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Soluble Fraction of *Trichosanthes diocia* Peroxidase in Decolorization of Reactive Orange 15

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Abstract

Salt fractionated proteins from *Trichosanthes diocia* was used to study decolorization of Reactive Orange 15 under multifarious experimental conditions like pH, temperature, time interval, enzyme concentration and in the presence of redox mediator 1-hydroxybenzotriazole (HOBT)/ vanillin. *T. diocia* peroxidase showed extensive decolorization of Reactive Orange15 in the presence of 1-hydroxybenzotriazole (HOBT) / vanillin. The dye was decolorized effectively with HOBT and so further studies were performed in the presence of HOBT. At an enzyme concentration of 0.45 EUmL^{-1} the peroxidase was able to remove the reactive dye up to a maximum of 94.6% with 1.0 mM 1-hydroxybenzotriazole. Maximum decolorization was achieved at a temperature of 40°C, pH 5.0 and with an incubation period of 90 min. Thus, the data indicates that *T. diocia* peroxidase could be a potential source for developing an inexpensive and efficient method for the treatment of recalcitrant Reactive Orange15 dyes that are potentially toxic.

1. Introduction

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 and 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 (Shaffiqu et al., 2002; Verma and Madamwar, 2002). 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. Here too microbial treatment has certain inherent limitations (Duran and Esposito, 2000; Torres et al., 2003).

It has been shown that peroxidases catalyze a variety of oxidation reactions and importantly dyes recalcitrant to peroxidase shows significant decolorization in the presence of redox mediators (Calcaterra et al., 2008). The redox mediated enzyme catalysis has wide application in degradation of polycyclic aromatic hydrocarbons which includes phenols, biphenyls, pesticides, insecticides etc. (Husain and Husain, 2008). The aim of the present study was to evaluate the competency of novel *Trichosanthes diocia* proteins in decolorizing industrially important Reactive Orange 15 in the presence of redox mediators under varying experimental conditions of pH, temperature, time intervals and enzyme. *T. diocia* commonly known as pointed gourd is widely planted in tropical areas. It was hitherto untested and our initial studies hinted towards its strong possibility for the treatment of recalcitrant hydrophobic textile dyes that are potentially toxic and even carcinogenic.

2. Materials and Methods

Dyes and chemicals

Reactive Orange15 (RO15), 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) and vanillin were obtained from SRL Chemicals (Mumbai, India). The pointed gourds were obtained from the local market.

Salt fractionation of *T. diocia* peroxidase

A 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 cheesecloth and centrifuged at 10,000 × g on a Remi C-24 Cooling Centrifuge for 15 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 collected by centrifugation at 10,000 × g on a Remi C-24 Cooling Centrifuge, dissolved in 100mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer (Akhtar et al., 2005).

Peroxidase activity of *T. diocia*

Protein concentration was estimated by 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) .

Treatment of Reactive dye solution

Reactive Orange15 was prepared in 100 mM glycine HCl buffer, pH 4.0. was independently incubated with pointed gourd peroxidase (PGP) (0.45 UmL^{-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 (λ_{494nm}) 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. Three sets of independent experiments were carried out in duplicate and the mean was calculated.

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

The dye Reactive Orange15 (5.0 mL) was incubated with PGP (0.45 UmL^{-1}) in the presence of redox mediator 1-hydroxybenzotriazole and vanillin (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 λ_{max} for each dye was recorded against untreated dye as control (100%).

To find out the optimum concentration of HOBT and vanillin a similar set of experiment as mentioned above was performed in the presence of varying concentrations of HOBT/ vanillin (0.05 to 2.0 mM). The reaction was terminated 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%).

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 1.0mM. The reaction was stopped by boiling the sample at 100°C for 10 min. The absorbance of the dye solution at λ_{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

Reactive Orange15 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 λ_{max} was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

Reactive Orange15 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. Reactive Orange15 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 1.0mM concentration.

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 λ_{max} for each dye was recorded against untreated dye as control (100%) and percent decolorization was calculated against untreated dye solution.

3. Result

Dye decolorization for Reactive Orange15 by PGP in the presence of different concentration of redox mediator viz., HOBT and vanillin is shown in Figure-1a and Figure 1b respectively. Although, the concentration of 1.0 mM HOBT was sufficient to result in maximum decolorization of 74.6%, there was no effect of HOBT on increasing it further to 1.5mM. At extreme lower concentration of HOBT the decolorization was less (61.3%) which was observed to be higher than in the absence of redox mediator. The dye decolorization

efficacy of *T.diocia* peroxidase was also evaluated in the presence of vanillin. Vanillin could enhance the decolorization of RO 15 but it could maximally remove the color upto 60.7% at a concentration of 1.0 mM. So we opted for HOBT as a better candidate to work as a redox mediator with *T. diocia* peroxidase. We had previously shown that the maximal decolorization was observed at PGP concentration of 0.45EUmL^{-1} and so the optimization of PGP concentration has not been shown. At this concentration of PGP the Reactive Orange 15 was decolorized to an extent of 94.6%. Figure-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 1.0 mM H_2O_2 that remained substantially unaffected till 1.2 mM H_2O_2. At concentrations of hydrogen peroxide beyond 1.2 mM there was slight decrease in the decolorization but at very low concentration of H_2O_2 (0.2mM) the extent of decolorization was also low.

Figure-1a: Reactive Orange15 dye decolorization as a function of HOBT. Other conditions were 0.8mM H_2O_2, 100mM glycine HCl buffer, pH 4.0 for 60 min at 37°C. (λ_{max} for Reactive Orange15 is 494nm)

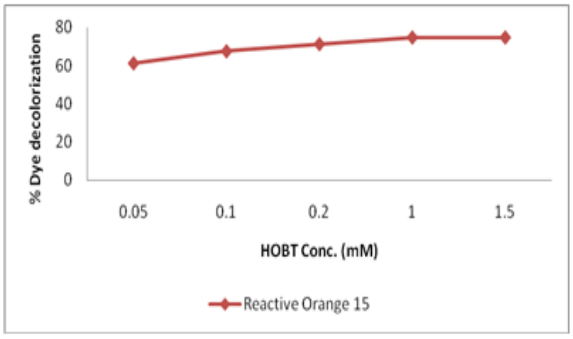


Figure-1b: Decolorization of Reactive Orange15 as a function of HOBT. Other conditions were 0.8mM H_2O_2, 100mM glycine HCl buffer, pH 4.0 for 60 min at 37°C. (λ_{max} for Reactive Orange15 is 494nm)

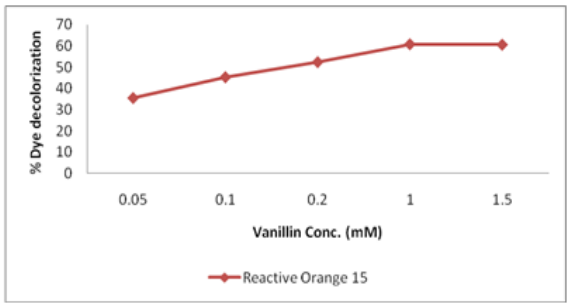


Figure-2: Decolorization of Reactive Orange15 at different concentrations of H_2O_2 (0.2 to 1.8 mM). (λ_{max} for Reactive Orange15 is 494nm)

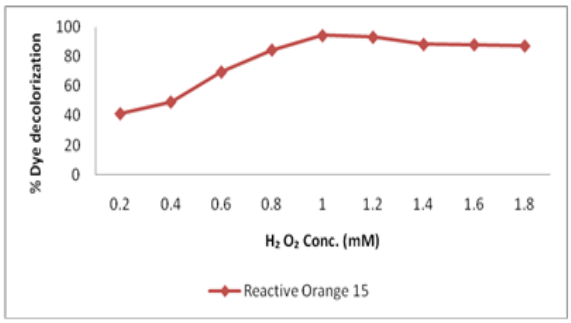


Figure-3: Decolorization of Reactive Orange15 as a function of temperature (20°C to 90°C). (λ_{max} for Reactive Orange15 is 494nm)

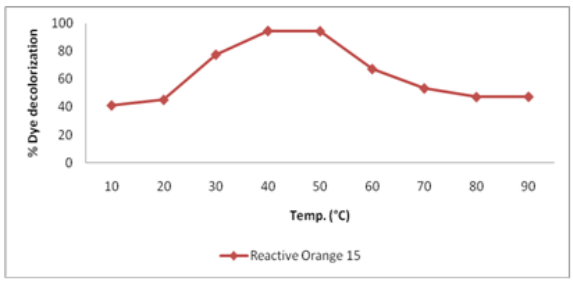


Figure-4: Decolorization of Reactive Orange15 as a function of pH (pH 2.0 to pH 10.0). The buffers were glycine-HCl (pH 2.0, 3.0 and 4.0), sodium acetate (pH 5.0), sodium phosphate (pH 6.0, 7.0 and 8.0), and Tris-HCl (pH 9.0 and 10.0). (λ_{max} for Reactive Orange15 is 494nm)

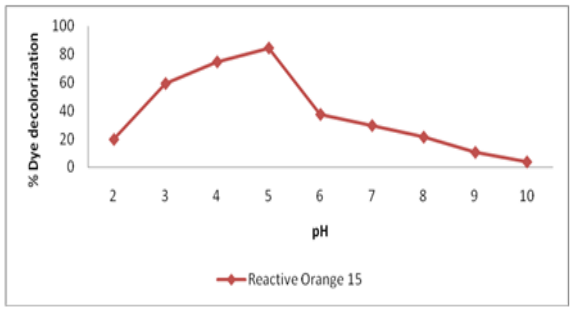
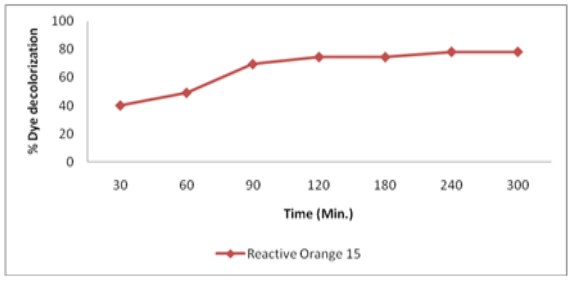


Figure-5: Decolorization of Reactive Orange15 as a function of time (30 min to 300 min). (λ_{max} for Reactive Orange15 is 494nm)



The result of temperature activity is shown in Figure-3. RO15 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-4. The optimum decolorization was recorded at pH 5.0. However, the extent of decolorization significantly decreased in an alkaline medium. The extent of decolorization of Reactive orange15 as a function of time is shown in Figure-5. The maximum decolorization was observed after 90 min of incubation and prolonging the time interval to 5hrs resulted in no further change of decolorization. There was a gradual progress in the rate of decolorization.

4. 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 by using simple techniques and subsequently used to study the dye decolorization of Reactive Orange15. Salt fractionated *T.diocia* proteins showing peroxidase activity has been used in this study. The dye Reactive Orange 15 was effectively decolorized and findings supportive to PGP has been demonstrated elsewhere with bitter gourd peroxidase (Satar and Husain, 2009). The Reactive Orange15 solutions were recalcitrant to HOBT, H_2O_2 or to the enzyme alone but in the presence of redox mediator *T. diocia* peroxidase showed higher efficiency in accomplishing decolorization, implying that dye decolorization was a redox mediated H_2O_2-dependent enzymatic interaction.

The 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. Reactive Orange15 underwent decolorization in the presence of redox mediator along with the formation of precipitate that could be removed by centrifugation. 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 due to 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 and vanillin as peroxidase mediators was examined. It was found that HOBT and vanillin both have mediating property for the decolorization of Reactive orange15 but HOBT exhibited better performance. This observation was in agreement with the earlier reports where HOBT was found to enhance decolorization of reactive and direct dyes drastically (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 Reactive Orange15 increased with increasing concentrations of HOBT, the maximum decolorization was observed to be 61.2%. Further addition of HOBT did not show any marked effect on decolorization. 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 Reactive Orange15 maximally in the presence of 1.0 mM H_2O_2 (Figure-2). Dye decolorization 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 but 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 reactive dyes is affected by temperature (Figure-3). The maximum decolorization for Reactive Orange15 was observed at 40°C. On increasing the temperature further 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, well supported by our findings (Jamal et al., 2010).

The extent of decolorization was also affected by pH and it was observed at an acidic range of pH 4.0 to pH 5.0 was suitable for maximum decolorization of Reactive Orange15 (Figure-4). 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 120min and remained almost unaffected till 5hrs (Figure-5). The extent of decolorization was not significantly affected by prolonging the incubation time. The findings

support the view that dye decolorization rate varies, depending upon the type of dye to be treated (Camarero et al., 2005).

Dye wastewater discharged from textile and dyestuff industries needs to be treated due to their impact on water bodies and to address growing public concern over their toxicity and carcinogenicity. Many different and complicated molecular structures of dyes make dye wastewater treatment difficult by conventional biological and physico-chemical processes. Therefore innovative treatment technologies need to be investigated. In this study, we have evaluated the effectiveness of *T.diocia* peroxidase in decolorization of Reactive Orange15 under varying set of conditions. The decolorization profile of pure dye solutions varied with enzyme concentration, pH, temperature and different concentration of HOBT/vanillin as redox mediators. HOBT worked as a better redox mediator than vanillin at very low concentration. The efficacy of decolorization drastically improved with HOBT and those dyes recalcitrant to *T.diocia* peroxidase exhibited remarkable results. The use of peroxidases can be extended to large-scale treatment of wide spectrum of structural dyes by using immobilized PGP along with relatively cheaper redox mediators. Thus, the study demonstrated that the peroxidase enzyme isolated from *T.diocia* can be coupled with redox mediator into a system that can serve as an effective biocatalyst for the treatment of effluents containing recalcitrant dyes from textile, dyeing and printing industries.

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References

Akhtar, S., Khan, A.A., and Husain, Q. (2005). Partially purified bitter gourd (*Momordica charantia*) peroxidase catalyzed decolorization of textile and other industrially important dyes Bioresour. Technol. 96, 1804–1811.

Baiocco, P., Barreca, A. M., Fabbrini, Galli, M.C., and Gentili, P. (2003). Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase-mediator systems. Org. Biomol. Chem. 1, 191–197.

Calcaterra, A., Galli, C., and Gentili, P. (2008). Phenolic compounds as likely natural mediators of laccase: A mechanistic assessment. J Mol Catal. B: Enzym. 5, 118-120.

Camarero, S., Ibarra, D., Martinez, M.J., and Martinez, A.J. (2005). Lignin derived compounds as efficient laccase mediators or decolorization of different types of recalcitrant dyes. Appl. Environ. Microbiol. 71, 1775–1784.

Duran, N., and Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl. Catal. B: Environ. 28, 83–99.

Fabbrini, M., Galli, C., and Gentili, P. (2002). Comparing the catalytic efficiency of some mediators of laccase. J. Mol. Cat. B 16, 231–240.

Husain, M., and Husain, Q. (2008). Applications of redox mediators in the treatment of organic pollutants by using oxidoreductive enzymes. Crit. Rev. Environ. Sci. Technol. 38, 1-42.

Husain, Q., and Jan, U. (2000). Detoxification of phenols and aromatic amines from polluted wastewater by using phenol oxidases. J. Sci.Ind. Res. 59, 286–293.

Jamal, F., Pandey, P.K., and Qidwai, T. (2010). Potential of Peroxidase Enzyme from *Trichosanthes diocia* to mediate Disperse Dye Decolorization in conjunction with Redox Mediators. J. Mol. Catal B: Enzym. 66, 177-181.

Kulshrestha, Y. and Husain, Q. (2007). Decolorization and degradation of acid dyes mediated by salt fractionated turnip (*Brassica rapa*) peroxidases. Toxicol. and Environ. Chem. 89, 155–267.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with Folin–phenol reagent. J. Biol. Chem. 193, 265–275.

Murugesan, K., Nam, I. H., Kim, Y. M., and Chang, Y. S. (2007).Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture. Enz. and Microbial Technol. 40,1662–1672.

Satar, R., and Husain, Q. (2009). Use of Bitter Gourd (*Momordica charantia*) peroxidase together with redox mediators to decolorize Reactive dyes. Biotechnol. Bioprocess Eng. 14,213–219.

Shaffiqu, T.S., Roy, J.J., Nair, R.A., and Abraham, T.E. (2002). Degradation of textile dyes mediated by plant peroxidases.Appl. Biochem.Biotechnol.102–103, 315–326.

Tatsumi, K., Wada, S., and Ichikawa, H. (1996). Removal of chlorophenols from wastewater by immobilized horseradish peroxidase. Biotechnol. Bioeng., 51,126–130.

Torres, E., Bustos-Jaimes, I., and Le Bogne, S. (2003). Potential use of oxidative enzymes for the detoxification of organic pollutants. Appl. Catal. B: Environ. 46, 1–15.

Vazquez-Duarte, H., Garcia-Almendarez, B., Regalado, C., and Whitaker, J. R. (2001). Purification and properties of a neutral peroxidase from turnip (*Brassica napus L var* purple top white globe) roots. J. Agri. Food Chem. 49, 4450–4456.

Verma, P., and Madamwar, D. (2002). Decolorization of synthetic textile dyes by lignin peroxidase of *Phanerochaete chrysosporium*. Folia Microbiol. 47, 283–286.

Wada, S., Ichikawa, H., and Tatsumi, K. (1995). Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and a coagulant. Biotechnol. Bioeng. 45,304–309.

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