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Mediator-assisted Decolorization and Detoxification of Textile Dyes/Dye Mixture by *Cyathus bulleri* Laccase

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Abstract Laccase from basidiomycete fungus *Cyathus bulleri* was evaluated for its ability to decolorize a number of reactive and acidic dyes in the presence of natural and synthetic mediators. The extent of decolorization was monitored at different mediator/dye concentrations and incubation time. Among the synthetic mediators, 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was effective at low mediator/dye ratios and resulted in 80–95% decolorization at rates that varied from 226±4 nmol min^{-1} mg^{-1} for Reactive Orange 1 to 1,333±15 nmol min^{-1} mg^{-1} for Reactive Red 198. Other synthetic mediators like 1-hydroxybenzotriazole and violuric acid showed both concentration- and time-dependent increases in percent decolorization. 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. The laccase–ABTS system also led to nearly 80% decolorization for the simulated dye mixture. No clear correlation between laccase activity on the mediator and its ability to decolorize dyes was found, but pH had a significant effect: Optimum pH for decolorization coincided with the optimum pH for mediator oxidation. The treated samples were also evaluated for toxicity in model microbial systems. The laccase–mediator system appears promising for treatment of textile wastewaters.

Keywords *Cyathus bulleri* · Dye decolorization · Mediator-assisted decolorization · Laccase · Detoxification

Introduction

It is estimated that approximately 10,000 different types of dyes and pigments are manufactured worldwide with the market of more than 7×10^5 tonnes per year [1]. Approximately 30% of reactive dyestuffs are lost and discharged with the effluents. Dyes, owing to their brilliance, are visible even at the lower concentrations, and their persistence

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in the environment is deleterious not only for the photosynthetic processes of the aquatic plants but also for all the living organisms. The chemical structures of the synthetic dye molecules are designed to resist fading on exposure to light or chemical attack, and this renders them recalcitrant. The various chemical and physical methods that can possibly be used for the treatment of textile effluent containing various dyes are not self-sufficient [2]. Among the biological treatment, aerobic bacteria are incapable of degrading these dyes, but the chromophoric group of azo dyes (the azo bond) can be acted upon by anaerobic bacteria, thus decolorizing the dyes [3]. However, the azo bond is reduced to amines, which are potentially carcinogenic [4].

White rot fungi, by virtue of their ability to degrade lignin in nature, produce enzymes like lignin peroxidases (LiPs; E.C 1.11.1.14), Mn peroxidases (MnPs; E.C 1.11.1.13), and laccases (EC 1.10.3.2) that are able to carry out oxidative decolorization of dyes thus bypassing the danger of formation of carcinogenic amines. However, due to the fact that many (LiP, MnP) of these enzymes are produced in low amounts by fungi and are dependent on metal ions and the cosubstrate hydrogen peroxide, these have not been considered for large-scale applications [5]. Laccases seem to be most promising candidates for enzyme-mediated remediation processes because of their broad substrate specificity, easy production, and rapid action at milder pH and temperature. These are multicopper oxidases, which catalyze one electron oxidation of a wide range of inorganic and organic substances, coupled with four-electron reduction of oxygen to water. The free radicals formed, due to laccase action, bypass the step involving the formation of carcinogenic amines [6] and, hence, can decolorize a wide range of industrial dyes.

Laccases cannot act on the nonphenolic components of aromatic compounds because of their low redox potential (0.5–0.8 V). Moreover, the complex high molecular substrates cannot penetrate the active site of the enzyme. However, small organic compounds (mediators) having high redox potentials (>0.9 V) can be oxidized and activated by laccases, and these enable degradation of the substrate [7, 8]. A number of natural and synthetic mediators have been reported to be effective [7, 8] in lignin depolymerization and on synthetic dyes. The aim of this study was to evaluate the efficacy of mediator (natural and synthetic)-assisted decolorization of various dyes and to identify parameters (such as mediator concentration, pH, time of incubation) affecting the decolorization process. The validity of these findings was confirmed by treating a simulated effluent (prepared by mixing these dyes in a certain proportion with additional salts added) and observing a decline in the color. Since the chromophoric groups add to the color, it was hypothesized that the removal (and/or breakdown) of these should lead to color removal and a decrease in toxicity. Thus, the treated dye mixture was also evaluated for respiratory toxicity and geno-toxicity. The previously characterized [9, 10] laccase from *Cyathus bulleri* was used in this investigation.

Materials and Methods

Dyes and Mediators

The dyes used in the study were Reactive Black 5, Reactive Red 198, Reactive Orange 7, Reactive Orange 1, and Acid Violet 17 (a kind gift from the Department of Textile Technology, IIT Delhi). Synthetic mediators [2,2′-azino-di-(-ethylbenzothiazoline-6-sulfonic acid)] (ABTS), 1-hydroxybenzotriazole (HOBT), hydroquinone, pyrogallol, 2–6 dimethoxyphenol, violuric acid, and syringic acid were purchased from Sigma Aldrich. Natural mediators vanillin, ethyl vanillin, acetovanillone, methyl vanillate, and *p*-coumaric acid were

from Himedia (Himedia Labs, Delhi) and were of the purest grade available. 3-Hydroxy anthranillic acid and guaiacol were from Sigma Aldrich.

Microbial Culture and Maintenance

C. bulleri 195062 from Canadian Type Culture Collection was a gift from Prof. R.C. Kuhad (Univ. of Delhi, South Campus). It was maintained as described previously [9] and subcultured every month.

Enzyme Production and Purification

Laccase was produced in basal liquid medium [11] in the presence of 2,6-dimethyl aniline as an inducer [9]. It was purified by 140-fold to homogeneity by the three-step method to a specific activity of 329 mg^{-1} protein [9]. The purity of the protein was confirmed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. A routine assay of laccase was carried out by monitoring the oxidation of 0.5 mM ABTS to its cation radical (ε_{420}, 36,500 M^{-1} cm^{-1}) in 100 mM acetate buffer, pH 5 [11]. One unit was defined as the amount of enzyme that released 1 μmol of product per minute.

Screening of Mediators

Mediator screening was carried out with all the dyes. The reaction was carried out in triplicates in a 25-ml conical flask containing 5 ml liquid, which contained fixed dye concentration of 50 μM in 50 mM sodium citrate buffer, pH 5.0, and fixed enzyme concentration of 100 mU/ml. Different mediators were used at a concentration of 100 μM. Incubation was done in an incubator shaker at 100 rpm, 30 °C. The change in optical density (OD) was recorded at the λ_{max} of the dyes at the end of 6 and 24 h and was used to compute percent decolorization.

Effect of Mediator Concentration on Decolorization

The effect of mediator concentration on dye decolorization was investigated. Synthetic mediators ABTS, 1-HOBT, and natural mediator vanillin were selected for this study. Dyes used were Reactive Black 5 and Acid Violet 17 as representatives of reactive and acidic dyes, respectively. The experimental conditions for dye decolorization were the same as reported in the previous section except that the mediator concentration was varied from 10 to 500 μM and the aliquots were withdrawn after 3, 6, and 24 h.

Laccase Activity on Various Mediators

The activity of laccase (100 mU/ml) was determined on various mediators. Reactions were carried out in a volume of 5 ml containing 100 mU/ml of laccase and 100 μM of the mediator. The activity on mediators was measured, and values were calculated based on their respective molar extinction coefficients [8, 11]. Results are reported relative to activity on ABTS, which was set to 100%.

Determination of the Rate of Dye Decolorization

The rate of dye decolorization was determined for individual dyes. The reaction mixture containing 50 μM dye, 50 μM ABTS (or 500 μM vanillin), and laccase (100 mU/ml) in a

volume of 20 ml was incubated shaken at 100 rpm at 30 °C. Aliquots were withdrawn after every 5 min initially and then after every 15 min to determine the decrease in OD at the λ_{max} of the individual dyes. Rates of dye decolorization were computed from the linear parts of the data for both ABTS and vanillin.

Preparation of Simulated Dye Effluent

The dyes were mixed at a concentration to reach OD values that matched with that of the actual effluent obtained from a local textile mill. The dyes used were Reactive Black 5, Reactive Red 198, Reactive Orange 7, Reactive Orange 1, and Acid Violet 17. Sodium sulfate was added to the dye mixture at a concentration of 2.5 g/l as per the specifications for dyeing wool. The decolorization was then performed as described above using mediators at their minimum effective concentrations.

Effect of Initial pH on the Decolorization of the Dye Mixture by Laccase

To study the effect of initial pH, both dye mixture (200 μM) and mediators (50 μM ABTS and 500 μM vanillin) were prepared in acetate buffer, the pH of which ranged from 3 to 6 and in phosphate buffer (pH 6–7). Laccase concentration and incubation period were kept as 100 mU ml^{-1} and 3 h, respectively. Color removal (%) at each pH value was calculated as the extent of decrease from the initial value of ∑OD at selected wavelengths.

Determination of Respiratory Toxicity

Toxicity of the enzyme–mediator-treated dye mixture was determined based on the decline in the oxygen consumption rate (OCR) of *Pseudomonas putida* [12]. The culture was procured from the National Collection of Industrial Microbes, NCIM 2650, at National Chemical Laboratory, Pune, India. The 16-h culture of *P. putida* was incubated with a specific concentration of untreated (or treated) dye mixture for a period of 2, 4, or 6 h. The culture was then mixed with sterile nutrient broth (purged with air for 30 min and containing same concentration of dyes) in the ratio of 1:5 (*v*/*v*) in an air-tight assembly with a fitted dissolved oxygen electrode (Oxi 315, WTW Weinheim, Germany). The culture was stirred at 250 rpm, and a periodic (in min) decline in dissolved oxygen was monitored to measure the OCR. The toxicity of the sample was correlated with its ability to cause a decline in OCR. The samples tested for toxicity were the dye mixture (0.20 mM) and dye mixture treated with laccase (100 mU/ml) and 0.1 mM ABTS/0.5 mM 1-HOBT/0.1 mM violuric acid/0.5 mM vanillin as mediators. Distilled water was used as a negative control and 4-chlorophenol (200 ppm) as a positive control. All the experiments were performed in triplicates.

Determination of Genotoxicity/Mutagenicity

The treated and the untreated dye mixture were also evaluated for toxicity using Ames test—the standard plate incorporation assay [13]. The tests were performed on *Salmonella typhimurium* TA 98 strain obtained from Microbial Type Culture Collection and Gene Bank at IMTECH, Chandigarh, India. All the tests were performed in triplicates, and the results were interpreted as per the standard specifications by determining the fold increase in the number of His^+ revertants (in the presence of the test chemical) with respect to the number of spontaneous revertants.

The dye mixture was tested at six different concentrations ranging from 0.05 to 0.5 mM. Other samples tested included the laccase-treated dye mixture (0.2 mM) using ABTS (0.1 mM)/1-HOBT (0.5 mM)/violuric acid (0.1 mM)/vanillin (0.5 mM). Distilled water was used as a negative control, and 4-nitro *o*-phenylenediamine was used as a positive control.

Statistical Analyses

All experiments were performed in triplicates. The results reported are an average of the three data points with standard deviations calculated.

Results

Decolorization of Individual Dyes

It was observed that for all the dyes studied, synthetic mediators were better in decolorization as compared to the natural mediators (Table 1). ABTS was most effective in achieving decolorization of all dyes in 6 h, which varied from 70% (for Reactive Black 5) to 85% (for Acid Violet 17). No significant increase in percent decolorization occurred on longer incubations except in the case of Reactive Orange 1. Violuric acid also mediated 75–90% decolorization of all the dyes except for Reactive Orange 1. As with ABTS, no significant increase in decolorization occurred on longer incubations except in the case of Acid Violet 17. In this study, there was increase in decolorization from 35% (after 6 h) to

Table 1 Screening mediators based on the dye (50 μM) decolorization monitored at specific wavelengths using mediator concentration of 100 μM in the presence of laccase at 100 mU/ml.

Mediators	Percent decolorization ± SD									
	Reactive Black 5 (599 nm)		Reactive Red 198 (515 nm)		Reactive Orange 7 (493 nm)		Reactive Orange 1 (490 nm)		Acid Violet 17 (542 nm)	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
ABTS	70±2	80±3	77±3	85±3	80± 1	82±1	45±2	75±3	85±1	95±2
1-HOBT	30±3	60±4	20±1	60±1	10±2	40±3	2±0.5	8±1	18±0.5	55±1
Hydroquinone	–	5±1	3±0.5	5±0.5	–	–	5±0.2	6±1	8±1	10±0.5
Pyrogallol	–	6±2	–	–	–	–	5±0.5	5±0.5	18±0.5	18±0.8
2–6 Dimethoxyphenol	7±1	9±1	10±1	12±2	–	–	7±1	8±1	38±2	20±1
Violuric acid	75±3	85±4	85±5	95±4	85±3	90±4	10±0.5	18±1	35±1	90±2
Syringic acid	5±0.5	10±1	2±0.5	10±1	–	–	12±0.8	13±1	25±1	17±2
Vanillin	40±6	50±8	30±2	40±3	30±1	35±2	–	–	12±2	28±2
Ethyl vanillin	38±3	45±2	20±4	35±3	25±2	32±2	–	–	30±2	22±1
Acetovanillone	32±2	55±3	40±2	50±2	40±4	50±3	15±2	18±3	32±5	40±5
Methyl vanillate	36±6	48±4	45±2	43±1	30±3	45±2	19±2	19±1	32±1	34±2
p– Coumaric acid	25±2	30±1	30±2	30±1	25±0.5	33±1	–	10±0.5	33±1	35±0.5
Guaiacol	–	5±2	8±1	10±1	–	–	18±1	19±0.5	10±2	12±1
3-HAA	–	–	–	–	–	–	–	–	–	–

Incubation was performed at 30 °C, 100 rpm.

– No decolorization

90% (after 24 h). 1-HOBT also effectively decolorized all the dyes except Reactive Orange 1. Hydroquinone, pyrogallol, 2–6 dimethoxyphenol, and syringic acid were not found to be effective for decolorization of any of the dyes.

Among the natural mediators, vanillin and its derivatives (ethyl vanillin, acetovanillone, and methyl vanillate) mediated decolorization from 20% to 60% for all the dyes, and there was a marginal difference between the samples removed after 6 or 24 h. Guaiacol and 3-HAA were found to be poor in their decolorization ability. *p*-Coumaric acid also decolorized by 25–35% all the dyes except Reactive Orange 1. The results showed good reproducibility with a standard deviation of less than 5% in most of the experiments (Table 1).

Laccase activity was measured on a fixed concentration of mediators, and the results are shown in Table 2. It was found that, relative to ABTS, laccase showed the next best activity on methyl vanillate followed by guaiacol, *p*-coumaric acid, acetovanillone, vanillin and 2–6 dimethoxyphenol. No clear correlation between laccase activity on different mediators and the ability of the oxidized mediator to decolorize dyes was observed.

Effect of Mediator Concentration

ABTS was able to decolorize dyes even at a low concentration of 10 μM. In case of Reactive Black 5, decolorization increased from 50% at 10 μM ABTS to 75% at 100 μM ABTS after 3-h incubation (Fig. 1a). A similar trend was observed for Acid Violet 17 where percent decolorization increased from 30% at 10 μM to 90% at 100 μM ABTS (Fig. 1b). The extent of decolorization increased marginally for both the dyes as the concentration of ABTS was increased from 100 to 250 μM. 1-HOBT showed concentration- (Fig. 1a,b) and time-dependent increases in percent decolorization. Vanillin also showed concentration dependence (Fig. 1a,b) but marginal time dependence on decolorization. A maximum decolorization of 40–45% was achieved with this mediator for both Reactive Black 5 and Acid Violet 17.

The rate of dye decolorization was computed for both ABTS and vanillin (Table 3). About twofold lower rates of decolorization were obtained for all the dyes (except for Reactive Black 5) with vanillin. ABTS was able to carry out decolorization at a rapid rate with more than 70% color removal in 10 min. Maximum decolorization was obtained for Reactive Red 198 followed by Reactive Orange 7, Reactive Black 5, Acid Violet 17, and Reactive Orange 1.

Table 2 Activity of *C. bulleri* laccase (100 mU/ml) on various mediators (100 μM) relative to ABTS, which was kept at 100%.

Compound	λ max (nm)	Molar extinction coefficient (M^{-1} cm^{-1}) [8, 11]	Percent activity ± SD
ABTS	420	36,500	100
Methyl vanillate	322	3,217	59.1±0.5
Guaiacol	470	6,400	33±2
p-Coumaric acid	312	11,110	23±0.7
Acetovanillone	304	7,100	19.2±1.5
Vanillin	308	9,200	17.5±0.5
2–6 Dimethoxyphenol	468	49,600	13.3±2
Ethyl vanillin	312	6,466	11.3±3

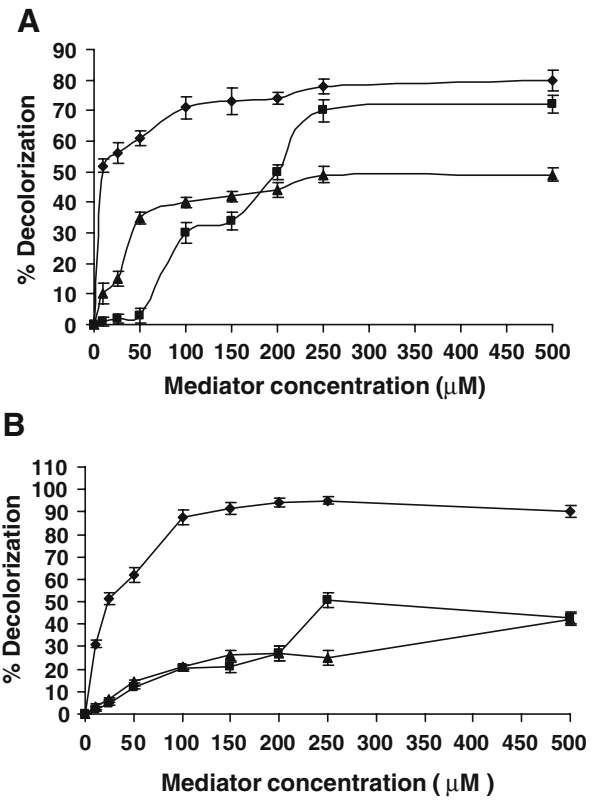

Fig. 1 Effect of mediator concentration on dye (50 μM) decolorization *by C. bulleri* laccase (100 mU/ml) monitored by decrease in absorbance after 6-h incubation using ABTS (*diamonds*), 1-HOBT (*squares*), and vanillin (*triangles*) as mediators. **a** Reactive Black 5, **b** Acid Violet 17

Decolorization of Dye Mixture

It was found that at individual dye concentration of 40 μM and dye mixture concentration of 200 μM, the OD profile (Table 4) was similar to that of the actual textile effluent (obtained from a local dyeing unit). The dye mixture was treated with laccase using different mediators at their minimum effective concentrations as determined from experiments involving individual dyes. The absorption spectrum of the treated dye mixture was recorded in the visible range and showed that both the synthetic mediators ABTS and 1-HOBT caused a substantial decline in OD. ∑OD was calculated from the sum of absorbances at each wavelength. Color removal (%) was calculated as the extent of

Table 3 Rate of individual dye decolorization (50 μM) using ABTS (100 μM) and vanillin (500 μM) as mediators.

Dye	Molar extinction coefficients (mM^{-1} cm^{-1}) (λ_{max} of dyes)	With ABTS (nmol min^{-1} mg^{-1}) ± SD	With Vanillin (nmol min^{-1} mg^{-1}) ± SD
Reactive Black 5	28 (599)	776±5	583.3±20
Reactive Red 198	9.3 (515)	1333±15	666±10
Reactive Orange 7	18.4 (490)	833±10	291.6±5
Reactive Orange 1	16.4 (493)	226±4	83.3±3
Acid Violet 17	16.3 (542)	444±6	208.3±10

Table 4 Visible range OD profile of the actual and simulated textile effluent.

	Actual affluent	Simulated textile effluent (OD ± SD)
OD at 400	0.96	0.64±0.08
OD at 450	1.11	0.83±0.09
OD at 500	1.49	1.20±0.05
OD at 550	1.49	1.22±0.1
OD at 600	1.56	0.82±0.12
OD at 650	0.87	0.60±0.04
OD at 700	0.13	0.15±0.01

decrease from the initial value of ∑OD. ABTS led to about 80% color removal, while 1-HOBT gave 69% decolorization. The effect of initial pH on the simulated effluent on percent decolorization showed that an initial pH value of 4.5 was optimal for decolorization using ABTS (Fig. 2a), and a pH value of 5.5–6.0 was optimal for using vanillin as a mediator for decolorization (Fig. 2b).

Respiratory Toxicity and Geno-toxicity Determination

The untreated and the treated dye mixtures were evaluated for toxicity (Table 5). The control cells exhibited an OCR of 0.86 mg l^{-1} min^{-1}. The addition of the dye mixture also did not affect this rate, while addition of a known inhibitor like 4-chlorophenol resulted in a fourfold drop in the OCR. The exposure of the cells to the laccase–mediator-treated dye mixture also did not lead to any drop in OCR indicating that the treated dyes, although decolorized, did not contain any byproducts that would inhibit OCR. No products were seen to accumulate either in the UV or in the visible region on the spectrophotometeric analysis. The treated dye samples exhibited a significant reduction in both toxicity and mutagenicity as evaluated by the Ames test (Table 6). The background lawn of *S. typhimurium* obtained after 48 h of incubation was observed under a microscope. The colony count was nearly 20 times less than that of the negative control, which contained the untreated dye mixture. The treated dye mixture was also found to give the background lawn of almost a similar density as that of the control. Moreover, when the number of His^+ revertants were calculated, based on the assumption that the dyes are not toxic, it was found that the untreated dye mixture was highly mutagenic (330 revertants), but the number of revertants in the treated dye mixture was not significantly higher (28 revertants). The results were statistically significant with a standard deviation of less than 3% (Table 6).

Discussion

It is well known that the substrate range of laccases ($E°$ 0.5–0.8 V) can be extended if a suitable redox mediator is added to the reactions. To fully exploit the potential of laccases as bioremediation agents, it is necessary to search for appropriate mediators. In view of varied chemical structures and redox potentials of the dyes, a large number of mediators need to be screened to select one that is best suited for all the dyes. Our aim was also to compare the parameters (such as pH, mediator concentration, time of incubation) affecting mediator (both the synthetic and natural mediators)-assisted laccase decolorization of a number of dyes and to see if the same would be effective in decolorizing the dye mixture (taking care that the simulated mixture is similar to the effluents generated in the textile

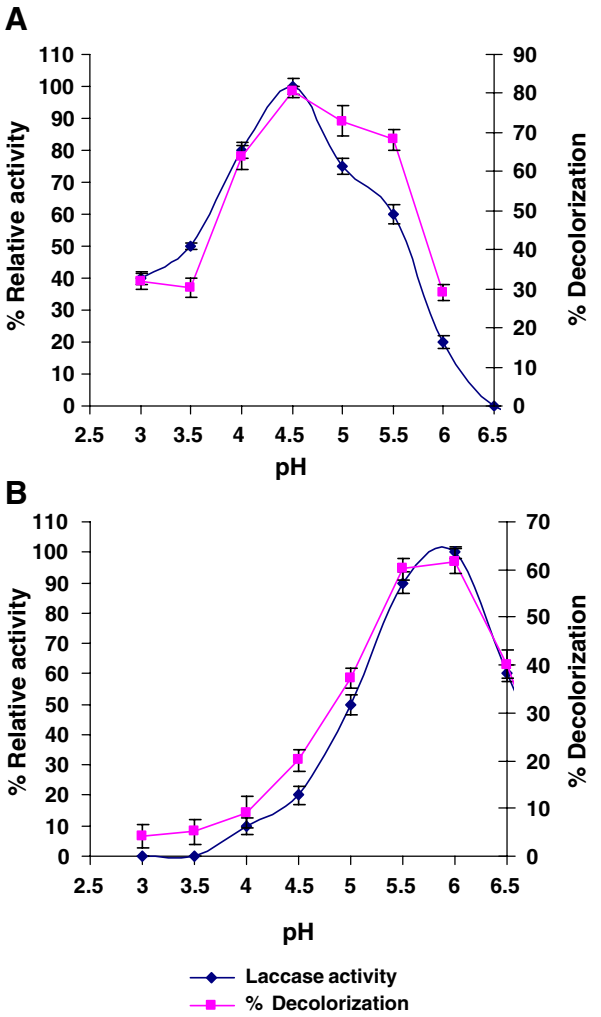

Fig. 2 Effect of initial pH on mediator (100 μM) oxidation and percent decolorization of dye mixture computed by decrease in ∑OD from the initial values of ∑OD. **a** ABTS, **b** vanillin

Table 5 Oxygen consumption rates of *P. putida* (fixed inoculum) after 4-h incubation with test compound monitored at 28 °C, 250 rpm.

Sample	Oxygen consumption rate ($mg\ l^{-1}\ min^{-1}$) ± SD
Dye mixture (0.2 mM)	0.85±0.2
Dye mixture (0.2 mM) treated with 100 mU/ml laccase–0.1 mM ABTS	0.87±0.1
Dye mixture (0.2 mM) treated with 100 mU/ml laccase–0.5 mM 1-HOBT	0.89±0.1
Dye mixture (0.2 mM) treated with 100 mU/ml laccase–0.1 mM violuric acid	0.90±0.3
Negative control (distilled water)	0.86±0.1
Positive control (200 ppm 4-chlorophenol)	0.21±0.5

Table 6 Mutagenic and toxic effects of treated and untreated dye mixture as depicted by Ames test.

Sample	Cell count/ plate	Number of revertants (observed)	Number of revertants if the colony count would have been equal to negative control
Negative control (DW)	3.6×10^6	20±2	20
Dye mixture (0.05 mM)	1.8×10^6	25±3	50
Dye mixture (0.5 mM)	1.7×10^5	16±1	330
Decolorized dye mixture (laccase–ABTS)	1.5×10^6	14±2	28
Decolorized dye mixture (laccase–Violuric acid)	1.2×10^6	16±2	48
Decolorized dye mixture (laccase–1-HOBT)	1.8×10^6	10±1	20
Decolorized dye mixture (laccase–vanillin)	1.4×10^6	12±1	30

mill). Our studies indicated that the well-known synthetic mediators such as ABTS, 1-HOBT, and violuric acid and the natural mediator vanillin differed in these parameters. However, all of them, irrespective of being natural or synthetic, reduced the physiological toxicity as well as geno-toxicity associated with the dye mixture.

Of all the mediators tested, ABTS was found to be the most effective, which was attributed to the property of laccase, which, in general, shows better activity on ABTS as compared to other laccase substrates [11]. Upon interaction with laccase, ABTS forms either $ABTS^{+}$ or $ABTS^{++}$ with $E°$ of 0.69 and 1.1 V, respectively. The latter form is proposed to be more effective in causing oxidation of a number of substrates and follows the electron transfer route; that is, an electron is abstracted from the substrate rather than a hydrogen atom, which is the case with mediators like 1-HOBT and violuric acid, which follow the hydrogen atom transfer mechanism [14]. Thus, the electron-donating substituents in the phenolic ring enhance the susceptibility of the dye to oxidation by laccase–ABTS and thus decolorization [6, 14]. The ABTS-mediated processes also showed rapid rates of dye decolorization with more than 70% color removal in 10 min. Very low concentrations (10 μM) were required to achieve effective decolorization. The optimum decolorization was achieved at 100 μM (dye/mediator ratio of 1:2), which is a desirable feature for a system to be cost effective. The inability of the mediators 1-HOBT and violuric acid to act on all the dyes and the reported inactivation of laccase induced by –NO– radical of 1-HOBT, on longer incubation periods, discourages the effectiveness of these mediators [15].

Among the natural mediators, vanillin and its derivatives acetovanillone, methyl vanillate, and ethyl vanillin showed approximately 40–65% decolorization of all the dyes. As opposed to ABTS, a higher concentration of vanillin was required for effective decolorization. Same results have been reported earlier with some other dyes [8]. The possible reasons for their lower efficiency are their higher redox potential and higher pK_a values. The rates of decolorization of individual dyes were about twofold lower than that with ABTS. Among the tested natural mediators, vanillin was found to be a good candidate for decolorization.

No clear correlation between laccase activity on the mediator and its ability to decolorize dyes was found. This is in contrast to that reported by Camarero et al. [8] where laccase activity on the mediator was directly correlated with its ability to decolorize dyes. In laccase–mediator systems, decolorization is mediated in two steps: First, the mediator is

oxidized by laccase, and then the oxidized mediator carries out the oxidation of the dye. Thus, even if a particular mediator is efficiently acted upon by laccase, the oxidized mediator may not efficiently act on dyes due to either the suboptimum difference in the redox potential (in case of electron transfer route oxidation) or the inability to access the oxidation site on the dye.

The typical dye effluent contains mixture of dyes and is found to have a typical visible range absorption profile [16]. Using the five dyes used in this study, we prepared a dye mixture whose absorption profile was similar to that of the actual effluent. The laccase–ABTS combination was able to decolorize this dye mixture to nearly 80%. The scan profile of the control dye mixture and the decolorized one in the visible range showed a substantial reduction in the absorbance at all wavelengths indicating a successful breakdown of the chromophoric groups. Other synthetic mediators like 1-HOBT and violuric acid also showed 65% and 79% decolorization, respectively. Many attempts have been made to decolorize the simulated textile dye effluent using mixed anaerobic consortia [17], aerobic consortia [18], and white rot fungi like *Coriolus versicolor* RC3 [19]. The use of microorganisms for treatment purposes has some inherent difficulties such as maintaining the optimum conditions for growth of microorganisms (supply of carbon and other nutrients, pH, moisture, oxygen tension, temperature, etc.). A slight fluctuation in any of the growth conditions may lead to substantial variation in the population of the microbe carrying out the degradation. Enzymes, on the other hand, are able to act in a wide range of environmental conditions and thus withstand the harsh conditions easily. Enzymes can be immobilized, and this makes them even more resistant to harsh conditions and enables them to be reused after their easy recovery. Claus et al. [20] have used laccase–1-HOBT for treating a mixture of synthetic dyes, and Mohorcic et al. [21] have used MnP for the treatment of artificial textile dye baths.

The initial pH of the dye mixture was found to have significant effect on decolorization with the optimum pH for mediator oxidation coinciding with the optimum pH for dye decolorization. This is justified as the more the extent of oxidation of the mediator is, the better the activity on the dyes will be, as long as the oxidized form of the mediator has the redox potential sufficient enough to oxidize the dye, as in the case of the laccase–ABTS system. Apparently, efficient decolorization will be accompanied by mediator oxidation by the enzyme. However, mediator oxidation alone need not result in dye decolorization. The oxidation of the phenolic group to the phenoxy radical is favored by the presence of the phenolate ion [22], and the phenolate ion concentration was higher when pH is above the pK_a. Vanillin was more efficiently oxidized at higher pH values, clearly indicating a higher pK_a value for vanillin.

The laccase–mediator-treated dye mixture did not show any respiration inhibition of *P. putida* indicating that no toxic byproducts accumulated. *P. putida*, in general, is found to exhibit some basal levels of resistance to the toxic environmental pollutants and is also known to degrade them. Thus, any molecule that is toxic enough to reduce the OCR (a measurable parameter) and not completely kill the cells could be well assessed for toxicity with this microbial system. In addition, the respiration inhibition is a better index as it reflects the total physiology of the cells. Dyes, in general, are found to have low aquatic toxicity. Clarke and Anliker [23] tested some 3,000 colorants and found that nearly 96% had an LC_{50} above 10 mg/l. Novotny et al. [24] did the toxicity analysis of azo and anthraquinonic dyes using three (bacteria, algal, protozoan) systems. While the bacterial system showed least sensitivity, especially toward the azo dyes, the algal system was the most sensitive. Nevertheless, it is not the dyes as such but their degradation products that are of major concern with respect to toxicity. It was found in this study that the

mutagenicity of the dye mixture was also substantially reduced by laccase–ABTS as well as laccase–vanillin treatment as indicated by the number of His^+ revertants (Table 6). The Ames *Salmonella* mutagenicity assay can also be used for preliminary toxicity determination [13], on the basis of the characteristic features of the background lawn of bacteria obtained after requisite incubation with the test compound. If the compound is toxic, at the dose investigated, then there is a thinning of the background lawn, and colonies appear bigger in size because the availability of histidine and biotin per cell increases. It was found that the untreated dye mixture led to the thinning of the background lawn indicating its toxicity. On the other hand, the treated dye mixture was found to have a background lawn similar to the negative control. With the recently reported expression of this laccase in *Escherichia coli* [25], it should be possible to test this enzyme for large-scale treatment of the dyes.

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