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Full Length Research Paper

Decolorization of the azo dye reactive black 5 using laccase mediator system

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Laccase mediator system (LMS) has been widely used in various fields. In this study, we tested the capacity of LMS in the decolorization of a recalcitrant azo dye Reactive Black 5 (RB5). Both acetosyringone and syringaldehyde demonstrated high mediating ability for RB5 decolorization and acetosyringone was proved to be the most efficient mediator. The decolorization process was not significantly affected at various temperature and pH, and the laccase mediator system still maintained high efficiency when the dye concentration increased. The optimized conditions for dye decolorization were 160 mg/L of RB5, 0.5 U/ml of laccase and 0.1 mmol/L of mediator at 40°C and pH 7.0. More than 92% of RB5 was decolorized with acetosyringone as mediator in only 30 min. The results show an enormous potential of this laccase mediator system in the treatment of azo dye effluents.

Key words: Azo dye, reactive black 5, mediator, laccase, decolorization.

INTRODUCTION

The rapid development of dye industry has accelerated dye production along with a series of environmental hazards. Azo dyes are the largest and most diverse group of synthetic dyes, and more than 50% of annually produced dyes are azo compounds (Szyguła et al., 2008). The hazards of azo dyes lie both in the high carcinogenicity of their cleavage products and their low biodegradability (Stolz, 2001). Reactive azo dyes are highly recalcitrant to conventional wastewater treatment processes, with as much as 90% of reactive dyes remaining unchanged after activated sludge treatment (Pierce, 1994). The reductive cleavage of the azo bond under anaerobic conditions usually leads to the formation of aromatic amines, which are highly toxic, mutagenic and carcinogenic (Pinheiro et al., 2004).

Lots of attempts have been made to remove azo dyes from the dye effluents. Traditional physical and chemical methods have technical and economical limitations, including high costs, low efficiency and ineffective for recalcitrant dyes (Srinivasan and Viraraghavan, 2010) as well as the production of large amounts of sludge (Borchert and Libra, 2001). In contrast, biological treatment provides a better alternative, and many micro-organisms have been reported to demonstrate the ability to degrade azo dyes (Stolz 2001). However, microbial treatment would result in biomass accumulation, which will expand the treatment scale (Murugesan et al., 2007), and the decolorization process is usually slow. Hence, the recent focus has shifted towards enzyme based treatment of colored wastewater. Researchers have isolated azoreductase from bacteria to decompose azo dyes by cleaving their azo bonds (Misal et al., 2011). Nevertheless, most reactions need to be conducted under strict anaerobic condition (Stolz, 2001). The degradation of azo dyes by azoreductase was incomplete due to their complex structures, and some of them might even be converted into toxic aromatic amines (O'Neill et al., 2000). An eco-friendly and non-specific enzyme, therefore, is in desperate need for degrading azo dyes.

Laccase (EC 1.10.3.2) has received much attention for its superiority in degrading various recalcitrant pollutants (Roriz et al., 2009). It is a type of oxidase widely distributed among plant, fungi and bacteria, and can catalyze a variety of aromatic compounds using oxygen as electron acceptor with water as the sole by-product (Cañas and Camarero, 2010). Some redox mediators can facilitate the catalytic activity of laccase and expand its

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substrates specificity to a much wider range (Cambria et al., 2008). Laccase mediator systems (LMS) have been widely used in different fields such as degrading organophosphorus compounds (OPs), insecticide, PHA, pulp biobleaching and dye decolorization (Trovaslet-Leroy et al., 2010; Kudanga et al., 2011).

In this work, we selected reactive black 5 (RB5), a widely used recalcitrant diazo dye, as a model to investigate the potential of a commercial laccase in azo dye decolorization. Several common mediators were tested for their efficiency in favoring the decolorization of RB5. Some factors which may affect the decolorization process were also optimized.

MATERIALS AND METHODS

Chemical reagents

Commercial laccase was kindly provided by Novozymes (Tianjin, China). Reactive black 5 (RB5), promazine (PZ), acetosyringone (Ace), violuric acid (VA), vanillin (Van), syringaldehyde (Syr), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 4-hydroxybenzoic acid (HBA) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Hydroxybenzotriazole (HBT) was obtained from Shanghai Medpep Co., Ltd (Shanghai, China). All other chemicals were of analytical grade.

Enzyme assays

Laccase activity was determined according to the method of Lu et al. (2007) with ABTS as the substrate. One activity unit was defined as the amount of enzyme that oxidized 1 µmol of ABTS per minute. All assays were carried out in triplicate.

Dye decolorization by laccase

Decolorization of RB5 was carried out in 50 ml baffled shake flasks at 40°C under mild shaking conditions. The reaction mixture consisted of 0.1 mol/L citrate–phosphate buffer (pH 3.0), laccase (0.5 to 10 U/ml) and RB5 (40 mg/L). Dye decolorization was determined at intervals by the decrease in absorbance under 597 nm and expressed in terms of percentage. Control experiments were performed under the same condition without the addition of laccase. Experiments were performed in triplicate. The decolorization percentage was defined as follows:

$$\text{Decolorization percentage (\%)} = (A_0 - A) / A_0 \times 100$$

Where, A_0 is the dye absorbance of the control, and A is the dye absorbance of the reaction sample.

Mediator screening

For laccase mediator screening, nine redox mediators were selected and added into the decolorization system with a final concentration of 0.1 mmol/L. The decolorization percentage of RB5 was measured after 2 h incubation at 40°C. Control experiments were performed under the same condition without mediator. Mediators that showed significant effect in enhancing dye decolorization were chosen for further study.

Optimization of RB5 decolorization by laccase mediator system

Parameters including pH, temperature, dye concentration and enzyme concentration that might affect reaction process were optimized in the presence of selected mediators. Dye decolorization was monitored after 2 h. The decolorization of RB5 under the optimized conditions was also performed. The control samples without enzyme were conducted in parallel under the same condition.

RESULTS AND DISCUSSION

Decolorization of reactive black 5 by laccase

The capacity of a commercial laccase in decolorization of RB5 was tested at pH 3, which is the optimum pH for ABTS oxidation. Laccase was able to decolorize RB5 without the addition of any mediator, however, the decolorization efficiency was relatively low (Figure 1). The increase in color removal was slow in proportion to the increase of laccase concentration. The maximum decolorization (25.16%) was observed at laccase concentration of 10 U/ml after an incubation of 2 h. The result indicates that RB5 was rather recalcitrant to degradation by laccase. The laccase from *Trametes modesta* (Nyanhongo et al., 2002) and *Daedalea quercina* (Baldrian, 2004) also showed a poor ability in decolorizing RB5, with only 9 to 18% decolorization of RB5 (Nyanhongo et al., 2002; Baldrian, 2004). In addition, laccases from *Pycnoporus cinnabarinus* and *Trametes villosa* failed to decolorize RB5 (Camarero et al., 2005). The resistance of RB5 to biodegradation may be due to the high redox potential as well as the steric hindrances of this dye (Camarero et al., 2005).

Mediator screening

The presence of some redox mediators could expand the catalytic activity of laccase towards many recalcitrant compounds (Dwivedi et al., 2011). Mediators are laccase substrates and act as electrons shuttles between the enzyme active site and the complex compounds, which enables the oxidation of target compounds (Riva, 2006). A moderate laccase activity (3 U/ml) was used to screen efficient laccase mediators for RB5 decolorization. Among the nine redox mediators tested, only acetosyringone (Ace) and syringaldehyde (Syr) could significantly enhance the decolorization of RB5, whereas the other mediators had little impact on color removal (Figure 2). Up to 65% decolorization was observed with the addition of Ace and Syr within 2 h, which is about six times higher than that of control sample.

The strong mediating potential of Ace and Syr were also demonstrated in other researches (Camarero et al., 2005; Dubé et al., 2008). These natural mediators usually have a strong mediating capability and generate stable

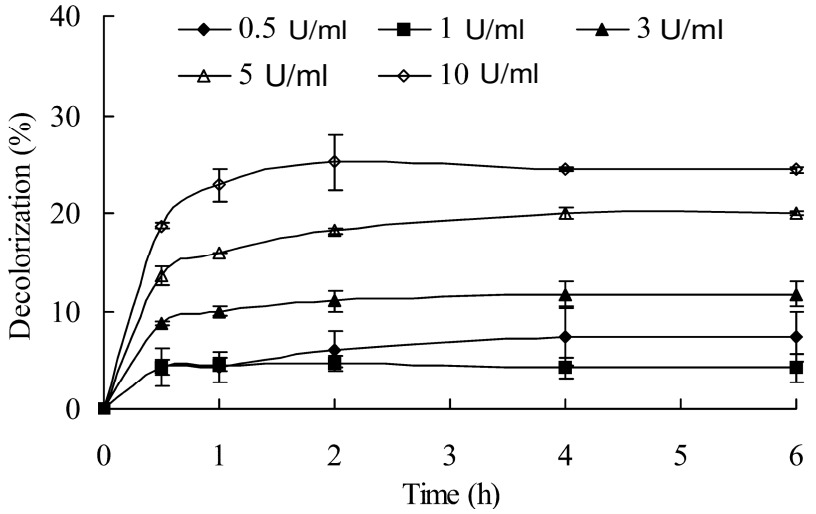

Figure 1. Decolorization of RB5 by laccase in 0.1 mol/L citrate-phosphate buffer (pH 3.0) at 40℃

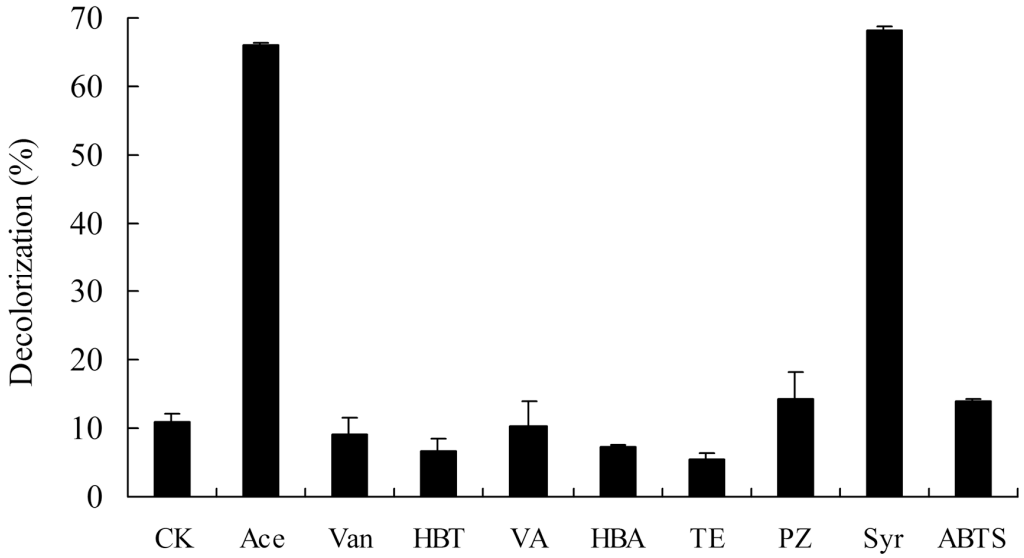

Figure 2. Decolorization of reactive black 5 by laccase in the absence and presence of redox mediators after 2 h incubation at 40℃ and pH 3.0.

radicals in reaction, which will keep the reaction functioning in a recycling way instead of gradual extinction (Cañas and Camarero, 2010). Although HBT is a frequently used laccase mediator and has been proved to be efficient in promoting the decolorization of RB5 (Nyanhongo et al., 2002; Claus et al., 2002; Murugesan et al., 2007), it failed to favor RB5 decolorization in this study and even slightly inhibited the decolorization process. The inactivation of laccase in dye decolorization caused by the toxic HBT radical was reported by Soares et al. (2001). Considerable redox mediators have been investigated to provide theoretical basis before industrial use, and most efficient mediators were artificial with the drawbacks of high price and toxicity, which limit their application extent (Majeau et al., 2010). Thus, natural laccase mediators, such as Ace and Syr, are more advantageous for industrial application.

Effect of pH on dye decolorization

The effect of pH on RB5 decolorization was investigated in 0.1 mol/L citrate phosphate buffers from pH 2.2 to 8.0. RB5 was mostly decolorized at pH 5.0 to 8.0 (Figure 3a). Maximum decolorization was observed at pH 7.0, with 93.16 and 90.98% color removal in the presence of Ace

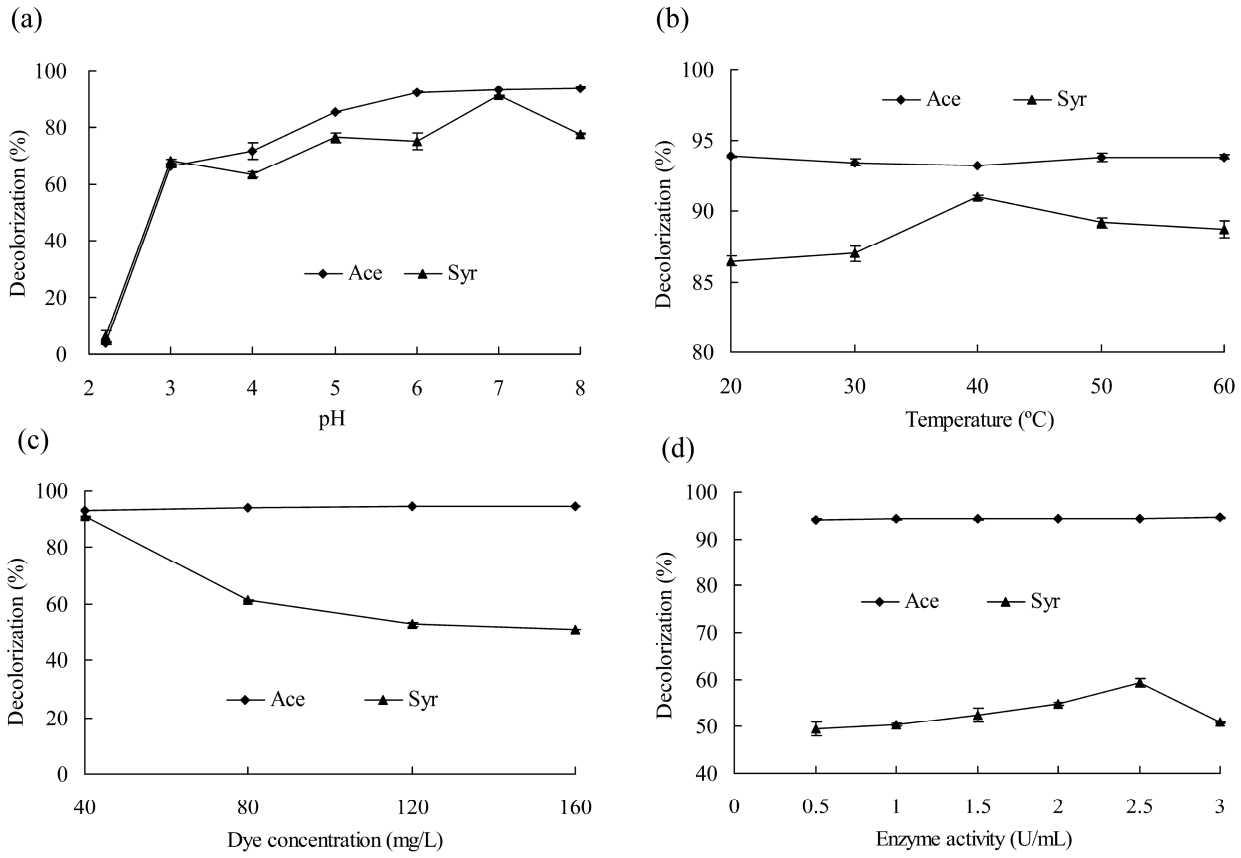

Figure 3. Effects of different factors on decolorization of reactive black 5 in the presence of 0.1 mmol/L mediator. (a) pH; (b) temperature; (c) dye concentration; (d) laccase activity.

and Syr, respectively. The high decolorization ability of this laccase-mediator system in neutral to alkaline pH range is very advantageous in wastewater treatment, since most fungal laccases are active in acid conditions for dye decolorization (Nyanhongo et al., 2002; Kokol et al., 2007).

Effect of temperature on dye decolorization

The result of RB5 decolorization at pH 7.0 under various temperatures is shown in Figure 3b. The treatment was almost not affected by changing temperatures from 20 to 50°C. About 93% of decolorization was obtained with Ace as mediator, and 86.47 to 90.98% of color removal was found for Syr after 2 h (Figure 3b). The optimal temperature for RB5 decolorization was observed at 40°C when Syr was used as the mediator. Therefore, further decolorization experiments were operated at 40°C.

Effect of dye concentration on dye decolorization

RB5 with a final concentration ranging from 40 to 160 mg/L was used to test the decolorization capacity of the laccase-mediator system in high dye concentrations. The commercial laccase in combination of Ace was found to be extremely efficient in decolorizing RB5 with concentration as high as 160 mg/L (Figure 3c). The extent of decolorization achieved 94.54% with Ace as mediator, whereas the decolorization percentage decreased with the increase of dye concentration when Syr was present (Figure 3c). However, there was still 50.94% of decolorization for 160 mg/L of RB5 in the presence of Syr. Industrial dye effluents usually contain high dye concentration, which would inhibit laccase activity due to their toxic effects (Katuri et al., 2009). Compared to Syr, Ace was more suitable for mediating the decolorization of synthetic azo dyes with high concentrations.

Effect of laccase activity on dye decolorization

We investigated the effect of laccase activity on decolorization of high concentration of RB5 (160 mg/L). The decolorization was almost unaffected at laccase activity of 0.5 to 3 U/ml when Ace was used, while the decolorization extent mediated by Syr slightly increased

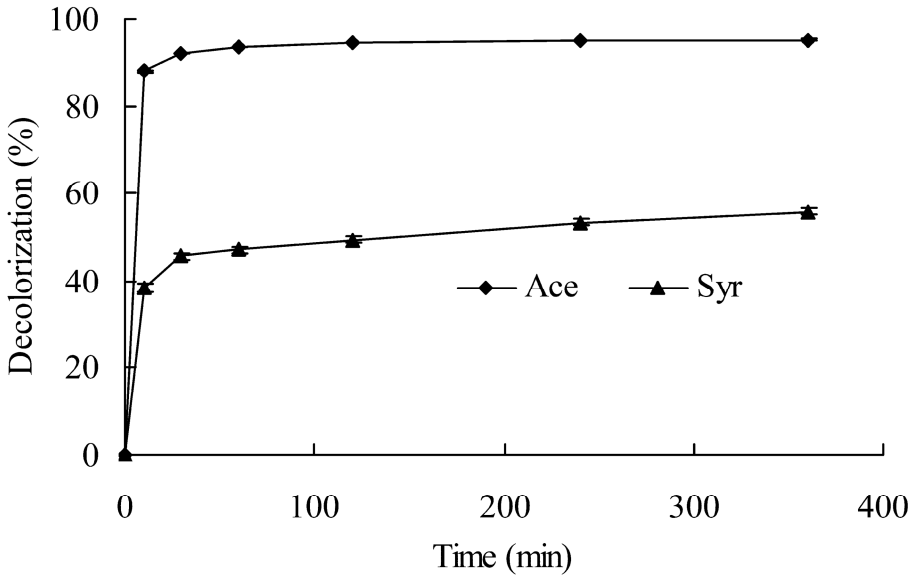

Figure 4. Decolorization of RB5 by laccase-mediator system under the optimized conditions.

in the presence of a higher laccase activity (Figure 3d). The decolorization percentage using 0.5 U/ml of laccase was 94.15 and 49.45% in the presence of Ace and Syr, respectively. The result indicates that a low laccase dosage could be used for RB5 decolorization by the LMS.

As shown in Figure 3, the mediators selected in the research could make LMS a more stable system for RB5 decolorization. Ace was more efficient in mediating RB5 decolorization than Syr under conditions of high dye concentration and low laccase activity. When added with mediator, the amount of laccase used could obviously be reduced to reach the same degrading effect, which would save the application cost and would be a notable predominance in industrial application.

Decolorization of reactive black 5 under optimal condition

The process of RB5 decolorization by LMS was performed in optimized conditions, which contained 160 mg/L of dye, 0.5 U/mL of laccase, 0.1 mmol/L of mediator, at 40℃, and pH 7.0. The reaction velocity increased sharply with the existence of mediator in the first 10 min (Figure 4). About 88% of RB5 was decolorized in 10 min with Ace as mediator, and the process became stable after 30 min. Meanwhile, the decolorization percentage gradually increased in the presence of Syr. RB5 was 75.57% decolorized with Syr as mediator after 6 h, which was less than the extent obtained in the presence of Ace. However, a final decolorization of about 92% was observed when the incubation time extended to 24 h. Compared with the decolorization by laccase alone, these two mediators accelerated the process rate by 14 to 22 fold in the first 30 min. The whole process almost remained stable after 8 h, and a much shorter degradation time was needed to obtain high decolorization percentage compared with other studies (Abadulla et al., 2000; Camarero et al., 2005; Couto, 2007).

Roriz et al. (2009) optimized the RB5 decolorization by laccase from *Trametes versicolor* using HBT as redox mediator, and the decolorization only reached about 60% in 20 min regardless of their high mediator concentration. Soares et al. (2001) also used laccase together with HBT or VA to decolorize the Remazol Brilliant Blue R and decolorization was enforced in optimal mediator concentration at 11 and 5.7 mmol/L for HBT and VA, respectively. The most limited factor to introduce LMS into industrial application would obviously be the cost of laccase and mediators. In this study, more than 92% of RB5 was decolorized when only 0.5 U/ml of laccase and 0.1 mmol/L of mediator (Ace) were used. Furthermore, the application of natural mediator in LMS could be much cheaper than the commonly used synthetic mediators such as HBT, ABTS and VA, and the use of natural mediator could also prevent the toxic effects of most synthetic mediators (Camarero et al., 2005).

Conclusion

In this work, we found that Ace and Syr demonstrated strong mediating abilities for the decolorization of the azo dye RB5, and Ace appeared to be even more efficient than Syr. The presence of these two mediators remarkably enhanced the decolorization rate and extent

for RB5. Temperature had little influence on decolorization by LMS. The decolorization by LMS remained its efficiency with a low laccase activity and high dye concentration. The results therefore indicate that this commercial laccase could be used in the practical treatment of azo dye wastewater in combination with Ace as the mediator.

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