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# DECOLORIZATION OF DISPERSE ORANGE 25 USING *TRICHOSANTHES DIOCIA* PROTEINS IN THE PRESENCE OF REDOX MEDIATOR

Farrukh Jamal*

*Department of Biochemistry, Dr. Ram Manohar Lohia Avadh University, Faizabad-224001, U.P., India*

## Abstract

This paper describes a peroxidase from *Trichosanthes diocia* to study decolorization of Disperse Orange 25 under different experimental conditions of pH, temperature, time interval and enzyme concentration in the presence of redox mediator 1-hydroxybenzotriazole (HOBT). *T. diocia* peroxidase showed remarkable decolorization of Disperse Orange 25 in the presence of 1-hydroxybenzotriazole. At an enzyme concentration of 0.45 $EUmL^{-1}$ the peroxidase could successfully decolorize the dye up to a maximum of 61.2% with 0.2 mM 1-hydroxybenzotriazole. Maximum dye removal was recorded at a temperature of 40°C and at pH 4.0. The time for achieving maximum decolorization was 120 min. Thus, the study suggests that *T. diocia* peroxidase could be a potential source for developing an inexpensive and efficient method for the treatment of recalcitrant Disperse Orange 25 dye which is potentially toxic.

Key words: Disperse Orange 25; 1-hydroxybenzotriazole; Decolorization; *Trichosanthes diocia* Peroxidase

## Introduction

The complex aromatic structure of the dyes are resistant to light, biological activity, ozone and other degradative environmental conditions. This renders conventional wastewater treatment ineffective. Anionic and non-ionic azo dyes release toxic amines due to the reactive cleavage of azo groups (Joshi et al., 2004). Presence of heavy metals like chromium, cobalt, nickel and copper (metallized dyes) in wastewater is also an environmental concern (Freeman et al., 1996). Till date scientists have been trying to develop a single and economical method for treatment of dyes in the textile wastewater but it still remains a big challenge (Santos et al., 2007). Various physico-chemical methods pertaining to treatment of textile wastewater for removal of dyes has been studied (Hao et al., 2000; Robinson et al., 2001; Forgacs et al., 2004). The major disadvantage associated are their high cost, low efficiency, limited versatility, interference by other wastewater constituents and handling of the waste generated.

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. 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. The redox mediated enzyme catalysis has wide application in degradation of polycyclic aromatic hydrocarbons which includes phenols, biphenyls, pesticides, insecticides etc. (Calcaterra et al., 2008; Husain and Husain, 2008).

The plant of our interest *Trichosanthes diocia* commonly known as pointed gourd is widely planted and abundantly available in tropical areas. The aim of the present study was to evaluate the effectiveness of peroxidase in decolorizing Disperse Orange 25 (DO25) under varying experimental conditions of pH, temperature, time interval and enzyme concentration on the basis of one-factor-at-a-time (OFAT) method.

## Materials and Methods

### Dyes and Chemicals

Dye Disperse Orange 25 (DO25), ammonium sulphate and Tween-20 were procured from Sigma Chemical Co. (St. Louis, MO, USA) and all other chemicals were of analytical grade. Redox mediator 1-hydroxybenzotriazole (HOBT) was obtained from SRL Chemicals (Mumbai, India). The pointed gourds were purchased from the local market.

### Partial purification of *T. diocia* by Ammonium Sulphate Precipitation

100 g of *T. diocia* fruit pulp was homogenized in 200 mL of 100mM sodium acetate buffer, pH 5.6. The homogenate was filtered through four layers of cheesecloth and then centrifuged at the speed of 10,000 × g on a Remi C-24 Cooling Centrifuge for 25 min at 4°C. By adding 20-80% (w/v) of ammonium sulphate, salt fractionation was carried out with the clear supernatant. The content was stirred overnight to get maximum precipitate at 4°C. The precipitate was

* Corresponding Author, Email: journal.farrukh@gmail.com

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 (Akhtar et al., 2005).

### *T. diocia* peroxidase activity assay

Protein concentration was estimated by taking BSA as a standard protein and following the procedure of Lowry et al. (1951). 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 (Akhtar et al., 2005) .

### Preparation and treatment of Disperse dye solution

Disperse Orange 25 was prepared in 100 mM glycine HCl buffer pH 4.0 and was independently incubated with pointed gourd peroxidase (PGP) (0.45 $EUmL^{-1}$) in 100 mM M glycine HCl buffer, pH 4.0 in the presence of 0.8 mM $H_2O_2$ for varying times at 37°C. The reaction was terminated by boiling at 100°C for 10 min. Dye decolorization was monitored by measuring the difference at the maximum absorbance for this dye ($\lambda_{457nm}$) as compared with control experiments without enzyme on UV-visible spectrophotometer (JASCO, Japan). Untreated dye solution (excluding the enzyme) 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 duplicate and the mean was calculated.

### Effect of redox mediator on *T.diocia* peroxidase mediated Disperse dye decolorization

The dye Disperse Orange 25 (5.0 mL) was incubated with PGP (0.45 $EUmL^{-1}$) in the presence of redox mediator 1-hydroxybenzotriazole (0.5 mM) and 0.75 mM $H_2O_2$ in 100mM glycine HCl buffer, pH 4.0 for 1 h at 37°C. The reaction was terminated by boiling the sample at 100°C for 10 min. The absorbance of the dye solutions at the respective $\lambda_{max}$ for each dye was recorded against untreated dye as control (100%).

To find out the optimum concentration of HOBT a similar set of experiment as mentioned above was performed in the presence of varying concentrations of HOBT (0.05 to 1.5 mM). The reaction was terminated by boiling the sample at 100°C for 10 min. The absorbance of the dye solutions at the respective $\lambda_{max}$ for each dye was recorded against untreated dye as control (100%).

### Decolorization with varying concentration of Enzyme (PGP) and $H_2O_2$

The dye was incubated with increasing concentrations of PGP (0.065 to 0.50 $EUmL^{-1}$) and $H_2O_2$ (0.2 to 1.8 mM) independently 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. The reaction was stopped by boiling the sample at 100°C for 10 min. The absorbance of the dye solution at $\lambda_{max}$ was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

### Decolorization as a function of temperature, pH and time

Disperse Orange 25 was incubated with PGP (0.45 $EUmL^{-1}$) at different temperatures (20°C to 90°C). Other reaction conditions were common. The reaction was stopped by boiling the sample at 100°C for 10 min. The absorbance of the dye solution at $\lambda_{max}$ was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

Disperse Orange 25 dye solution was made in different buffers each of 100mM and in the range of pH 2.0 to pH 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). The dye was treated with PGP (0.45 $UmL^{-1}$) in buffers of varying pH and in the presence of 1.0 mM $H_2O_2$ for 1 h at 37°C. Disperse Orange 25 was treated with PGP (0.45 $EUmL^{-1}$) 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 at concentrations of 0.2 mM.

In each of the above experimental protocol reaction was stopped by boiling the sample at 100°C for 10 min. The absorbance of the dye solutions at the respective $\lambda_{max}$ for each dye was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

### Results

Dye decolorization for Disperse Orange 25 by PGP in the presence of different concentration of redox mediator is shown in Figure 1. At HOBT concentration of 0.2mM maximum decolorization (61.2%) was observed. Upon increasing the HOBT concentration to 1.5mM, there was no remarkable variation in the extent of decolorization. At extreme lower concentration of HOBT the decolorization was less (56.1%) which was observed to be higher than in the absence of redox mediator. Figure 2 shows the extent of decolorization of Disperse orange 25 with varying concentrations of PGP. Maximal decolorization was observed at PGP concentration of 0.45$EUmL^{-1}$. At concentrations above this, no remarkable change in dye decolorization was observed. Figure 3 shows the extent of percent dye removal improved with the increasing concentration of

$H_2O_2$. The maximum percent decolorization was observed at a concentration of 0.8 mM $H_2O_2$ that remained substantially unaffected till 1.2 mM. At concentrations of hydrogen peroxide beyond 1.2 mM there was slight decrease in the extent of decolorization. However, at very low concentration of $H_2O_2$ (0.2 mM) the observed decolorization was as low as 40%.

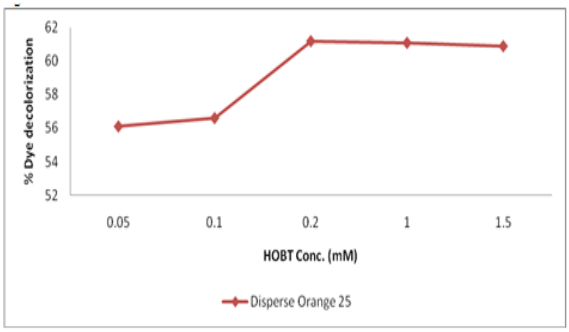


Figure I. Disperse Orange 25 dye decolorization as a function of redox mediator HOBT (0.05 to 1.5 mM). Other conditions were 0.8mM $H_2O_2$, 100mM glycine HCl buffer pH 4.0, incubation for 60 min at 37°C. ($\lambda_{max}$ for Disperse Orange 25 is 457nm).

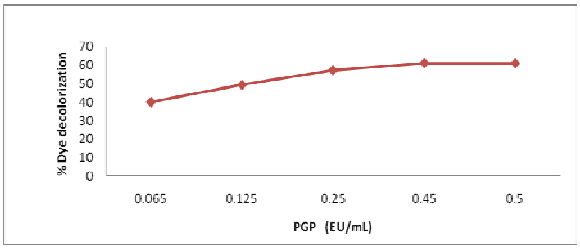


Figure 2. Disperse Orange 25 dye decolorization as a function of enzyme (PGP) concentration (0.065 to 0.50 $EUmL^{-1}$). ($\lambda_{max}$ for Disperse Orange 25 is 457nm).

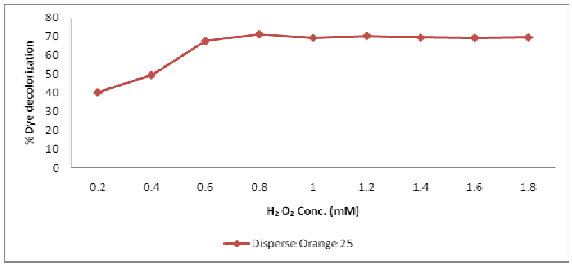


Figure 3. Disperse Orange 25 dye decolorization at different concentrations of $H_2O_2$ (0.2 to 1.8 mM). ($\lambda_{max}$ for Disperse Orange 25 is 457nm).

The results of temperature activity are shown in Figure 4. The disperse dye exhibited sufficient decolorization at 40°C which remained unaffected till 50°C. Buffers in the range of pH 2.0 to pH 10.0 were used to find out the range of pH in which significant decolorization was observed. The results of pH activity are shown in Figure 5. The pH range 4.0 to 5.0 was better suited for dye decolorization. The pH at which optimum decolorization recorded was pH 4.0. The decolorization of the dye significantly decreased in an alkaline medium.

The extent of decolorization of Disperse orange 25 as a function of time is shown in Figure 6. Maximum dye removal was recorded at 120 min of incubation.

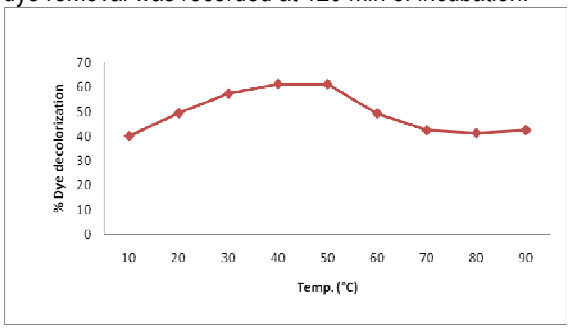


Figure 4. Disperse Orange 25 dye decolorization as a function of temperature (20°C to 90°C). ($\lambda_{max}$ for Disperse Orange 25 is 457nm).

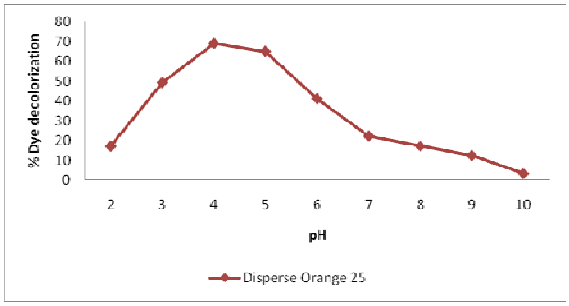


Figure 5. Disperse Orange 25 dye decolorization as a function of pH (pH 2.0 to pH 10.0). The buffers were glycine-HCl (pH 2.0, 3.0 and 4.0), sodium acetate (pH 5.0), sodium phosphate (pH 6.0, 7.0 and 8.0), and Tris-HCl (pH 9.0 and 10.0). ($\lambda_{max}$ for Disperse Orange 25 is 457nm).

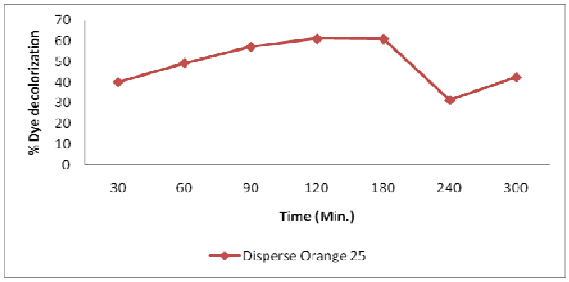


Figure 6 Disperse Orange 25 dye decolorization as a function of time (30 min to 300 min). ($\lambda_{max}$ for Disperse Orange 25 is 457nm).

Prolonging the time interval resulted in decrease of decolorization. There was gradual progress in the extent of decolorization.

## Discussion

Traditional wastewater treatment technologies have proven to be markedly ineffective for handling wastewater of synthetic textile dyes due to the chemical stability of these pollutants. Therefore, establishment of result oriented enzyme based cheap

and ecofriendly techniques holds considerable promise. The enzyme pointed gourd peroxidase (PGP) has been partially purified and used to study the dye decolorization of Disperse Orange 25 by using simple techniques. The emphasis on enzyme purification has been ignored due to its enormous cost and salt fractioned *T.diocia* proteins showing peroxidase activity has been used in this study. Findings supportive to PGP has been demonstrated with bitter gourd peroxidase (Satar and Husain, 2009). The Disperse Orange 25 solutions were recalcitrant to HOBT, $H_2O_2$ or to the enzyme alone but in the presence of redox mediator PGP showed higher efficiency in accomplishing decolorization, implying that dye decolorization was a redox mediated $H_2O_2$-dependent enzymatic interaction.

Redox mediator has the potential to mediate an oxidation reaction between a substrate and an enzyme (Baiocco et al., 2003). The mediation efficiency is governed by redox potential of the redox mediator and the oxidation mechanism of the substrate. Disperse Orange 25 underwent decolorization in the presence of 0.2 mM HOBT. This finding supports earlier reports that treatment of phenols and aromatic amines by peroxidases resulted in formation of large insoluble aggregates (Wada et al., 1995; Tatsumi et al., 1996; Husain and Jan, 2000; Duran and Esposito, 2000). Oxidation of substrate occurs through 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 (Fabbrini et al., 2002). In this study, redox-mediating property of HOBT as peroxidase mediators was examined and found to have mediating property for the decolorization of Disperse orange 25. This observation was in agreement with the earlier reports where HOBT was found to enhance decolorization of reactive and direct dyes drastically (Matto and Husain, 2007; Jamal et al., 2010).

The pointed gourd peroxidase was effective in decolorizing the dye at low concentrations of HOBT (Figure 1). The extent of decolorization of Disperse Orange 25 increased with increasing concentrations of HOBT, the maximum decolorization was observed to be 61.2%. Further addition of HOBT resulted in a slow and insignificant decrease in decolorization of the dye. The dosage of redox mediator is an important factor contributing for the enzyme-mediated decolorization under the given set of conditions. The enzyme was able to decolorize Disperse Orange 25 maximally in the presence of 0.8 mM hydrogen peroxide (Figure 3). Dye removal at similar concentrations has been reported for soybean peroxidase, bitter gourd peroxidase (BGP) and turnip peroxidase (Kulshrestha and Husain, 2007). Higher concentrations of $H_2O_2$ irreversibly oxidized the enzyme ferri-heme group essential for peroxidase activity consequently inhibiting peroxidase activity. Our results are consistent and very near to values reported earlier for maximum functional concentration of $H_2O_2$ (Vazquez-Duarte et al., 2001). The decolorization of disperse dyes are influenced by temperature (Jamal et al., 2010). The maximum decolorization for Disperse Orange 25 was observed at 40°C (Figure 4). On increasing the temperature there was no major effect upto 50°C but further increase in temperature contributes towards decrease in the extent of decolorization perhaps due to denaturation of the proteins. However, decolorization maxima are generally effective at 40°C (Satar and Husain, 2009), well supported by our findings (Jamal et al., 2010).

Decolorization to a large extent was observed in the acidic range of pH 4.0 to pH 5.0 but maximum decolorization of Disperse Orange 25 was achieved at pH 4.0 (Figure 5). It has earlier been reported that the degradation of industrially important dyes by peroxidases from different sources operates to a maximum level in the buffers of acidic pH. The incubation period is an important parameter to study the extent of decolorization (Akhtar et al., 2005; Murugesan et al., 2007). Time activity plot exhibited maximum decolorization at 120 min and remained almost unaffected till 180 min (Figure 6). The extent of decolorization decreased over prolonging the incubation time upto 5h. It is not clear as to why this decline takes place however the accumulation of the degraded product could be an important factor contributing to the inhibitory effect of the peroxidase. The rate of dye decolorization varies, depending upon the type of dye to be treated (Camarero et al., 2005).

The salt fractionated peroxidase from *T.diocia* significantly catalyzed the decolorization/degradation of synthetic textile dyes. Decolorization enhanced remarkably in the presence of redox mediator HOBT which was effective at low concentration. Disperse Orange 25 by itself is recalcitrant to degradation / decolorization with the peroxidase or redox mediator but when acted upon by this peroxidase in the presence of HOBT, extensive decolorization was achieved. Thus, this study demonstrated that this novel *T. diocia* peroxidase in its salt fractionated state can be coupled with low concentration of redox mediator to cause effective decolorization of synthetic and recalcitrant disperse dyes.

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