ORIGINAL ARTICLE

Enzymatic decolourisation of Methyl Orange and Bismarck Brown using crude peroxidase from *Armoracia rusticana*

Mugdha Ambatkar · Usha Mukundan

Abstract The decolourisation of Methyl Orange (MO) and Bismarck Brown (BB) by crude peroxidase from *Armoracia rusticana* (Horseradish) was studied by varying different reaction parameters. The pH of the reaction mixture, initial dye concentration, amount of enzyme and hydrogen peroxide concentration were optimised for ambient temperatures (30 ± 2 °C). The optimum pH for decolourisation was 4.0 (72.95 %) and 3.0 (79.24 %) for MO and BB, respectively. Also it was found that the Chemical Oxygen Demand of the enzyme-treated sample was significantly lower than that of the untreated controls for both dyes. The addition of a complex iron salt like Ferric EDTA was found to enhance the decolourisation of both dyes at pH 6.0, showing an increase of 8.69 % and 14.17 % in the decolourisation of MO and of BB, respectively. The present study explores the potential of crude peroxidase from horseradish to decolourise representative monoazo and diazo dyes, MO and BB, respectively. An attempt has been made to utilise a crude enzyme with appreciable activity obtained after minimal processing for the decolourisation of the aforesaid dyes. The findings of this study would find application in the enzymatic treatment of wastewater containing azo dyes.

Keywords Peroxidase · Decolourisation · Azo dyes · *Armoracia rusticana* · Ferric salt

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Introduction

The impending global water crisis has necessitated practices to conserve and replenish our existing water resources. The anthropogenic requirement of water for domestic and industrial purposes, and the wastewater consequently generated, is immense. Treatment of wastewater before it enters receiving water bodies has become exceedingly important (Mohapatra 2006).

Industries that utilise dyes in the manufacturing process of their products contribute coloured effluents to wastewater streams. Azo dyes are among the most widely used class of dyes in a variety of industries such as textile, leather, paper and even food (Vijaykumar et al. 2006). The presence of colour in an effluent is more than an aesthetic problem. Absorption of solar radiation is hindered by the chromophores of the dye molecules. This adversely affects the productivity of photosynthetic primary producers of the food chain that exists in the receiving water body (Abo-Farah 2010). Azo dyes are relatively recalcitrant and are not easily biodegradable. It is, however, preferable to degrade azo dyes by oxidation rather than by reduction. Since the reduction of the azo (-N=N-) bond leads to the formation of aromatic amines (Karim and Husain 2009), of which several have been found to be toxic (Zille et al. 2004).

Treatment methods used for dyestuff containing effluents include many oxidation processes collectively called advanced oxidation processes (AOPs). Some of the AOPs, like the Fenton process and its modifications, are efficient for treating a wide variety of pollutants and are cost effective as well. However, the continuous requirement of iron salts and highly acidic conditions (pH 2–3) of operation can sometimes limit their application.

Enzymatic degradation of pollutants in wastewater is fast gaining importance as an alternative or a supplement to conventional effluent treatment practices (Vijaykumar et al. 2006; da Silva et al. 2010). Enzymes are efficient biological catalysts and can operate under mild conditions of pH and temperature. The fact that enzymes are biodegradable makes them environmentally acceptable. Oxidoreductases such as peroxidases (Husain et al. 2010; Karim et al. 2012) and laccases (Chivukula and Renganathan 1995; Vijaykumar et al. 2006) are of special importance in wastewater treatment. The performance of purified enzymes is definitely superior to that of crude enzymes. Using purified enzymes in wastewater treatment is not feasible due to the prohibitive cost involved in such a large scale of operation; however, crude enzymes are more suitable for such use. It is necessary to develop treatment processes that use crude enzymes with appreciable activity, obtained after minimal processing.

In the present study, decolourisation of representative monoazo and diazo dyes, Methyl Orange (MO) and Bismarck Brown (BB), respectively, was studied at a laboratory scale using crude peroxidase extracted from in vitro grown *Armoracia rusticana* plantlets. This plant, commonly called horseradish, is the commercial source of the enzyme horseradish peroxidase (HRP) (EC 1.11.1.7). Reaction parameters like pH, dye concentration, hydrogen peroxide concentration and enzyme concentration were optimised. The Chemical Oxygen Demand (COD) of the dye solutions with and without enzymatic treatment was determined. The effect of adding Fe(III)-EDTA to the reaction mixture, on the enzymatic decolourisation of the two dyes was also observed.

Materials and methods

Chemicals used

The dyes used for the study were Methyl Orange (MO) and Bismarck Brown R (BB) representing monoazo and diazo dyes, respectively. MO was obtained from S.D. Fine Chemicals (Boisar), India, while BB was obtained from Burgoyne Burbridges and Co. (India), Mumbai. Stock solutions of 1 mg mL^{-1} of both were prepared in distilled water. Different volumes of these stock solutions were utilised as required in the study. Guaiacol (2-methoxy phenol) was purchased from Sisco Research Laboratories, India. Hydrogen peroxide was purchased from Qualigens Fine Chemicals, Navi Mumbai, India. Chemicals for buffer preparation, such as KH_2PO_4, KOH, Citric acid, and Trisodium citrate, were purchased from S.D. Fine Chemicals (Boisar), India, while Tris–

HCl was purchased from Loba Chemie, Mumbai. The Fe(III)-EDTA used for the experiments was obtained from Loba Chemie, Mumbai. Standard HRP (250 U mg^{-1}) was obtained from Hi-Media, Mumbai.

Multiplication of in vitro cultures of *A. rusticana* plantlets

Plantlets of *Armoracia rusticana* were subcultured every 4–6 weeks on modified Murashige and Skoog's (MS) medium (Dodd and Roberts 1985) containing 3 % sucrose and 0.5 mg L^{-1} Indole-3-acetic acid (IAA). The leaf, petiole and node of the source plantlet were used as explants.

Preparation of crude peroxidase extract

A known weight of fresh plant petiole tissue (1.0 g) was macerated in a pre-chilled mortar pestle with 10 mL of cold 0.1 mol L^{-1} phosphate buffer (pH 5.8). The suspension was cold centrifuged at 2,500 rpm for 10 min. The supernatant was used as the source of crude enzyme. The protein content of the crude extract was estimated (Lowry et al. 1951). Since the crude extract contained chlorophylls, the absorbance due to chlorophylls was compensated for using an appropriate colour correction.

Determination of guaiacol activity of crude peroxidase extract

The activity of the crude peroxidase extracts was estimated using JASCO V-530 spectrophotometer by a method described by Kim and Yoo (1996). In this assay, the coloured product tetraguaiacol ($\varepsilon = 26,600$ L mol^{-1} cm^{-1}) is estimated at 470 nm. The reaction mixture (3.0 mL) contained 0.975 mL of phosphate buffer, 0.025 mL enzyme extract, 1.0 mL (15 mmol L^{-1}) guaiacol and 1.0 mL (3.0 mmol L^{-1}) hydrogen peroxide.

The activity of the crude enzyme towards guaiacol was expressed in units per mL (U mL^{-1}). The definition of 1 unit (U) is as follows: the amount of enzyme required to convert 1 μmole of substrate to product in 1 min. The activity of the crude extract was calculated using the formula shown in Equation 1:

$$\frac{U}{ml} = \frac{\Delta OD}{min} \times \frac{RmV}{EV} \times \frac{df}{\varepsilon_{470}} \tag{1}$$

where, $\Delta OD/min$ is the increase in absorbance per minute (min^{-1}), RmV is the reaction mixture volume (mL), df is the dilution factor, ε_{470} is the molar absorptivity of tetraguaiacol at 470 nm (mL μmol^{-1} cm^{-1}), EV is the enzyme extract volume (mL)

Enzymatic dye decolourisation

Decolourisation was assessed by measuring initial and final absorbance using JASCO V-530 spectrophotometer. The final reaction volume was 3.0 mL, the duration of observation was 24 h unless stated otherwise and all the decolourisation experiments were performed at ambient temperature (30 ± 2 °C). Positive control samples were prepared for MO (20 ppm) and BB (30 ppm) using HCl to obtain pH 3.0, ferrous ammonium sulphate (0.70 mmol L^{-1}) and H_2O_2 (500 mg L^{-1}) as used in the modified Fenton process (Habib et al. 2012). All samples were centrifuged at 2,500 rpm for 10 min before reading absorbance at 460 and 400 nm for MO and BB, respectively. The percentage of decolourisation was calculated using the formula given below in Equation 2 (Singh et al. 2011):

$$\% \text{ Decolourisation} = \frac{(A_i - A_f) \times 100}{A_i} \qquad (2)$$

where, A_i is the initial absorbance; A_f is the final absorbance.

Optimisation of pH of reaction mixture

This study was carried out using crude and standard horseradish peroxidase enzyme (HRP). The pH was varied in the range pH 3–9 at intervals of 1 pH unit. The purpose of this study was to compare the performance of both standard HRP and crude peroxidase at different pH and that of the modified Fenton process which operates optimally for azo dyes at pH 3. Citrate buffers (0.1 mol L^{-1}) were used to obtain pH 3–5, phosphate buffers (0.1 mol L^{-1}) for pH 6–8 and Tris–HCl buffer was used to obtain pH 9. The reaction mixtures contained 0.2 mL of crude peroxidase (guaiacol activity 0.932 ± 0.014 U mL^{-1}). The reaction mixtures with standard HRP contained 0.02 mL of 0.5 mg mL^{-1} enzyme solution. The concentrations of dyes were 20 and 30 mg L^{-1} for MO and BB, respectively. The final concentration of hydrogen peroxide was maintained at 2 mmol L^{-1}. Since the colour of MO is affected by pH, the absorbance of MO containing reaction mixtures was read at the respective λ_{max} for each pH. The absorbance of reaction mixtures containing BB was read at 400 nm for all pH since pH did not affect the λ_{max} of BB.

Optimisation of amount of enzyme

Different volumes of enzyme suspension in the range 0.025–0.4 mL of crude peroxidase enzyme (guaiacol activity 0.854 ± 0.006 U mL^{-1}) were used for these experiments. The pH was maintained at pH 4.0 for MO and pH 3.0 for BB. The concentrations of dyes in the reaction mixture were 20 and 30 mg L^{-1} for MO and BB, respectively. The final concentration of hydrogen peroxide was maintained at 2 mmol L^{-1}.

Optimisation of initial dye concentration

The initial concentration of MO and BB was varied in the range 10–60 mg L^{-1}. The pH was maintained at pH 4.0 and 3.0 for MO and BB, respectively. The final concentration of hydrogen peroxide was maintained at 2 mmol L^{-1}. The volumes of crude enzyme used were 0.2 and 0.1 mL (guaiacol activity, 0.913 ± 0.024 U mL^{-1}) for MO and BB, respectively. The change in the absorbance was observed over 48 h.

Optimisation of hydrogen peroxide concentration

The concentration of hydrogen peroxide was varied in the range 1–10 mmol L^{-1}. The pH was maintained at pH 4.0 and 3.0 for MO and BB, respectively. The concentrations of dyes were 20 and 30 mg L^{-1} for MO and BB, respectively. The volumes of crude enzyme used were 0.2 and 0.1 mL (guaiacol activity 0.913 ± 0.024 U mL^{-1}) for MO and BB, respectively.

Effect of enzymatic treatment on COD

For these experiments, 30 mL of the reaction mixtures was prepared and analysed using a method mentioned by Roth et al. (1989). The concentrations of dyes were 20 and 30 mg L^{-1} for MO and BB, respectively. The optimum concentrations of hydrogen peroxide and enzyme extract were used for the COD experiments. Estimation of COD was done after allowing the decolourisation reaction to occur for 24 h.

Effect of Fe-EDTA on decolourisation of dyes at pH 6.0

The pH optimisation experiments for decolourisation showed that pH 4.0 and 3.0 were optimum for the decolourisation of MO and BB, respectively. Both dyes show relatively lower decolourisation at pH 6.0. The crude enzyme, however, has maximum activity at pH 6.0. The presence of Fe-EDTA (0.23 mmol L^{-1}) significantly improves the activity of the enzyme. Hence, decolourisation of MO and BB in the presence of 0.23 mmol L^{-1} Fe-EDTA was carried out at pH 6.0. The concentrations of dyes were 20 and 30 mg L^{-1} for MO and BB, respectively. The final concentration of hydrogen peroxide was maintained at 2 mmol L^{-1}. The volumes of crude enzyme used were 0.2 and 0.1 mL (guaiacol activity 0.908 ± 0.03 U mL^{-1}) for MO and BB, respectively.

Statistical analysis of data

The reported values are mean ± SD ($n = 3$). All the statistical analyses of the data obtained from the studies described above were performed using SPSS version 19. The results of the analysis were obtained for $p < 0.05$. In cases where ANOVA has been performed, multiple comparisons were made using Duncan's multiple range test (DMRT). Unless stated otherwise, the 'MO' series has been assigned groups represented by upper case letters (A>B>C…) and the 'BB' series has been assigned groups represented by lower case letters (a>b>c…). The means (within a single series, i.e. MO or BB) that have been assigned the same letter(s) are not significantly different from each other at $p < 0.05$. The same representation was adopted for cases where treatments (COD mg of oxygen L^{-1} and Ferric EDTA treatment) were compared using the unpaired t test at $p < 0.05$.

Results

As seen in Fig. 1, the maximum decolourisation of MO using either standard HRP (87.30 %) or crude peroxidase (72.95 %) occurred at pH 4.0. The decolourisation by the modified Fenton process (96.51 %) was higher than that achieved by standard HRP. The modified Fenton process was carried out only at its optimum pH for azo dye decolourisation viz., pH 3.0 (Habib et al. 2012). The means of standard HRP series are represented by capital letters (A, B, C…) and the means of the crude peroxidase series are represented by lower case letters (a, b, c…).

The maximum enzymatic decolourisation of BB occurred at pH 3.0, as seen in Fig. 2. Standard HRP caused higher decolourisation (82.65 %) than crude peroxidase (79.24 %). The modified Fenton process brought about relatively lower decolourisation (26.51 %) of BB than the

enzymatic treatment. The means of standard HRP series are represented by capital letters (A, B, C…) and the means of the crude peroxidase series are represented by lower case letters (a, b, c…).

As seen in Fig. 3, the decolourisation of MO increased as the amount of crude enzyme was increased, the maximum being at 0.342 U (41.98 %) The decolourisation of BB increased with the amount of enzyme present in the reaction mixture only up to 0.043 U at which it was maximum (73.01 %) and beyond which a steady decrease in decolourisation was observed. In case of both the dyes, the decolourisation due to the bleaching effect of hydrogen peroxide alone (shown as 0 U of crude peroxidase) was significantly lower than in the enzyme-treated samples (2.65 % for MO and 14.73 % for BB).

The decolourisation of MO was significantly higher at 10 mg L^{-1} (45.79 %) than at higher concentrations, as seen in Fig. 4. The decolourisation of BB is significantly higher at 30 mg L^{-1} (81.21 %) and beyond than at lower concentrations of the dye. The BB molecules appear to form polymers on enzymatic oxidation and form a precipitate. As a result, the upper solution (supernatant) contained a relatively lower concentration of the dye at the end of the reaction.

As seen in Fig. 5, the optimum concentration of hydrogen peroxide for the decolourisation (63.13 %) of MO was 1 mmol L^{-1}. Beyond this concentration, there was a steady decrease in the decolourisation of MO. The decolourisation of BB at 4 mmol L^{-1} (71.90 %) of hydrogen peroxide and beyond was significantly higher than at lower concentrations. Beyond 4 mmol L^{-1}, the decolourisation of BB did increase with the concentration of hydrogen peroxide, but not significantly. The decolourisation of both dyes in the absence of added hydrogen peroxide was significantly lower than the treated samples. This decolourisation (1.22 % for MO and 34.09 % for BB) may be attributed to the hydrogen peroxide formed

Fig. 1 Comparison of decolourisation of Methyl Orange (20 mg L^{-1}) using crude HRP, standard HRP at different pH and the modified Fenton process (pH 3.0)
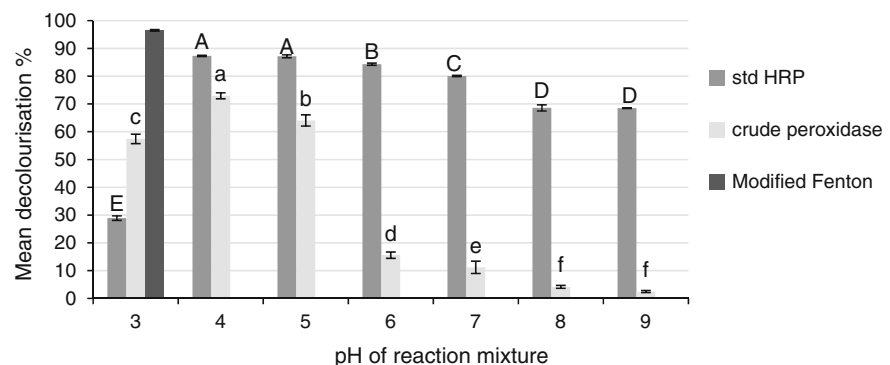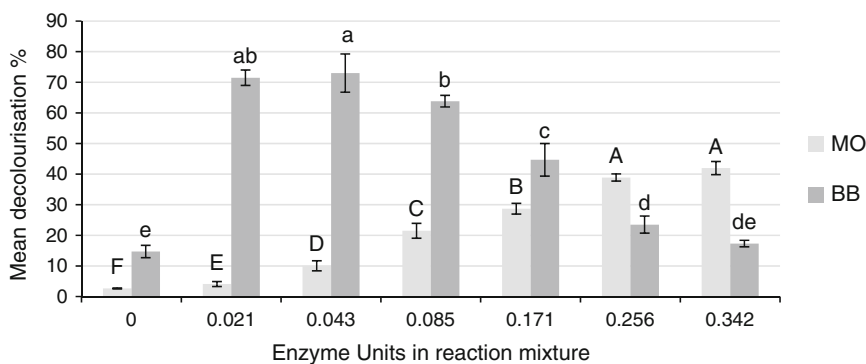
Means that are assigned different letters in a given series (A>B>C>D for std HRP and a>b>c… for crude peroxidase) are statistically significantly different from each other at $p < 0.05$.

Fig. 2 Comparison of decolourisation of Bismarck Brown (30 mg L^{-1}) using crude HRP, standard HRP at different pH and the modified Fenton process (pH 3.0)
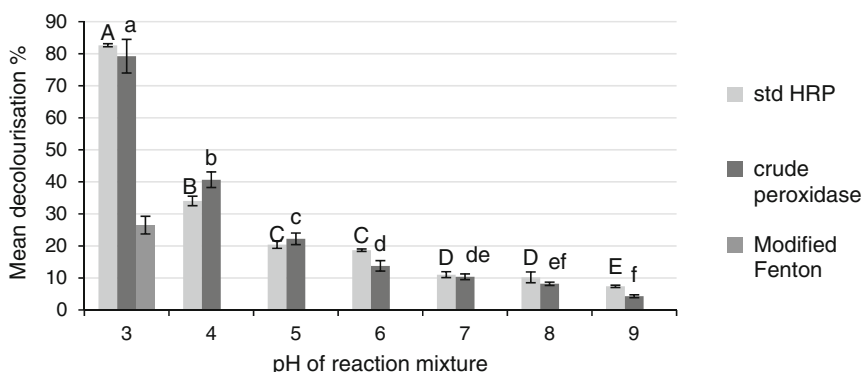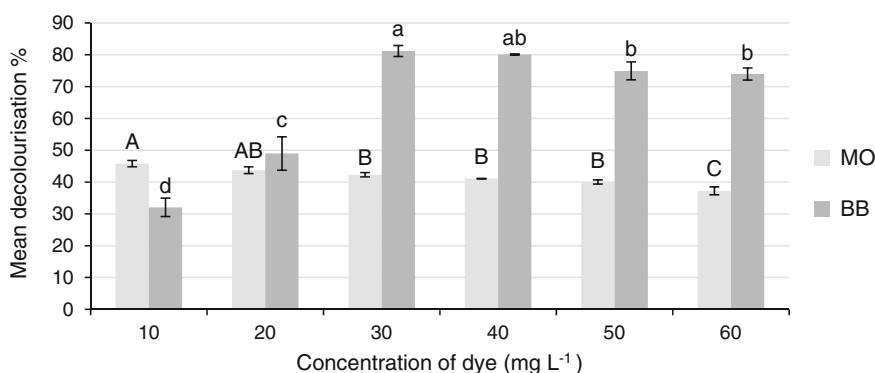

Means that are assigned different letters in a given series (A>B>C>D for std HRP and a>b>c… for crude peroxidase) are statistically significantly different from each other at $p < 0.05$.

Fig. 3 Decolourisation of Methyl Orange (20 mg L^{-1}) and Bismarck Brown (30 mg L^{-1}) using different amounts of crude peroxidase

Means assigned different letters in a given series (A>B>C… for MO and a>b>c… for BB) are statistically significantly different at $p < 0.05$

Fig. 4 Decolourisation of different concentrations of Methyl Orange and Bismarck Brown by crude horseradish peroxidase

Means assigned different letters in a given series (A>B>C… for MO and a>b>c… for BB) are statistically significantly different at $p < 0.05$

indigenously in the plant tissue (Chen and Schopfer 1999) from which the crude extract had been prepared.

The Chemical Oxygen Demand of solutions of MO and BB is significantly lower when subjected to enzymatic decolourisation than untreated solutions, as seen in Fig. 6. The COD of MO decreased from 4,060 to 3,666.67 mg of oxygen L^{-1} on treatment. Similarly, COD of BB decreased from 4,220 to 4,066.67 mg of oxygen L^{-1} when treated. This shows that the enzymatic

decolourisation can decrease the COD of the solution under treatment.

As seen in Fig. 7, the decolourisation of MO increased from 27.99 % in the absence of Ferric EDTA to 36.68 % in its presence. Similarly, the decolourisation of BB increased from 3.43 to 17.60 % when Ferric EDTA is included in the reaction mixture. The increase in decolourisation of MO and BB was found to be 8.69 and 14.17 %, respectively, in the presence of Ferric EDTA.

Fig. 5 Decolourisation of Methyl Orange (20 mg L^{-1}) and Bismarck Brown (30 mg L^{-1}) by crude horseradish peroxidase at different concentrations of hydrogen peroxide
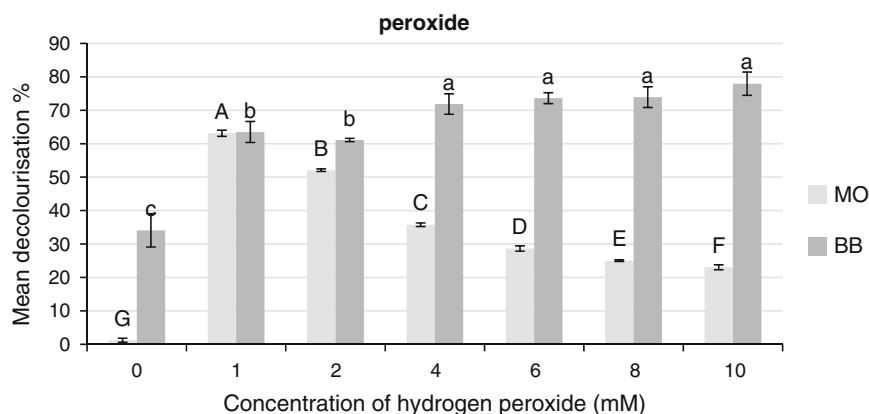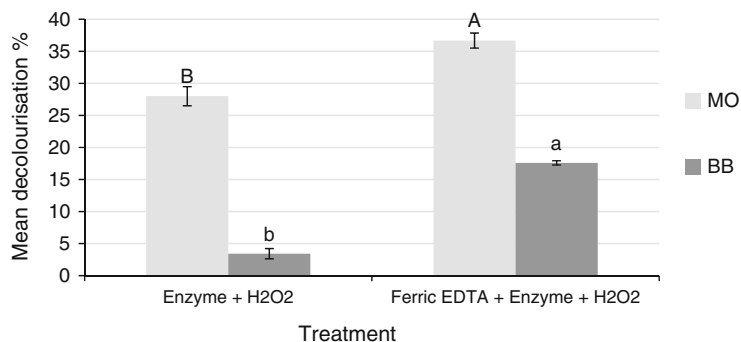

Means assigned different letters in a given series (A>B>C… for MO and a>b>c… for BB) are statistically significantly different at $p < 0.05$

Fig. 6 Comparison of Chemical Oxygen Demand (COD) of solutions of Methyl Orange (20 mg L^{-1}) and Bismarck Brown (30 mg L^{-1}) with and without enzymatic decolourisation treatment
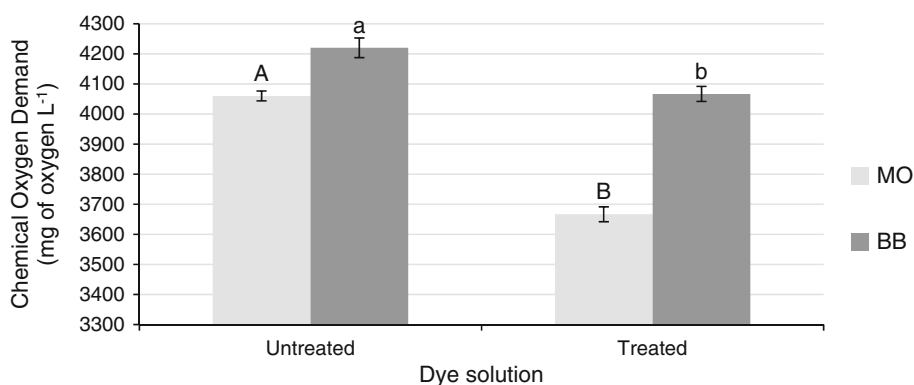
Means assigned different letters in a given series (A>B for MO and a>b for BB) are statistically significantly different at $p < 0.05$

Fig. 7 Decolourisation of Methyl Orange (20 mg L^{-1}) and Bismark Brown (30 mg L^{-1}) by crude horseradish peroxidase in the presence and absence of Ferric EDTA at pH 6.0

Means assigned different letters in a given series (A>B for MO and a>b for BB) are statistically significantly different at $p < 0.05$

Discussion

The use of enzymes in wastewater treatment is gaining momentum (Singh et al. 2011). Most often microbial enzymes (Franciscon et al. 2009) or fungal enzymes (Singh et al. 2010) are used for this purpose. The potential of plant enzymes for effluent treatment is relatively less utilised. Electron-rich molecules like azo dyes are likely to be substrates for oxidative enzymes like Peroxidase and Laccase (Vijaykumar et al. 2006). Horseradish peroxidase (HRP) is selective about the electron acceptor, i.e. hydrogen peroxide, but is relatively less selective about the electron donor. Consequently, HRP is a versatile enzyme with a relatively wide range of substrates (Kersten et al. 1990). Considering that HRP catalyses redox reactions, the redox potential of a molecule is one of the most critical

parameters that decides whether or not it can be oxidised (Zille et al. 2004) by HRP.

The present study is an attempt to assess on a laboratory scale the potential of crude HRP to decolourise monoazo and diazo dyes using MO and BB as respective representatives. Doubtlessly, standard HRP causes significantly greater decolourisation than does its crude counterpart. However, the cost of using standard or purified enzymes on a large scale is prohibitive. A major drawback of using crude extract is that the activity of crude enzyme may vary with each batch of extract. Throughout the study, however, extracts showing relatively consistent peroxidase activity towards guaiacol were used.

In the pH optimisation experiment, the decolourisation capacity of standard HRP and crude peroxidase (CP) was compared. For both dyes, the optimum pH for HRP and CP was identical. Both the dyes MO and BB are decolourised under acidic conditions. The decolourisation obtained for MO and BB was compared to that obtained by the modified Fenton process (Habib et al. 2012). The Fenton process is one of the most widely used advanced oxidation processes (AOP) for the treatment of coloured effluents. The Fenton process was modified such that ferrous ammonium sulphate (FAS) was used instead of ferrous sulphate in the present study. The modified Fenton process was considered the positive control for decolourisation, and it works optimally for azo dye decolourisation at pH 3.0 (Habib et al. 2012). Our preliminary studies (data not shown) revealed that the pH optimum for the crude peroxidase is pH 6.0. But the maximum decolourisation of MO and BB was found to be at pH 4.0 and 3.0, respectively. The pH of the reaction mixture will affect the substrate molecule (Ulson de Souza et al. 2007) in terms of redox potential (Coen et al. 2001), extent of protonation and the net charge, thereby making it more or less susceptible to enzymatic attack. The conformation of the enzyme is also affected by pH which results in increased or decreased activity of the enzyme (Nelson and Cox 2004).

MO is a pH sensitive dye, red–orange under acidic conditions and yellow in neutral and alkaline conditions. Decolourisation caused a change in colour from orange to yellow. However, measurement of pH before and after decolourisation with HRP and CP revealed that the pH change was negligible and the colour change was indeed due to the probable oxidation of the MO molecule. A similar change in colour was observed with the modified Fenton reaction. The pH of the reaction mixture containing BB was also measured before and after decolourisation with HRP and CP. The pH change in this case too was negligible, suggesting the possible oxidation of BB molecules in the course of decolourisation. On oxidation, the BB molecules in the solution seem to polymerise as reported for phenols and anilines (Hollmann and Arends

2012). The polymerised dye tends to form a precipitate, as reported in case of phenols (Nicelle et al. 1995). The extent of polymerisation increases with the concentration of substrate (Xavier et al. 2011) i.e. BB.

The decolourisation of MO increases steadily with the amount of crude enzyme present in the reaction mixture ($R^2 = 0.94$). The decolourisation of BB, however, decreases if the amount of enzyme exceeds 0.043 U. During the enzyme optimisation experiments, the concentration of H_2O_2 was kept constant. In the first step of the oxidative mechanism of Horseradish peroxidase, the heme Fe(III) of peroxidase is oxidised by H_2O_2 (Veitch 2004). The enzyme used in the experiment is in the crude form and is a mixture of various peroxidases. Some of the peroxidases like Ascorbate peroxidase (APx) which is most often present in such crude peroxidase extracts have a much higher affinity for H_2O_2 ($K_m = 3$ μM H_2O_2 for APx) than HRP ($K_m = 0.27$ mM) (Penel and Castillo 1991). In such a situation, the peroxidase with higher affinity for H_2O_2 will utilise it and hence the H_2O_2 is insufficient or unavailable for HRP to react with. This decreases the extent of decolourisation. At lower concentrations of the crude enzyme in the reaction mixture, the HRP and APx still do compete for H_2O_2; however, because the H_2O_2 is in excess, the decolourisation occurs by the action of HRP. As the amount of crude enzyme is increased (keeping the concentration of H_2O_2 constant), the enzyme with higher affinity succeeds in utilising the H_2O_2 rendering it unavailable for reaction with HRP. Hence, the concentration of H_2O_2 limits the extent of decolourisation.

The competition between enzymes for H_2O_2 can be rectified by increasing the concentration of H_2O_2. As seen in Fig. 5, the decolourisation of BB increases with an increase in the concentration of H_2O_2. The crude enzyme may require a higher concentration of H_2O_2 during the oxidation of BB as compared to MO. Also, in a case where an additive like Fe-EDTA is present in the reaction mixture, there is no competition between HRP and Fe-EDTA for H_2O_2, and there are more sites of oxidation. Hence, the extent of decolourisation is greater in the Fe-EDTA treated samples (see Fig. 7).

At higher concentrations of BB (30 mg L^{-1} and above) and H_2O_2 (4 mmol L^{-1} and above), the decolourisation does not change significantly. The H_2O_2 requirement of MO is lower than that of BB since it appears to be more easily oxidised than BB. At higher concentrations of H_2O_2, the decolourisation of MO is lower since the crude enzyme may have become inactivated (Veitch 2004; Hollmann and Arends 2012) when the substrate is MO. It has been indicated in the graph that there is some decolourisation even in the absence of added H_2O_2. This could be due H_2O_2 endogenously present in the plant material which has leached out during the preparation of the crude enzyme

extract. Hydrogen peroxide (H_2O_2) is a product of normal oxidative metabolism in practically all aerobic living systems. In plants, the release of H_2O_2 may occur even without a causative pathogenic stimulus (Chen and Schopfer 1999). In fact, it is one of the major physiological roles of the plant peroxidases to breakdown the endogenously produced H_2O_2 to prevent oxidative damage to the plant cells. The H_2O_2 thus produced inside the plantlets from which the crude enzyme extracts were prepared, had become available for reaction with the target dyes, when the extracts were added in the reaction mixture. Hence, there is some amount of decolourisation observed even in those reaction mixtures in which H_2O_2 had not been added externally.

It is likely that the BB molecule is less easily oxidised than MO due to the presence of two azo linkages present in it. The decolourisation of azo dyes is dependent on the structure of the target dye (Franciscon et al. 2009). The azo linkages are electron-withdrawing in nature and can hinder the oxidation (Maddhinni et al. 2006) of the molecule in which they are present. The experiments for optimisation of initial dye concentration were carried out for 48 h since no clear trends were discernable at the end of 24 h.

It is possible to propose an outline of a probable mechanism of action of the crude peroxidase on the dyes. The decolourisation of MO may be thought to occur by the oxidation of the molecule by the crude peroxidase with H_2O_2. This oxidation is brought about by the enzyme by two sequential one-electron transfers (Veitch 2004). This oxidation causes the formation of dye radicals and related chemical species that comprise the decolourised or bleached form of the dye (Coen et al. 2001). The susceptibility of the MO molecules to enzymatic oxidation would also depend on the pH of the reaction mixture.
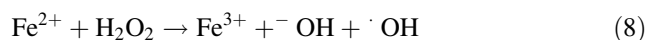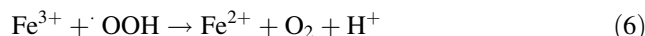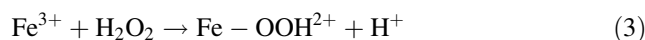
In the case of BB, a precipitate is observed if the reaction mixture is allowed to stand undisturbed for several hours. This would indicate that in the case of BB, the enzymatic oxidation of the BB molecule (in presence of H_2O_2) probably leads to the formation of radicals that initiate a polymerisation reaction. These polymers grow in length by the addition of newly oxidised dye radicals. The large size of the polymerised dye and its poor solubility in aqueous solutions cause precipitation (Hollmann and Arends 2012). The pH of the reaction mixture would be one of the factors governing the susceptibility of BB molecules to enzymatic oxidation.

Alternatively, the crude peroxidase may react with H_2O_2 to form hydroxyl radicals ($^\bullet OH$) (Chen and Schopfer 1999), which are highly reactive oxidising agents (Abo-Farah 2010). It has been found that $^\bullet OH$ can be generated from H_2O_2 in plant cells by peroxidases (Almagro et al. 2009) The hydroxyl radical is likely to be instrumental in oxidising the dye molecules to form dye radicals, which in

turn may polymerise as in the case of BB, or form the bleached dye species as in the case of MO.

The treatment of effluent with materials of biological origin may lead to an increase in the COD of the effluent (Mohapatra 2006). The results from the present study have shown that the enzymatic treatment was able to reduce the COD significantly. This may be because the enzymatic treatment generates oxygen which negates the COD increasing effect of adding biological material.

The resting state (Fe^{3+}) of the heme group of Horseradish peroxidase may be considered similar to the Fe^{3+} that participates in Fenton-like oxidations (Chen and Schopfer 1999). The Fenton process mainly involves the reaction of ferrous ions with hydrogen peroxide, forming highly reactive hydroxyl radicals ($^\bullet OH$) under strongly acidic conditions (pH 2–4). These radicals can oxidise a wide variety of organic compounds including azo dyes (Habib et al. 2012). The term 'Fenton-like' refers to reactions wherein a ferric salt reacts with hydrogen peroxide leading to the formation of hydroxyl radicals. The sequence of reactions of the Fenton-like oxidation is as described by Abo-Farah (2010).

$$Fe^{3+} + H_2O_2 \rightarrow Fe-OOH^{2+} + H^+ \tag{3}$$

$$Fe-OOH^{2+} \rightarrow Fe^{2+} + \cdot OOH \tag{4}$$

$$Fe^{2+} + \cdot OOH \rightarrow Fe^{3+} + {}^-OOH \tag{5}$$

$$Fe^{3+} + \cdot OOH \rightarrow Fe^{2+} + O_2 + H^+ \tag{6}$$

$$H_2O_2 + \cdot OH \rightarrow H_2O + \cdot OOH \tag{7}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^-OH + \cdot OH \tag{8}$$

As seen in reaction, molecular oxygen is evolved during the course of the reaction. The action of peroxidase enzyme and hydrogen peroxide may also have a similar result, thereby leading to a decrease in the COD.

The oxidative mechanism of HRP involves the Fe(III) of the heme group. In the catalytic cycle, the first reaction which leads to the formation of Compound I occurs between H_2O_2 and the resting Fe(III) state of the heme group. Compound I is a high oxidation state intermediate containing an oxoferryl (Fe IV) centre which is two oxidising equivalents above the resting state. The substrate of the enzyme provides an electron (the first of two single electron transfers) that reduces Compound I to form Compound II. Compound II is also an oxoferryl compound which is one oxidising equivalent above the resting state. Both Compound I and Compound II have redox potentials of approximately +1 V (Veitch 2004).

There are similarities in the mechanism of oxidation of a substrate by the heme group of HRP (Compound III) and ferrous chelates. In fact, the ferrous ion of Compound III can be thought of as the Fenton reagent (Chen and

Schopfer 1999). Compound III is produced by the action of excess hydrogen peroxide on the resting state of the enzyme (Veitch 2004). It has also been suggested that the Fe ion in the heme group of peroxidase may act in a manner similar to the chelated Fe^{2+} in Fe^{2+}-EDTA (Chen and Schopfer 1999) or Fe^{3+} in Fe^{3+}-EDTA (Nam et al. 2001). The role of Fe-EDTA was that of an additive to minimise the use of enzyme utilised in the reaction. Since the Ferric ion of Fe-EDTA in the presence of H_2O_2 functions in a manner similar to that of the Ferric ion in the heme group of the active site of enzyme peroxidase. It is an additive that mimics the action of the enzyme during the reaction. Since HRP contains Fe^{3+} in the (resting) catalytic heme group, Ferric EDTA was selected as an additive to the reaction mixture. It was hypothesised that the presence of the chelated Ferric ions may mimic the Fenton-like process and can enhance decolourisation. It was found from the present study that the addition of an Iron chelate to the reaction mixture improved the decolourisation of both dyes at pH 6.0. Adding a Fe salt to the reaction mixture is like adding more sites of oxidation. Horseradish peroxidase can reduce H_2O_2 to $^\bullet OH$, and ultimately to H_2O (Chen and Schopfer 1999) in the presence of a suitable reducing agent, using a mechanism similar to that used by chelated Fe^{3+} ions or Fe^{3+} ions in the Fenton-like process (Abo-Farah 2010). This explains the increased decolourisation of MO and BB by CP in the presence of Ferric EDTA. Over time (after 48 h), the decolourisation of the dyes by Fe-EDTA alone (i.e. in the control samples that are devoid of enzymes) is comparable to or is even greater than that seen in the samples containing both enzyme and Fe-EDTA (data not shown). The probable reason for this is the inactivation of enzyme over a period of time. The rate of oxidation of a substrate by a chelated iron salt in the presence of H_2O_2 is lower than the oxidation rate obtained when a free (non-chelated) iron salt like ferrous sulphate is used. The presence of a chelating agent lowers the oxidation rate by simply limiting the availability of the iron ions (Li 2007). The decolourisation enhancing effect of Ferric EDTA on crude peroxidase at pH 6.0 is significant because in recent times, effluent treatment research is directed towards being able to modify existing methods to work in neutral and near neutral conditions (Li 2007).

The ability of enzymes to decolourise various azo dyes has been tested by several researchers in the past. Microorganisms like *Staphylococcus* have been shown to oxidatively decolourise (at least 97 %) solutions of azo dyes like direct blue 71 and reactive yellow 107 (Franciscon et al. 2009). The use of extracted enzymes as in the present work, however, offers an advantage in the fact that an enzyme is not affected by the toxicity of the dye. Horseradish peroxidase obtained from fresh tubers of the horseradish plant and partially purified by dialysis was shown to successfully decolourise the monoazo

dye direct yellow (Maddhinni et al. 2006) in synthetic waste water. The present work uses a very crude peroxidase extract and has shown appreciable decolourisation of both representative dyes. The present work also offers a comparison between the decolourisation of mono and diazo dyes by crude peroxidase.

The role of the heme group of the peroxidases like Horseradish peroxidase in the oxidation of aromatic compounds has been studied in detail (Veitch 2004). The oxidation of various organic compounds by a combination of an iron salt and hydrogen peroxide (Fenton and Fenton-like process) has been well documented (Abo-Farah 2010). The oxidative decolourisation of azo dyes using Ferric EDTA with hydrogen peroxide has also been reported (Nam et al. 2001). In the literature surveyed by the authors, there has not been any report of using Ferric EDTA as an additive to enhance azo dye decolourisation by crude peroxidase enzyme. The present work has explored the use of Ferric EDTA as an additive that can enhance the action of the enzyme and even minimise the quantity of enzyme used in the reaction, since it appears to mimic the action of the peroxidase during azo dye decolourisation.

Model dyes, monoazo (MO) and diazo (BB), can both be decolourised appreciably in solution, on treatment with crude peroxidase enzyme. The treatment also significantly decreases the COD of the dye solutions. Decolourisation of both dyes can be enhanced at near neutral conditions using an iron chelate like Ferric EDTA. Immobilising the crude enzyme on a suitable support would be one of steps in our future work. Immobilisation would serve to localise the enzyme and increase the local concentration of the enzyme. The higher concentration of the crude enzyme would also help to decrease the reaction time. If the enzymatic decolourisation method given in the present work is suitably modified for effluent treatment on a large scale, the quality of the effluent released into the environment can be improved considerably.

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