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Decolorization Potential of Some Reactive Dyes with Crude Laccase and Laccase-Mediated System

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Abstract In this study, decolorization of dyestuffs, such as Reactive Red 198, Rem Blue RR, Dylon Navy 17, Rem Red RR, and Rem Yellow RR was studied using laccase and laccase-mediated system. The laccases are known to have an important potential for remediation of pollutants. Among these dyestuffs, decolorization of Rem Blue RR and Dylon Navy 17 was performed with crude laccase under optimized conditions. Vanillin was selected as laccase mediator after screening six different compounds with Rem Yellow RR, Reactive Red 198, and Rem Red RR as substrates. However, Rem Yellow RR was not decolorized by either laccase or laccase-mediated system. It is observed that the culture supernatant contained high laccase activity after treatment with catalase that was responsible for the decolorization. Besides, culture supernatant with high laccase activity as enzyme source was treated with catalase; in this way, the hypothesis that laccase was the enzyme responsible for decolorization was supported. The Rem Blue RR was decolorized with 64.84% under the optimum conditions and Dylon Navy 17 with 75.43% with crude

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laccase. However, using the laccase and vanillin, the decolorization of Reactive Red 198 and Rem Red RR was found to be 62% and 68%, respectively. Our study demonstrated that the decolorization abilities of laccase and/or laccase mediator systems were based on the types of mediator, the dye structure, and the standard experimental conditions. Also, the electrochemical behaviors of some samples were studied. The redox potentials of these samples were determined using cyclic voltammetry on glassy carbon electrode in phosphate buffer (pH 6) solution.

Keywords Laccase · Dyestuffs · Mediator · Decolorization · *Trametes versicolor*

Introduction

Dyestuffs used in textile industry have severe hazardous impact because of discharge of these pollutants to the environment, especially aquatic regions. When industrial effluents containing colored wastewaters are released to aquatic systems, sunlight penetration in this natural life reduces; in this way, photosynthetic activity decreases, and oxygen concentration is also affected negatively [1]. These environmental pollution-caused recalcitrant chemicals have been among the main ecological problems, and public health risk such as toxic, mutagenic, and carcinogenic effects have occurred [2]. Dyes have different chemical structures composed of azo, nitro, or sulfo groups [3]. In particular, reactive dyes used frequently in the textile industry are not recyclable, and physical and chemical technologies (coagulation, membrane technologies, flocculation, ozonation, Fenton's reactive, reverse osmosis, cucurbutyril, electrochemical degradation, active carbon) treating these dyestuffs are costly, produce large amounts of sludge and toxic byproducts, consume excessively energy, and are not adapted to all wastewaters containing various substituents [1–5]. With respect to these, biological treatments of wastewaters including dyestuffs will be both cost-effective and eco-friendly.

Decolorization and detoxification of many dyes cannot be achieved by natural organisms. However, owing to the fact that white rot fungi at degrading toxic compounds have broad specificity, they can metabolize and degrade a lot of chemicals including dyestuff compounds [6]. One of the extracellular enzymes, laccase excreted from white rot fungi, has important potential in decolorization of textile dyestuffs [7].

Laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2), belonging to copper-containing oxidases, catalyzes the reduction of molecular oxygen to water, with no hydrogen peroxide formation. Laccase alone has a limited effect on textile dyestuff degradation owing to its specificity for phenolic compounds; however, the range of laccase substrates can be extended to non-phenolics by addition of mediators in a reaction medium. Some natural and synthetic molecules, 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) [8, 9], 1-hydroxybenzotriazole (HBT) [10], 2,2,6,6-tetramethylpiperidin-1-yloxy [11], polyoxometalates [12, 13], osmium-based redox polymers [14], violuric acid [4], lignin-derived compounds such as vanillin, vanillyl alcohol, vanillic acid, acetosyringone, syringaldehyde [15], compounds naturally produced by fungi [16], phenolic compounds [17], have been evaluated in various applications as mediators with laccase.

Increasing the range of substrates of laccase is possible by adding redox mediators to the reaction medium, first described by Bourbonnais and Paice [18], thereby oxidizing nonphenolic compounds. Mediator molecules, by mediating or increasing the enzyme action, can widen the range of compounds affected by laccase. This property acquired by

the enzyme provides an advantage for biotechnological applications [19]. In recent years, the laccase and/or mediator systems have attracted much interest for decolorization in the textile industry [20–27] and the pulp and paper industries [28] for application in eco-friendly processes.

The aim of the present investigation was to study the reaction conditions which affected the percentage of decolorization of Reactive Red 198 (RR 198), Rem Blue RR, Dylon Navy 17, Rem Red RR, and Rem Yellow RR with laccase and/or laccase mediator systems. To improve the decolorization, pH, initial dye concentration, enzyme amount, temperature, and incubation time were studied for laccase alone for Rem Blue RR and Dylon Navy 17. Six different mediators were investigated for the other dyestuffs that could not decolorize with crude laccase. In the case of the study of laccase-mediated system, besides the optimization parameters, mediator concentration and amount were also studied.

In this paper, laccase was used for decolorizing Rem Blue RR and Dylon Navy 17, and the decolorization of RR 198 and Rem Red RR was achieved using vanillin as a redox mediator together with crude laccase.

Materials and Methods

Production of Laccase and Determining Laccase Activity

Trametes versicolor ATCC200801, as a laccase producer, was grown on submerged cultures in potato dextrose broth (PDB) (Acumedia) using wheat bran as inducer purchased from the local market of Eskişehir, Turkey, according to Gedikli [29]. For the laccase production, the fungal strain was previously grown on PDB for 4 days at 30 °C, and the fungal pellets were homogenized and were used as inoculums. Four milliliters homogenate was transferred into 100 mL PDB including 3% (*w*/*v*) wheat bran in a 250-mL flask for agitated culture conditions (150 rpm) at 30 °C for 12 days of incubation. All experiments were carried out in aseptic conditions; the culture supernatant was obtained after filtering, and this was used as a source of crude laccase for all the dyestuff decolorization.

To determine the laccase activity of the supernatant, 0.1 mL of culture supernatant was added to 4.9 mL sodium acetate buffer (50 mM, pH 4.5), containing 0.1 mM guaiacol as substrate and incubated at 37 °C in water bath for 15 min.

One activity unit was defined as the amount of enzyme that oxidized and increase in A465 of 0.1 absorbance unit per minute [30]. Incubations with denatured laccase served as a control. Absorbance was measured with a UV–Vis spectrophotometer (Jasco V530).

Dye Decolorization

Dyes used in all experiments except for RR 198 were supplied with Dyestar and Dylon as a commercial product. Dyes used in all the experiments were of analytical grade. A chemical structure of RR 198 was given in Fig. 1. At first, spectrum screening of each dyestuff was performed to determine wavelength giving peak. Standard curves were prepared for every dyestuff at these absorbance values obtained. Dye decolorization was determined by monitoring the decrease in the absorbance at the wavelength of maximum absorption for each dye: Rem Blue RR (λ_{max}=604 nm), Dylon Navy 17 (λ_{max}=586 nm), RR 198 (λ_{max}=525 nm), Rem Red RR (λ_{max}=521 nm), and Rem Yellow RR (λ_{max}=418 nm). A UV–visible spectrophotometer (Jasco V530) was used in all experiments. Decolorization is calculated as:

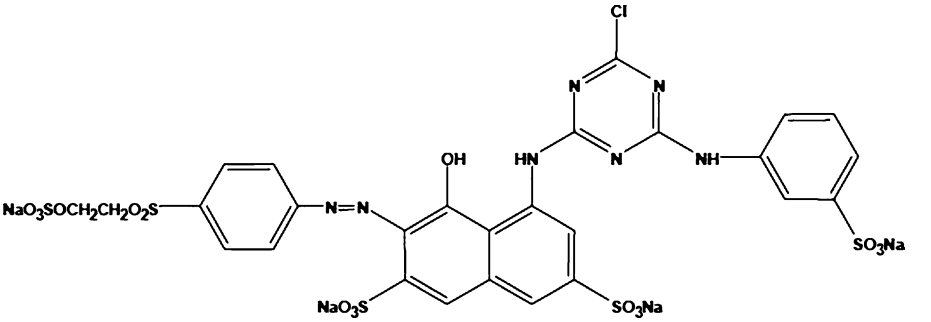

Fig. 1 Chemical structure of RR 198

$(C_i - C_f)/C_i \times 100$, where C_i is the initial dyestuff concentration and C_f is the final dyestuff concentration.

Optimization of Decolorization Parameters

To improve the decolorization efficiency of laccase produced by *T. versicolor* ATCC200801 with dyes Rem Blue RR and Dylon Navy 17, the effects of varying pH, initial dye concentration, enzyme amount, temperature, and incubation time were investigated. In each experiment, one factor was varied by keeping the previously optimized condition constant. For dyes RR 198, Rem Red RR, and RemYellow RR not decolorized by laccase alone, optimization experiments including parameters pH, mediator concentration, initial dye concentration, temperature, incubation time, enzyme amount, and mediator amount were performed accompanied with vanillin, which is a laccase mediator molecule to increase the percentage of decolorization. All experiments were performed in triplicate.

Defining Optimum Conditions of Decolorization of Rem Blue RR and Dylon Navy 17 by Culture Supernatant with High Laccase Activity

Decolorization tubes containing dye solutions were adjusted at varying pH (pH 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, and 8) at 35 °C, enzyme amount of 1 mL, and 9 mL of dyestuff (50 mg L^{-1}) for 30 min. For pH 3–5, sodium acetate buffer adjusted pH with acetic acid; for pH 6–8, Na_2HPO_4–NaH_2PO_4 buffer was used. Decolorization cuvettes were measured at 604 and 586 nm, respectively, for Rem Blue RR and Dylon Navy 17. Instead of enzyme, denatured enzyme and dye solution were used by maintaining constant other conditions as control group.

In order to investigate the tolerance of the laccase against the dyestuff, the decolorization was carried out with varying concentration solutions (5–150 mg L^{-1}) of two dyestuffs. The reaction mixture contained 1 mL of enzyme solution and 9 mL of the dye and was incubated at 30 °C for the following two dyes in a total volume of 10 mL. The pH of 5 and 5.5 were maintained for the reaction mixtures containing Rem Blue RR and Dylon Navy 17 dyes, respectively.

The above reaction mixture was incubated at varying temperatures from 20 to 45 °C for 30 min at optimized conditions.

To investigate the effect of incubation time on enzymatic decolorization, the reaction mixture was incubated for 5, 15, 30, 45, 60, and 120 min and 12 and 24 h under standard assay conditions as described above.

Decolorization experiments were also performed with varying enzyme amounts (0.5–4 mL). For this, the initial dye concentration used was 50 mg L^{-1}, then incubated at 35 °C for 30 min with different pH of 5 and 5.5 for dyes Rem Blue RR and Dylon Navy 17, respectively.

Defining Optimal Conditions of Decolorization of RR 198, Rem Red RR, and Rem Yellow RR by Culture Supernatant with High Laccase Activity and Vanillin

Small molecular weight components, namely mediator compounds including dimetoxyphenol (DMP), phenol, vanillin, guaiacol, veratryl alcohol, and L-tyrosine at different concentrations, were tested in the reaction mixture. All mediators were purchased from Sigma-Aldrich. According to screening experiments, vanillin was the most effective mediator molecule; therefore, vanillin was used for further optimization studies. Among Reactive Red 198, Rem Red RR, and Rem Yellow RR examined, only RR 198 and Rem Red RR were decolorized by laccase-mediated system.

Laccase alone as a mediator did not decolorize RR 198 and Rem Red RR. Therefore, the effects of pH, initial dye concentration, enzyme amount, incubation time, and temperature were examined for all the dyes including Rem Blue RR and Dylon Navy 17. This, along with the concentration (25–1,000 μM) and volume (0.5–3 mL) of the mediator, was also varied to examine the effective decolorization.

Inspection of Activities of Peroxidase Group Enzymes on Decolorization by Adding Catalase to Culture Supernatant

Although the culture supernatant contained high laccase activity, the culture supernatant may also contain peroxidases, which was tested with catalase (Sigma) treatment.

Electrochemical Studies

The electrochemical behavior of vanillin, laccase, vanillin+laccase+RR 198, and vanillin+laccase+Rem Red RR mixtures were investigated at 35 °C using Gamry model Reference 600 Potentiostat/Galvanostat. The electrochemical cell used was of the three-electrode type with separate compartments for the reference electrode (Ag/AgCl, sat.) and the counter electrode (a Pt wire). The GC electrode (Bas 3.0 mm diameter) was used as a working electrode. Before each experiment, the surface of the glassy carbon electrode was polished using diamond polishing pad then cleaned in ethanol–acetone solution using ultrasonic bath. Electrochemical investigation of the samples was performed by cyclic potential sweeping in the potential range between −0.1 and +1.1 V (vs. Ag/AgCl, sat.) at a scan rate of 50 mV s^{-1}. The voltammograms were recorded in phosphate buffer (pH 6) solution.

Results and Discussion

Screening of Mediator

The decolorization of five dyes was studied, out of which Rem Blue RR and Dylon Navy 17 were decolorized when the crude laccase was used alone. The other three dyes, Rem Yellow RR, RR 198, and Rem Red RR were not decolorized with laccase alone. For that

reason, in the decolorization of these three dyes, the potential performance of crude laccase with different mediators in reaction medium was evaluated. Reasons might be that the redox potential of the three reactive dyes was higher than that of crude laccase or the dyes cannot access the active site of the enzyme because of their structure. Therefore, the use of mediators such as dimetoxyphenol, phenol, vanillin, guaiacol, veratryl alcohol, and L-tyrosine was preferentially studied.

The redox mediators used in this work were previously established as appropriate mediators of laccase [15, 31]. Six mediators were examined, and among them, vanillin provided the highest decolorization rates. The effectiveness order for redox mediators at low concentration is: vanillin > phenol > dimetoxyphenol > veratryl alcohol > guaiacol for RR 198 and vanillin > veratryl alcohol > phenol > L-tyrosine for Rem Red RR (Table 1). Since vanillin has low cost and non-toxic characteristics besides being the most effective mediator examined, it was preferred for further experiments related to mediator usage.

Chhabra et al. [26] studied mediator-assisted decolorization and detoxification of textile dye mixture by *Cyathus bulleri* laccase. Among the synthetic mediators, ABTS was effective at low mediator/dye ratios and resulted in 80–95% decolorization for Reactive Orange 1 to 1,333±15 nmol min^{-1} mg^{-1} for RR 198. Natural mediators like vanillin, on the other hand, were found to be less effective on all the dyes except Reactive Orange 1. Computed rates of decolorization were about twofold lower than that with ABTS. According to Murugesan et al. [24], the rate of malachite green transformation in the presence of these natural compounds was many fold higher than that of HBT or ABTS and was similar to the effect of natural phenolic compounds on the decolorization of some synthetic dyes [15]. Our results clearly demonstrated the efficiency of phenolic compounds for Rem Red RR and RR 198 decolorization, indicating that natural phenolic compounds could potentially replace synthetic redox mediators for decolorization.

Natural mediators have significant advantages over synthetic mediators. Oxidation of phenolic compounds by laccase occurred with phenoxy radicals similar to the production of NO– radical from N–OH compounds [32]. The half-life of the NO– radical of HBT is short because it is highly reactive and decays rapidly into benzotriazole. In addition, it deactivates the laccase activity [33]. However, radicals of phenolic compounds have long half-lives and reversible reactions [34].

Table 1 Screening of mediator with RR 198 and Rem Red RR (experimental conditions for all examined dyes; pH 5, initial dye concentration, 50 mg L^{-1}; incubation time, 30 min; enzyme amount, 1 mL; enzyme activity, 24–26 U mL^{-1}; incubation temperature, 35 °C; mediator amount, 1 mL for RR 198 and Rem Red RR).

	DMP	Phenol	Vanillin	Veratryl alcohol	Guaiacol	L-Tyrosine	Time (min)
RR 198	9.03	11.83	11.77	8.15	0	8.35	5th
	8.99	12.1	12.79	8.19	0	9.13	15th
	8.39	12.42	9.17	8.3	0	8.64	30th
	9.13	12.64	20.11	9.1	0	9.31	60th
	9.98	12.86	25.9	8.75	0	9.39	120th
Rem Red RR	0	7.52	0	8.74	0	6.28	5th
	0	7.68	2.9	8.55	0	3.62	15th
	0	7.96	10.46	8.65	0	3.96	30th
	0	8.79	18.32	10.39	0	4.69	60th
	0	9.46	25.87	8.51	0	3.88	120th

As seen in Fig. 1, RR 198 contains –NH and –OH groups. These groups are the most vulnerable to laccase attack. Also, when using mediators, the steric hindrance may be reducing the accessibility of these reactive groups to laccase. For RR 198, the discrepancy between our results and those of Borchert and Libra [35] and Munari et al. [36] could lie in the difference in fungal strains for enzyme production in laccase activity provided, in the culture conditions used for growing the fungus strain when laccase was produced, and whether using a mediator molecule.

Wong and Yu [37] studied decolorization of three synthetic dyes with typical chromophores (anthraquinone, azo, and indigo) with *T. versicolor* laccase and demonstrated that azo and indigo dyes were not the substrates of laccase, and small molecule metabolites mediated the interaction between the dyes and the enzyme, while anthraquinone dye was an enzyme substrate that was directly oxidized by laccase.

The dissimilarities in dye decolorization can be attributed to structural differences, especially the position of substituents on the aromatic ring known as steric effect, which could obstruct the correct docking of a dye molecule in the active site of the laccase.

The results obtained showed that Rem Yellow RR was not decolorized by laccase nor laccase-mediated system. This dye showed much more resistance to decolorization under these experimental conditions established.

pH Optimization

The results related to pH optimization are plotted in Fig. 2. Optimum pHs were at 5, 5.5, 6, and 6, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR. The optimum growth pH for decolorization by laccase varied around pH 4–6, which showed

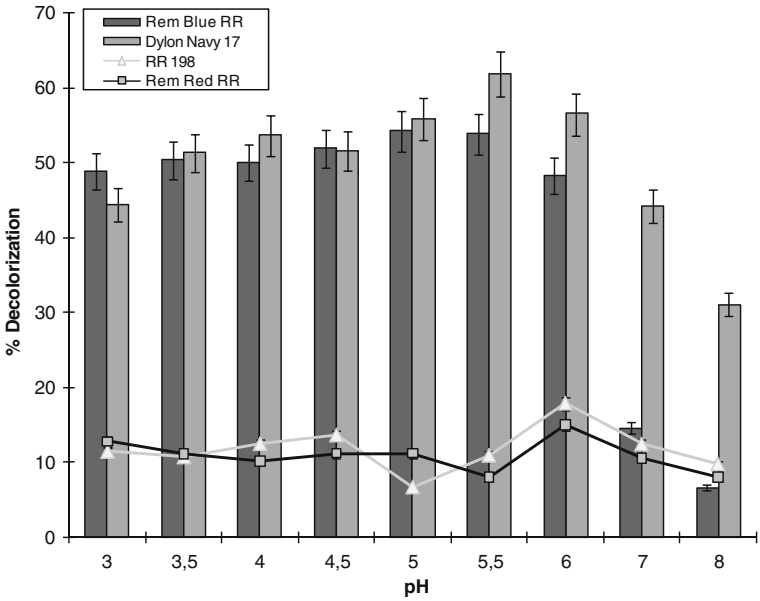

Fig. 2 Effect of pH on enzymatic decolorization (initial dye concentration, 50 mg L^{-1} for Rem Blue RR and Dylon Navy 17 and 10 mg L^{-1} for RR 198 and Rem Red RR; incubation time, 30 min; enzyme amount, 1 mL; enzyme activity, 24–26 U mL^{-1}; incubation temperature, 35 °C; mediator amount, 1 mL, and mediator concentration, 100 μM for RR 198 and Rem Red RR)

laccase activity at this range. The reason of finding different optimum pH for every dyestuff tested can be medium composition and chemical structure of dyes present in decolorization medium [38, 39]. Mechichi et al. [40] reported 97% degradation of Remazol Brilliant Blue R by *Trametes trogii* laccase having 0.2 U mL^{-1} at pH 5. The medium pH exhibited a variety of effects depending on the properties of dyestuffs examined.

Initial Dye Concentration Optimization

On account of the fact that dyes are toxic compounds and can tolerate different chemical structures, these dyes were decolorized to variable extents by extracellular enzymes produced by white rot fungi [41].

As shown in Fig. 3, initial dye concentration was chosen at 50, 50, 25, and 25 mg L^{-1} for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR, respectively. Initial dye concentration tolerated laccase at 50 mg L^{-1} or laccase-mediated system at 25 mg L^{-1}. Then, a gradual decrease in decolorization was observed. Sadhasivam et al. varied initial dye concentration from 1 to 5 mg L^{-1}. The rate of dye decolorization gradually increased with time and attained equilibrium at 18 h in both crude and partially purified laccase [21].

Temperature Optimization

The most suitable temperatures were selected at 30, 45, and 55 °C, respectively, for Rem Blue RR, Dylon Navy 17, and dyes decolorized by laccase and a mediator molecule (Fig. 4). Optimum temperature can depend on the type of dye and the strain used to produce

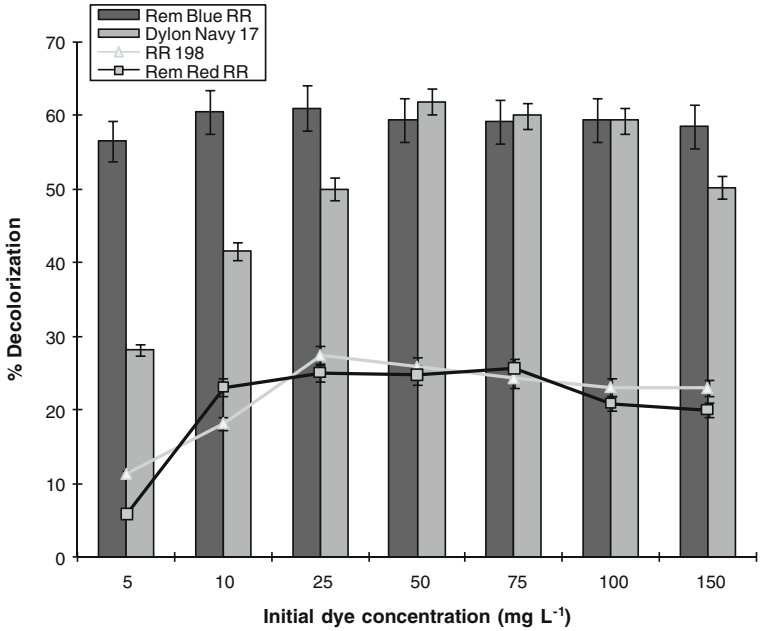

Fig. 3 Effect of initial dye concentration on enzymatic decolorization (pH 5, 5.5, 6, and 6, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR; incubation time, 30 min; enzyme amount, 1 mL; enzyme activity, 24–26 U mL^{-1}; incubation temperature, 35 °C; mediator amount, 1 mL for RR 198 and Rem Red RR; mediator concentration, 200 and 500 μM, respectively, for RR 198 and Rem Red RR)

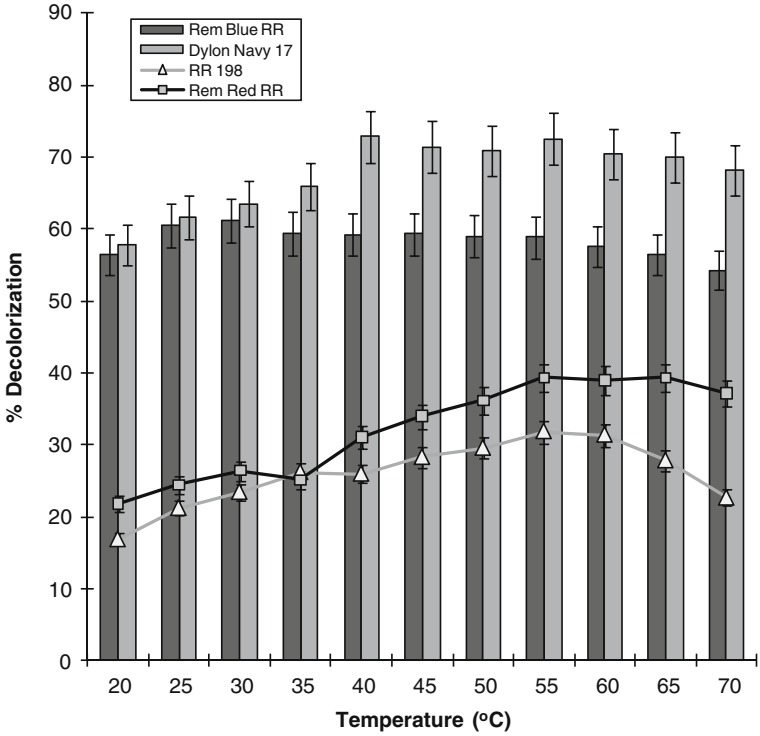

Fig. 4 Effect of temperature on enzymatic decolorization (pH 5, 5.5, 6, and 6, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR; initial dye concentration, 50 mg L^{-1} for Rem Blue RR and Dylon Navy 17; 25 mg L^{-1} for RR 198 and Rem Red RR; incubation time, 30 min; enzyme amount, 1 mL; enzyme activity, 24–26 U mL^{-1}; mediator amount, 1 mL for RR 198 and Rem Red RR; mediator concentration, 200 μM for RR 198 and 500 μM for Red RR)

laccase. According to technical literature from DeniLite, the best temperature for the decolorization of indigo in the denim bleaching process is 60–70 °C. Zhang et al. [42] observed that the optimum temperature for decolorization of Acid Green 27, Acid Violet 7, and Indigo Carmine with laccase and ABTS was 70 °C. These experimental findings showed that optimum temperature of mediated decolorization was higher than decolorization with laccase alone.

Incubation Time Optimization

According to the results obtained in the optimization of incubation time, optimum incubation time was found as 120 and 1,440 min; in all dyes decolorized by laccase alone and dyes decolorized by laccase and a mediator molecule (Fig. 5).

The maximum decolorization (96%) of Amaranth (100 mg L^{-1}) was achieved in 8 h with laccase from *Ganoderma* sp. WR-1 [43]. The similar results showed that the dye decolorization was dependent on initial dye concentration as well as the chemical structure of the individual dye. Murugesan et al. studied decolorization of Remazol Brilliant Blue R with laccase alone and in the presence of 0.6 mM copper ion; the decolorization reached 100% after 6 h. Although thymol and VIO can improve the reactive efficiency of RBBR

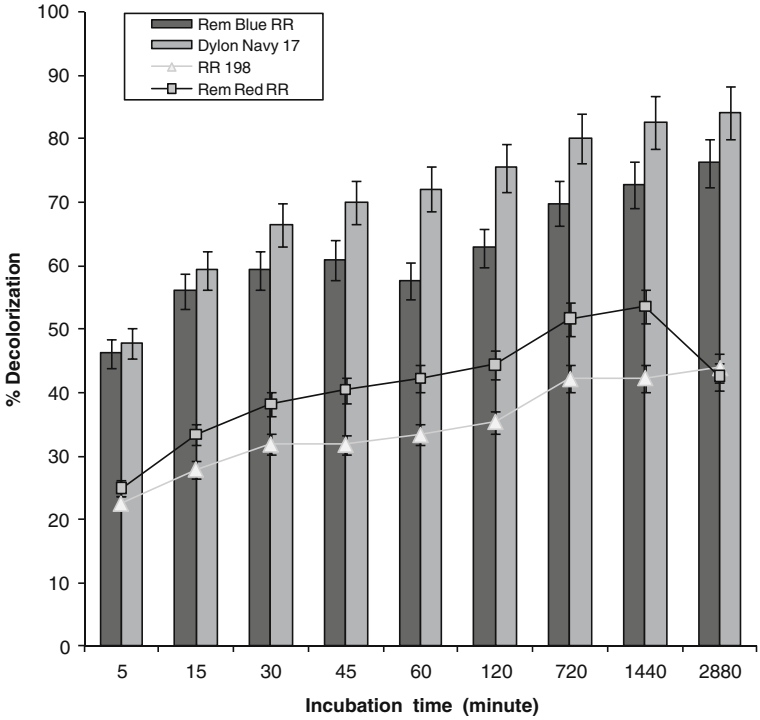

Fig. 5 Effect of time on enzymatic decolorization (pH 5, 5.5, 6, and 6, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR; initial dye concentration, 50 mg L^{-1} for Rem Blue RR and Dylon Navy 17; 25 mg L^{-1} for RR 198 and Rem Red RR; enzyme amount, 1 mL; enzyme activity, 24–26 U mL^{-1}; incubation temperature, 30, 45, 55, and 55 °C, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR; mediator amount, 1 mL for RR 198 and Rem Red RR; mediator concentration, 200 μM for RR 198 and 500 μM for Red RR)

(100% decolorization in 2 h), in order to have less impact on the environment, it was better to oxidize RBBR by laccase without mediator [24].

The limiting step in the oxidation of phenols by laccase is the first electron transfer from the substrate to T1 copper. This situation originates from the differences in redox potential between the substrate and the enzyme [15]. Incubation time of decolorization can alter as to abilities of mediators to change redox potential. Ciullini et al. [44] noted that 85% of decolorization of Acid Blue 324 was achieved thanks to HBT as mediator after 4 h.

Enzyme Amount Assays

As it can be seen in Fig. 6, when the enzyme amount was increased, the rate of enzymatic decolorization was enhanced. In this context, the most decolorization was provided in the presence of 4 mL of enzyme. Notwithstanding, further experiments used 1 mL amount of enzyme because 4 mL, observed as the optimum value, caused dilution in reaction tubes; in this manner, diluted reaction volume is at a ratio of 40%.

Table 2 shows the evaluation of decolorization with respect to all optimum parameters found for decolorized dye with laccase alone.

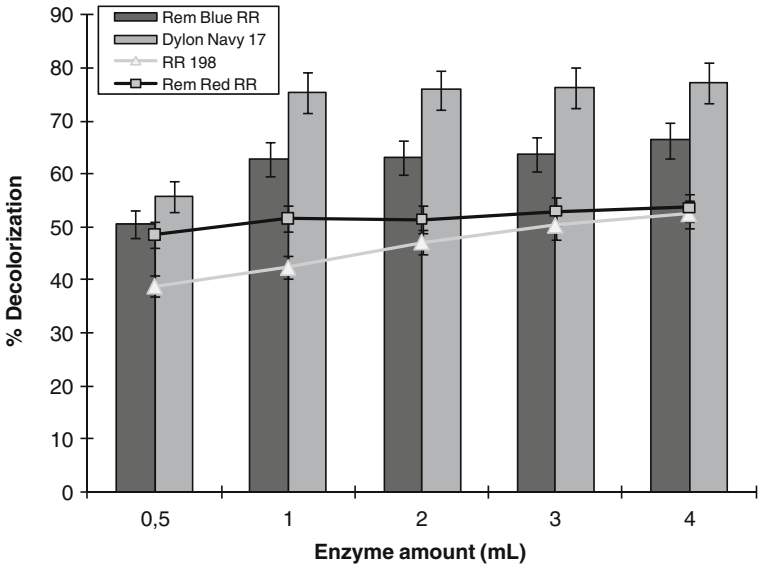

Fig. 6 Effect of enzyme amount on enzymatic decolorization (pH 5, 5.5, 6, and 6, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR; initial dye concentration, 50 mg L^{-1} for Rem Blue RR and Dylon Navy 17, 25 mg L^{-1} for RR 198 and Rem Red RR; incubation time, 120 min for Rem Blue RR and Dylon Navy 17, 1,440 min for RR 198 and Rem Red RR; enzyme activity, 24–26 U mL^{-1}; incubation temperature, 30, 45, 55, and 55 °C, respectively, for Rem Blue RR, Dylon Navy 17, RR 198, and Rem Red RR; mediator amount, 1 mL for RR 198 and Rem Red RR; mediator concentration, 200 μM for RR 198 and 500 μM for Red RR)

Investigation Effects of Mediator Concentration and Amount on Decolorization

The influences of the mediator concentrations on the decolorization of RR 198 and Rem Red RR were shown in Table 3. From the different concentrations examined, the best results were obtained at vanillin concentrations of 200 and 500 μM for RR 198 and Rem Red RR. Decolorization percentages of about 21.16% and 23.13% in 30 min were obtained, respectively, for RR 198 and for Rem Red RR. Due to their high reactivity, the radicals can undergo chemical reactions with the aromatic amino acid side chains of the laccase enzyme thereby inactivating the enzyme [45]. Reducing or keeping stable the percentage of decolorization at above a certain mediator concentration in our study could

Table 2 Evaluation of decolorization with respect to optimum parameters found (for decolorized dye with laccase alone).

	Parameters of dyes decolorized with laccase alone					
	pH	Initial dye concentration (mg L^{-1})	Enzyme amount (mL)	Temperature (°C)	Incubation time (min)	Optimum decolorization (%)
Rem Blue RR	5	50	1	30	120	64.84
Dylon Navy 17	5.5	50	1	45	120	75.43

Table 3 Evaluation of decolorization in respect of optimum parameters found (for decolorized dye with laccase-mediated system).

	Parameters of dyes decolorized with laccase mediator such as vanillin										
	pH	Mediator concentration (μM) values examined and % decolorization of dye									Initial dye concentration (mg L^{-1})
RR 198	6	25 μM	100 μM	150 μM	200 μM	300 μM	400 μM	500 μM	750 μM	1,000 μM	
		15.6%	18.1%	19.4%	21.1%	21.3%	21.6%	21.2%	20.9%	20.6%	25
Rem Red RR	6	25 μM	100 μM	150 μM	200 μM	300 μM	400 μM	500 μM	750 μM	1,000 μM	25
		15.8%	14.9%	17.3%	17.4%	19.5%	21.6%	23.1%	22.8%	21.6%	
Rem Yellow RR	–	–									–

	Parameters of dyes decolorized with laccase mediator such as vanillin									
	Temperature (°C)	Incubation time (min)	Enzyme amount (mL)	Mediator amount (mL) values examined and % decolorization of dye						Optimum decolorization (%)
RR 198				0.5 mL	1 mL	1.5 mL	2 mL	2.5 mL	3 mL	
	55	1,440	1	32.1%	41.6%	48.1%	54.1%	58.4%	62.4%	62.40
Rem Red RR	55	1,440	1	0.5 mL	1 mL	1.5 mL	2 mL	2.5 mL	3 mL	68.00
				41.5%	51.6%	56.8%	61.3%	64.7%	68.0%	
Rem Yellow RR	–	–	–	–						–

depend on this phenomenon. Moldes and Sanroman [46] found also that increase of HBT mediator concentration led to a higher decolorization rate, but the increase of HBT concentration above 2 mM improved neither the decolorization rate nor the decolorization degree.

To determine the influence of mediator amount on enzymatic decolorization, an experiment was conducted at the range of 0.5–3 mL, and enzymatic decolorization showed parallelism to mediator amount (Table 3).

Using vanillin has an advantage for being a natural mediator. Utilizing natural compounds as mediators has attained more and more interest by researchers in recent years inasmuch as several benefits could be attained through these small molecules, such as being less toxic and environmentally safe compounds, can be found easily, and reduction in treatment cost and energy consumption [46]. For this reason, the objective for the scientific community was to test natural mediators on reactive dyestuffs or to find new ones.

Because of the fact that there are a lot of factors affecting laccase-mediated systems, which are the difference of redox potential between laccase and the mediator; the properties of the oxidized form of the mediator such as the stability, the type, and position of substituents in the mediator; inactivation of laccase capability; and substrate affinity, estimation of the behavior of redox mediator is difficult [16, 47, 48].

Culture supernatant with high laccase activity as enzyme source was treated with catalase, in this way, the hypothesis that laccase was the enzyme responsible for decolorization was supported. Besides other extracellular enzymes excreted by *T. versicolor*, peroxidases involved in dye decolorization and degradation work in the presence of hydrogen peroxide.

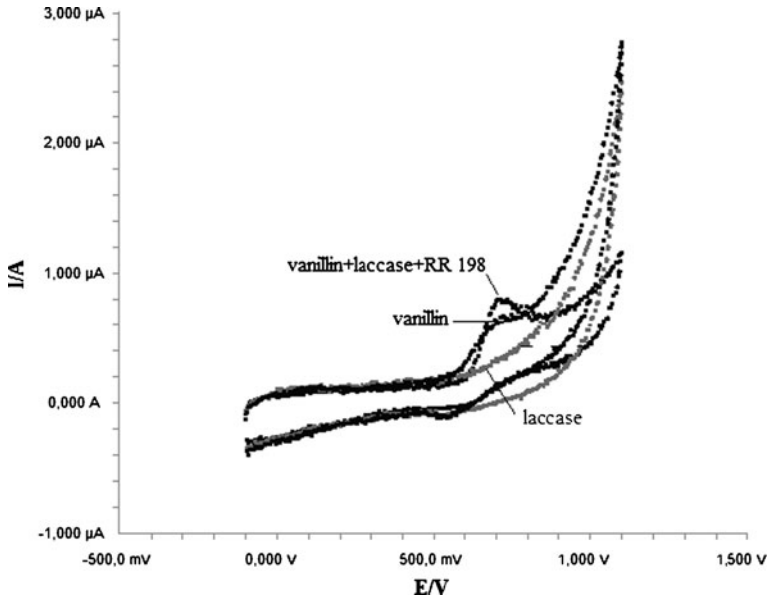

Fig. 7 Cyclic voltammograms of vanillin (200 µM), laccase, and vanillin+laccase+RR 198 in phosphate buffer (pH=6) solution at scan rate of 50 mV s^{-1}

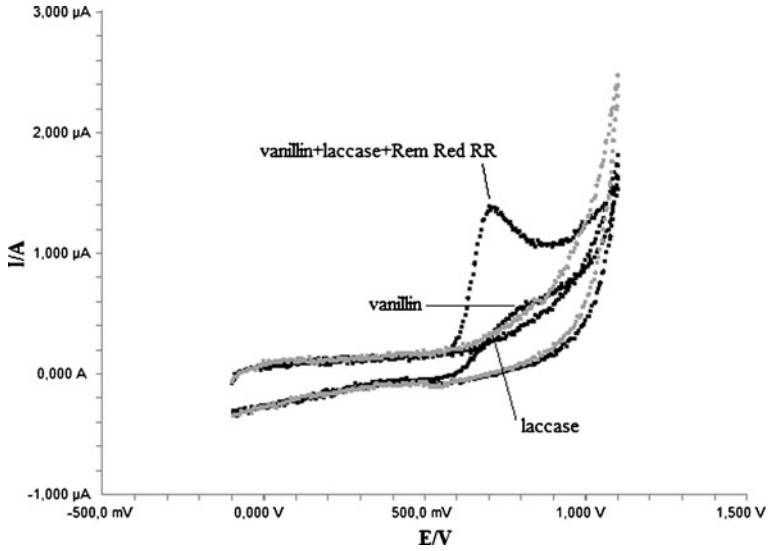

Fig. 8 Cyclic voltammograms of vanillin (500 µM), laccase, and vanillin+laccase+Rem Red RR in phosphate buffer (pH=6) solution at scan rate of 50 mV s^{-1}

Electrochemical Behaviors of Dye Solutions

To obtain information about the redox potentials of vanillin, laccase, vanillin+laccase+RR 198, and vanillin+laccase+Rem Red RR mixtures, their cyclic voltammograms were recorded in phosphate buffer solution. The changes of redox potentials and currents of vanillin (200 µM), laccase, and vanillin (200 µM)+laccase+RR 198 can be seen in Fig. 7. The redox potentials are 706, 639, and 727 mV for vanillin (200 µM), laccase, and vanillin (200 µM)+laccase+RR 198, respectively. Figure 8 shows the electrochemical behaviors of vanillin (500 µM) and vanillin (500 µM)+laccase+Rem Red RR. The redox potentials are 660 and 694 mV for vanillin(500 µM) and vanillin (500 µM)+laccase+Rem Red RR, respectively. The redox potentials of solution shifted to anodic potential when RR 198 and Rem Red RR were added to buffer solution containing laccase and vanillin. This result can be related to degradation of RR 198 and Rem Red RR.

Conclusions

In view of the results provided, it can be concluded that *T. versicolor* laccase can be used for decolorization of Rem Blue RR and Dylon Navy 17. On the other hand, the inclusion of vanillin as redox mediator was a prerequisite for decolorization to occur. Our study showed that submerged wheat bran culture extract showed decolorization as effective as vanillin at the stage of experiment of mediator screening.

According to literature screening done and results obtained, in decolorization of Dylon Navy 17 and Rem Blue RR by laccase and Rem Red RR by laccase and vanillin, a mediator molecule contributes to the reaction. The decolorization of dyes with laccase alone and mediated system appears promising for treatment of textile wastewaters.

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