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Potential of peroxidase enzyme from *Trichosanthes diocia* to mediate disperse dye decolorization in conjunction with redox mediators

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ABSTRACT

Disperse dyes are frequently used worldwide for dyeing polyester, nylon, cellulose acetate and acrylic fibers. The highly variable and complex chemical structure of the dyes makes them difficult to remove using conventional wastewater treatment systems. Redox mediated enzymatic catalysis has wide applications including degradation of polycyclic compounds, phenols, aromatic amines, biphenyls, pesticides, insecticides etc. We investigated the effect of *Trichosanthes diocia* peroxidase along with redox mediators on decolorization of water insoluble disperse dyes; Disperse Red 19 and Disperse Black 9. Nine different redox mediators; bromophenol, 2,4-dichlorophenol, guaiacol, 1-hydroxybenzotriazole, m-cresol, quinol, syringaldehyde, vanillin and violuric acid were evaluated. Results indicated that among the chosen mediators, 1-hydroxybenzotriazole (HOBT) was most effective for decolorization. At a concentration of 0.45 U mL^{-1} the peroxidase could decolorize Disperse Red 19 to a maximum of 79% with 0.2 mM 1-hydroxybenzotriazole whereas Disperse Black 9 decolorized upto 60% with 0.5 mM 1-hydroxybenzotriazole. The time, pH and temperature at which maximum decolorization were recorded was 60 min, 4 and 42 °C. Data were analyzed by one-way ANOVA and Mann Whitney test. The study suggests that peroxidase from *T. diocia* could be a cheap, easy, effective source and along with redox mediators be used to treat recalcitrant synthetic dyes.

1. Introduction

Many chemical dyes have been used increasingly in textile and dyeing industries because of their ease and cost effectiveness in synthesis, firmness and variety in color compared to that of natural dyes. Approximately 50% of the dyes are released in the industrial effluents. It is known that 90% of reactive textile dyes entering activated sludge sewage treatment plants will pass through unchanged and will be discharged to rivers. There is a great environmental concern about the fate of these dyes, as large amounts of unbound dye are discharged in the effluent [1].

Color is usually the first contaminant to be recognized in wastewater, as very small amounts of synthetic dyes in water (10–15 mg L^{-1}) are highly visible, affecting the aesthetic merit, transparency and gas solubility of water bodies.

Disperse dyes have low solubility in water. They constitute largest group of colorants used in industry. The dyes chosen for this study are commonly present in industrial effluents; resist aerobic and short-term anaerobic treatment and are difficult to remove by chemical treatment. They are mainly used is the dyeing of polyesters, and find minor use in dyeing cellulose acetates and polyamides. Dye removal from wastewaters with traditional physico-chemical processes, such as coagulation, adsorption and oxidation with ozone is expensive, can generate large volumes of sludge and usually require the addition of environmentally hazardous chemical additives [2]. On the other hand, most of the synthetic dyes are xenobiotic compounds which are poorly removed by the use of conventional biological aerobic treatments [3]. Decolorization of dye wastewater is an area where innovative treatment technologies need to be investigated. The focus in recent times has shifted towards enzyme based treatment of colored wastewater/industrial effluents. The processes involving physical and chemical treatment for decolorization of textile wastewater have numerous operational problems, secondary pollution problems and high cost [4]. Currently, bioremediation is becoming important because of its cost effectiveness, environmental friendliness and production of less sludge as compared to the chemical and physical decomposition processes [5]. Peroxidases catalyze a variety of oxidation reactions and dyes recalcitrant to peroxidase action undergo decolorization to a significant extent in the presence of redox mediators [6]. The redox mediated enzyme catalysis has wide application in degradation of polycyclic aromatic hydrocarbons which includes phenols, biphenyls, pesticides, insecticides

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etc. [7,8]. The aim of the present study was to identify and isolate peroxidase enzyme from commonly available plant sources and tests its effectiveness under different conditions; which could be used for developing an inexpensive and efficient method for treatment of hydrophobic disperse dyes that are potentially toxic and even carcinogenic.

2. Experimental methods

2.1. Dyes and chemicals

The disperse dyes namely Disperse Red 19 (DR19) Disperse Black 9 (DB9), ammonium sulphate, and Tween-20 were procured from Sigma Chemical Co. (St. Louis, MO, USA) and all other chemicals were of analytical grade. Redox mediators viz., bromophenol, 2,4-dichlorophenol, guaiacol, 1-hydroxybenzotriazole (HOBT), m-cresol, quinol, syringaldehyde, vanillin and violuric acid were obtained from SRL Chemicals (Mumbai, India). The pointed gourds were purchased from the local market.

2.2. Enzyme purification by ammonium sulphate precipitation

Briefly, 300 g of pointed gourd was homogenized in 600 mL of 100 mM sodium acetate buffer, pH 5.6. The homogenate was filtered through multi-layers of cheese cloth and then centrifuged at the speed of 10,000 × *g* on a Remi C-24 Cooling Centrifuge for 30 min at 4 °C. The clear solution thus obtained was subjected to 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 collected by centrifugation 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) [9].

2.3. Protein estimation and measurement of peroxidase activity

Protein concentration was estimated by taking BSA as a standard protein and following the procedure of Lowry et al. [10]. 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 and treatment of synthetic dye solution

The disperse dyes were solubilized in 0.025% (v/v) Tween 20 prepared in 0.1 M glycine HCl buffer, pH 3.0. DR19 (25 mg L^{-1}) and DB9 (50 mg L^{-1}) were independently incubated with pointed gourd peroxidase (PGP) (0.45 U mL^{-1}) in 0.1 M 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 7 min. Dye decolorization was monitored by measuring the difference at the maximum absorbance for each dye (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively) as compared with control experiments without enzyme on UV–visible spectrophotometer (JASCO, 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.

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

Five independent experiments were carried out in duplicate and the mean was calculated with average standard deviations, <5%.

2.5. PGP mediated dye decolorization in the presence of different redox mediators

Each of the two dyes (5.0 mL) were incubated with PGP (0.45 U mL^{-1}) in the presence of each redox mediators viz., bromophenol, 2,4-dichlorophenol, guaiacol, 1-hydroxybenzotriazole, m-cresol, quinol, syringaldehyde, vanillin and violuric acid (0.5 mM) and 0.75 mM H_2O_2 in 0.1 M glycine HCl buffer, pH 4.0 for 1 h at 37 °C. The final reaction volume was kept 10 mL. The reaction was stopped by boiling the sample at 100 °C for 7 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%). A similar set of experiment was performed in the presence of varying concentrations of HOBT (0.05–0.60 mM).

2.6. Dye decolorization with varying concentration of enzyme (PGP) and H_2O_2

Each of the two dyes were incubated with increasing concentrations of PGP (0.02–0.95 U mL^{-1}) and H_2O_2 (0.2–1.8 mM) in 0.1 M glycine HCl buffer, pH 4.0 in the presence of 0.75 mM H_2O_2 for 1 h at 37 °C. HOBT used as a redox mediator at concentrations of 0.2 mM and 0.5 mM for DR 19 and DB9, respectively. The reaction was stopped by boiling the sample at 100 °C for 7 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

2.7. PGP mediated dye decolorization as a function of temperature and pH

The dye solutions were made in different buffers each of 100 mM and in the range of pH 2.0–10.0. The buffers were glycine–HCl (pH 2.0, 3.0 and 4.0), sodium acetate (pH 5.0), sodium phosphate (pH 6.0, 7.0 and 8.0), and Tris–HCl (pH 9.0 and 10.0). Each of the two dye was treated with PGP (0.45 U mL^{-1}) in buffers of varying pH and in the presence of 0.8 mM H_2O_2 for 1 h at 37 °C. The decolorization of each dye DR19 and DB9 was also performed in the presence of 0.2 mM and 0.5 mM HOBT, respectively. The reaction was stopped by boiling the sample at 100 °C for 7 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

Each of the two dyes was incubated with PGP (0.45 U mL^{-1}) at different temperatures (20–90 °C). Other reaction conditions were common. The reaction was stopped by boiling the sample at 100 °C for 7 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

2.8. PGP mediated dye decolorization as a function of time

The individual dye was treated with PGP (0.45 U mL^{-1}) in the presence of 0.8 mM H_2O_2 in 0.1 M glycine HCl buffer, pH 4.0 at 37 °C for varying time intervals. HOBT was used as a redox mediator as mentioned above. The reaction was stopped by boiling the sample at 100 °C for 7 min. The absorbance of the dye solutions at the respective λ_{max} for each dye was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

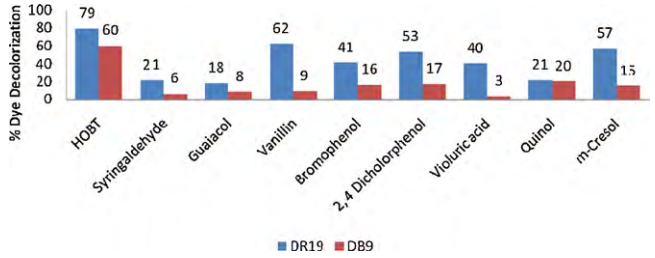

Fig. 1. Percent dye decolorization as a function of different redox mediators. The dyes DR19 (25 mg L^{-1}, 5.0 mL) and DB9 (50 mg L^{-1}, 5.0 mL) solutions were incubated independently with PGP (0.45 U mL^{-1}) in the presence of 0.5 mM concentration of each redox mediators; other conditions were 0.8 mM H_2O_2, 100 mM glycine HCl buffer, pH 4.0 for 60 min at 37 °C. (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.) DR19 (blue) and DB9 (red); the colors are kept in all the subsequent figures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.9. Statistical analysis

Five independent experiments were carried out in duplicate and the mean was calculated. The data expressed in various studies was plotted using Microsoft Excel2007 and Prism Graph Pad 5.0. Data was analyzed by one-way ANOVA and Mann Whitney test. *P*-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. PGP mediated dye decolorization in the presence of different redox mediators

The effect of different redox mediators on the dye decolorization by PGP is shown in Fig. 1 Among the nine different redox mediators studied for dye decolorization, HOBT was the most effective in decolorizing the dyes under study. The extent of decolorization was 79.1% and 60% for DR19 and DB9 respectively; followed by vanillin (62% for DR19) and quinol (20% for DB9). The other redox mediators were relatively less effective. Table 1 shows the effect of increasing concentrations of HOBT (0.05–1.0 mM) on DR19 and DB9. With the increasing concentration of HOBT there was an increase in the extent of decolorization of both dyes. The decolorization in the presence of HOBT was substantial and a maximum decolorization of 79.1% was observed with 0.25 mM HOBT (at 0.20 mM HOBT, 79%) for DR19 and 60% for DB9 in the presence of 0.5 mM HOBT. There was a slight decrease in percent decolorization of the dyes above these concentrations of HOBT.

Table 1
Percent dye decolorization at different concentrations of HOBT.

HOBT Conc. (mM)	Percent dye decolorization	
	DR19	DB9
0.05	65	32
0.10	70	37
0.15	76	43
0.20	79	46
0.25	79.1	48
0.30	78	52
0.35	74	56
0.40	73	57
0.45	72	58
0.50	70	60
0.55	69	56
0.60	68	54

The dyes DR19 and DB9 solutions were incubated independently with PGP (0.45 U/mL), 0.8 mM H_2O_2 and in the presence of HOBT (0.05–0.60 mM) for DR19 and DB9 respectively; other conditions were 100 mM glycine HC1 buffer, pH 4.0 for 60 min at 37 °C. (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.)

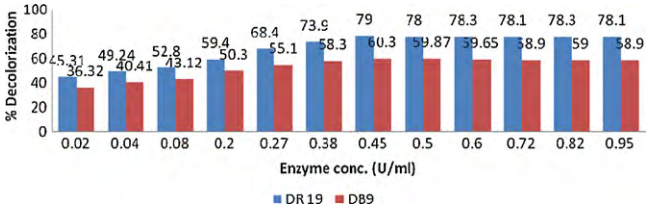

Fig. 2. Percent dye decolorization as a function of different enzyme (PGP) concentrations. The dyes DR19 (25 mg L^{-1}, 5.0 mL) and DB9 (50 mg L^{-1}, 5.0 mL) solutions were incubated independently with PGP (0.02–0.95 U mL^{-1}) in the presence of 0.2 mM and 0.5 mM concentration of HOBT for DR19 and DB9 respectively; other conditions were 0.8 mM H_2O_2, 100 mM glycine HCl buffer, pH 4.0 for 60 min at 37 °C. (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.)

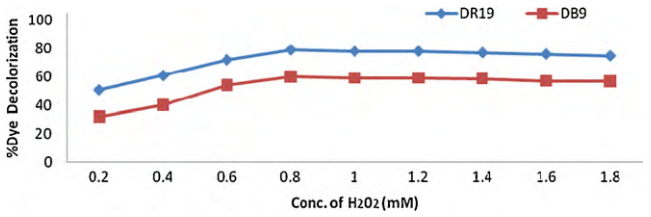

Fig. 3. Percent dye decolorization at different concentrations of H_2O_2. The dyes DR19 (25 mg L^{-1}, 5.0 mL) and DB9 (50 mg L^{-1}, 5.0 mL) solutions were incubated independently with H_2O_2 concentrations and PGP (0.45 U mL^{-1}) in the presence of varying amounts of H_2O_2 (0.2–1.8 mM) for DR19 and DB9 respectively; and other conditions were 100 mM glycine HCl buffer, pH 4.0 for 60 min at 37 °C. (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.)

3.2. Dye decolorization with varying concentration of enzyme (PGP) and H_2O_2

Fig. 2 shows the extent of decolorization of DR19 and DB9 with varying concentrations of PGP. The maximal decolorization for these two dyes was observed at PGP concentration of 0.45 U mL^{-1}. Dye decolorization was not significantly exhibited with any further increase of PGP. Fig. 3 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 H_2O_2 and remained substantially unaffected till 1.2 mM H_2O_2.

3.3. PGP mediated dye decolorization as a function of temperature and pH

The percent decolorization was plotted as a function of temperature and the results are shown in Fig. 4. Both the dyes (DR19 and DB9) showed maximum decolorization at 42 °C. 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 decolorization is shown in Fig. 5. An acidic range of pH (3.0–6.0) was better suited for dye decolorization. A pH optimum recorded for the dyes was pH 4.0. There was significant decrease in the extent of decolorization in an alkaline medium.

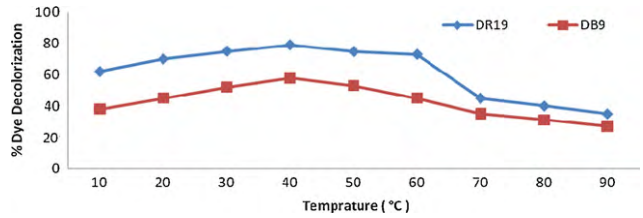

Fig. 4. Percent dye decolorization as a function of temperature. The dyes DR19 (25 mg L^{-1}, 5.0 mL) and DB9 (50 mg L^{-1}, 5.0 mL) solutions were incubated independently with PGP (0.45 U mL^{-1}) in the presence of HOBT, 0.8 mM H_2O_2, 100 mM glycine HCl buffer, pH 4.0 for 60 min at temperatures (20–90 °C). (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.)

Table 2
The results of ANOVA and Mann Whitney test are presented in tabulated form.

S. No.	% Dye decolorization at different parameters	*P*-value DR19	*P*-value DB9	Type of test
1.	Enz. Conc.:H_2O_2:pH	0.0109*	0.007*	ANOVA (Kruskal–Wallis)
2.	Enz. Conc.:H_2O_2:HOBT	0.9707	0.3156	ANOVA (Kruskal–Wallis)
3.	HOBT:Temp.:Time	0.1327	0.0224*	ANOVA (Kruskal–Wallis)
4.	H_2O_2:pH	0.0106*	0.0181*	Mann Whitney test
5.	Enz. Conc.:H_2O_2	1.0000	0.3736	Mann Whitney test

* *P*-values < 0.05 were considered statistically significant.

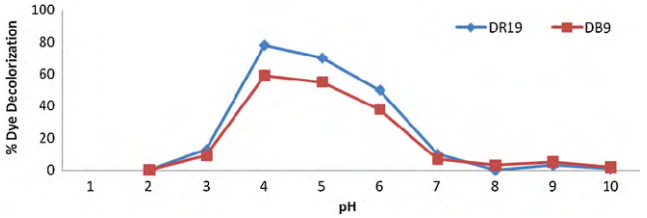

Fig. 5. Percent dye decolorization as a function of pH. The dyes DR19 (25 mg L^{-1}, 5.0 mL) and DB9 (50 mg L^{-1}, 5.0 mL) solutions were incubated independently with PGP (0.45 U mL^{-1}) in the presence of HOBT, 0.8 mM H_2O_2, 100 mM glycine HCl buffer at different pH (2, 3, 4, 5, 6, 7, 8, 9 and 10) for 60 min at 37 °C. (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.)

3.4. PGP mediated dye decolorization as a function of time

The extent of decolorization of both dyes DR19 and DB9 as a function of time is shown in Fig. 6. Maximum decolorization of both the dyes was observed within 1 h of incubation at 42 °C. However, no effective increase was observed when the dyes were further incubated for longer times. Sufficient amount of DR19 was decolorized within 20 min while the decolorization of DB9 was slow in the same time interval.

3.5. Statistical analysis

Data analyzed by one-way ANOVA and Mann Whitney test. *P*-values < 0.05 were considered statistically significant and presented as Table 2.

4. Discussion

Colored industrial effluents from the dyeing industries represent major environmental problems. Unbound reactive dyes undergo hydrolysis due to temperature and pH values during the dyeing processes. The strong color of discharged dyes even at very small concentrations has a huge impact on the aquatic environment caused by its turbidity and high pollution strength; in addition toxic degradation products can be formed. The decolorization of the colored contaminants is very much needed and emphasis is being given on developing simple, cheap and ecofriendly systems employing enzyme based treatment. As compared to the conventional method of dye removal that have their own limitations and drawbacks, the isolation and evaluation of the efficacy of enzymes isolated from plant sources is better and more promising. In the present study the dye decolorization ability of peroxidase isolated from *Trichosanthes diocia* has been evaluated in the presence of redox mediators under different sets of conditions standardized in our laboratory. The enzyme pointed gourd peroxidase has been partially purified and used to study the dye decolorization of water insoluble disperse dyes by using simple techniques. Not much emphasis has been given on enzyme purification due to its enormous cost. This is the first study with PGP and studies of similar nature had earlier been carried out with peroxidases from other natural sources [11]. It was observed that the dye solutions were recalcitrant upon exposure to HOBT, H_2O_2 or to the enzyme alone. The enzyme in the presence of redox mediators was much effective in performing the decolorization of the dyes, implying dye decolorization was a result of redox mediated H_2O_2-dependent enzymatic reaction.

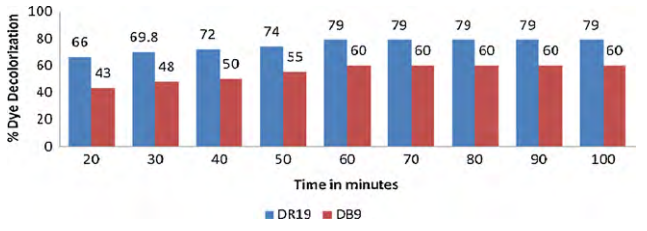

Fig. 6. Percent dye decolorization as a function of time. The dyes DR19 (25 mg L^{-1}, 5.0 mL) and DB9 (50 mg L^{-1}, 5.0 mL) solutions were incubated independently with PGP (0.45 U mL^{-1}) in the presence of HOBT, 0.8 mM H_2O_2, 100 mM glycine HCl buffer, pH 4.0 and for time 20–100 min at 37 °C. (λ_{max} for DR19 and DB9 are 495 nm and 461 nm respectively.)

It has already been reported that redox mediators have the potential to mediate an oxidation reaction between a substrate and an enzyme [12]. Different redox mediators have different mediation efficiency which is governed by redox potential of the mediator and the oxidation mechanism of the substrate [13]. Oxidation of substrate occurs by free radical formation by the mediator. The free radicals can be formed either by one-electron oxidation of substrate or by abstraction of a proton from the substrate [14,15]. In this study, redox-mediating property of nine different compounds as peroxidase mediators was evaluated (Fig. 1). Among the nine investigated compounds, HOBT was found to have the best mediating property for the decolorization of DR19 and DB9. This observation was in agreement with the earlier reports where HOBT was found to enhance decolorization of reactive and direct dyes drastically [16]. It is well documented that the redox potential of enzymes varies depending upon the source of the enzyme.

The pointed gourd peroxidase was effective in decolorizing the dyes at low concentrations of HOBT (Table 1). Although the extent of decolorization of DR19 and DB9 increased with increasing concentrations of HOBT, the maximum decolorization was observed to be 79% and 60% with 0.2 mM and 0.5 mM for DR19 and DB9, respectively. Further addition of HOBT resulted in a slow decrease in decolorization of both the dyes. This inhibition could likely be due to the high reactivity of HOBT radical, which might undergo chemical reactions with side chains of aromatic amino acid by enzyme thereby; inactivating it [12,13]. Hence, the dosage of redox mediator is an important factor for the enzyme-mediated decolorization [17].

The enzyme reacted well to decolorize both the dyes in the presence of 0.8 mM H_2O_2 (Fig. 3). The maximum decolorization was obtained at 0.8 mM H_2O_2 which is slightly higher than reported for soybean peroxidase, bitter gourd peroxidase (BGP) and turnip peroxidase [18,11,19]. Although the concentration of H_2O_2 greater than 0.75 mM acted as an inhibitor of peroxidase activity by irreversibly oxidizing the enzyme ferri-heme group essential for peroxidase activity; our results are consistent and very near to values reported earlier for maximum functional concentration of H_2O_2 [20]. The reaction temperature is an important parameter

which effects the decolorization of dyes. The maximum decolorization for both the dyes DR19 and DB9 was at 42 °C (Fig. 4). It has been reported that BGP mediated disperse dye decolorization was optimal at 40 °C [11].

The maximum decolorization of DR19 and DB9 was obtained at an acidic of pH 4.0 (Fig. 5). 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. DR19 and DB9 were maximally decolorized within 20 min of incubation (Fig. 6) [9,17]. There was slow and gradual enhancement of decolorization upto 60 min of incubation. It was also evident from the observation that DR19 was decolorized to a greater extent within 20 min in the presence of only 0.2 mM HOBT. However, DB 9 was decolorized maximally in the presence of 0.5 mM HOBT and decolorization rate was slow. This data is consistent with reports that decolorization rate varies, depending upon the type of dye to be treated [21].

One-way ANOVA and Mann Whitney test was used to compare the variance in percent dye decolorization at different parameters. Analysis of the percent decolorization at different concentrations of enzymes, H_2O_2 and pH gives statistically significant value ($P < 0.05$) for both the dyes. A non-significant *P*-value was obtained for DR19 at different concentrations of HOBT, varying time and temperature; whereas for DB9 *P*-value was significant for decolorization under the same conditions. Thus, the decolorization of DR19 and DB9 with the peroxidase under study shows significant variations under different conditions. The results of one-way ANOVA and Mann Whitney test are tabulated in Table 2.

5. Conclusions

Dye wastewater discharged from textile and dyestuff industries have to be treated due to their impact on water bodies and growing public concern over their toxicity and carcinogenicity. Many different and complicated molecular structures of dyes make dye wastewater difficult to be treated by conventional biological and physico-chemical process. Therefore, innovative treatment technologies need to be investigated. This study demonstrated that the disperse dyes present in the wastewater can be efficiently treated with enzyme isolated from cheap and easily available plant sources and can be coupled with redox mediators to cause effective decolorization of the dyes.

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