBBR concentrations up to 400 mg/l; however, concentrations above 400 mg/l were inhibitory. The effect of enzyme concentration on RBBR decolourization was studied by increasing laccase activity from 0.5 U/ml to 1.5 U/ml. It can be seen in Fig. 3B, that 88% of RBBR was decolourized within 48 h of incubation when 1.5 U/ml of laccase was used. We can also notice that decolourization rate of RBBR increased with enzyme concentrations up to 0.75 U/ml.

Effect of pH, temperature and non-ionic surfactants on RBBR decolorization

The effect of pH was studied at pH values between 3 and 7.

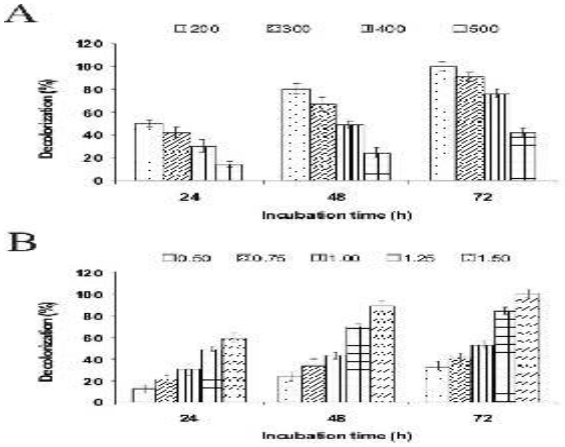

Figure 4. Effect of pH (A) and temperature (B) on the decolorization of RBBR by the laccase of *Polyporus* sp. S133.

Fig. 4A shows that decolorization of RBBR was optimal at pH 5. No decolorization was observed at pH values of 7. However, slow decolorization was achieved at pH 3 and 6. The decolorization of RBBR were also affected by pH and temperature of the extracellular enzyme. These results confirms that the pH optimum of this laccase is substrate dependent as purified laccases showed optimum pH values, estimated in 100 mM tartrate buffer, at 2.5 and 3 for the oxidations of DMP (Zouari-Mechichi *et al*., 2006). However it was only stable at neutral pH values. The effect of temperature was studied by incubating the reaction mixture at temperatures between 4 and 60°C. The optimal temperature for decolourization was 50°C (Fig. 34B). The laccase was stable for 24 h at 50°C but it lost 90% of its activity at 60°C (Zouari-Mechichi *et al*., 2006).

Effect of non-ionic surfactants on RBBR decolorization

In order to study whether the non-ionic surfactants influenced the decolorization, the influence of three representative non-ionic surfactants was studied using the laccase. These were of the polysorbate type (Tween 80), sorbitol esters type (Tween 20), and polyoxyethylene esthers (Brij 35).

Figure 5 shows the decolorization obtained after treatment with surfactants for 30 min.

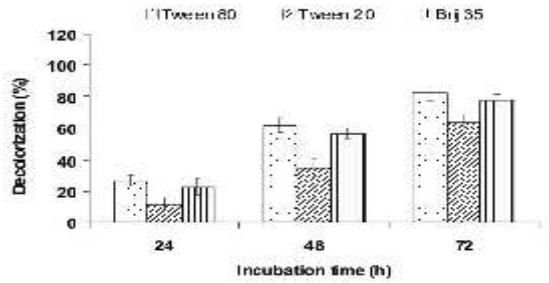

Figure 5. Effect of non-ionic surfactants on the decolorization of RBBR by the laccase of *Polyporus* sp. S133.

These did not enhance RBBR decolorization. In some cases an inhibitory effect was seen and this was particularly pronounced using Tween 20. The three different types of non-ionic surfactants studied in the present work did not enhance decolorization. The addition of non-ionic surfactants was not affected in enhancing the decolorization. In some case an inhibitory effect was seen which may be due to inhibition of laccase activity (Eriksson *et*

al., 1990). This suggests that the main role of the non-ionic surfactant present in the formulation may be in the solubilization of indigo in the textile finishing process as well as to stabilize the formulation.

In a view result obtained in the present work, it can be concluded that laccase from *Polyporus* sp. S133 is a powerful tools for the decolorization of textile dye. The purified laccase was found to decolorize efficiently the dye at a concentration of 200 mg l^{-1} in the present 1.5 U ml^{-1} of enzyme. In addition, decolorization was optimal at pH 5 and at a temperature of 5 °C. The effect of HBT was also assayed. Addition of HBT to purified laccase increases the decolorization. The effect of non-ionic surfactants in the decolorization of RBBR was tested with Tween 80, Tween 20 and Brij 35 at concentration of 0.5%. It was demonstrated that Tween 20 was inhibiting substrate for the decolorization while Tween 80 and Brij 35 were not inhibiting for the decolorization.

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