LIST OF PUBLICATIONS AND PRESENTATIONS


4. Kulshrestha, Y., Husain, Q. Decolorization and degradation of textile carpet industrial effluent by using immobilized turnip (Brassica rapa) and tomato (Lycopersicon esculentum) peroxidases. 2007 (communicated).


Direct immobilization of peroxidase on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd (Momordica charantia)

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Abstract

The direct immobilization of peroxidases on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd has been investigated. The activated DEAE cellulose was quite effective in high yield immobilization of peroxidases from bitter gourd and it could bind nearly 590 enzyme units per g of the matrix. Bitter gourd peroxidase immobilized on this anion exchanger showed very high effectiveness factor ($\eta$) as 0.95. BGP bound very strongly to the DEAE cellulose, as it did not detach even in the presence of 0.5 M NaCl. Immobilized bitter gourd peroxidase preparation was more stable to the denaturation induced by pH, heat, urea, proteolytic enzyme, detergents (Surf Excel and Rin powder), Triton X 100 and water-miscible organic solvents (dioxane, dimethyl sulphoxide and $n$-propanol). Peroxidase adsorbed on the matrix exhibited very high resistance to proteolysis mediated by the trypsin treatment. DEAE cellulose bound bitter gourd peroxidase lost 45% of its initial activity after treatment with 2.5 mg trypsin per ml of incubation mixture for 1 h at 37 °C while the soluble enzyme lost nearly 65% of the initial activity under similar incubation conditions.

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Keywords: Adsorption; Direct; DEAE cellulose; Bitter gourd; Immobilization; Peroxidase; Stabilization; $n$-Propanol

1. Introduction

Recently, it has been reported that peroxidases can be used in the detoxification and biotransformation of several phenols, aromatic amines, biphenyls, bisphenols and dyes present in polluted wastewater/industrial effluents coming out from several industries [1–5]. The soluble enzyme cannot be exploited at the large-scale due to some inherent limitations to treat the huge volume of effluents. On the other hand, the immobilized enzyme has offered several advantages, such as enhanced stability, easier product recovery and purification, protection of enzymes against denaturants, proteolysis and reduced susceptibility to contamination. Several methods have been used for the immobilization of peroxidases from various sources but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports [6,7]. These factors were affecting the cost of the immobilized enzyme system [8]. These expensive immobilized systems could not meet the requirements for the treatment of huge volume of effluents. However, among the techniques used for the immobilization of enzymes, adsorption on the insoluble supports has several merits over the other known methods. It is a simple procedure and can be exploited for the direct immobilization of enzymes even from the crude cell homogenates.

Adsorption procedures are significantly useful for the immobilization of enzymes directly from the crude homogenate and thus avoiding the high cost of enzyme purification [9,10]. Ease of immobilization, lack of chemical modification and usually accompanying enhancement in stability are some of the advantages offered by the adsorption procedures. Several investigators have described the immobilization of enzymes on the bioaffinity supports for the specific
immobilization of enzymes directly from partially purified enzyme preparation but these procedures require the use of expensive ligands, such as antibodies or lectins [6,11,12]. In order to minimize the cost of immobilization, the adsorption of proteins directly from partially purified enzyme preparation on an anion exchanger can be performed. These supports have already appeared in high yield and stable immobilization of enzymes.

Here, an effort has been made to immobilize the peroxidases on an anion exchanger, diethyl aminoethyl (DEAE) cellulose directly from the salt fractionated proteins of bitter gourd and dialyzed bitter gourd proteins. DEAE cellulose adsorbed bitter gourd peroxidase (BGP) preparation was compared with its soluble counterpart for its stability against pH, heat, urea, detergents, water-immiscible organic solvents and proteolytic enzyme (trypsin). DEAE cellulose adsorbed BGP preparation was significantly stable against several tested physical and chemical parameters.

2. Materials and methods

2.1. Materials

DEAE cellulose 11 was the product of SRL Chemicals, Mumbai, India. o-Dianisidine-HCl was purchased from the Center for Biochemical Technology, New Delhi, India. Ammonium sulphate, dioxane, dimethyl sulphoxide, n-propanol and Triton X 100 were obtained from the SRL Chemicals Mumbai, India. Surf Excel and Rin powder were purchased from the local market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (50 g) was homogenized in 100 ml of 50 mM sodium acetate buffer, pH 5.6. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 x g on a Remi R-24 cooling centrifuge for 10 min at 4°C. The clear supernatant was subjected to salt fractionation by adding 20–80% (w/v) (NH₄)₂SO₄. It was stirred overnight at 4°C and the obtained precipitate was collected by centrifugation at 10,000 x g on a Remi R-24 cooling centrifuge for 10 min at 4°C [6]. The collected precipitate was redissolved in 50 mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer.

2.3. Activation of DEAE cellulose

DEAE cellulose (5.0 gm) was added to 100 ml of distilled water and was stirred slowly, kept overnight for swelling. Swelled DEAE cellulose was filtered on a Buchner funnel and was incubated with 100 ml of 0.5N HCl for 1 h. It was collected by filtration on Buchner funnel and was washed with distilled water continuously till it attained pH 7.0. Hundred milliters of 0.5N NaOH was added to HCl treated DEAE cellulose and it was stirred on a magnetic stirrer for 1 h at room temperature 25°C. It was washed again with distilled water till it attained neutral pH. Further, it was suspended and stored in 100 ml of distilled water at 4°C.

2.4. Adsorption of BGP on activated DEAE cellulose

BGP (5535 units) was added to 5.0 g of DEAE cellulose and stirred overnight at 4°C. Unbound BGP was removed by extensive washing with the assay buffer.

2.5. Effect of ion concentrations on the DEAE cellulose adsorbed BGP

The adsorbed BGP preparation was incubated with increasing concentration of NaCl (0.1–1.0 M) in 0.1 M sodium acetate buffer, pH 5.6 for 1 h at 37°C. In order to monitor the effect of long-time exposure of immobilized enzyme with ions, the adsorbed BGP was also incubated with 0.1 M NaCl up to 24 h.

2.6. Effect of trypsin mediated proteolysis on the activity of soluble and immobilized BGP

Soluble and immobilized BGP preparations (1.25 units) were incubated with 0.25–2.5 mg trypsin/ml of incubation mixture at 37°C for 1 h [6]. The activities of soluble and immobilized BGP in assay buffer without any trypsin treatment were taken as control (100%), for the calculation of percent activity. Peroxidase activity was determined according to the standard procedure.

2.7. Effect of Surf Excel and Rin powder on the activity of soluble and DEAE cellulose adsorbed BGP

Soluble and immobilized BGP preparations (1.25 units) were incubated independently with varying concentration of Surf Excel and Rin powder (0.1–1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37°C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent were taken as control (100%), for the calculation of percent activity.

2.8. Treatment of soluble and immobilized BGP with Triton X 100

Soluble and immobilized BGP (1.25 units) preparations were incubated with increasing concentration of Triton X 100 (0.5–5%, v/v) in 50 mM sodium acetate buffer, pH 5.6 at 37°C for 1 h. After exposure the peroxidase activity was determined according to the procedure described in the text.
2.9. Effect of water-miscible organic solvents on the activity of soluble and immobilized BGP

Soluble and immobilized BGP preparations (1.25 units) were incubated with 10–60% (v/v) of water-miscible organic solvents; dioxane/dimethyl sulphoxide (DMSO)/n-propanol in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated organic solvent concentrations after the incubation period. Other assay conditions were the same as described in the text.

2.10. Measurement of peroxidase activity

Peroxidase activity was estimated from the change in the optical density (A440 nm) at 37 °C by measuring the initial rate of oxidation of o-dianisidine-HCl in the presence of hydrogen peroxide, using both the substrates in saturating concentrations. The immobilized preparation was continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparations [7].

One unit of peroxidase activity was defined as the amount of enzyme protein that catalyzes the formation of 1 μmol of o-oxidized product of o-dianisidine–HCl in the presence of hydrogen peroxide per minute at 37 °C.

2.11. Determination of protein concentration

The protein concentration was determined by the procedure of Lowry et al. [14]. Bovine serum albumin was used as standard.

3. Results

3.1. Adsorption of BGP on DEAE cellulose

DEAE cellulose is commonly used for the purification and immobilization of variety of enzymes and proteins. In view of DEAE cellulose property to adsorb proteins on the basis of ionic interactions, this property has been exploited for directly binding the enzymes from the dialyzed salt fractionated bitter gourd proteins by simply incubating DEAE cellulose with ammonium sulphate fractionated dialyzed proteins overnight at 4 °C. After binding proteins on DEAE cellulose, the complex was washed with assay buffer till the traces of unbound proteins were removed. Unbound proteins were removed by extensive washing with assay buffer. DEAE cellulose adsorbed 590 units of peroxidase per g of the matrix. The effectiveness factor ‘η’ of the immobilized enzyme preparation was 0.95 (Table 1). High effectiveness factor of immobilized bitter gourd peroxidase preparation suggested that immobilized preparation was quite porous and effective in catalysis.

| Amount of Enzyme loaded (X) (units) | 1107 |
| Amount of Enzyme activity in washes (Y) (units) | 482 |
| Activity bound/g of DEAE cellulose (units) | 625 |
| Theoretical (X – Y = A) (A) | 590 |
| Actual (B) | 0.95 |
| Effectiveness factor (η) (B/A) | 95 |

Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%. Peroxidase activity was assayed according to the procedure given in the text.

3.2. Effect of ion concentrations on the DEAE cellulose adsorbed BGP activity

The wastewaters may also contain several types of ions; therefore it was necessary to examine the detachment of BGP from its support in the presence of various concentrations of ions. The exposure of adsorbed enzyme with increasing concentration of NaCl (0.1–1.0 M) for 1 h exhibited retention of very high enzyme activity even in the presence of 1.0 M NaCl. The incubation of immobilized BGP upto 0.5 M NaCl for 1 h had no detachment of enzyme activity (Fig. 1). In order to monitor the effect of long exposure of immobilized enzyme with ions, the adsorbed BGP was incubated with 0.1 M NaCl upto 24 h. The incubation of immobilized enzyme with 0.1 M NaCl for 24 h resulted in a slight loss of only 13% of the initial activity (data not given). These observations suggested that the binding of BGP with DEAE cellulose was quite strong and such type of immobilized enzyme preparations can be easily exploited for its use in the treatment of wastewater containing aromatic pollutants.
3.3. Stability properties of soluble and DEAE cellulose-bound BGP preparations

The stability of soluble and DEAE cellulose-bound BGP preparations was monitored against various physical and chemical parameters because these parameters can affect the activity of the enzymes used for the treatment of organic pollutants present in the wastewater.

3.3.1. pH-activity profiles of soluble and DEAE cellulose-bound BGP preparations

DEAE cellulose-bound BGP showed broadening in the pH-activity profile as compared to the native enzyme (Fig. 2). Immobilized enzyme retained significantly higher enzyme activity on both sides of pH-optimum in comparison to free enzyme. pH-optimum of immobilized enzyme had no difference from pH 5.0 to 6.0, although soluble enzyme showed pH-optimum at pH 5.0.

3.3.2. Temperature-activity profiles of soluble and DEAE cellulose-bound BGP preparations

DEAE cellulose-bound BGP preparation had no change in temperature-optima as compared to its soluble counterpart. Both the preparations exhibited temperature-optima at 40°C. However, DEAE cellulose-bound BGP retained significantly greater fractions of catalytic activity at high temperatures (Fig. 3).

3.3.3. Thermal denaturation plot of soluble and DEAE cellulose-bound BGP preparations

Fig. 4 demonstrates the thermal denaturation of soluble and immobilized BGP at 60°C for 2 h. Soluble BGP incubated at 60°C for 2 h retained 43% of its initial enzyme activity while the immobilized enzyme incubated under similar conditions was significantly more stable to heat inactivation. The immobilized BGP exhibited 60% of the original activity after 2 h of heat treatment.

3.3.4. Urea-mediated denaturation of soluble and DEAE cellulose-bound BGP preparations

DEAE cellulose-bound BGP was more resistant to inactivation induced by 4.0 M urea compared to its soluble counterpart. Exposure of soluble enzyme with 4.0 M urea for 2 h resulted in the loss of 53% activity whereas the immobilized enzyme retained more than 70% of the initial enzyme activity (Fig. 5).

Fig. 2. pH-activity profiles of soluble and DEAE cellulose-bound BGP. The appropriate amounts of soluble and immobilized BGP were taken for the preparation of pH-activity profile. The reaction mixture was incubated at 37°C for 15 min in buffers of pH ranges from 3.0 to 10.0. The buffers used were 50 mM glycine-HCl for the pH 3.0, sodium acetate for the pH 4.0, 5.0, sodium phosphate for the pH 6.0, 7.0, 8.0 and Tris-HCl for the pH 9.0, 10.0. Symbols indicate the soluble (○) and immobilized (●) enzyme.

Fig. 3. Temperature-activity profiles of soluble and DEAE cellulose-bound BGP. The activity of soluble and immobilized BGP (1.25 units) was monitored at various indicated temperatures. Activity expressed at 40°C was taken as control for calculating percent activity. For symbols refer to Fig. 2.

Fig. 4. Thermal denaturation of soluble and DEAE cellulose adsorbed BGP. Soluble and immobilized BGP (1.25 units) were incubated at 60°C for varying times in 50 mM sodium acetate buffer, pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined as described in the text. Un-incubated samples at 60°C were taken as 100% for the calculation of remaining percent activity. For symbols refer to Fig. 2.
Fig. 5. Effect of 4.0 M urea on soluble and DEAE cellulose adsorbed BGP. Soluble and immobilized BGP (1.25 units) were incubated in 4.0 M urea in 50 mM sodium acetate buffer, pH 5.6. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the percent activity untreated samples were considered as 100%. For symbols refer to Fig. 2.

3.3.5. Protease mediated inactivation of soluble and DEAE cellulose bound BGP preparations

Fig. 6 shows the stability of soluble and immobilized BGP in the presence of increasing concentration (0.25–2.5 mg) of trypsin/ml of incubation volume. Soluble BGP was rapidly inactivated in the presence of increasing concentrations of trypsin and retained 36% of the initial activity after 1 h incubation with 2.5 mg trypsin/ml at 37 °C while the immobilized BGP was remarkably more stable against proteolysis mediated by trypsin. However, the immobilized BGP showed over 55% of the original enzyme activity under similar treatment conditions.

3.3.6. Effect of detergents on the activity of soluble and immobilized BGP

In this study, three different detergents have been selected for comparative stability of soluble and immobilized BGP. Surf Excel and Rin powder are very commonly used detergent in every household and laundry. Unused detergents are normally present in the wastewater coming out of municipal waste. Somewhere such wastewater can mix with the effluents released by the industries. In order to make immobilized enzyme preparation more efficient for wastewater treatment, we have investigated the effect of Triton X 100, Surf Excel and Rin powder on the activity of soluble and immobilized BGP. Soluble BGP was more sensitive to the Surf Excel exposure and lost nearly 67% enzyme activity after 1 h incubation with 0.5% (w/v) detergent. Moreover, the immobilized BGP was markedly more resistant to inactivation induced by Surf Excel and retained over 70% of the initial activity (Table 2).

Table 2 further demonstrates the effect of increasing concentration of (0.1–1.0%) Rin powder on the activity of soluble and immobilized BGP. The soluble enzyme retained a marginal activity of 58% after 1 h exposure to 0.5% Rin powder. However, the immobilized BGP exhibited 85% of the original activity under similar exposure conditions.

Moreover, the immobilized BGP preparation was more resistant to denaturation induced by Triton X 100, this preparation retained 60% of the initial activity even in the presence of 5.0% (v/v) Triton X 100 whereas the soluble BGP retained only 38% of the original activity under similar exposure (Fig. 7).

Table 2

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<th>Detergent concentration (%)</th>
<th>Percent remaining BGP activity</th>
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<tr>
<td>Surf Excel</td>
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<td>Rin powder</td>
<td>Soluble</td>
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Soluble and immobilized BGP preparations (1.25 units) were incubated with varying concentration of Surf Excel and Rin powder (0.1–1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent was taken as control (100%), for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

Fig. 6. Effect of trypsin concentration on the activity of soluble and DEAE cellulose adsorbed BGP. Soluble and DEAE cellulose adsorbed BGP (1.25 units) were independently incubated with increasing concentration of trypsin (0.25–2.5 mg) in a total volume of 1.0 ml of 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Activity of enzyme was assayed according to the procedure described in text. For symbols refer to Fig. 2.

Table 2

Effect of detergents on the activity of soluble and DEAE cellulose adsorbed BGP

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Soluble and immobilized BGP preparations (1.25 units) were incubated with varying concentration of Surf Excel and Rin powder (0.1–1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent was taken as control (100%), for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.
4. Discussion

Several methods have been used for the immobilization/stabilization of enzymes but very few can meet the requirement of enzyme immobilization directly from the crude homogenate [11]. In this manuscript, an effort has been made to immobilize the peroxidases directly from the amonium sulphate fractionated proteins of bitter gourd on DEAE cellulose. It is well documented that anion exchangers can be used for the purification of large number of proteins [15]. BGP was immobilized in very high yield on DEAE cellulose and it bound 590 units of BGP/g of the ion exchanger. The preparation thus obtained was highly active and exhibited very high effectiveness factor, as 0.95 (Table 1). Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure [16]. In this case, the yield of immobilization was quite superior over other methods used for the immobilization of peroxidases [17–19]. DEAE cellulose adsorbed BGP was tightly retained as it has no significant detachment even in the presence of 0.5 M NaCl (Fig. 1). BGP bound to DEAE cellulose support exhibited very high stabilization against pH, heat and urea denaturation (Figs. 2–5). Several earlier investigators have also reported the use of DEAE cellulose support for high yield and stable immobilization of enzymes and proteins [20,21]. The immobilized BGP preparation exhibited broadening in pH–activity profiles (Fig. 2). We have earlier

Table 3

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<th>Organic solvent (% v/v)</th>
<th>Dioxane</th>
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<th>n-Propanol</th>
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<td>Immobilized</td>
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Soluble and immobilized BGP (1.25 units) preparations were incubated with increasing concentration of dioxane/DMSO/n-propanol (0–60%, v/v) in 50 mM sodium phosphate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any water-miscible organic solvent were taken as control (100%) for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%. 

Several industrial effluents and wastewaters sometimes also contain organic solvents together with other aromatic pollutants and such compounds can affect the activity of enzymes during wastewater treatment. However, it is necessary to investigate the role of water-miscible organic solvents on the activity of immobilized enzymes. The exposure of soluble enzyme with varying concentrations of DMSO (10–60%, v/v) resulted in the loss of greater fraction of enzyme activity while the immobilized enzyme was quite resistant to inactivation induced by DMSO. Exposure of immobilized enzyme preparation with 50% (v/v) DMSO for 1 h retained 63% of its activity, although the soluble enzyme lost nearly 59% of its original activity under similar treatment (Table 3). The incubation of soluble and immobilized BGP with increasing concentrations of dioxane resulted in a continuous loss of enzyme activity. However, the immobilized enzyme was more resistant to inactivation mediated by dioxane. The treatment of soluble BGP with 60% (v/v) dioxane for 1 h resulted in a loss of 84% of the initial activity while the DEAE cellulose bound BGP exhibited stabilization against similar treatment and retained more than 35% of its original activity (Table 3). Table 3 summarizes the treatment of soluble and immobilized BGP with increasing concentration of n-propanol. The incubation of soluble enzyme with 60% (v/v) concentration of n-propanol resulted in decreasing more than 50% activity while immobilized enzyme showed retention of 70% of the initial activity.
shown that the Con A-Sepharose bound BGP had similar type of broadening in pH-activity profiles [6]. DEAE cellulose bound BGP was remarkably stable against proteolysis mediated by trypsin (Fig. 6). It has been earlier reported that Con A-Sepharose bound BGP was quite resistant to proteolysis induced by trypsin [6].

Wastewater coming out from various elimination sites contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of polluted wastewater. In order to use such enzymes for the removal of aromatic pollutants from wastewater it becomes necessary to monitor the stability of enzymes in presence of denaturants. The effect of detergents on the enzyme activity of immobilized BGP must be investigated prior to its application in the treatment of wastewater contaminated with hazardous organic compounds. Our observations suggested that DEAE cellulose bound BGP preparation was markedly more stable against the exposure caused by very high concentrations of several detergents (Table 2; Fig. 7). DEAE cellulose bound enzyme could work more efficiently on industrial effluents containing compounds like soap and detergents. DEAE cellulose bound BGP was quite resistant against denaturation induced by detergents, such as Triton X 100 (Fig. 7), Rin powder and Surf Excel (Table 2). Lower concentrations of detergents enhanced the activity of immobilized BGP. These observations indicated the presence of lower concentrations of detergents is not harmful to the enzyme native conformation. The enhancement of enzyme activity by lower concentrations of detergents and stabilization of bioaffinity bound BGP against high concentrations of such type of detergents had already been reported by some earlier workers [6].

Organic solvents are also very common pollutants together with aromatic compounds and their presence can affect the structure of enzymes. Enzymes employed for the treatment of wastewater containing pollutants would be affected by the presence of such solvents. Due to presence of organic solvents in wastewater it necessitates the investigation of the stability of enzymes against inactivation induced by the exposure of such organic solvents. The DEAE cellulose bound BGP was markedly more stable when it exposed to dioxane, DMSO and propanol (Table 3). There have been reports that immobilization of enzymes by multipoint attachment protects them from denaturation induced by organic solvents in cosolvent mixtures [22,23]. These workers have described that potato polyphenol oxidase adsorbed on chitin behaved differently compared to soluble enzyme in aqueous-organic cosolvent mixtures. Moreover, they have evaluated that enzymes; polyphenol oxidase, peroxidase, trypsin and acid phosphatase showed stimulation of enzyme activity within a specific concentration range of water-miscible organic solvents present in the medium [24]. Enzyme immobilized by adsorption on Eudragit S-100, chitin and chitosan exhibited enhanced activity in organic cosolvent mixtures when the concentration of the organic solvent is around 10–20% (v/v) [9,10]. More recently, in our laboratory it has been shown that enzymes immobilized on protein supports were also quite resistant to denaturation induced by various water-miscible organic solvents [25,26].

DEAE cellulose bound BGP preparation has pronounced stability against pH, heat, urea, proteolysis, detergents and water-miscible organic solvents. Several earlier investigators have described that the immobilization of enzymes on DEAE cellulose support resulted in the stabilization of enzymes against various forms of denaturation [13,15,20]. Protease resistance is an additional attribute to the adsorption of BGP on an anion exchanger. It is expected that DEAE cellulose bound BGP preparation has a great future in the treatment of organic pollutants present in industrial effluents. DEAE cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it leads to the desorption of enzyme from the support. Adsorbed enzyme could be cross-linked by using bifunctional or multifunctional reagents in order to prevent the dissociation/desorption of enzyme from the ion exchanger [13,27].

5. Conclusion

Adsorption procedures are significantly useful in the immobilization of enzymes directly from the crude homogenate and thus avoiding the high cost of enzyme purification. The treatment of organic pollutants present in industrial effluents based on peroxidases has attracted considerable interest since the past two decades. However, practical applications of large-scale enzymatic removal have always been limited due to high cost and lower operational stability of peroxidases. Moreover, this procedure emphasized the immobilization of BGP directly from the crude homogenate or ammonium sulphate precipitated proteins. It has further reduced the cost of immobilized enzyme preparation. BGP adsorbed on DEAE cellulose support showed very high yield of immobilization and markedly high stabilization against several types of denaturants. In near future, the enzyme reactors containing such inexpensive immobilized enzyme preparations could be exploited for the treatment of wastewater containing toxic and hazardous compounds.

Acknowledgements

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References

Bioaffinity-based an inexpensive and high yield procedure for the immobilization of turnip (Brassica rapa) peroxidase

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Abstract

This study demonstrates the immobilization of carbohydrate containing turnip peroxidase on an inexpensive bioaffinity adsorbent, Concanavalin A-cellulose support. The bioaffinity support was prepared simply by incubating cellulose powder with jack bean extract at 4 °C. Cellulose powder adsorbed 30 mg concanavalin A/g of the matrix. Concanavalin A adsorbed cellulose has been employed for the simultaneous purification and immobilization of glycoenzymes directly from ammonium sulphate fractionated proteins of turnip. The obtained bioaffinity support was quite effective in high yield immobilization of peroxidase from turnip and it retained 672 U/g. Turnip peroxidase immobilized on concanavalin A-cellulose support retained 80% of the initial activity. Immobilized turnip peroxidase preparation was quite resistant against the denaturation mediated by pH, heat, urea, guanidinium-HCl, Surf Excel, cetyltrimethylammonium bromide and water-miscible organic solvents; dimethyl formamide, dioxane and n-propanol. Low concentration of detergents like Surf Excel and cetyltrimethylammonium bromide enhanced the activity of soluble and immobilized turnip peroxidase.

Keywords: Bioaffinity support; Concanavalin A; Cellulose; Immobilization; Peroxidase; Purification; Stabilization; Turnip roots

1. Introduction

Peroxidase (E.C. 1.11.1.7) are ubiquitous heme-proteins, which utilize hydrogen peroxide to catalyze the oxidation of a wide spectrum of organic and inorganic substrates (Duran et al., 2002; Daarte-Vazquez et al., 2003). Plant peroxidases are receiving increasing attention due to their extensive potential applications in clinical, biochemical, biotechnological, industrial and in the synthesis of useful compounds (Ryu et al., 1993; Lebarzewska and Ginalska, 1995; Kim and Moon, 2005; Duran and Esposito, 2000). These enzymes could also be exploited for the detoxification and remediation of various aromatic pollutants such as phenols, aromatic amines, 2,4,6-trinitrotoluene and dyes, etc. present in wastewater/industrial effluents coming out from several industries such as textile, dyes, printing, paper and pulp (Husain and Jan, 2000; Akhtar et al., 2005a,b; Lee et al., 2003; McEldon and Dordick, 1996). The use of soluble enzymes has some inherent limitations whereas their immobilized form has several advantages over the soluble enzymes such as enhanced stability, easier product recovery and purification, protection of enzymes against denaturants, proteolysis and reduced susceptibility to contamination.

Numerous efforts have been made to develop the procedures for the immobilization of peroxidase from various sources but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports which increased the cost of the processes (Husain and Jan, 2000; Akhtar et al., 2005a; Tischer and Kasche, 1999). However, such immobilized enzyme preparations could not be exploited for the treatment of large volume of effluents coming out of the industrial sites. Among the techniques used for the immobilization of enzymes, bioaffinity supports have attracted the attention of the enzymologists due to several merits over the other known classical methods. Researchers have shown remarkable interest in the immobilization of enzymes on bioaffinity supports due to ease of immobilization, lack of chemical modification and usually accompanying an enhancement in stability (Saleemuddin and
Husain, 1991; Akhtar et al., 2005c; Saleemuddin, 1999). Besides the mentioned advantages offered by the bioaffinity-based procedures, there is some additional benefit, such as proper orientation of enzyme on the support (Turkova, 1999; Mislovicová et al., 2000; Khan et al., 2005). These supports have been used for high yield and stable immobilization of glycoenzymes/ enzymes. A large number of bioaffinity-based procedures have already been developed for the immobilization of enzymes directly from the crude homogenate or partially purified enzyme preparation (Saleemuddin and Husain, 1991; Akhtar et al., 2005c; Saleemuddin, 1999; Khan et al., 2005).

In this article an effort has been made to select an inexpensive and easily available source of peroxidase, turnip. The purpose of this study was to find a cheaper and easily available alternative for the commercially available enzymes and its immobilization and utilization at large scale. Con A-cellulose immobilized turnip peroxidase (TP) preparation was compared with its soluble counter-part for its stability against various physical and chemical parameters.

2. Materials and methods

2.1. Materials

Methyl α-D-mannopyranoside was the products of Sigma Chem. Co. (St. Louis, MO), USA. Jack bean meal was procured from the Loba Chem. Co., India. o-Dianisidine-HCl was obtained from the Center for Biotechnological Technology, New Delhi, India. Celtrimethylammonium bromide, dioxane, dimethyl formamide and n-propanol were obtained from the SRL Chemicals, Mumbai, India. Cellulose powder (0.02-0.15 mm) was obtained from Centron Research Labs, Mumbai, India. Surf Excel was the product of Hindustan Lever Ltd., Mumbai, India. Turnip roots were purchased from the local vegetable market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of turnip proteins

Turnip root (200 g) was homogenized in 200 ml of 0.1 M sodium acetate buffer, pH 5.5. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 × g on a Remi Cooling Centrifuge C-24. The clear solution thus obtained was subjected to salt fractionation by adding 20-80% (v/v) (NH4)2SO4. The mixture was stirred overnight at 4 °C to obtain the maximum precipitate. The precipitate was collected by centrifugation at 10,000 × g on a Remi Cooling Centrifuge C-24. The obtained precipitate was re-dissolved in 0.1 M sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer (Matzo and Husain, 2006).

2.3. Preparation of bioaffinity support

Cellulose (5.0 g) was incubated and stirred with 100 ml of clear solution of jack bean extract prepared in 0.1 M sodium phosphate buffer, pH 6.2 overnight at 4 °C. The unbound proteins were removed by extensive washing with assay buffer (Akhtar et al., 2005c). The specific binding of Con A with cellulose was confirmed by eluting the bound lectin using 1.0 M methyl α-D-mannopyranoside.

2.4. Measurement of peroxidase activity

Peroxidase activity was determined from a change in the optical density (A460 nm) by measuring the initial rate of oxidation of 6.0 mM o-dianisidine-HCl in the presence of 18 mM hydrogen peroxide in 0.1 M sodium acetate buffer, pH 5.5 for 15 min at 37 °C. The immobilized preparation was continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparations (Musthapa et al., 2004).

One unit of peroxidase activity (U) was defined as the amount of enzyme protein that catalyzes the oxidation of 1 (mol of o-dianisidine-HCl per min at 37 °C into colored product (εmax = 30,000 M−1 L−1).

2.5. Immobilization of TP on Con A-cellulose support

TP (7240 U) were added to 5.0 g of Con A-cellulose support and stirred in sodium phosphate buffer, pH 6.2 at 4 °C overnight. The unbound TP was removed by extensive washing with the assay buffer (Akhtar et al., 2005c).

2.6. Effect of pH on soluble and immobilized TP

The activities of soluble and immobilized TP preparations (1.15 U) were measured in buffers of various pH values (3.0-10.0). The molarity of each buffer was 0.1 mol L−1.

2.7. Effect of temperature on soluble and immobilized TP

The activities of soluble and immobilized TP preparations (1.15 U) were measured at various temperatures (20-80 °C) under standard assay conditions. The activity obtained at 30 °C was taken as 100% for the calculation of percent activity. Soluble and immobilized TP preparations (1.15 U) were incubated at 60 °C in 0.1 M sodium acetate buffer, pH 5.5. Aliquots of each preparation were removed at each indicated time interval and activity was measured. The activity obtained without incubation at 60 °C was taken as control (100%) for the calculation of percent activity.

2.8. Effect of detergents on soluble and immobilized TP

Surf Excel (0.1-1.0%, w/v) and CTAB (0.2-2.0%, w/v) were used as final assay concentration to observe the effect of detergents on the activity of TP. Soluble and immobilized TP preparations (1.15 U) were incubated with the detergents in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated detergent concentrations. The activity obtained without exposure to detergent was taken as 100% for the calculation of percent activity.

2.9. Effect of water-soluble organic solvents on soluble and immobilized TP

Soluble and immobilized TP preparations (1.15 U) were incubated with 10-60% (v/v) of water-miscible organic solvents; DMF/dioxane/n-propanol prepared in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated organic solvent concentrations after the incubation period (Jan et al., 2001). Other assay conditions were the same as described in the text.

2.10. Determination of protein concentration

The protein concentration was determined according to the procedure described by Lowry et al. (1951). Bovine serum albumin was used as standard.

3. Results

3.1. Preparation of bioaffinity support and immobilization of TP

Cellulose adsorbed nearly 30 mg protein/g cellulose powder from jack bean extract. Con A-cellulose matrix was selected as a bioaffinity media for the direct immobilization of
glycoenzymes from ammonium sulphate fractionated turnip proteins. It has already described that some of the isoenzymes of turnip peroxidases are glycosylated in nature (Duarte-Vazquez et al., 2003). In view of the glycoproteinic nature of turnip peroxidases, these enzymes could be directly immobilized on Con A-cellulose support from ammonium sulphate fractionated proteins or from the crude homogenate of turnip. The unbound proteins were removed by extensive washing with assay buffer. Con A-cellulose adsorbed 672 U of peroxidase per g of the matrix (Table 1).

The stability of soluble and Con A-cellulose bound TP preparation was monitored against various physical and chemical parameters because these parameters can influence the activity of the enzymes used for the treatment of organic pollutants present in the wastewater.

3.2. pH-activity profile

Fig. 1 shows the pH-activity profile of soluble and Con A-cellulose bound TP. Soluble and immobilized both TP preparations showed same pH-optima at pH 5.0. However, the immobilized TP preparation exhibited higher percent of activity at alkaline side of pH-optima.

3.3. Effect of temperature

Bioaffinity bound TP preparation exhibited a marginal broadening in temperature-activity profile. Soluble and Con A-cellulose bound TP preparations showed same temperature-optima at 30 °C. Con A-cellulose adsorbed TP retained greater fractions of catalytic activity at higher temperatures as compared to the free enzyme (Fig. 2). Soluble TP retained a marginal activity of 22% at 70 °C whereas the immobilized enzymes exhibited more than half of the maximum activity.

Fig. 3 demonstrates the thermal denaturation of soluble and immobilized TP at 60 °C. Immobilized TP incubated at 60 °C
for 2 h retained 54% of the initial enzyme activity while the soluble enzyme lost nearly 83% of the original activity under similar incubation conditions.

### 3.4. Effect of urea and guanidinium-HCl

Con A-cellulose bound TP was more resistant to inactivation induced by 4.0 M guanidinium-HCl compared to its soluble counterpart. Immobilized enzyme preparation retained more than half of the initial enzyme activity when exposed to 4.0 M guanidinium-HCl for 1 h while the soluble TP exhibited marginally 15% of the initial activity under similar incubation conditions (Table 2).

Table 2 further demonstrates the treatment of soluble and immobilized TP preparations with 4.0 M urea for various time periods. Immobilized TP was significantly more stable against the denaturation mediated by urea and this preparation retained over 85% of the enzyme activity after 2 h exposure with 4.0 M urea. However, the soluble enzyme was more sensitive to urea treatment and lost more than 65% of the initial TP activity after 2 h treatment with 4.0 M urea concentration (Table 2).

### 3.5. Effect of detergents

Wastewater coming out from various industrial sites contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of polluted wastewater. In order to use such enzymes for the treatment of aromatic pollutants from wastewater it becomes necessary to monitor the stability of enzymes in the presence of some detergents. In this study different detergents have been selected for comparative stability of soluble and immobilized TP. Surf Excel is a very common detergent used in household and laundry. Unused detergent is normally present in the wastewater coming out of municipal waste. This wastewater contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of aromatic compounds from wastewater and laundry. Unused detergent is normally present in the wastewater coming out of municipal waste. This wastewater contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of aromatic compounds from wastewater and laundry.

In view of their presence in industrial effluents, it became more important to examine the effect of some water-miscible organic solvents on the activity of TP, soluble and immobilized TP preparations were further incubated with 0.2–2.0% (w/v) CTAB, a cationic detergent for 1 h at 37 °C. Pre-incubation of soluble and immobilized enzyme preparations with 2% (w/v) CTAB for 1 h exhibited enhanced activity of 131% and 225% of the original enzyme activity, respectively (Fig. 5).

### 3.6. Effect of water-miscible organic solvents

Wastewater coming out from industrial sites also contains several types of solvents along with other aromatic pollutants. In view of their presence in industrial effluents, it became more important to examine the effect of some water-miscible organic solvents on the activity of immobilized enzymes. The exposure of soluble enzyme with varying concentration of DMF (10–60%, v/v) resulted in the loss of greater fraction of enzyme activity.

![Fig. 4. Effect of Surf Excel on the activity of soluble and immobilized TP](image-url)
activity while the immobilized enzyme was quite resistant to inactivation induced by DMF. Con A-cellulose bound TP preparation retained nearly 70% of the original activity after 1 h exposure with 60% (v/v) DMF whereas the soluble enzyme lost nearly 90% of the activity under similar treatment conditions (Table 3).

The exposure of soluble and Con A-cellulose adsorbed TP with (10–60%, v/v) dioxane showed a continuous decrease in peroxidase activity. Immobilized TP had retained greater fraction of catalytic activity at the exposure of various concentrations of dioxane (Table 3). The incubation of soluble and immobilized TP with increasing concentration of n-propanol resulted in a continuous loss of enzyme activity. Moreover, the immobilized enzyme preparation exhibited more resistant to inactivation induced by n-propanol. The treatment of soluble TP with 60% (v/v) n-propanol for 1 h resulted in a loss of 88% of the initial activity while the Con A-cellulose bound TP exhibited significantly higher stabilization against similar treatment and retained more than 50% of the initial activity (Table 3).

4. Discussion

Several methods have been described for the immobilization/stabilization of enzymes but very few of them meet the requirement of enzyme immobilization directly from the crude homogenate or partially purified enzyme preparations. Here a simple and an elegant approach have been applied to immobilize TP directly from ammonium sulphate fractionated proteins of turnip roots on Con A-cellulose. It is now well recognized that polysaccharides could be used for bioaffinity-based purification of Con A from the jack bean extract (Saleemuddin and Husain, 1991). However, such property has been exploited for the preparation of bioaffinity media for the immobilization of enzymes from crude preparations. TP was immobilized in very high yield on Con A-cellulose support and it retained 672 U of TP/g of the adsorbent. The immobilization yield was quite superior over other methods used for the immobilization of peroxidases (Akhtar et al., 2005c; Husain et al., 1992). Con A-cellulose bound TP exhibited very high stabilization against the inactivation induced by pH, heat, urea and guanidinium-HCl denaturation (Figs. 1–3 and Table 2). Several earlier investigators have also reported about the use of Con A support for high yield and stable immobilization of glycoenzymes (Saleemuddin and Husain, 1991; Saleemuddin, 1999).

Lower concentrations of various detergents enhanced activity of soluble and immobilized TP. These experiments indicated that the presence of lower concentrations of detergents is not harmful to the enzyme function. Such enzymes can work more efficiently on industrial effluents containing compounds, i.e. detergents. Con A-cellulose adsorbed TP was quite resistant against denaturation induced by detergents such as Triton X 100, Tween 20 (data not given), Surf Excel and CTAB (Figs. 4 and 5). Numerous detergents normally flow in the municipal wastewater and these could affect the activity of enzymes. Our observations suggested that Con A-cellulose bound TP preparation was remarkably more stable against the exposure caused by high concentration of several detergents. Potential applications of this enzyme could be used for the treatment of wastewater containing hazardous aromatic pollutants.

Organic solvents are also very common pollutants along with aromatic compounds and their presence can influence the structure of enzymes. Enzymes exploited for the treatment of wastewater containing aromatic pollutants would be affected by the presence of water-miscible organic solvents. The Con A-cellulose bound TP was remarkably more resistant against the inactivation mediated by DMF, dioxane and n-propanol (Table 3). It has been already reported that immobilization of enzymes by multipoint attachment protects them from

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Table 3

<table>
<thead>
<tr>
<th>Organic solvent (v/v)</th>
<th>Percent remaining TP activity</th>
<th>DMF (S-TP, I-TP)</th>
<th>Dioxane (S-TP, I-TP)</th>
<th>n-Propanol (S-TP, I-TP)</th>
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Soluble and immobilized TP preparations (1.15 U) were independently incubated with increasing concentrations of DMF/dioxane/n-propanol (10–60%, v/v) in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations. The activity of soluble and immobilized TP in assay buffer without any organic solvent was taken as control (100%) for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.
denaturation mediated by water-miscible organic solvents (Mozhaev et al., 1990; Fernandez-Lafuente et al., 1995; Batra and Gupta, 1994). Akhtar et al. (2005c) have demonstrated that bitter gourd peroxidase immobilized on Con A-Sephadex support behaved differently compared to soluble enzyme in aqueous–organic co-solvent mixtures. More recently in our laboratory it has been shown that enzymes immobilized on protein supports were also quite resistant to denaturation induced by various water-miscible organic solvents (Matto and Husain, 2006; Jan et al., 2001; Jan and Husain, 2004). Fernandes et al. (2003) improved HRP organic solvent tolerance by immobilization, though to a lesser extent since at 20% organic solvent concentration HRP-PANIG retained between 60% (acetone) and 19% (acetonitrile) activity. In a more recent study Magri et al. (2005) have also reported that immobilized soybean seed coat peroxidase shows full activity over the organic solvent concentration range (5–70%, v/v) assayed whereas the free enzyme was almost inactive in 50% (v/v) of the solvents assayed. Enzymatic catalysis in organic solvents is possible if the active-site structure is not substantially disturbed by the organic solvent and if enough water is present (Ryu and Dordick, 1992); it seems that in the case of Con A-cellulose bound TP both conditions are met.

Con A-cellulose bound TP preparation has pronounced stability against pH, heat, urea, guanidinium-HCl, detergents and water-miscible organic solvents. Earlier reports described that the immobilization of glycoenzymes on Con A support resulted in the stabilization of enzymes against several types of denaturation (Saleemuddin and Husain, 1991; Mislovicova et al., 2000). It suggested that Con A-cellulose bound TP preparation has great potential in the treatment of organic pollutants present in industrial effluents. Con A-cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it led to the desorption of enzyme or Con A or both from the support. Cross-linking of bioaffinity adsorbed enzyme could be done by using bifunctional or polyfunctional reagents to prevent the dissociation/desorption of enzyme or Con A-glycoenzyme complex from the cellulose support (Jan et al., 2006).

5. Conclusion

The procedure for the immobilization of proteins, developed in this study exhibited its own merits due to use of crude jack bean extract, the source of lectin and ammonium sulphate fractionated turnip proteins, source of enzyme. Moreover, this procedure emphasized the immobilization of TP directly from the crude homogenate or ammonium sulphate fractionated proteins on bioaffinity support. TP adsorbed on Con A support showed very high yield of immobilization and markedly high stabilization against several forms of denaturants. In near future, the reactors containing such types of inexpensive immobilized enzyme preparations could be exploited for the treatment of wastewater containing toxic and hazardous compounds.

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Decolorization and degradation of acid dyes mediated by salt fractionated turnip (Brassica rapa) peroxidases

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Abstract
Peroxidases from turnip roots (524 U g⁻¹ of vegetable) were highly effective in decolorizing acid dyes having wide spectrum chemical groups. Dye solutions, containing 40–170 mg dye L⁻¹, were treated by turnip peroxidases (TP) (specific activity of 122.0 U mg⁻¹ proteins). These enzymes were able to decolorize most of the acid dyes in the presence of 2.0 mM 1-hydroxybenzotriazole (HOBT). Increasing concentration of enzyme and time in the absence of HOBT did not influence dye decolorization. The rate of decolorization was significantly enhanced when HOBT was added to the decolorizing solutions. The decolorization of all the used dyes was maximum at pH 5.0 and 40°C. Complex mixtures of dyes were significantly decolorized when treated with enzyme in the presence of HOBT (2.0 mM). Phytotoxicity test based on Allium cepa root growth inhibition has shown that majority of the TP-treated dye product were not more toxic than their parent dye. Kinetic parameters of the TP with various dyes showed that this enzyme has highest affinity for Acid Yellow 42. This study demonstrates that the peroxidase/mediator system was an effective biocatalyst for the treatment of industrial effluents from textile, dye manufacturing, dyeing and printing industries or complex mixtures of dyes.

Keywords: Turnip, peroxidases, 1-hydroxybenzotriazole, dye, degradation

Abbreviations: TP, Turnip peroxidases; HOBT, 1-hydroxybenzotriazole; HRP, horseradish peroxidase; BGP, bitter gourd peroxidase

Introduction
Pollution of communal water bodies by waste dyestuff released from textile plants and dye houses represents a major environmental concern. There is currently a considerable
environmental interest for color removal from a wide range of wastewater. In case of the textile manufacturing industry, up to 50% of the dyes are lost after dyeing process which are disposed out in the effluents [1,2]. Most of the dyes used in textile industry are classified as cationic, anionic or non-ionic type. Anionic dyes are the direct, acid and reactive dyes [3]. Acid dyes comprise of one of the classes of water soluble synthetic dyes with greatest variety of colors and structures. Although presently a wide range of physical and chemical methods is available to decolorize dye-contaminated effluents [4], but these methods are outdated due to some unresolved problems. However alternative procedures based on biotechnological principles are attracting increasing interest [2,5].

Dyes are removed by a wide variety of aerobic or anaerobic organisms, which are preferably employed as mixed cultures because of their relative robustness and versatility against xenobiotic compounds [1,6,7]. Recently, a pilot plant containing a combination of anaerobic/aerobic organisms has been developed for the treatment of colored textile effluents [5]. One major advantage of such systems is the complete mineralization often achieved due to synergistic action of different organisms [8].

However, the biological procedures have their own limitations, such as the non-biodegradability of the xenobiotic compounds due to lack of requisite enzymes in the biological treatment plant [2,7]. Often the environment of the microorganisms is not optimal for rapid degradation of pollutants [1,2]. There is a need to find alternative procedures for their treatment that are effective in removing dyes from large volume of effluents and are low in cost [9].

Recently, enzymatic approach has attracted much interest in the removal of phenolic pollutants from aqueous solutions as an alternative strategy to the conventional chemical as well as microbial treatments that pose some serious limitations [10–12]. Oxidoreductive enzymes; peroxidases and polyphenol oxidases are participating in the degradation/removal of aromatic pollutants from various contaminated sites [13]. These enzymes can act on a broad range of substrates that can also catalyze the decolorization and decontamination of organic pollutants, even if they are present in a very low concentration at the contaminated site. In view of the potential of the enzymes in treating the phenolic compounds several microbial and plant oxidoreductases have been employed for the treatment of dyes, but none of them has been exploited at large scale due to low enzymatic activity in biological materials and high cost of enzyme purification [13–16].

In this study, we have investigated the role of partially purified turnip peroxidases (TP) for the degradation/decolorization of acid dyes, having a wide spectrum of chemical groups, currently being used by the textile industries. The majority of the tested dyes were recalcitrant to decolorization/degradation by TP. However, the addition of 2.0 mM 1-hydroxybenzotriazole (HOBT), a redox mediator, to the reaction mixture enhanced the rate of decolorization of these dyes several folds. The mixtures of dyes were also successfully decolorized by TP. Kinetic parameters of the TP with various dyes were also determined in order to examine the affinity of the enzyme for dyes.

**Materials and methods**

**Materials**

Acid dyes were a gift from Atul Chemicals, Ltd. India. Ammonium sulfate and HOBT were purchased from SRL Chemicals, Mumbai, India. o-Dianisidine-HCl was obtained from the IGIB, New Delhi, India. Turnip used in the study was purchased from a local...
vegetable market. The chemicals and other reagents employed were of analytical grade and were used without any further purification.

Ammonium sulfate fractionation of turnip proteins

Turnip (50 g) was homogenized in 100 mL of 100 mM sodium acetate buffer, pH 5.5. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 g on a Remi C-24 Cooling Centrifuge. The clear supernatant thus obtained was subjected to salt fractionation by adding 20-80% (w/v) (NH₄)₂SO₄. The mixture was stirred overnight at 4°C to obtain maximum precipitate. The precipitate was collected by centrifugation at 10,000 g on a Remi C-24 Cooling Centrifuge. The obtained precipitate was re-dissolved in an appropriate volume of 100 mM sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer [17].

Treatment of dyes with increasing concentration of TP

The dyes (40-170 mg L⁻¹) were prepared in 100 mM sodium acetate buffer, pH 5.0. Each dye was incubated with increasing concentration of TP “0.117-0.352 U mL⁻¹” of reaction volume” in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H₂O₂ for 1 h at 37°C. HOBT (2.0 mM) was used as a redox mediator for the selected experiments. Dye decolorization by TP was monitored at their respective wavelength maxima in the presence and absence of 2.0 mM HOBT. The percent decolorization was calculated by taking untreated dye solution as control (100%).

Treatment of dyes with fixed concentration of TP for varying times

Each dye was incubated with TP (0.235 U mL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 at 37°C in the presence of 0.75 mM H₂O₂ for varying time intervals; 15 min to 2 h. Decolorization was also performed in the presence of 2.0 mM HOBT under other similar experimental conditions. The decrease in absorbance was monitored at predetermined intervals at the respective λmax of each dye. The percent decolorization was calculated by taking untreated dye solution as control (100%).

Allium cepa test for TP-treated dyes

The Allium cepa bioassay for the treated dye sample was carried out according to the method of Fiskesjo [18]. For this test small onions of equal size are taken and by using a sharp knife the yellowish brown outer scales and brownish bottom plates are removed carefully leaving the ring primordial intact. Boiling tubes filled with treated dye sample consisting of 0.235 U mL⁻¹ of TP in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H₂O₂ and 2.0 mM of HOBT are used to remain in contact with onion bulbs. Aqua guard water is used as control in all experiments, and the experiments are performed in dark conditions.

One onion is placed at the top of each tube with root primordial downward touching the liquid. After a gap of 12 h the same samples are added in the tube to fill up to the top and care is taken that there should be no gap between onion bulb and sample present in the test tube.

The treatment is continued for 7 days. After completion of the time of treatment onions are taken out, and for each sample root length are measured. Inhibitions in the growth of A. cepa roots are considered as an index for the degree of toxicity [18].
Effect of pH on the decolorization of dyes by TP

In this experiment the dyes were prepared in the buffers of different pH values (3.0–10.0). Each dye was treated with TP (0.235 U mL⁻¹) in the buffers of various pH values in the presence of 0.75 mM H₂O₂ for 1 h at 37°C. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization. Dye decolorization by TP was monitored at the respective wavelength maxima of each dye. The percent decolorization was calculated by taking each untreated dye in specific buffer as control (100%).

Effect of temperature on the decolorization of dyes by TP

Each dye was incubated with TP (0.235 U mL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 and 0.75 mM H₂O₂ at 30–80°C for 1 h. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization. Decrease in color of dyes after treatment with TP was monitored at specific wavelength maxima of each dye. The percent decolorization was calculated by taking untreated dye solution incubated at each temperature as control (100%).

Decolorization of mixture of dyes by TP

Dye mixtures were prepared by mixing different dyes in equal proportions in terms of absorbance. The mixtures of dyes were treated with TP (0.235 U mL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H₂O₂ and 2 mM of HOBT for 1 h at 37°C. Decrease in absorbance in each TP-treated dye mixture was monitored at specific wavelength maxima of the mixture. The percent decolorization was calculated by taking untreated dye mixture as control (100%).

Determination of $K_m$ and $V_{max}$ of the TP with respect to tested dyes

The initial rates of enzymatic dye degradation were measured at various concentrations of the dye. In this experiment, the solutions having different dye concentrations ranging from 15–200 mg L⁻¹ were treated with TP (0.235 U mL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 and 0.75 mM H₂O₂ at 37°C for 1 h. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization.

UV–Visible spectral analysis

Procedure for the dye decolorization was followed by UV–Vis spectral analysis. Spectra for the control and TP-treated dye samples were taken on Cintra 10e UV–Vis spectrophotometer.

Assay of TP activity

Peroxidase activity was measured from the change in the optical density (A₄₆₀ nm) at 37°C by measuring the initial rate of oxidation of o-dianisidine-HCl by H₂O₂ using the two substrates in saturating concentrations [19].

One unit of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of o-dianisidine-HCl in the presence of H₂O₂ into 1.0 μmol of chromophoric complex ($ε_m = 30,000$ M⁻¹ cm⁻¹) per min at 37°C.
Determination of protein concentration

The protein concentration was determined by the procedure described by Lowry et al. [20]. Bovine serum albumin was used as standard.

Results and discussion

Turnip root is a good source of peroxidase, and because of their kinetic and biochemical properties have a high potential as an economic alternative to the other commercially available plant and microbial peroxidases.

TP catalyzed polymerization and precipitation of various phenolic compounds is well-documented in the literature [21,22]. However, the decolorization and degradation of dyes by TP has not yet been reported. Therefore, for the first time, we have investigated the role of TP in the decolorization of industrially important acid dyes used in textile industry. Ammonium sulfate precipitated proteins from turnip root were taken for the treatment of a number of acid dyes. Partially purified preparation of TP was obtained by adding 20–80% ammonium sulfate, and this preparation exhibited a specific activity of 122.0 U mg⁻¹ proteins. The experiments were designed to investigate the dye decolorization in the presence of H₂O₂ and partially purified TP. The dye solutions were found to be stable upon exposure to H₂O₂ or to the enzyme alone.

Treatment of dyes by varying TP concentration

Table I summarizes the decolorization of five acid textile dyes by using increasing concentration of TP (0.117–0.352 U mL⁻¹ of reaction volume) for 1 h at 37°C. An increase in the enzyme activity has resulted in a continuous enhancement in the rate of dye decolorization. Acid Blue 92 was decolorized 61% with 0.352 U of TP mL⁻¹ of reaction mixture whereas Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were recalcitrant to the TP action (Table I).

<table>
<thead>
<tr>
<th>Name of the dye</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>0.117</th>
<th>0.235</th>
<th>0.352</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Blue 92</td>
<td>564</td>
<td>13</td>
<td>21</td>
<td>61</td>
</tr>
<tr>
<td>Acid Red 97</td>
<td>497</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acid Yellow 42</td>
<td>408</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acid Black 1</td>
<td>619</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acid Black 210</td>
<td>456</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Each dye was treated with increasing concentrations of TP (0.117–0.352 U mL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (-) and (+) signs indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.
Table II. Treatment of acid dyes with fixed concentration of TP for varying times.

<table>
<thead>
<tr>
<th>Name of the dye</th>
<th>Acidity Blue 92</th>
<th>Acidity Red 97</th>
<th>Acidity Yellow 42</th>
<th>Acidity Black 1</th>
<th>Acidity Black 210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decolorization (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>15</td>
<td>30</td>
<td>45</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>(-)</td>
<td>68</td>
<td>67</td>
<td>76</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>(+)</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(-)</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>(+)</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(-)</td>
<td>0</td>
<td>91</td>
<td>0</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>(+)</td>
<td>91</td>
<td>0</td>
<td>0</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>(-)</td>
<td>73</td>
<td>75</td>
<td>77</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(+)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td>(-)</td>
<td>37</td>
<td>41</td>
<td>62</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>(+)</td>
<td>37</td>
<td>41</td>
<td>62</td>
<td>62</td>
<td>62</td>
</tr>
</tbody>
</table>

Each dye was treated with TP (0.235 U mL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 at 37°C for varying time periods. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (-) and (+) signs indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

Treatment of dyes for varying times with fixed concentration of TP

Acid dyes were incubated with 0.235 U of TP mL⁻¹ of reaction volume for increasing time period. Out of five, only Acid Blue 92 were decolorized on treatment with TP for 2 h at 37°C. Although more color disappeared when dye was incubated for longer durations, the rate of decolorization was slow (Table II). Rest of the four dyes; Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were fully recalcitrant to decolorization by TP even after a longer period of incubation under similar conditions.

Treatment of dyes in the presence of redox-mediator

Five acid dyes used in this study were treated with TP in the presence of 2.0 mM HOBT and 0.75 mM H₂O₂ at 37°C. Presence of HOBT drastically enhanced the rate of decolorization of recalcitrant dyes (Table I). Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were recalcitrant to decolorization in the absence of HOBT. However, these dyes were decolorized up to 92, 91, 87 and 65%, respectively by the action of 0.352 U mL⁻¹ of reaction volume in the presence of 2.0 mM HOBT at 37°C for 1 h.

Role of HOBT in the decolorization of recalcitrant dyes is further noticeable in Table II. The decolorization of dyes with 0.235 U of TP mL⁻¹ of reaction volume at 37°C for 1 h in the presence of 2.0 mM HOBT was 86% for Acid Red 97, 91% for Acid Yellow 42, 87% for Acid Black 1 and 62% for Acid Black 210. It was evident from the results that 1 h of the reaction time was sufficient for the maximum removal of dye (Table II). After 1 h of incubation time, a marginal amount of dye removal was noticed up to remaining 2 h of the incubation time. Acid Yellow 42 was decolorized up to 91% within 15 min of incubation time. Recently, Mohan et al. [23] demonstrated decolorization of Acid Black 10 BX by horseradish peroxidase (HRP), and dye decolorization was maximum in 45 min.

Decolorization of textile reactive dyes in the absence of HOBT was followed by the formation of precipitate, which settled down and could be removed by centrifugation. Several earlier investigators have shown that the treatment of phenols and aromatic amines by peroxidases and tyrosinases resulted in the formation of large insoluble aggregates [10,11]. However, the decolorization of acid dyes by TP in the presence of 2.0 mM HOBT appeared without the formation of any precipitate. It suggested that the decolorization of
dyes took place via degradation of aromatic ring of the compounds or by cleaving certain functional groups. These results were in agreement with earlier published findings [14,15,24]. Hence, HOBT could have a dual role, first as a mediator in increasing the substrate range of dyes for TP and second enhancing the rate of oxidation of various substrates.

### Determination of phytotoxicity of decolorized product

In order to examine the toxicity of TP decolorized product of acid dyes, we performed phytotoxicity experiment by using *A. cepa* test with all the used dyes and their decolorized product. Table III shows the growth of *A. cepa* in terms of length in cm and percent inhibition brought about by dye solutions (untreated and treated). When *A. cepa* was incubated with dye solutions for 7 days, the maximum growth inhibition was recorded to be 94.54% for Acid Red 97 (untreated), the average root length was recorded to be 0.30 cm compared with 5.50 cm in control. The minimum inhibition in root length was 65.45% for TP-treated Acid Yellow 42 product as compared to control.

Table III also shows the effect of Acid Black 1 on the *A. cepa* root inhibition which further demonstrates the formation of non-toxic dye degraded products. However the degradation of Acid Blue 92 and Acid Black 210 brought about a growth inhibition of 84.54 and 85.09%, respectively, which is high in comparison with untreated dye solutions.

However, the product of some TP-treated acid dyes was more toxic as compared to parent dye. In order to get rid of toxic product, we investigated the decolorization of acid dyes in the presence of phenol and it produced insoluble product, which could be easily removed by centrifugation. Thus, it can fully minimize the risk of the product toxicity (data not given).

### Effect of pH on the decolorization of dyes with TP

Five acid textile dyes were treated with TP in the buffers of different pH values (Figure 1). Most of the dyes were maximally decolorized at pH 5.0. As pH of the decolorizing sample was increased up to pH 10.0, the rate of decolorization decreased in all the treated dyes. Further, at higher pH Acid Red 97 and Acid Black 210 were marginally decolorized. However, Acid Blue 92, Acid Black 1 and Acid Yellow 42 showed no decolorization in
Effect of pH on the TP mediated decolorization of acid dyes. Each dye was treated with TP (0.235 U of TP mL$^{-1}$) in buffer of different pH value (3.0-10.0) at 37°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%. Symbols indicate (•) Acid Blue 92, (○) Acid Red 97, (▼) Acid Yellow 42, (▼) Acid Black 1 and (■) Acid Black 210.

Figure 2 shows the effect of different temperatures (30-80°C) on the decolorization of acid dyes. The decolorization of dyes was maximum at 40°C in case of all the tested dyes.

alkaline range (Figure 1). In an earlier study it has been shown that HRP and bitter gourd peroxidase (BGP) could decolorize and degrade dyes maximally at pH 2.5 and 3.0 [14,25].

Effect of temperature on the decolorization of dyes with TP

Figure 2 shows the effect of different temperatures (30-80°C) on the decolorization of acid dyes. The decolorization of dyes was maximum at 40°C in case of all the tested dyes.
Table IV. Decolorization of polluted water containing mixture of dyes.

<table>
<thead>
<tr>
<th>Mixture of acid dyes</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>(+) HOBT (%)</th>
<th>(-) HOBT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Red 97 + Acid Yellow 42 + Acid Black 1 + Acid Blue 92</td>
<td>541</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>Acid Yellow 42 + Acid Black 1 + Acid Black 210 + Acid Blue 92</td>
<td>582</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Acid Red 97 + Acid Black 210 + Acid Black 1 + Acid Blue 92</td>
<td>580</td>
<td>95</td>
<td>4</td>
</tr>
<tr>
<td>Acid Red 97 + Acid Yellow 42 + Acid Blue 92 + Acid Black 210</td>
<td>531</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>Acid Red 97 + Acid Yellow 42 + Acid Black 1 + Acid Black 210</td>
<td>439</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>Acid Red 97 + Acid Yellow 42 + Acid Blue 92 + Acid Black 1 + Acid Black 210 + Acid Blue 92</td>
<td>536</td>
<td>57</td>
<td>0</td>
</tr>
</tbody>
</table>

The mixtures of dyes were treated with TP (0.235 UmL⁻¹) in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (—) and (+) signs indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

Above and below this temperature rate of decolorization was decreased. These results were in agreement with earlier reported results; the decolorization of reactive dyes by BGP was maximum at 40°C [25].

Treatment of mixtures of dyes with TP

To understand the decolorization of dyes present in industrial effluent, we prepared complex mixtures of various acid dyes by mixing different dyes in equal proportions and incubated with 0.235 EU of TP mL⁻¹ in the presence of 2.0 mM HOBT and 0.75 mM H₂O₂ for 1 h at 37°C (Table IV). The $\lambda_{\text{max}}$ for each dye mixture was pre-determined and decolorization was monitored after incubation period at their $\lambda_{\text{max}}$. The mixtures were decolorized to a varying extent (37-95%) in the presence of 2.0 mM HOBT, whereas the complex mixtures were also recalcitrant to the TP action in the absence of redox-mediator.

Kinetics of acid dye decolorization

In order to determine the kinetic parameters of enzyme for various used dyes, an experiment with different dye concentrations, ranging from 15 to 200 mg L⁻¹ was performed. The plot of initial rate versus dye concentration for all five acid dyes followed the hyperbolic pattern as expected for Michaelis–Menten kinetics (data not given). Moreover, the Lineweaver-Burk plot, inverse of initial rate versus inverse of substrate (dye) concentration is also found to be linear. The values of specific dye decolorization rate ($r_{\text{dyemax}}$) estimated from the experimental data was 7.5 mg L⁻¹ h⁻¹ for Acid Blue 92, 79 mg L⁻¹ h⁻¹ for Acid Red 97, 6.5 mg L⁻¹ h⁻¹ for Acid Yellow 42, 4.2 mg L⁻¹ h⁻¹ for Acid Black 1 and 20.5 mg L⁻¹ h⁻¹ for Acid Black 210. The value of apparent Michaelis constant ($K_m$) was 89 mg L⁻¹ for Acid Blue 92, 97.5 mg L⁻¹ for Acid Red 97, 6.2 mg L⁻¹ for Acid Yellow 42, 35.8 mg L⁻¹ for Acid Black 1 and 16 mg L⁻¹ for Acid Black 210 (Table V). The $K_m$ value was lowest for the Acid Yellow 42, it showed the highest affinity of the enzyme for this dye. This is also evident from Table II that in the presence of HOBT the dye was decolorized 91% in 15 min. However, other dyes took more time to achieve the same decolorization.
Table V. Kinetics constants of TP for decolorization of dyes.

<table>
<thead>
<tr>
<th>Name of the dye</th>
<th>$\text{rate}_{\text{max}}$ (mg L$^{-1}$ h$^{-1}$)</th>
<th>$K_m$ (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Blue 92</td>
<td>7.5</td>
<td>89</td>
</tr>
<tr>
<td>Acid Red 97</td>
<td>79</td>
<td>97.5</td>
</tr>
<tr>
<td>Acid Yellow 42</td>
<td>6.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Acid Black 1</td>
<td>4.2</td>
<td>35.8</td>
</tr>
<tr>
<td>Acid Black 210</td>
<td>20.5</td>
<td>16</td>
</tr>
</tbody>
</table>

Independent dye was treated as described in the text. Each value represents the mean for three independent experiments performed in duplicate, with average SD <5%.

Figure 3. UV-Vis absorption spectra of Acid Blue 92. The dye was incubated with TP (0.235 U mL$^{-1}$) and 0.75 mM H$_2$O$_2$ in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h in the presence and absence of 2.0 mM HOBT. Spectra for the control and TP-treated dye solution were taken on Cintra 10e UV-Vis spectrophotometer. Untreated dye solution (A), treated dye solution in the absence of HOBT (B) and treated dye solution in the presence of HOBT (C).

UV-Vis spectral analysis

Figures 3 and 4 demonstrate the decolorization and degradation of Acid Blue 92 and a mixture of Acid Red 97, Acid Yellow 42, Acid Blue 92, and Acid Black 1, respectively by TP in the presence of 2.0 mM HOBT. These figures evidently demonstrate the rapid disappearance of absorption peaks in the visible region in the presence of HOBT. The disappearance of absorption peak in the presence of HOBT in visible region was due to the breakdown of chromophoric group present in the dyes [14]. The decolorization of Direct Fast Scarlet 4 BS in the microbial consortium composed of Pseudomonas 1–10 and White-rot fungus 8–4* also indicated the formation of intermediates with phenyl ring as the major components in the UV region, and its content was suggested to be more than in the original solution [26]. The absorbance peaks appeared in the UV region after TP treatment was quite comparable to the results explained by Fang et al. [26].
Figure 4. UV-Vis absorption spectra of a mixture of dyes. The dye mixture (Acid Red 97, Acid Yellow 42, Acid Blue 92 and Acid Black 1) was incubated with TP (0.235 μM L⁻¹) and 0.75 mM H₂O₂ in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h in presence of 2 mM HOBT. Spectra for the control and TP-treated dye mixture were taken on Cintra 10e UV-Vis spectrophotometer. Untreated dye mixture (A) and treated dye mixture in the presence of HOBT (B).

Bourbonnais and Paice [27] described for the first time the use of redox mediators by allowing laccase to oxidize non-phenolic compounds thereby expanding the range of substrates that can be oxidized by this enzyme. The mechanism of action of laccase mediator system has been extensively studied and it is used in textile industry in the finishing process for indigo stained materials. Several workers have demonstrated that use of enzyme/redox mediator system enhanced the rate of dye decolorization by several folds, but these mediators were required in very high concentrations (5.7 mM violuric acid/laccase system, 11.6 mM of HOBT/laccase system) [9,28]. In this study, for the first time, we have shown decolorization of dyes by TP by using very low concentration of HOBT (2.0 mM), which enhanced the rate of acid dyes decolorization by 5–92 folds. There were several reports about the enhancement of laccase activity by redox-mediators [9,28,29]. Most recently Akhtar et al. [24] have demonstrated the use of redox-mediator in the decolorization of dyes by using a peroxidase from bitter gourd. These results further support that peroxidases from other sources could also be used for the decolorization of recalcitrant dyes in the presence of redox mediators.

The potential application of TP for dye degradation was tested using a number of chemically diverse commercially available acid dyes. Dye solutions, were successfully treated with TP in the presence and absence of redox mediator, HOBT. Decolorization rate was drastically increased when industrially important acid dyes were treated with TP in the presence of 2.0 mM HOBT. In order to understand the application of TP in effluent treatment, we prepared complex mixtures of dyes. The complex mixtures of dyes treated with TP in the presence of HOBT were significantly decolorized. The application of peroxidase that is easily available and inexpensive can overcome its limitations in wastewater treatment. The use of peroxidases can be extended to the large-scale treatment of a wide spectrum of structural dye by using immobilized TP and relatively cheaper redox-mediators. This as well as the scale up of enzymatic processes is the subject of further study.
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References


