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*Full Length Research Paper*

# Biodecolorization of Reactive Black 5 by laccase-mediator system

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**Reactive azo dyes are widely used as textile colorants, typically for cotton dyeing, due to their variety of color shades, and minimal energy consumption. In the present study, commercial laccase from *Trametes versicolor* was used for the biodecolorization of Reactive Black 5 (RB-5) dye using different redox mediators viz, N-hydroxybenzotriazole (HBT), 2,2′-azino-bis-(3-ethylbenzthiazoline- 6-sulfonic acid (ABTS), 2,6-dimethoxy phenol (DMP), syringaldehyde, vanillin, aceto-vanillone, *p*-coumaric acid and catechol. Commercial laccase alone did not show any considerable decolorization of RB-5. However, the laccase in the presence of syringaldehyde showed the strongest decolorization rate (98%), followed by vanillin (55.21%), aceto-vanillone (53.25%), ABTS (42.78%), *p*-coumaric acid (41.9%), DMP (39%), and catechol (36.33%); while least decolorization was observed with HBT at dye/mediator ratio of only 1:5 after 30 min. Therefore, syringaldehyde performance was evaluated at different mediator/dye ratios (1:1, 1:5 and 1:10) using commercial laccase and it was compared with that of synthetic mediator like HBT. It was found that the presence of syringaldehyde was essential for biodecolorization of RB-5. Moreover, it was observed that syringaldehyde was an effective natural redox mediator as compared to synthetic HBT. Enhanced decolorization (98%) of RB-5 by laccase was observed with 1:5 syringaldehyde and dye ratio for 30 min but maximum removal (22%) of RB-5 was recorded with HBT at 1:1 after 40 min. Thus, the study reveals that the phenolic compounds could be used as potential redox mediators for enhanced laccase-mediated decolorization of azo dyes.**

**Key words: Reactive** Black 5 (RB-5), redox mediators, laccase, biodegradation, azo dyes.

## INTRODUCTION

Wastewaters of the textile industries contain considerable amounts of non-fixed dyes especially azo-dyes. Reactive azo dyes are mostly used in textile dyeing due to their variety of color shades, high wet fastness profiles, ease of application, brilliant colors, minimal energy consumption, high photolytic stability, and resistance to microbial degradation. The release of such colored wastewaters in the ecosystem is a dramatic source of esthetic pollution, eutrophication and perturbations in thaquatic life (Lachheb et al., 2002).

Most physicochemical dye removal methods, which are generally used for effluent treatment have many limitations (Balcioglu and Arslan, 2001; Ghoreishi and Haghighi, 2003). In recent years, biological decolorization method has been considered as an alternative and eco-friendly method to dye degradation and color removal (Hafiz et al., 2008; Asgher et al., 2009; Bibi et al., 2011). Dyes biodegradation is usually carried out by white rot fungi by their ligninolytic enzymes such as lignin peroxidases, manganese peroxidases, and laccases (Asgher et al., 2008). A number of white rot fungi have been explored for decolourization of various industrial dyes and treatment of dye effluent (Bhatti et al., 2008; Asgher et al., 2010; Bibi et al., 2010). Majority of these studies were carried out with fungal mycelia. One of the

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**Abbreviations: RB-5,** Reactive Black 5; **HBT,** N-hydroxybenzotriazole; **ABTS,** 2,2′-azino-bis-(3-ethylbenzthiazoline- 6-sulfonic acid; **DMP,** 2,6-dimethoxy phenol.

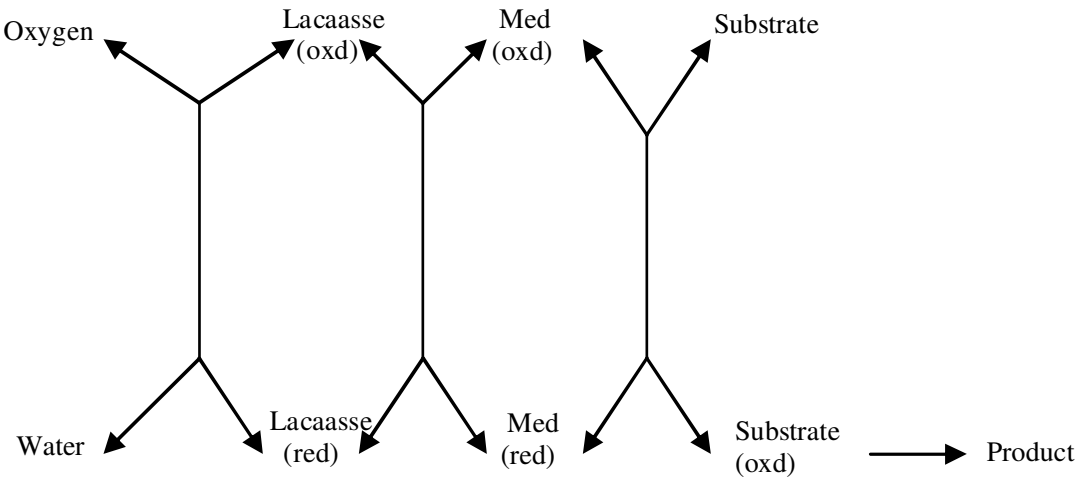


**Figure 1.** Mediator system of laccase (Gochev and Krastanov, 2007).

major disadvantages of using fungal cultures for dyes decolourization is the accumulation of biomass, which would cost the wastewater treatment in industrial scale. To overcome this disadvantage, the application of isolated enzymes for dye decolourization has increased in recent years (Kandelbauer et al., 2004; Bibi et al., 2011).

Laccases are oxidoreductases which belong to the multinuclear copper-containing oxidases and are able to decolorize and detoxify industrial dyes (Zouari-Mechichi et al., 2006). These oxidative enzymes are particularly abundant in white rot fungi and have been purified and characterized extensively from many white rot fungi (Jordaan et al., 2004). In recent years, laccase based treatment method has received much attention in the treatment of various recalcitrant pollutants because the laccase production is constitutive in most of the white rot fungi and it can be easily enhanced by chiefly available laccase inducers. Many studies have been demonstrated for dye decolourization using both crude and purified forms of laccase (Baldrian et al., 2004). However, some of the dyes cannot be oxidized, or partly oxidized by laccase, because they are too large to penetrate into the enzyme active site or have a particularly high redox potential. In recent years, some natural phenolic compounds, including syringaldehyde and acetosyringone (AS), have been described as efficient and eco-friendly laccase mediators for textile and environmental applications. Cho et al. (2007) have intensified the range of compounds that can be oxidized by these enzymes. The mechanism by which redox mediators play a role in laccase-mediated oxidation reactions is now well characterized. When a substrate is oxidized by a laccase the redox mediator forms cation radicals (short-lived intermediates) which co-oxidize the substrate. These cation radicals can be formed by two mechanisms; the redox mediator can perform either a one-electron oxidation of the substrate to a radical cation (Xu et al., 2001) or it abstracts an H-atom from the substrate converting it into a radical (Fabbrini et al., 2002). According to Gochev and Krastanov (2007), the mechanism of laccase mediators system is given in Figure 1.

The rationale behind the present study was to evaluate the potential of different naturally occurring phenolic compounds to mediate the oxidative reactions catalyzed by laccase with the aim of identifying cheaper, more competent and ecofriendly mediators for the decolorization of recalcitrant dyes and for other industrial and environmental applications. Also in enzymatic dye decolourization, optimization of the concentrations of redox mediator and dye is an important criterion for successful decolourization. To our knowledge, there has been no study for the optimization of dye/mediator ratio in enzymatic dye decolourization using fungal laccase. To evaluate the mediating capabilities of these compounds, we used a test based on the decolorization of Reactive Black 5 (RB-5), a recalcitrant dye that is not oxidized by commercial laccase alone.

## MATERIALS AND METHODS

### Chemicals and microorganism

Reactive Black 5 (RB-5), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), syringaldehyde (SYD), N-hydroxybenzotriazole (HBT), 2,6-dimethoxy phenol (DMP), catechol, *p*-coumaric acid, vanillin and aceto-vanillone were purchased from Sigma-Aldrich Co. USA. Laccase (from *Trametes versicolor* EC: 1.10.3.2) was obtained from Fluka, USA. The Chemical structure of Reactive Black 5 dye is shown in Figure 2.

### Screening of redox mediators

Screening experiments were carried out to investigate the effect of redox mediators on the decolorization of RB-5 dye by commercial laccase. For this purpose, 10 µl of *T. versicolor* laccase and

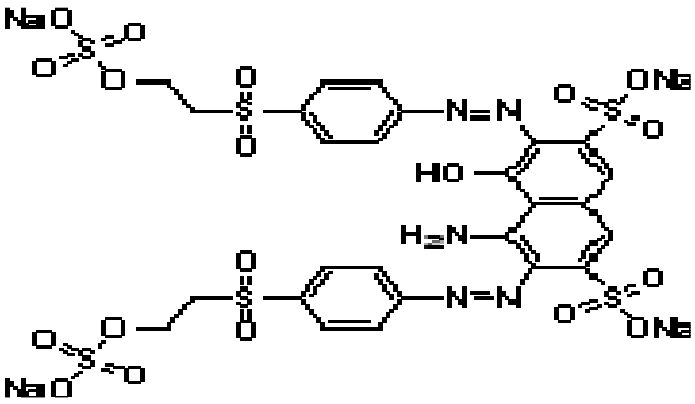

**Figure 2.** Chemical structure of Reactive black 5 dye (RB-5) (C.I. 20505; M.W= 991).

Reactive Black 5/redox mediator at 1:5 were incubated in the citrate-phosphate buffer (pH 4.5) for 30 min in Eppen-dorf tube at 25°C. Absorbance of each sample was noted after 30 min at $\lambda_{max}$ (595 nm) of the respective dye in order to determine the percentage decolorization.

#### Screening of dye/ mediator ratio

Decolorization experiments were carried out in 2 ml Eppen-dorf tube. Reaction mixture (1 ml) containing 100 mM citrate-phosphate buffer (pH 4.5), laccase concentration (10 µl), and dye: syringaldehyde ratio 1:1, 1:5 and 1:10 were prepared. The reaction tubes were incubated at 25°C under dark and the decolourization was monitored spectrophotometrically (Cary 3 Bio, Varian, UK) after different time intervals by recording the absorbance at the $\lambda_{max}$ of the dye. Different sets of control were also run parallel to see the effect of mediator.

#### UV-Vis spectral analysis

RB-5 decolorization was determined by measuring the decrease in optical density of the dye at the wavelength of maximum absorbance ($\lambda_{max}$ 595 nm) in a UV–Vis spectrophotometer (Cary 3 Bio, Varian, UK) and expressed in percentage. Different control samples were also run parallel and contained the reaction mixture i) with-out enzyme (RB-5 only), ii) SYD+RB-5 and iii) HBT+RB-5. The percentage decolorization was calculated as follows:

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

All experiments were conducted in triplicates and data were presented as mean±SD.

## RESULTS AND DISCUSSION

### Effect of various redox mediators on the decolorization of RB-5 dye by laccase of *T. versicolor*

Laccase mediator systems are important bioremediation agents as the rate of reactions could be enhanced in the presence of the mediators. In order to observe the effect of redox mediators on the biodecolorization of RB-5 dye, different laccase mediators (phenolic alcohols, aldehydes, ketones, acids, etc.) were used. The effect of the natural and synthetic mediators on the decolorization of Reactive Black 5 (30 min treatment) is shown in Figure 3. The results reveal that laccase showed strongest decolorization rate (97.45%) in the presence of syringaldehyde followed by vanillin (55.21%), aceto-vanillone (53.25%), ABTS (42.78%), *p*-coumaric acid (41.9%), DMP (39%), and catechol (36.33%). However, least decolorization rate (5.67%) was observed with HBT at a dye/mediator ratio of 1:5 only, while laccase alone did not decolorized RB-5 dye.

The specificity of mediators towards different functional groups must also be taken into account in laccase-mediator reactions. For example, laccase- ABTS is not reactive towards benzylic ethers or alkylbenzenes, but it is effective on benzyl alcohols (Baiocco et al., 2003; Cantarella et al., 2003) and oxidized HBT and VIO also seem to be more competent towards some specific groups (Soares et al., 2002). In the present study, different decolorization rates were observed depending on the type of mediator used. Finally, syringaldehyde seemed to be highly competent to mediate the oxidation of the tested dye. Recently, two different mechanisms for the oxidation of non-phenolic compounds by laccase mediator systems have been proposed: i) an electron transfer route for mediators such as ABTS and ii) a radical hydrogen atom transfer route for mediators of the -NOH- type (Baiocco et al., 2003). It is very likely that the phenoxy radicals formed during the oxidation of natural mediators by laccase act similar to the -NO·- radicals from -NOH- compounds, that is, they extract a hydrogen atom from the substrate (Acunzo and Galli, 2003). Therefore, the dissociation energy of the corresponding bond should govern their reaction with the laccase mediators.

The feasibility of the laccase-mediator systems in bio-transformation reactions depends on redox reversibility of the radical-substrate reaction, as well as on the balance

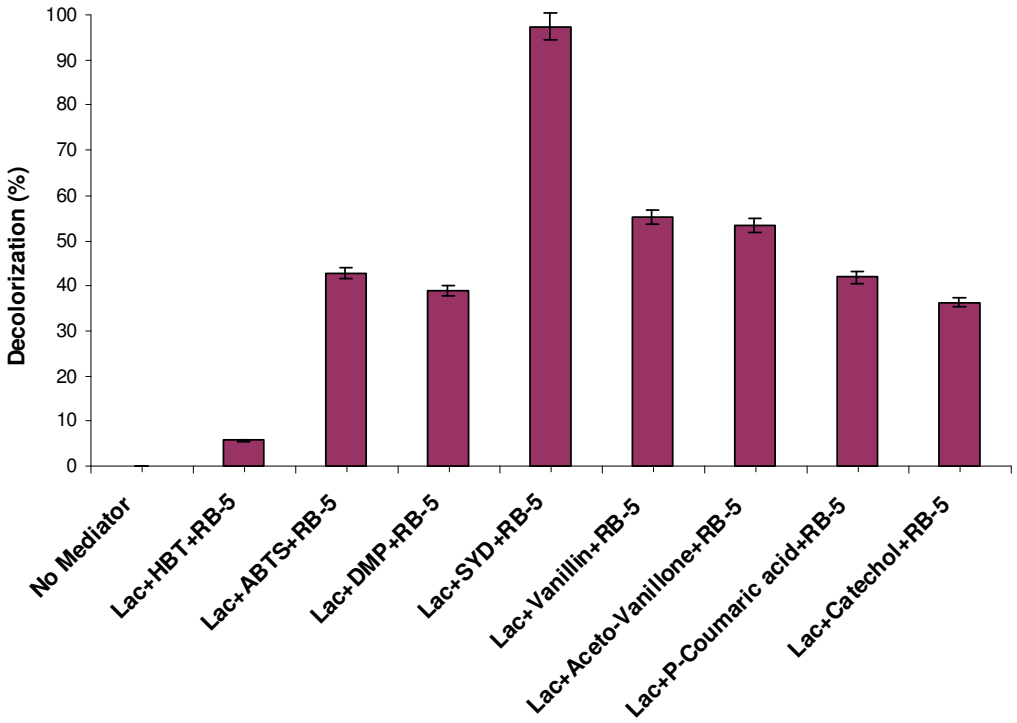


**Figure 3.** Effect of various redox mediators on the decolorization of RB-5 dye by laccase of *Trametes versicolor*.

between the stability and reactivity of the mediator radical which, in addition, should not inhibit enzyme activity (Camarero et al., 2007).The two former conditions have been confirmed with syringaldehyde, since voltametric and electron paramagnetic resonance studies have shown the true reversibility of the substrate reaction (Fernandez-Sanchez et al., 2002) and the long half-life of its phenoxy radical (Marzullo et al., 1995). In contrast, the -NO·- radical from HBT inactivates laccase, and due to its high reactivity, decays rapidly to benzotriazole and other inactive compounds (Li et al., 1998). Results showed that syringaldehyde is more easily oxidized by laccase than vanillin, ABTS, HBT, DMP, catechol and aceto-vanillone due to its high redox potential. This S-type phenols also form more stable radicals due to the presence of two methoxyl groups on the aromatic ring that prevent the formation of biphenyl-type structures by radical condensation. The higher stability of syringaldehyde phenoxy radicals is due to slightly acidic aqueous media (Caldwell and Steelink, 1969) as we carried out this reaction in phosphate buffer (pH 4.5).

## Optimization of redox mediator/dye ratio for enhanced decolorization of RB-5 by *T. versicolor* laccase

It was observed experimentally that the presence of syringaldehyde is essential for decolourization of RB-5 dye because laccase alone did not decolorize the dye (Figure 3). Therefore, in screening trials, different ratios of dye and redox mediator (SYD and HBT) were run to find out the most optimum ratio for accelerated decolorization of RB-5 dye. For this purpose, HBT and SYD were selected to compare the effect of synthetic and natural redox mediators on the decolorization of RB-5 dye by laccase of *T. versicolor*. It was noted that 1:5 (which we already used in mediator screening trials) was the optimum with SYD and 1:1 with HBT but much efficient decolorization (98%) of RB-5 dye was achived at 1:5 with SYD just within 30 min (Figure 5) while in case of HBT, the rate of decolorization was very slow and after 40 min, the maximum decolorization was only 22% at 1:1 dye: mediator ratio (Figure 6). Therefore, syringaldehyde was found to be an effective natural redox mediator as compared to synthetic HBT. Figure 5 shows the effect of 1:5 dye and mediator ratio on the decolorization of RB-5 dye by laccase. It clearly shows that the rate of decolorization was increased by increasing the dye: mediator ratio and reached a maximum of up to 98% with 1:5 but by further increase in this ratio (1:10) (Figure 4), the rate of decolorization was dramatically decreased.

Figure 7 clearly indicates that the extent of color removal of RB-5 dye was maximum, whereas, there is no significant removal in the presence of HBT at 1:5 dye: mediator ratio. However, in the presence of a large amount of mediator (500 µM), the enzyme concentration exhibited a negative effect on the rate of decolorization.

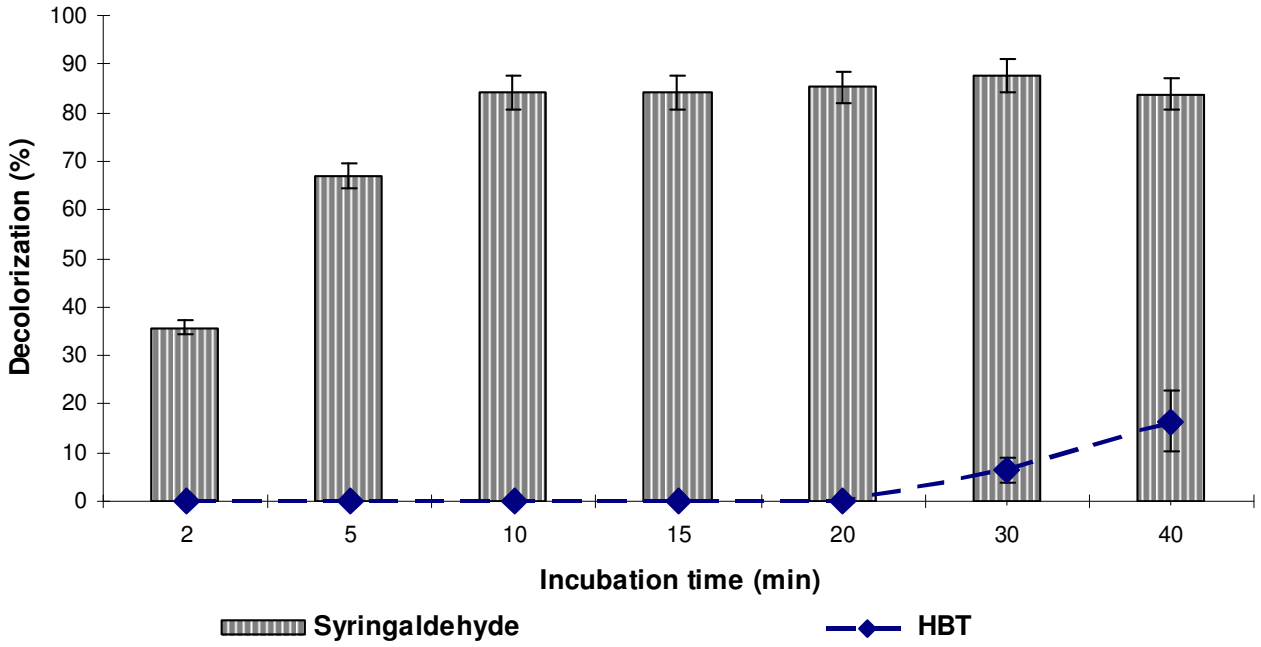


**Figure 4.** Decolorization of RB-5 dye by laccase with 1:10 (dye: redox mediator (SYD /HBT) ratio.

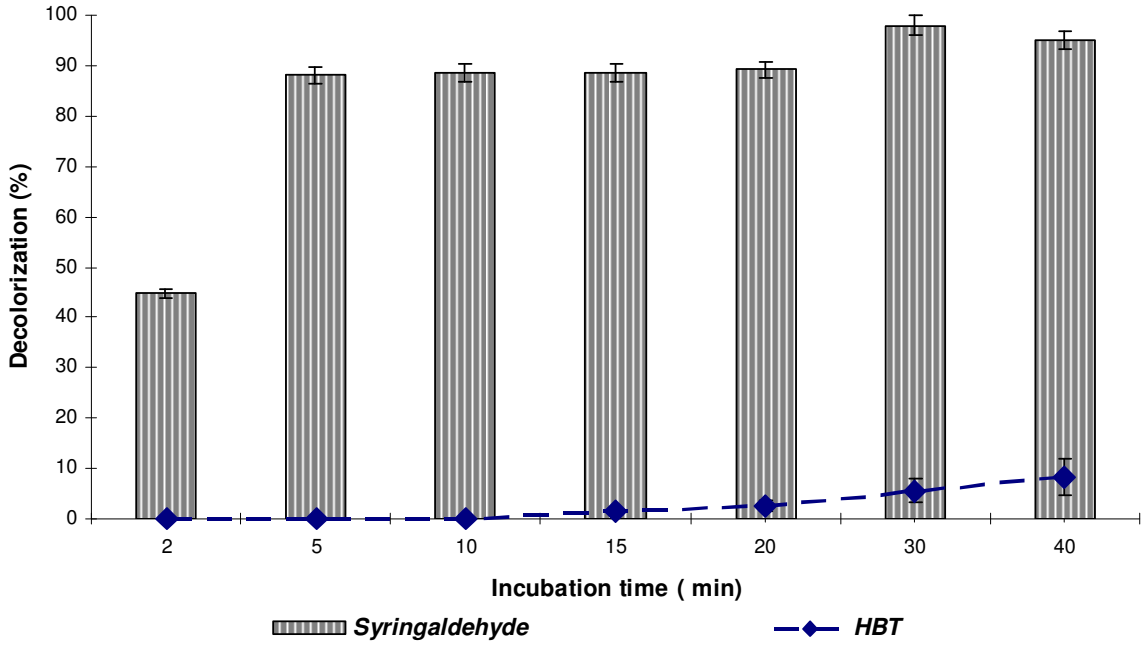


**Figure 5.** Decolorization of RB-5 dye by laccase with 1:5 (dye: redox mediator (SYD /HBT) ratio.

Wong and Yu, (1999) also reported that the efficiency of laccase-mediator systems in the decolorization reaction depended principally on the mediator concentrations and laccase activity used. Some fungal laccases as well as laccase mediator systems are efficient in dye decolorization. Figure 8 shows the UV-Vis absorption spectra of RB-5 dye at different dye: mediator ratio, which clearly indicated that at 1:5 dye: mediator ratio, the rate of color removal was maximum.

Different dyes were decolorized by different laccases at different rates. The decolorization rate depends on the structure and the redox-potential of the enzyme as well as the dye structure (Soares et al., 2002; Maalej-Kammoun et al., 2009). Preliminary results showed that

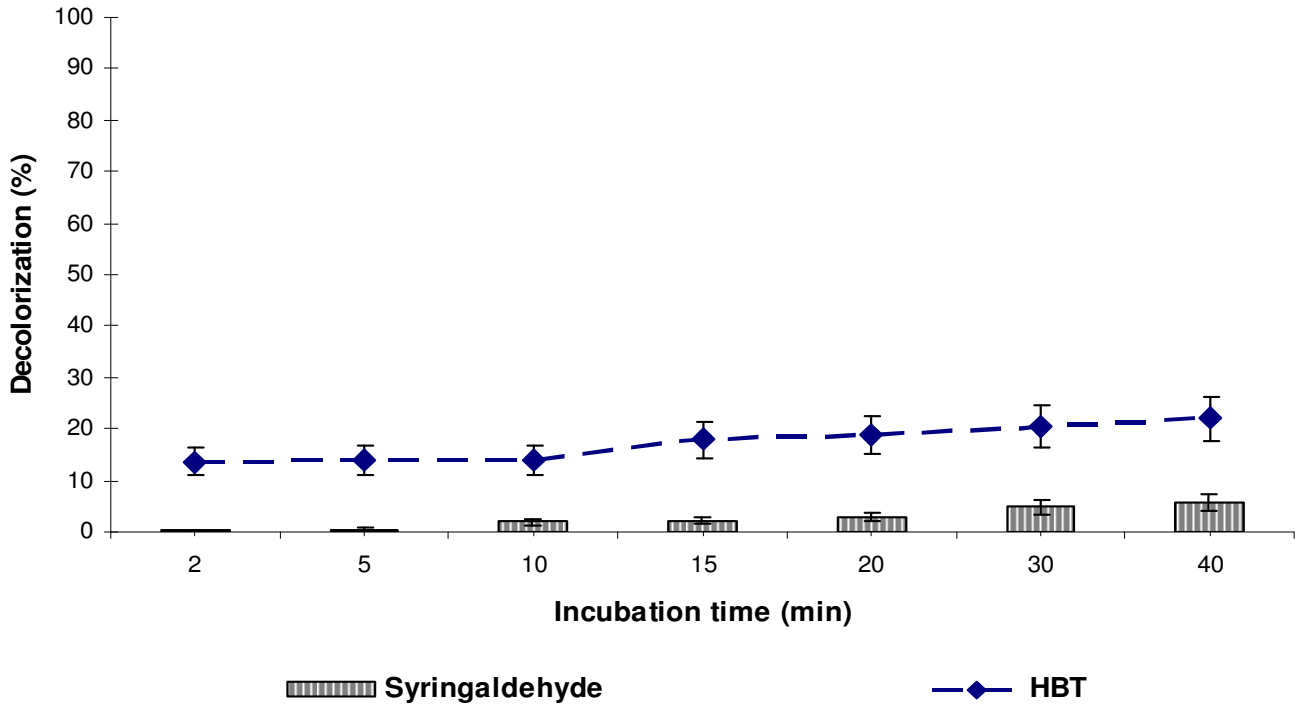


**Figure 6.** Decolorization of RB-5 dye by laccase with 1:1 (dye: redox mediator (SYD /HBT) ratio.

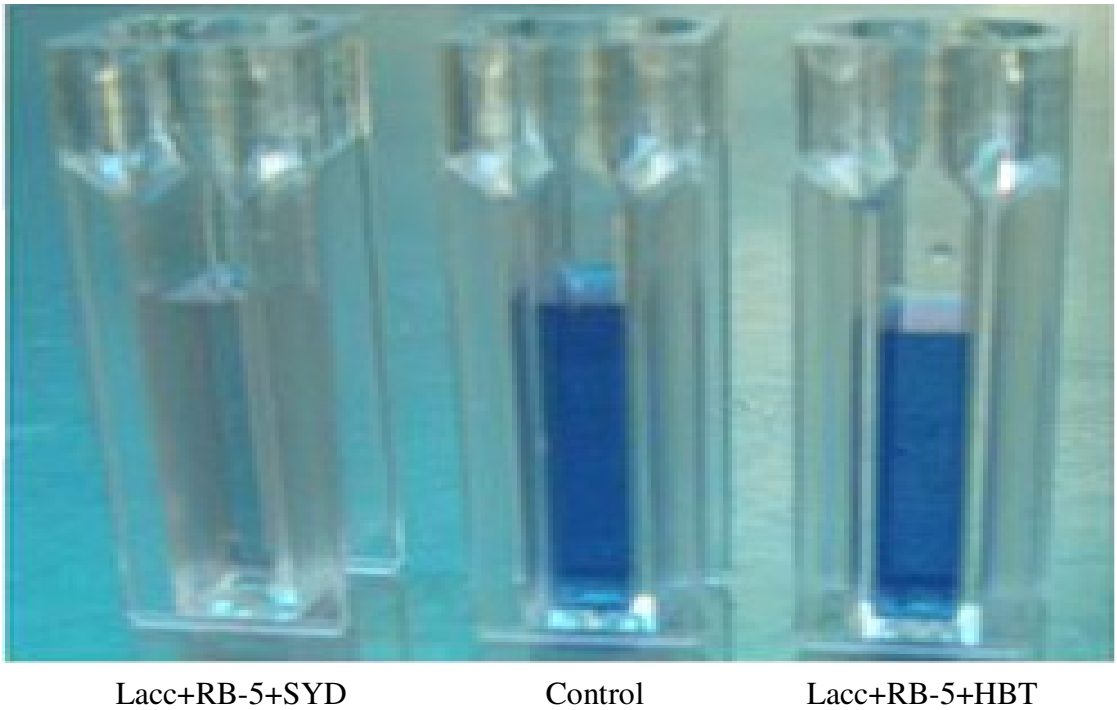


**Figure 7.** Decolorization of RB-5 dye by laccase in the presence of SYD and HBT at 1:5 dye: mediator ratio.

*T. versicolor* laccase did not decolorize RB-5, indicating that the presence of a mediator is required. Similarly, reports from literature show that laccase alone does not decolorize some types of textile dyes (Rodriguez et al., 2005; Hu et al., 2009). The reason might be that the redox potential of the dye is higher than that of type 1 Cu

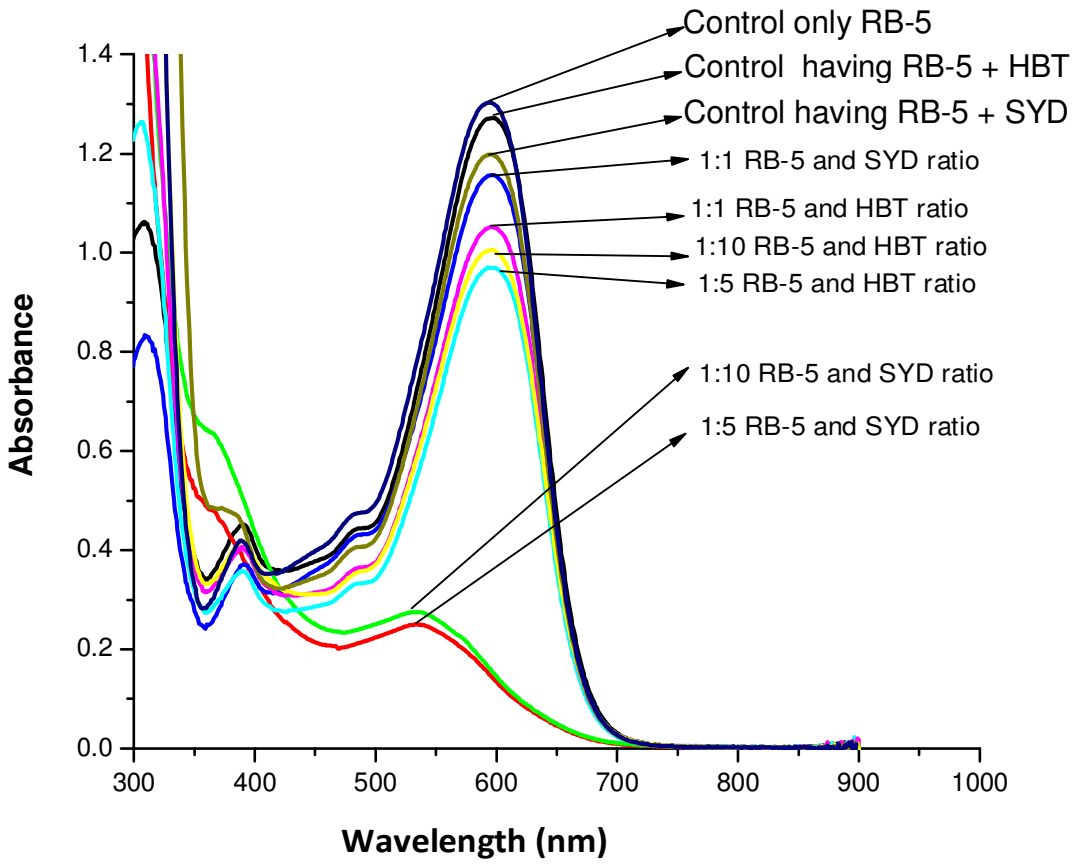


**Figure 8.** UV-Vis absorption spectra of RB-5 dye at different dye: mediator ratio treated with laccase of *T. versicolor* obtained after 30 min of incubation.

of the laccase or the dye could not access the type 1 Cu active site because of its steric hindrance. However, such dyes can be oxidized by laccase in the presence of some redox mediators (Rodriguez et al., 2005; Murugesan et al., 2007).

## Conclusions

In this study, biodecolorization of Reactive Black 5 by commercial laccase from *T. versicolor* was studied in batch mode. The biodecolorization of RB-5 was strongly affected by redox mediators. The results show that the presence of a natural mediator, syringaldehyde (SYD) was essential for the decolorization of RB-5 dye by *T. versicolor* laccase. The concentration of the SYD proved to be the principal factor that affected the yield of the dye decolorization. Enhanced decolorization (98%) was obtained using commercial laccase under the optimal conditions (1:5 dye: mediator ratio).

It was concluded that lignin-derived phenols (such as syringaldehyde) represent ecofriendly alternatives to synthetic (HBT) mediators for laccase degradation of di-azo dye and other recalcitrant compounds in terms of both efficiency and velocity of oxidation.

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