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Optimization of internal conditions for biocatalytic dye color removal and a comparison of redox mediator's efficiency on partially purified *Trichosanthes dioica* peroxidase

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ABSTRACT

The decolorization proficiency of partially purified *Trichosanthes dioica* peroxidase was examined for the degradation/decolorization of textile, non-textile dyes and dye mixtures. Internal conditions of pH, temperature, time intervals and enzyme concentration with selected redox mediator was optimized to obtain a cost effective decolorization setup for recalcitrant dyes.

Among the tested redox mediators, 1-hydroxybenzotriazole (HOBT) acted as a better electron transfer agent by contrast to vanillin for both textile and non-textile dyes. Maximum decolorization for reactive and disperse dyes was achieved with optimum conditions of 0.45 EU/ml, 1.0 mM HOBT, pH 5.0, 50 °C, 2 h and 0.20 EU/ml, 0.2 mM HOBT, pH 4.0, 40 °C, 1 h, respectively. The dye color change was related to its structure and consequently reduction/oxidation mediated by HOBT. To study the performance of biocatalysis in a redox mediated heterogeneous system, dye mixtures simulating industrial effluents were used which exhibited more than 82% decolorization with 1-hydroxybenzotriazole.

Result indicates the use of inexpensive peroxidase from easily available natural resources in overcoming the limitations in current wastewater treatment strategies. Such heterogeneous biocatalytic system can be extended on to large-scale treatment of wide spectrum of structurally complicated dyes by using immobilized peroxidases along with relatively cheaper redox mediators. A comparison of the efficiency with other redox mediators has also been discussed.

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1. Introduction

Along with the growing commercial availability of dyes, their range and scope of application is also expanding. Consequently, a large amount of unused dyes are released in the industrial effluents. A major fraction of textile dyes entering modern activated sludge sewage treatment plants pass through unchanged. There is an intense environmental concern about the fate of these unbound dyes. These discharged dyes form toxic products and their strong color causes turbidity which even at very low concentrations has a huge impact on the aquatic environment.

For accomplishing dye color removal, the focus in recent times has shifted towards an ecofriendly and sustainable enzyme based treatment of colored wastewater/industrial textile effluents. The peroxidase and polyphenol oxidases participate in the degradation of a broad range of substrate even at very low concentration. Further, these peroxidases and polyphenol oxidases have been used for treatment of dyes but large-scale exploitation has not been achieved due to their low enzymatic activity in biological materials and high cost of purification [1–3]. Bioremediation is a viable tool for restoration of contaminated subsurface environments. It is gaining importance due to its cost effectiveness, environmental friendliness and production of less sludge as compared to chemical and physical decomposition processes [4–7].

It has been shown that peroxidases catalyze a variety of oxidation reactions and importantly dyes recalcitrant to peroxidase shows enhanced decolorization in the presence of redox mediators [8]. The redox mediated enzyme catalysis has wide application in degradation of polycyclic aromatic hydrocarbons which includes phenols, biphenyls, pesticides, insecticides, etc. [8,9]. The current study optimized the internal factors of redox mediated dye decolorization of industrially important textile and non-textile dyes by partially purified *Trichosanthes dioica* proteins in conjunction with redox mediators. Also, the effect of redox mediator on color changes is related to the molecular structure of dye. *T. dioica*, popularly

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known as pointed gourd, is widely planted in tropical areas and consumed as vegetables.

2. Experimental methods

2.1. Chemicals

All the textile dyes and non-textile dyes, ammonium sulphate and Tween-20 were procured from Sigma Chemical Co. (St. Louis, MO, USA). Redox mediators viz., 1-hydroxybenzotriazole (HOBT) and vanillin were obtained from SRL Chemicals (Mumbai, India). All other chemicals were of analytical grade. The pointed gourds were procured from Narendra Dev University of Agriculture and Technology, Faizabad, UP, India. The samples were aseptically transferred into sterilized plastic bags.

2.2. Partial purification of T. dioica peroxidase enzyme

Briefly, 90 g of pointed gourd was homogenized in 180 ml of 100 mM sodium acetate buffer, pH 5.6. The homogenate was filtered through multi-layers of cheese cloth and centrifuged at 10,000 × *g* on a Remi C-24 cooling centrifuge for 30 min at 4 °C. The clear solution thus obtained was used for salt fractionation by adding 10–80% (w/v) $(NH_4)_2SO_4$. The proteins were precipitated by continuously stirring at 4 °C overnight. The precipitate was centrifuged at 10,000 × *g* on a Remi C-24 cooling centrifuge, dissolved in 100 mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer (0.1 M glycine HCl buffer, pH 4.0) [10].

2.3. Protein estimation and measurement of T. dioica peroxidase activity

Protein concentration was estimated by taking BSA as a standard protein and following the procedure of Lowry et al. [11]. Peroxidase activity was determined by a change in the optical density (A_{460} nm) at 37 °C by measuring the initial rate of oxidation of 6.0 mM o-dianisidine HCl in the presence of 18.0 mM H_2O_2 in 0.1 M glycine-HCl buffer, pH 4.0, for 20 min at 37 °C. One unit of activity was defined as the amount of enzyme that transformed 1 μmol of o-dianisidine HCl as substrate per min.

2.4. Preparation of dye solutions and calculation of percent decolorization

The dyes (45–210 mg/l) were solubilized in 100 mM glycine HCl buffer, pH 4.0. Each dye was independently incubated with pointed gourd peroxidase (PGP) (0.45 EU/ml) in 100 mM glycine HCl buffer, pH 4.0 in the presence of 0.80 mM H_2O_2 for varying times at 37 °C. The reaction was stopped by boiling at 100 °C for 10 min. Dye decolorization was monitored by measuring the difference at the maximum absorbance for each dye as compared with control experiments without enzyme on UV–visible spectrophotometer (JASCO V-550, Japan). Untreated dye solution (inclusive of all reagents except the enzymes) was used as control (100%) for the calculation of percent decolorization. The dye decolorization was calculated as the ratio of the difference of absorbance of treated and untreated dye to that of treated dye and converted in terms of percentage. Five independent experiments were carried out in triplicate and the mean was calculated.

2.5. Decolorization of textile and non-textile dyes in the presence of redox mediators

Each of the textile and non-textile dyes (5.0 ml) was incubated with PGP (0.45 EU/ml) in the presence of two different redox mediators viz., 1-hydroxybenzotriazole and vanillin at varying concentrations (0.05–1.5 mM) along with 0.8 mM H_2O_2 in 100 mM glycine HCl buffer, pH 4.0 for 2 h at 37 °C. The final reaction volume was kept 10 ml.

In the following sets of experiments of this section the reaction was stopped by boiling the sample at 100 °C for 10 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%).

2.6. Effect of T. dioica peroxidase concentration on decolorization of textile and non-textile dyes

Each of the two categories of individual dyes was incubated with increasing concentrations of PGP (0.065–0.50 EU/ml) in 100 mM glycine HCl buffer, pH 4.0 in the presence of 0.8 mM H_2O_2 for 1 h at 37 °C. HOBT used as a redox mediator at concentrations of 1.0 mM for reactive dyes and 0.2 mM for disperse dyes.

2.7. Effect of H_2O_2 and pH on decolorization of textile dyes

Each of the textile dyes was incubated with increasing concentrations of H_2O_2 (0.2–1.8 mM) in 100 mM glycine HCl buffer, pH 4.0 in the presence of HOBT at 1.0 mM for reactive dyes and 0.2 mM for disperse dyes for 1 h at 37 °C. The dye solutions were made in different buffers each of 100 mM and in the range of pH 2.0–10.0 [12].

2.8. Effect of temperature and time on decolorization of textile dyes

Each of the dyes was incubated with PGP (0.45 EU/ml) at different temperatures (20–90 °C). Other reaction conditions were common. The reaction was stopped by boiling the sample at 100 °C for 10 min and absorbance was recorded at λ_{max} of each dye.

2.9. Effect of HOBT on decolorization of dye mixtures

Dye mixtures were prepared by mixing each dye in equal proportions in terms of absorbance. The mixtures of dyes were treated with 0.45 EU/ml of PGP in 100 mM sodium acetate buffer, pH 4.0 in the presence of 1.0 mM H_2O_2 and 1.0 mM of HOBT for 1 h at 37 °C.

3. Results

3.1. Effect of redox mediators on decolorization profile of textile and non-textile dyes

The effect of different redox mediators on dye color removal by PGP is shown in Table 1. Among the two different redox mediators studied for dye decolorization, HOBT was more effective in dye color removal. The extent of decolorization (measured as percent decolorization) by PGP in the presence of HOBT was in the range of 98.6–69.8% for the reactive dyes whereas the disperse dyes exhibited low color change in the range of 79.2–61.2%. The operational HOBT concentration was 1.0 mM and 0.2 mM for reactive and disperse dyes, respectively. On the other hand, vanillin supplementation could decolorize reactive dyes (71.2–60.2%) and disperse dyes (55.3–34.5%) at 1.0 mM concentration. Table 1 shows the effect of increasing concentrations of HOBT and vanillin (0.05–1.5 mM) on textile and other dyes. Although both the redox mediators promoted decolorization; the dye color change of each textile dye was profound at each of the varying concentration of HOBT

Table 1
Percent dye decolorization of different textile and non-textile dyes in the presence *T. dioica* peroxidase along with two different redox mediators (HOBT and Vanillin).

	Percent dye decolorization in the presence of *T. dioica* peroxidase plus redox mediators at varying concentrations (mM) incubated for 2 h									
	HOBT					Vanillin				
	0.05	0.10	0.20	1.0	1.5	0.05	0.10	0.20	1.0	1.5
Textile dyes (λ_{max})										
Reactive Blue 15 (RB15) (675 nm)	86.5	89.7	97.6	98.6	98.5	46.6	56.7	64.8	71.2	71.1
Reactive Orange 16 (RO16) (494 nm)	61.3	67.5	71.2	74.6	74.6	35.4	45.2	52.3	60.7	60.5
Reactive Red 4 (RR4) (517 nm)	61.6	65.4	67.9	68.2	68.1	32.2	43.2	53.1	61.2	61.1
Reactive Yellow 2 (RY 2) (404 nm)	63.1	65.6	67.6	69.8	69.8	37.6	42.3	54.3	67.2	67.1
Disperse Black 9 (DB 9) (464 nm)	54.2	58.7	69.1	68.7	69.1	15.2	24.2	34.5	55.3	55.2
Disperse Orange 25 (DO 25) (457 nm)	56.1	56.6	61.2	61.1	60.9	28.9	37.8	43.7	45.7	45.6
Disperse Red 19 (DR 19) (495 nm)	67.1	69.9	79.2	78.9	79.1	43.6	56.7	61.2	67.9	67.8
Disperse Yellow 7 (DY 7) (385 nm)	58.9	61.3	67.3	67.2	67.3	15.6	24.8	37.9	37.9	37.8
Non-textile dyes (λ_{max})										
Celestine Blue (CB) (642 nm)	46.2	52.3	66.8	79.6	79.3	39.7	46.8	61.3	74.9	74.2
Coomassie Brilliant (COB) Blue R250 (553 nm)	56.3	69.4	88.9	98.9	98.1	42.1	61.2	76.2	79.8	79.3
Methylene Blue (MB) (664 nm)	60.1	67.8	89.3	98.6	98.5	56.7	68.7	71.2	73.2	73.2
Eriochrome Black T (EBT) (503 nm)	59.3	68.7	90.2	92.5	92.2	45.3	54.5	68.9	79.8	79.7
Evans Blue (EB) (611 nm)	67.5	76.4	87.7	97.1	96.8	43.8	61.3	69.9	78.3	78.2
Martius Yellow (MY) (430 nm)	6.4	15.6	35.3	39.1	39.1	5.1	13.4	20.9	33.8	33.7
Methyl Orange (MO) (505 nm)	64.2	75.6	87.9	98.4	98.4	55.6	67.3	66.7	78.5	78.4
Naphthol Blue Black (NBB) (618 nm)	57.6	69.7	78.9	97.5	97.2	51.2	62.9	72.1	84.9	84.3
Rhodamine 6G (R6G) (524 nm)	62.3	69.8	74.3	98.9	98.6	54.3	58.9	68.9	89.4	89.1

than vanillin. The non-textile dyes reacted well with 1.0 mM HOBT supplemented PGP than vanillin to effectively undergo color change. However, at concentrations of redox mediators beyond 1.0 mM percent change in decolorization was largely unaffected.

3.2. *T. dioica* peroxidase mediated decolorization of textile and non-textile dyes

Fig. 1a shows the extent of decolorization of reactive and disperse dyes with increasing concentration of PGP. The maximal

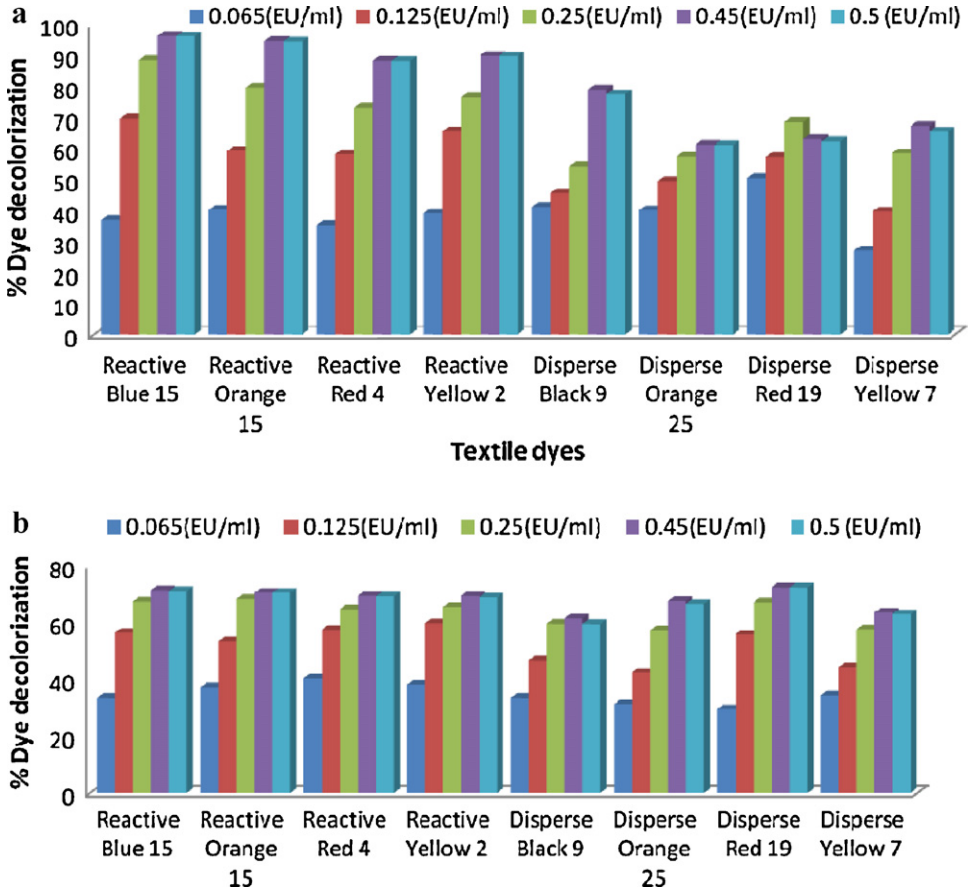

Fig. 1. (a) Percent dye decolorization of textile dyes in the presence of fixed concentration of HOBT [0.2 mM for reactive dyes and 1.0 mM for Disperse Dye] and increasing concentration of *T. dioica* peroxidase enzyme (EU/ml). Please see Table 1 for λ_{max} of each dye. (b) Percent dye decolorization of textile dyes in the presence of fixed concentration of vanillin (1.0 mM) and increasing concentration of *T. dioica* peroxidase enzyme (EU/ml). Please see Table 1 for λ_{max} of each dye.

Table 2
Decolorization of non-textile dyes by *T. dioica* peroxidase at different enzyme concentration, incubation period with fixed concentration of HOBT, H_2O_2.

Non-textile dyes (λ_{max}) nm	0.065 EU/ml		0.125 EU/ml		0.250 EU/ml		0.45 EU/ml		0.50 EU/ml	
	−HOBT (60 min)	+HOBT (60 min)	−HOBT (120 min)	+HOBT (120 min)	−HOBT (160 min)	+HOBT (160 min)	−HOBT (180 min)	+HOBT (180 min)	−HOBT (240 min)	+HOBT (240 min)
Celestine Blue (642 nm)	46.6	57.3	49.7	68.7	54.3	69.3	55.6	79.5	58.9	79.6
Coomassie Brilliant Blue R250 (553 nm)	47.6	92.4	48.2	93.2	51.3	98.9	51.2	98.7	51.1	98.7
Methylene Blue (664 nm)	2.5	67.2	3.7	78.5	3.6	98.4	3.5	98.6	3.2	98.9
Eriochrome Black T (503 nm)	48.7	67.7	51.6	77.4	55.4	91.3	55.3	92.0	55.1	92.2
Evans Blue (607 nm)	86.4	94.3	87.5	95.4	87.8	96.7	87.2	97.1	86.7	97.2
Martius Yellow (433 nm)	10.2	33.2	12.4	34.5	13.2	35.6	13.5	35.1	12.3	35.2
Methyl Orange (464 nm)	12.3	87.2	13.2	89.2	13.2	98.5	13.1	98.7	12.2	98.6
Naphthol Blue Black (618 nm)	47.2	78.8	49.2	86.2	56.6	97.4	57.2	97.3	57.9	97.1
Rhodamine 6G (525 nm)	11.2	78.4	13.2	86.3	13.2	99.7	14.2	99.6	13.2	99.5

decolorization for the textile and non-textile dyes was observed at PGP concentration of 0.45 EU/ml after an incubation time of 2 h. However, on increasing the PGP concentration further, there was no remarkable dye color change. The addition of 0.2 mM and 1.0 mM HOBT to soluble PGP facilitated decolorization of disperse and reactive dyes, respectively. Reactive Blue 15 and Reactive Orange 15 decolorized to the extent of 96.2% and 94.6% whereas the others, Reactive Red 4 and Reactive Yellow 2 showed color change up to 88.2% and 89.8%, respectively. Among the disperse dyes only Disperse Black 9 was effectively decolorized up to 79% whereas the others showed disappearance of color below 69.3%.

In the presence of 1.0 mM vanillin, partially purified *T. dioica* peroxidase at 0.45 EU/ml catalyzed decolorization of reactive and disperse dyes in the range of 61.4–71.3% after 2 h of incubation (Fig. 1b). Although the dyes showed remarkable color change after the first increase in peroxidase concentration, however there was no profound change on decolorization on further increasing the peroxidase concentration or the incubation time.

Nine different non-textile dyes were studied (Table 2). These dyes were treated with different amount of *T. dioica* peroxidase (0.065–0.50 EU/ml) and incubated for varying time interval with and without the redox mediator HOBT (1.0 mM) at 37 °C. The results indicated that few non-textile dyes viz., Methylene Blue, Martius Yellow, Methyl Orange, Rhodamine 6G were highly recalcitrant to decolorization by *T. dioica* in the absence of HOBT after an incubation of 2 h. However, on supplementation with 1.0 mM HOBT the dye color change achieved for Methylene Blue, Martius Yellow and Methyl Orange, was 98.6%, 35.1% and 98.7%, respectively; whereas Rhodamine 6G almost decolorized completely. For other dyes, the maximum decolorization exhibited after 3 h was 98.7%, 97.3%, 97.1%, 67.8% for Coomassie Brilliant Blue R 250, Naphthol Blue Black, Evans Blue and Eriochrome Black T, respectively.

3.3. H_2O_2 and pH activity profile of decolorization of textile dyes

Fig. 2 shows that the percent decolorization improved with the increasing concentration of H_2O_2 and the maximum decolorization was observed at a concentration of 0.8 mM and 1.0 mM of H_2O_2 for disperse and reactive dyes, respectively which remained substantially unaffected till 1.2 mM H_2O_2.

To find out the range of pH in which significant decolorization was observed; buffers in the range of pH 2.0–10.0 were used. The percent dye color change is shown in Fig. 3. An acidic range of pH (3.0–6.0) was better suited for dye decolorization. Maximum decolorization was observed at pH 4.0 and pH 5.0 at fixed concentration of *T. dioica* peroxidase and HOBT for disperse and reactive dyes, respectively. The extent of dye color removal decreased in an alkaline medium and at pH 10.0 the decolorization action of the enzyme was almost insignificant/lost.

3.4. Temperature and time activity profile of decolorization of textile dyes

The percent decolorization was plotted as a function of temperature and the results are shown in Fig. 4. Among the textile dyes the reactive dyes exhibited maximum decolorization at 50 °C whereas the disperse dyes showed maximum dye color change at 40 °C in the presence optimum concentration of 1.0 mM and 0.2 mM HOBT, respectively. The decolorization achieved for Reactive Blue 15 (96.1%), Reactive Orange 15 (94.4%), Reactive Red 4

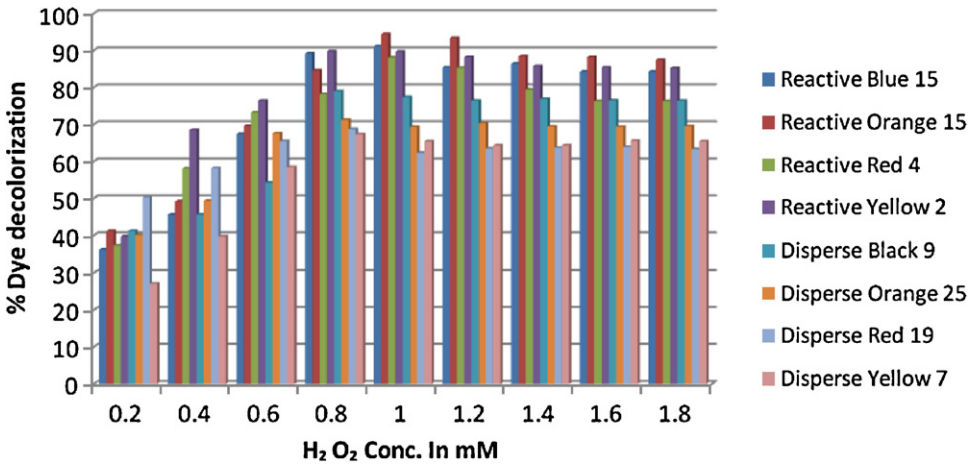

Fig. 2. Percent dye decolorization of textile dyes in the presence of fixed concentration of HOBT, *T. dioica* peroxidase enzyme (0.45 EU/ml) and varying concentration of H_2O_2. Please see Table 1 for λ_{max} of each dye.

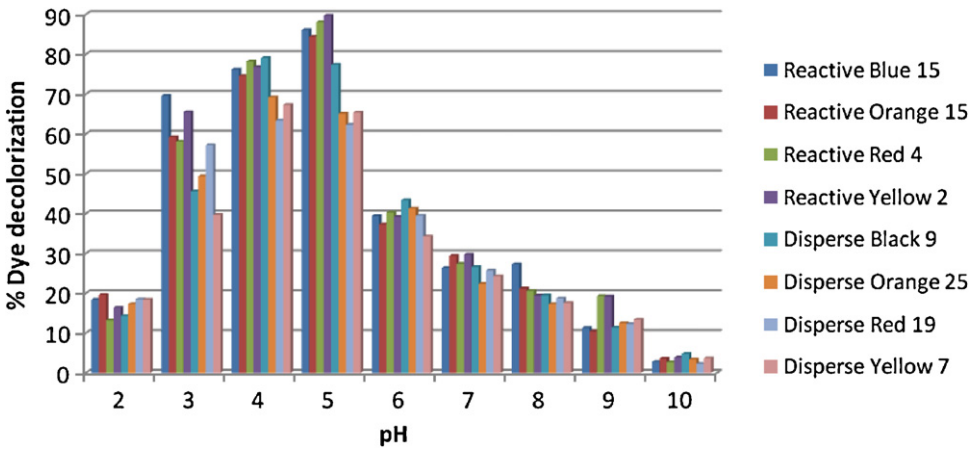

Fig. 3. Percent dye decolorization of textile dyes in the presence of fixed concentration of HOBT, *T. dioica* peroxidase enzyme (0.45 EU/ml) and varying pH. Please see Table 1 for λ_{max} of each dye.

(85.2%) whereas disperse dyes underwent dye color change in the range of 61.2–79%.

The extent of decolorization of textile dyes as a function of time is shown in Fig. 5. Maximum decolorization for reactive and disperse dyes was observed within 1 h of incubation at 50 °C and 40 °C with 0.45 EU/ml of PGP and 1.0 mM and 0.2 mM HOBT, respectively. However, further decolorization of these dyes progressed slowly up to 4 h, although no effective increase was observed even when the dyes were further incubated for longer times. Among the reactive dyes Reactive Blue 15 decolorized almost completely and Reactive Orange 15 up to 78.3% at 4 h incubation, whereas Reactive Red 4 and Reactive Yellow 2 showed decolorization up to 86.3% and 81.2% under similar conditions. The disperse dyes were comparatively resistant to decolorization and only in the presence of redox mediators Disperse Red 19, Disperse Yellow 7, Disperse Black 9 decolorized to 79.5%, 72.3% and 71.6%, respectively. Disperse Orange 15 was comparatively weakly degraded and decolorized to a lesser extent under similar conditions with a maximum of 61.2%.

3.5. HOBT mediated decolorization of different dye mixtures by T. dioica peroxidase

To simulate the decolorization of dyes from industrial effluent, complex mixtures of textile dyes including reactive, disperse and non-textile dyes were prepared by mixing four different dyes in equal proportions and incubated with 0.45 EU/ml of *T. dioica* peroxidase in the presence of 1.0 mM HOBT and 1.0 mM H_2O_2 for 3 h at 40 °C. The decolorization was recorded at wavelength maxima of each mixture determined spectrophotometrically. The combinations of different dyes showed decolorization by more than 82% (Fig. 6). The rate of decolorization of dye mixture was slower in comparison to that of individual dyes both in the presence and absence of HOBT. However, the HOBT mediated dye decolorization was more effective and profound.

4. Discussion

In this paper we optimized the internal conditions of decolorization of a wide spectrum of industrially important textile dyes as well as some non-textile dyes using redox mediated peroxidase catalytic system. In order to make the process of decolorization cost effective and feasible we ignored the degree of purity of enzyme and opted to use ammonium sulphate precipitated protein fractions from *T. dioica*. PGP was partially purified using 10–80% ammonium sulphate which retained a specific activity of 96.0 U/mg of protein. The experiments were performed at different enzyme concentration, pH, temperature, incubation period and redox mediators namely, HOBT and vanillin. The data obtained for performing color removal of a wide variety of dyes are interesting and suggestive

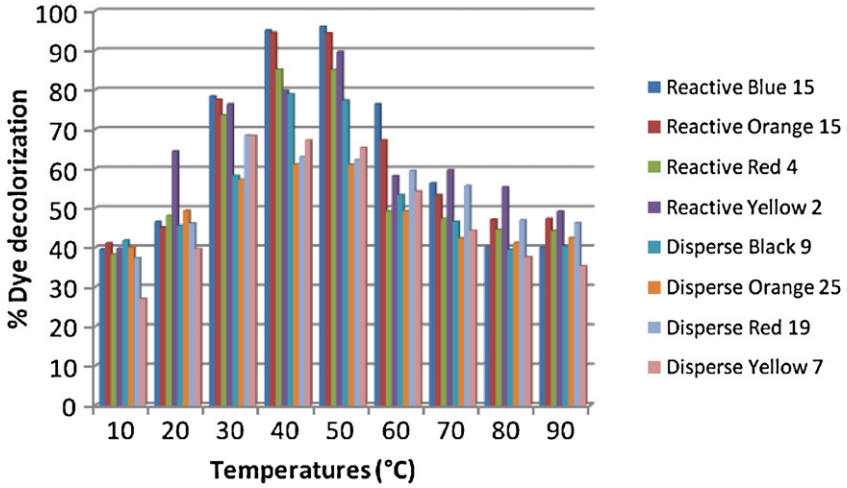

Fig. 4. Percent dye decolorization of Textile Dyes in the presence of fixed concentration of HOBT, *T. dioica* peroxidase enzyme (EU/ml) at different temperatures. Please see Table 1 for λ_{max} of each dye.

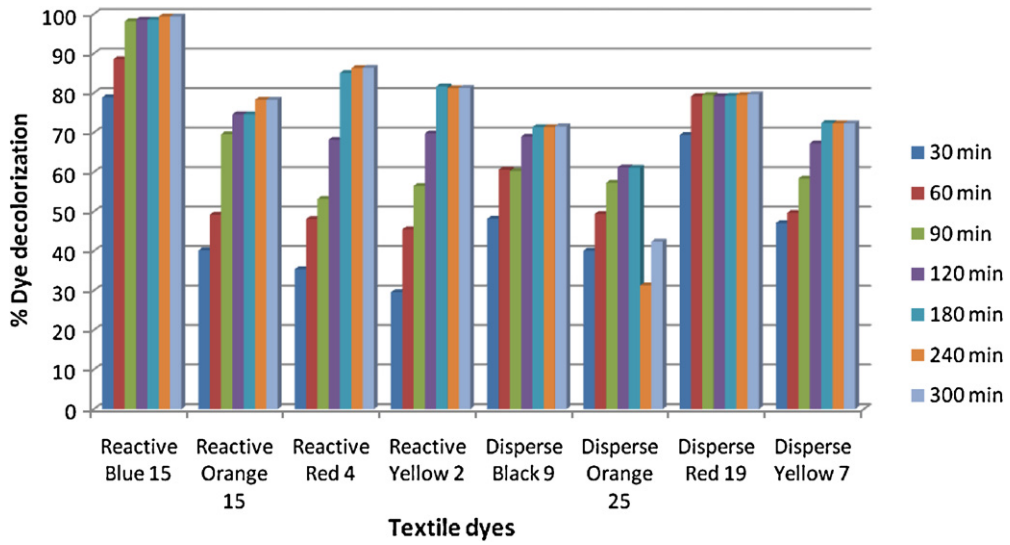

Fig. 5. Percent dye decolorization of textile dyes in the presence of fixed concentration of HOBT, *T. dioica* peroxidase enzyme (0.45 EU/ml) at different time interval. Please see Table 1 for λ_{max} of each dye.

of redox mediated biocatalysis to treat wastewater contaminated with these dyes.

4.1. Redox mediator and dye decolorization

The decolorization profile of all the dyes increased significantly at HOBT concentration of 0.2 mM (Table 1). However, the dye color change was marginal on increasing the HOBT concentration further to 1.5 mM. The optimum requirement of HOBT for reactive and disperse dyes were different. The reactive dyes exhibited decolorization maximally at 1.0 mM HOBT whereas by contrast, disperse dyes showed maximum decolorization at lower values of HOBT. The structural complexity and perhaps the source of the peroxidase may account for fivefold higher requirement of HOBT to facilitate maximum decolorization. Nevertheless, the redox mediator vanillin was able to decolorize both the reactive and disperse dyes at 1.0 mM concentration but the extent of decolorization was inadequate and slow in comparison to HOBT for certain dyes like Reactive Blue 15, Reactive Orange 16 and most of the experimented disperse dyes. Disperse Yellow 7 decolorized slowly (37.9%) with *T. dioica* peroxidase in the presence of relatively higher concentration of vanillin. Thus, the HOBT served as a better electron transfer agent than vanillin and that decolorization of dyes took place via degradation of aromatic ring of the compounds or by cleaving certain functional groups [10,15]. All other textile dyes showed insignificant or no decolorization with *T. dioica* peroxidase alone when studied under optimum conditions of dye decolorization in the absence of HOBT (data not shown as the extent of dye decolorization is inadequate and slow without redox mediators). The non-textile dyes exhibited remarkable decolorization in presence of both the redox mediators at 1.0 mM concentration. The formation of some amount of soluble aggregates following color removal by reactive dyes is supported by reports that treatment of phenols and aromatic amines by peroxidases resulted in formation of large insoluble aggregates [13,14]. On the contrary other dyes studied showed no

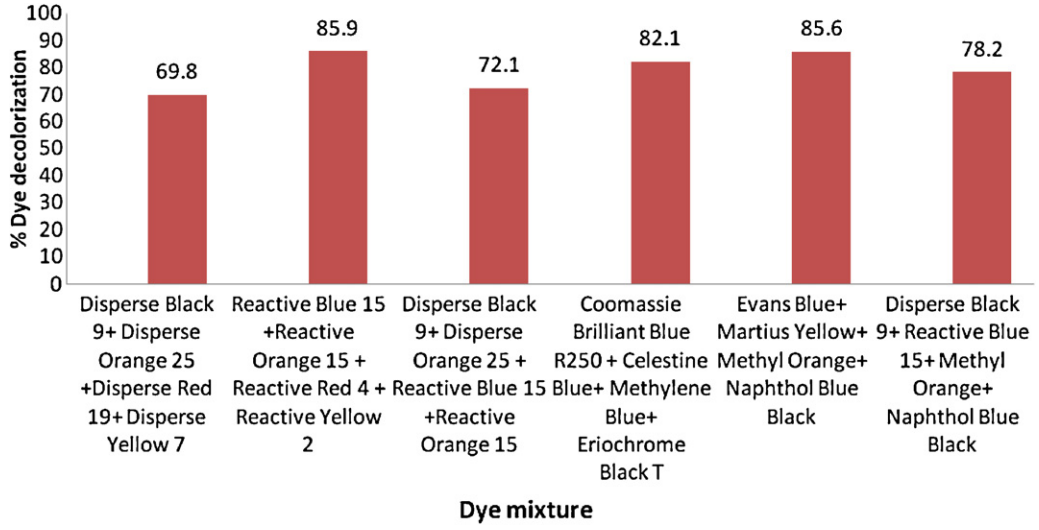

Fig. 6. Percent dye decolorization of dye mixture by *T. dioica* in the presence of HOBT/H_2O_2 to mimic dyes in industrial effluents [Disperse Black 9 + Disperse Orange 25 + Disperse Red 19 + Disperse Yellow 7 (λ_{461}); Reactive Blue 15 + Reactive Orange 15 + Reactive Red 4 + Reactive Yellow 2 (λ_{531}); Disperse Black 9 + Disperse Orange 25 + Reactive Blue 15 + Reactive Orange 15 (λ_{521}); Coomassie Brilliant Blue R250 + Celestine Blue + Methylene Blue + Eriochrome Black T (λ_{595}); Evans Blue + Martius Yellow + Methyl Orange + Naphthol Blue Black (λ_{541}); Disperse Black 9 + Reactive Blue 15 + Methyl Orange + Naphthol Blue Black (λ_{571})].

formation of precipitate during decolorization in the presence of HOBT.

Redox mediators have the potential to mediate oxidation reaction between a substrate and an enzyme [16]. The redox potential of redox mediators governs the mediation efficiency and oxidation mechanism of the substrate [17]. Oxidation of substrate occurs by free radical formation by the mediator. These free radicals can be formed either by one-electron oxidation of substrate or by abstraction of a proton from the substrate [18,19]. Laccase have been used with redox mediators to oxidize non-phenolic compounds [20]. The mechanism of action of laccase mediator system has been extensively studied and it is used in the textile industry in the finishing process for indigo stained materials. Several workers have demonstrated that the use of redox mediator system enhanced the rate of dye decolorization by several folds but these mediators were required in very high concentrations [20–22]. Over here we have shown the decolorization of both textile and non-textile dyes as well as dye mixtures mediated by *T. dioica* peroxidase under the influence of low concentration of redox mediators.

HOBT mediated reactions operate via an electron transfer mechanism and the rates are strongly dependent on the redox potential of oxidized substrates. It may also extend the substrate specificity of peroxidases towards substrates of higher redox potential. Peroxidases generate reactive radical species which subsequently undergo further oxidation either to cleavage or oligomerization products. Azo dye decomposition proceeds first via two sequential electron abstractions. This is followed by an attack of the nucleophile water on the resulting resonance stabilized cation. Subsequently, breakdown of the dye molecule takes place concomitantly with the release of one proton and one molecule of N_2 yielding chinoid aromatics and transient hydroperoxides, respectively [23]. The resulting chinones and radicals could undergo coupling reactions yielding oligomeric and polymeric structures.

4.2. Reaction parameters and dye color removal

The peroxidase reacted well to facilitate decolorization at concentration of 0.8 mM and 1.0 mM of H_2O_2 for disperse and reactive dyes, respectively and remained substantially unaffected till 1.2 mM (Fig. 2). Although higher concentration of H_2O_2 acts as an inhibitor of peroxidase activity by irreversibly oxidizing the enzyme ferri-heme group essential for peroxidase activity but in this study we observed a relatively higher working concentration of H_2O_2 which could perhaps be due to the source of peroxidase [15,24].

The enzyme on supplementation with redox mediator efficiently catalyzed decolorization of the dyes, implying dye color change was a redox mediated H_2O_2-dependent enzymatic reaction. The enzyme performance was optimum in an acidic medium of pH range 3–6, whereas its decolorizing/degrading activity was adversely affected in alkaline medium (Fig. 3). The data not only support the earlier view of disperse dye decolorization in acidic medium by *T. dioica* peroxidase but further indicate that acid medium favors catalysis of even reactive dyes. It has earlier been reported that the degradation of industrially important dyes by enzymes such as horse radish peroxidase, polyphenol oxidase, BGP and laccase was also maximum in the buffers of acidic pH [12,25].

The reaction temperature is an important parameter which affects the decolorization of dyes. The maximum decolorization for both the reactive and disperse dyes were in the temperature range of 40–50 °C and in the presence of fixed concentration of HOBT (Fig. 4). The extent of decolorization was remarkable for Reactive Blue 15, Reactive Orange 16, Reactive Red 4 at 50 °C whereas disperse dyes could be decolorized in the range of 61.2–79% at 40 °C. The decolorization varies with the nature of the dyes but redox mediated decolorization with *T. dioica* peroxidase was a better solution for effective decolorization of recalcitrant compounds. The rate of decolorization varied with time and maximum decolorization in the presence of HOBT was observed within 1 h of incubation for Reactive Blue 15 and Disperse Red 19 (Fig. 5). However, for other dyes the extent of decolorization progressed slowly and reached a plateau after 4 h of incubation. These data are consistent with reports that decolorization rate varies, depending upon the type of dye to be treated [26].

The non-textile dyes studied for decolorization with *T. dioica* peroxidase exhibited enhanced decolorization in the presence of 1.0 mM HOBT, whereas in the absence of redox mediator decolorization was inadequate and much slow. Coomassie Brilliant Blue R250, Methylene Blue, Eriochrome Black T, Martius Yellow, Methyl Orange, Naphthol Blue Black and Rhodamine were extensively decolorized by *T. dioica* peroxidase under the influence HOBT (Table 2). The decolorization of Evans blue was not significantly affected by the presence of redox mediator, although in the presence of HOBT decolorization achieved was higher. The performance of this system was maximal during 160 min of incubation. Celestine blue was decolorized to 79.5% in the presence of HOBT at higher concentration of enzyme as well as longer incubation time. The data in Table 2 are suggestive of *T. dioica* peroxidase in conjunction with low concentration of HOBT to be a wonderful decolorization/degradation system for non-textile dyes as well. Further, dye mixtures simulating industrial effluents exhibited more than 82% decolorization with 1-hydroxybenzotriazole (Fig. 6). However, the rate of decolorization of dye mixture was slower in comparison to that of individual dyes both in the presence and absence of HOBT.

4.3. Dye structure and degradation

Decolorization efficiency is related to the different chemical structures of the dyes and potential of the redox mediators employed for study. Although, it is difficult to suggest the actual molecular mechanism operational in the heterogeneous mixture, dye structure and mediation efficiency of redox mediators contributes largely to peroxidase mediated catalysis. Almansa et al studied the influence of structure on dye degradation of closely related twenty-two model azo dyes subjected to catalyzed oxidation using redox mediator [27]. These model dyes were based on the molecular framework of 2,7-dihydroxy-1-phenylazonaphthalene-3,6-disulphonic acid and differed in the nature and position of the substituent on the phenyl ring in the *ortho*-, *meta*-, or *para*-position with respect to the azo linkage. The study reflected that enzymatic degradation without the assistance of an electron mediator took place only with the hydroxy-substituted azo dyes whereas the other dyes except for the trifluoromethyl-substituted ones were degraded only when catalytic amounts of redox mediator were present in the reaction mixture. The partially purified PGP decolorized the structurally diverse commercial textile dyes (RB15, RO16, RR4, RY2, DB9, DR25, DR19 and DY7) in the presence of redox mediator. Textile dyes experimented for dye color change had a diazo group (–N=N–) except RB15 (Fig. 7a and b). RO16, RR4, RY2 are hydroxy-substituted azo dyes and easily undergo catalytic oxidation leading to decolorization in the presence of PGP with redox mediator. All the tested dyes exhibited dye color removal to >∼60% in the presence of redox mediator HOBT. Scanty information is available in the literature which could quantitatively describe the effects of dye chemical structure on the reactivity towards peroxidase oxidation in presence of mediators. From the decolorization studies of model azo dyes it can be concluded that dyes carrying a hydroxy group either in the *ortho*- or *para*-position relative to azo-bond were the most reactive and susceptible to oxidation when treated with peroxidase in conjunction with redox mediators. Kandelbauer et al., have reported the susceptibility of enzymatic oxidation of hydroxy-substituted dyes which led to its complete

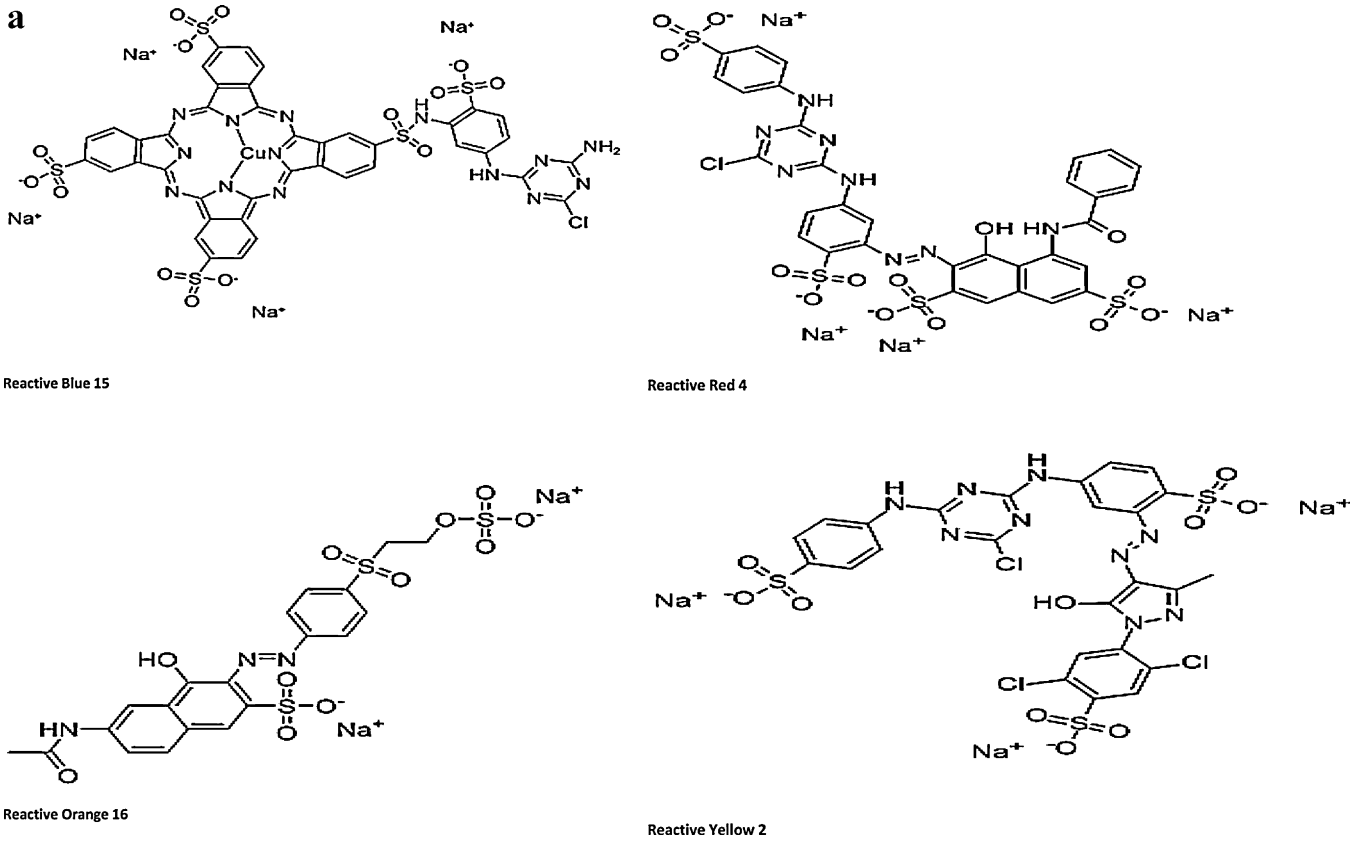

Fig. 7. (a) Molecular structures of reactive dyes. (b) Molecular structures of disperse dyes.

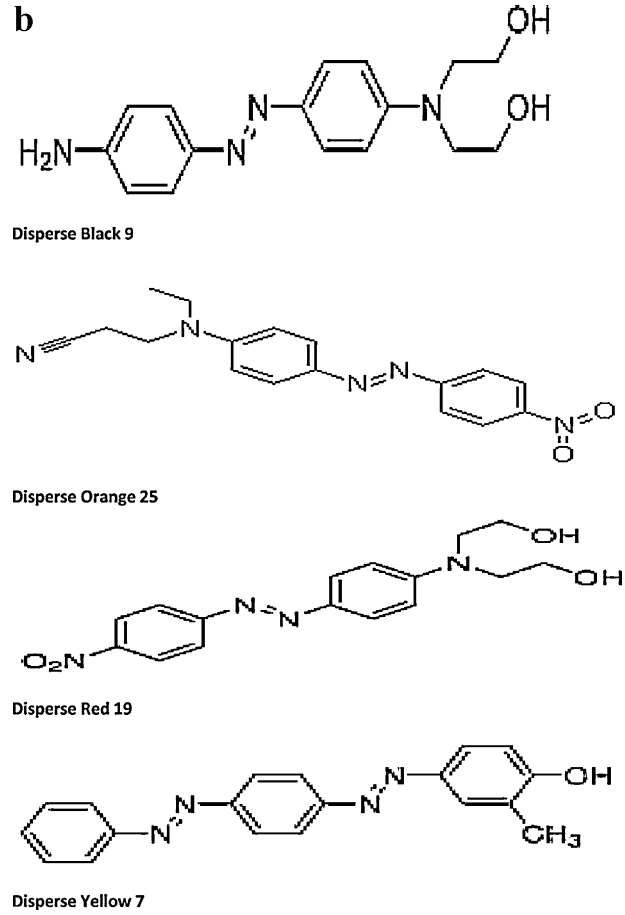

Fig. 7. (*Continued*)

decolorization [28]. In the present study complete decolorization could not be achieved but since these dyes were recalcitrant to decolorization alone by PGP or redox mediator, the combined influence was effective in achieving sufficient decolorization.

Upon substitution most of the substrates become less susceptible towards oxidation. Electron-withdrawing substituents generally were found to diminish reaction rates whereas electron-donating groups enhanced the susceptibility of the dye towards oxidative attack. Further, electron-withdrawing substituent may contribute towards recalcitrance of dyes which undergo redox mediated enzymatic decolorization. The main function of the peroxidase/redox mediator thus consists of oxidatively rendering the azo-dye more susceptible to further nucleophilic attack and nitrogen is eliminated in molecular form. By choosing appropriate reaction conditions it should also be possible to shift the chemical equilibria between the enzymatically activated radical species towards oligomerization reactions. Dyes with sulfonate group (RB15) exhibit a strong electron-withdrawing effect and thus exhibited low overall reactivity. Such dyes underwent dye color change in the presence of redox mediator indicating the enhancement of catalytic oxidation of PGP.

HOBT addition enhanced dye oxidation in all cases and compared to vanillin, was a more suitable and potent redox mediator that effectively influenced decolorization by soluble *T. dioica* peroxidase. Moreover, the redox mediating properties of HOBT were superior to vanillin, possibly due to the difference in redox potential. The dyes tested may have a narrow redox potential range and perhaps a correlation exists between redox potential and dye reduction/oxidation rates. It is known that closer the redox potential is between dye and redox mediator, the faster is dye reduction in a non-peroxidase mediated catalytic system, because electron transfer is facilitated due to the low potential difference. However, on the contrary soluble peroxidase mediated catalysis in the

presence of H_2O_2/redox mediator is an oxidative event leading to dye color change. Consequently such behavior might explain the better catalytic properties of HOBT as compared to vanillin. Moreover, peroxidase mediated dye oxidation rate is not only determined by redox potential, but also by other factors such as chemical structure, environmental conditions and anaerobic sludge affinity and concentration.

In another study it has been shown that azo dyes such as Congo Red, Reactive Red 2, Reactive Red 120 and Reactive Black 5 exhibited remarkable dye color change when PGP was supplemented with either riboflavin or AQDS (antraquinone-2,6-disulfonate) at similar concentrations [29]. However, Reactive Orange 16 showed color change more than 90% with both riboflavin and AQDS whereas with HOBT only up to 71.2% decolorization could be achieved. In all the cases vanillin mediated catalysis by PGP was slow and inadequate (Reactive Orange 16; 52.3%). This comparison of redox mediator suggests that riboflavin would serve as an effective redox mediator for achieving remarkable decolorization of reactive dyes whereas disperse dyes can undergo dye color change on supplementing PGP with 1-hydroxybenzotriazole.

5. Conclusions

To address the growing public concern over the toxicity and carcinogenicity of synthetic and recalcitrant dyes innovative treatment technologies need to be investigated. The oxidative transformation of dyes depends on their chemical structure. The presence of *ortho*-hydroxy groups with respect to the azo link enhances the decolorization rates of azo dyes with peroxidases whereas nitro- and sulphonate groups stabilized the dye molecules. In this study, we have studied dye color removal and optimized conditions suitable for wide spectrum of textile, non-textile dyes and dye mixtures. Decolorization of dyes by contrast, drastically improved with HOBT and dyes/dye mixtures recalcitrant to *T. dioica* peroxidase alone exhibited remarkable decolorization. Research activities should focus on optimization of the reaction conditions in order to achieve maximum oxidative coupling of primarily formed dye fragments. Together with introduction of substituents which enhances peroxidase catalyzed decolorization, novel "biodegradable" dyes could be designed. The study provides a lead in using inexpensive peroxidase from easily available natural resources in overcoming the limitations in current wastewater treatment strategies. Such simple peroxidase system can be extended on to large-scale treatment of wide spectrum of structural dyes by using immobilized PGP along with relatively cheaper redox mediators.

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