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Ferric Chelate Enhances Azo Dye Decolourisation by Crude Horseradish Peroxidase

Mugdha Ambatkar[a] and Usha Mukundan[a]

[a] Plant Biotechnology Research Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai- 400086 INDIA

Corresponding author: umukundan@hotmail.com

Abstract

The mechanisms of oxidation of azo dyes by the Fenton-like process and the enzyme Horseradish peroxidase are similar. Both processes occur between pH 2.0-4.0. Horseradish peroxidase is a heme containing enzyme. Ferric-Ethylenediamine tetraacetic acid (Ferric-EDTA) which resembles the chelated Ferric ion of the heme group was selected as an additive for the decolourisation of three azo dyes- Methyl Orange, Bismarck Brown and Tartrazine by Horse peroxidase. The decolourisation was computed using the difference between initial and final absorbance of the dye solutions over 24 hours. It was found in the present study, that the addition of Ferric-EDTA improved the decolourisation of Methyl Orange and Bismarck Brown at pH 6.0 and Tartrazine at pH 9.0; by 10.33%, 13.41% and 1.58%, respectively. The enhanced decolourisation may be because Ferric-EDTA mimics the oxidative decolourisation action of peroxidase on the tested azo dyes. The activity of crude peroxidase was enhanced using 0.23 mmol L^{-1} and 0.56 mmol L^{-1} at pH 6.0 and 9.0 respectively. Methyl Orange and Bismarck Brown are decolourised at relatively mildly acidic condition, pH 6.0 as against pH 2.0-4.0 in presence of the additive. The findings of the present study are significant in the enzymatic decolourisation of wastewater containing azo dyes. Enzymatic wastewater treatment requires large amounts of enzymes. An additive that can mimic the action of the enzyme and enhance its performance can reduce the total amount of enzyme used in the treatment. This may even help to reduce the running cost of enzymatic wastewater treatment.

Keywords: Additive; Azo Dyes; Decolourisation; Ferric-EDTA; Peroxidase

1.0 Introduction:

Dyestuff containing effluents are a potential threat to the water bodies they may contact. The presence of dyes in the water body adversely affects the utilisation of sunlight by photosynthetic aquatic organisms. Azo dyes are among the most widely used colourants in various industries (Abo-Farah, 2010). Azo dyes, contribute greatly to the colour of effluents due to their high tinctorial strength, hence the Fenton process and its many modifications are important in decolourising effluents containing azo dyes. The Fenton process and its variations are commonly used Advanced Oxidation Processes (AOPs) that have been used for the oxidative decolourisation of coloured effluents. The classical Fenton reagent is a combination of hydrogen peroxide and an iron (II) salt such as ferrous sulphate. Later, a modification of the Fenton reagent that involved iron (III) salts with hydrogen peroxide came to be used and was called 'Fenton-like reagent'. The reaction using the ferric salt reagent was called the Fenton-like reaction.

In recent years, the use of enzymes for the treatment of effluents is steadily gaining importance (Kandelbauer *et al.*, 2004; Franciscon *et al.*, 2009; da Silva *et al.*, 2010; Singh *et al*., 2011). Enzymatic treatment is often preferred over other means of bioremediation (Ambatkar and Mukundan, 2012). It is relatively easier to determine the optimum operating conditions for an isolated enzyme than to do the same with microbial cultures (Karam and Nicell, 1997). The biological origin and degradability of enzymes makes their use in effluent treatment environmentally acceptable (Adam *et al.*, 1999). The use of purified enzymes for a large-scale application like wastewater treatment is not economically viable (Karam and Nicell, 1997). Hence, the potential of crude and non-purified enzymes from various sources for use in effluent treatment is being assessed (Johnson and Pokora, 1994; Naghibi *et al.*, 2003; Ambatkar and Mukundan, 2014).

Table 1: List of dyes amenable to enzymatic decolourisation

Substrate	Enzyme	Enzyme Source	Reference
Drimarene Blue X-3LR, Drimarene Blue X-BLN, Drimarene Rubinol X-3LR and Drimarene Rubinol CL-R	Horseradish Peroxidase	*Armoracia rusticana*	da Silva *et al.*, 2010
Reactive Yellow, Reactive Black, Reactive Red and Direct Blue	Azoreductase	*Staphylococcus arlettae*	Franciscon *et al.*, 2009
Reactive Red 141	Lignin Peroxidase	*Rhizobium radiobacter*	Ghodake *et al.*, 2009
Tartrazine and Ponceau	Azoreductase	Green algae	Omar, 2008
Crystal Violet and Malachite Green	Laccase	*Fusarium solani*	Abedin, 2008
Remazol Turquoise Blue G 133 and Lanaset Blue 2R	Horseradish Peroxidase	*Armoracia rusticana*	Ulson de Souza *et al.*, 2007
Acid Orange 6, Acid Orange 7, Methyl Orange and Methyl Red	Mixture of bacterial oxidoreductases	Sludge Methanogens	Kalyuzhnyi *et al.*, 2006
Direct Yellow	Horseradish Peroxidase	*Armoracia rusticana*	Maddhinni et al., 2006
Acid Blue	Laccase	*Cladosporium cladosporioides*	Vijaykumar *et al.*, 2006
3-(4 dimethyl amino-1 phenylazo) Benzene Sulphonic Acid	Laccase	*Trametes villosa*	Zille *et al.*, 2005

Peroxidases are oxidoreductases that are present in a wide variety of life-forms (Welinder, 1992; Hiraga *et al*., 2001). These enzymes have been conserved throughout evolution (Morgenstern et al., 2008) across different classes of organisms. The enzyme Horseradish Peroxidase (HRP) is also an oxidoreductase and has a Fe ion at its reactive catalytic centre (Veitch, 2004). This ion is present in the Fe (III) oxidation state as part of the heme group. Oxidations mediated by HRP involve the Fe (III) of the heme group. In the catalytic cycle the first reaction occurs between H_2O_2 and the resting Fe (III) state of the heme group. This reaction forms Compound I, which is a high oxidation state intermediate containing an oxoferryl (Fe IV) centre which is two oxidising equivalents above the resting state. The electron rich substrate of the enzyme provides an electron. This is 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 state above the resting state. Both, Compound I and Compound II have redox potentials of approximately +1 V (Veitch, 2004). The action of excess hydrogen peroxide on the resting state of the enzyme has been shown to produce Compound III [represented as Fe (II)-O_2]. This generally occurs in the absence of a reducing substrate (Rodriguez-Lopez, 1997).

The enzyme HRP has been studied with enormous interest for its ability to oxidize several electron rich organic substrates (Klibanov and Morris, 1981). The wide substrate range may be attributed to the absence of a specific binding site for the substrate (Kersten *et al.*, 1990) on the enzyme. There are similarities in the mechanism of oxidation of a substrate by the heme group of HRP (Compound III) and by ferrous chelates. In fact, the ferrous ion of Compound III has been likened to the Fenton reagent (Chen and Schopfer, 1999).The heme Fe (III) can be thought of as being similar to a Fe ion in chelated or complexed form. It has also been suggested that while oxidising organic substrates, 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). In the presence of hydrogen peroxide, Fe^{3+} of Fe^{3+} EDTA may also behave like the Fe ion in the heme group of peroxidase (Walling *et al.*, 1975). Since HRP contains Fe^{3+} in the (resting) catalytic heme group, Ferric EDTA (hence forth referred to as Fe-EDTA) was selected as an additive to the reaction mixture.

The objective of the present work was to study the effect of adding a Ferric chelate on the oxidation of azo dyes by crude HRP. The study was designed to answer the question, 'Does the addition of Ferric-EDTA augment the oxidation of azo dyes by crude HRP?' Based on the similarities in the mechanism of oxidation, it was hypothesised that the addition of Fe-EDTA to the reaction mixture would increase the oxidation of the substrate, since there would be a greater number of sites for

oxidation. The hypothesis was tested by a series of experiments. Initially, the optimum pH of the crude enzyme was determined. The effect of adding Fe-EDTA on the activity of the crude enzyme at low (pH 3.0), near-neutral (pH 6.0) and high (pH 9.0) pH was observed. The concentration of Fe-EDTA was varied at the pH at which there was no inhibition of crude enzyme activity. The optimum concentration of the Fe-EDTA additive at the tested pH was determined. Finally, the ability of the crude enzyme to decolourise the three azo dyes Methyl Orange, Bismarck Brown and Tartrazine, in the presence of Fe-EDTA was compared to controls without Fe-EDTA. Since Fe-EDTA appears to mimic the action of the crude enzyme, it may be possible to decrease the amount of enzyme required in large scale applications like enzymatic wastewater treatment, by using Fe-EDTA as an additive. The novelty of the present work lies in the fact that the similarity between the mechanism of oxidation mediated by a heme containing enzyme and a complex Ferric salt has been utilised for an application like wastewater treatment to reduce the amount of enzyme required for such a large scale operation. This is the first report of a Ferric chelate salt being used to augment the activity of an enzyme. The salt Fe-EDTA salt acts like the enzyme and enhances the extent of oxidation of substrates. The chelated form of Ferric ion prevents it from precipitating at pH 6.0 and beyond (Li, 2007). The chelated form also controls the rate of consumption of Hydrogen peroxide, thereby circumventing the need for frequent dosing with Hydrogen peroxide (Li, 2007).

2.0 Materials and Methods:

2.1 Chemicals

The azo dyes used for the study were Methyl Orange (MO) and Bismarck Brown R (BB) and Tartrazine (TZ). MO was obtained from S.D. Fine Chemicals (Boisar), India; while BB was obtained from Burgoyne Burbridges and Co. (India), Mumbai. Tartrazine was kindly provided by Neelikon Food Dyes and Chemicals, (Roha) India. Stock solutions of 1 mg mL^{-1} of the dyes were prepared in distilled water. Different volumes of these stock solutions were utilized 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, like KH_2PO_4, KOH, Citric acid, Trisodium citrate and HCl 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.

2.2 Preparation of Extract

Approximately 0.2 g of petiole tissue from *in vitro Armoracia rusticana* (horseradish) plantlets (**Figure a**) was macerated in a pre-chilled mortar and pestle, with 2.0 mL of phosphate buffer (0.1 mol L^{-1}, pH 5.8). The mixture was centrifuged at room temperature for 10 min 3000 rpm. The supernatant was used as the source of crude peroxidase.

Figure a: *In vitro* plantlet of *Armoracia rusticana*

2.3 Determination of optimum pH of crude peroxidase:

The activity assay of the crude peroxidase extract at different pH towards guaiacol was performed using a spectrophotometric method (Kim and Yoo, 1996). The reaction mixture contained 0.95 mL of buffer of pH (pH 3.0 to 9.0), 0.05 mL of enzyme extract, 1.0 mL of guaiacol (15 mmol L^{-1}) and 1.0 mL of H_2O_2 (3 mmol L^{-1}). All measurements of absorbance were performed using JASCO V-530 UV-VIS spectrophotometer. Throughout the study, the activity (in U mL^{-1}) of the crude enzyme extracts was calculated using the formula given below-

Equation 1: $$\mathrm{UmL^{-1}} = (\Delta\ \mathrm{OD/min}) \times (\mathrm{RmV/CeV}) \times (\mathrm{df}/\varepsilon_{470})$$

where,
ΔOD/min = increase in absorbance per minute (min^{-1})
RmV = reaction mixture volume (mL)
df = dilution factor
ε_{470} = molar absorptivity of tetraguaiacol at 470 nm (mL μmol^{-1} cm^{-1})
CeV = crude enzyme volume (mL)

2.4 Effect of Ferric-EDTA on the activity of crude peroxidase at different pH:

The effect of adding Ferric EDTA to the reaction mixture at pH 3.0, 6.0 and 9.0 was observed. These three pH were chosen to represent the entire range of pH tested low or acidic (pH 3.0), medium or near-neutral (pH 6.0) and high or alkaline (pH 9.0). The reaction mixture for crude peroxidase assay contained Ferric EDTA at a concentration of 0.12 mmol L^{-1}. The reaction mixture composition was as follows- 1.77 mL buffer (pH 3.0, 6.0 and 9.0), 0.1 mL crude enzyme extract, 0.03 mL Fe-EDTA (4.3 g L^{-1}), 1.0 mL guaiacol (15 mmol L^{-1}) and 1.0 mL H_2O_2 (3mmol L^{-1}).

2.5 Effect of various concentrations of Fe-EDTA on the activity of crude peroxidase:

The concentration of Fe-EDTA in the assay reaction mixture was varied between 0.12 and 0.56 mmol L^{-1} along with control mixture in which Fe-EDTA was not added. The assay was carried out at pH 6.0. The buffer volumes were adjusted according to the volume of the Fe-EDTA (4.3 g L^{-1}) added. The final reaction volume was 3.0 mL. A similar assay was performed at pH 9.0.

2.6 Effect of Fe-EDTA on azo dye decolourisation by crude peroxidase;

The decolourisation of mono azo dye Methyl Orange (MO) and diazo dye Bismarck Brown (BB), using crude peroxidase in the presence of 0.23 mmol L^{-1} Fe-EDTA was carried out at pH 6.0. The concentrations of dyes were 20 mg $L^{-1}$and 30 mg L^{-1} for MO and BB respectively. The concentration of hydrogen peroxide was 2 mmol L^{-1}. The volumes of crude enzyme (0.39 U mL^{-1}) used were 0.2 mL and 0.1 mL for MO and BB respectively. The decolourisation of the two azo dyes was observed after 24 hours.

The decolourisation of Tartrazine (TZ 10 mg L^{-1}) using crude peroxidase was assessed at pH 9.0. Earlier pilot studies had shown that crude peroxidase brought about significantly higher decolourisation of TZ, at pH 9.0 than at any other pH. The volume of crude enzyme used was 0.2 mL and the concentration of hydrogen peroxide was 2 mmol L^{-1}. The effect of the presence of 0.56 mmol L^{-1} Fe-EDTA on decolourisation of TZ was observed after a reaction time of 24 hours. Decolourisation was assessed reading initial and final absorbance at 460 nm, 400 nm and 425 nm for MO, BB and TZ respectively. The percentage of decolourisation was calculated using the formula described below (Singh *et al.*, 2011):

Equation 2: $$\% \text{ Decolourisation} = \frac{(Ai - Af) \times 100}{Ai}$$

where,
A*i* = Initial absorbance
A*f* = Final absorbance after 24 h

2.7 Statistical analysis of data:

The reported values are means ± SD. All the statistical analysis 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). In a given series, means that have been assigned the same letter(s) (a>b>c....) are not significantly different from each other at $p = 0.05$. In **Table 2** the control and treated samples have been compared at each pH using the unpaired t-test. The means of control and treated samples that have been assigned the same letters (as superscripts) are not significantly different from each other at $p = 0.05$, at a given pH of reaction mixture. In the decolourisation graph (**Figure e**), the means of the control and treated populations have been compared using the unpaired t-test. Since more than one series has been represented on a single graph, each series has been assigned a different set of letters (A>B....., P>Q..... and X>Y...) to aid the comparison of means *within* the series.

3.0 Results and Discussion:

The activity of crude peroxidase towards guaiacol at different pH is shown in **Figure b**. Crude peroxidase was most active in the pH range 5.0-8.0. The activity was highest at pH 6.0 (0.39 U mL^{-1}). At pH 3.0, 4.0 and 9.0, the activity was significantly lower.

The guaiacol activity of crude peroxidase was tested in the presence and absence of Fe-EDTA (0.12 mmol L^{-1}) at pH 3.0, 6.0 and 9.0. As seen in the **Table 2**, the activity of crude peroxidase was not affected significantly by the presence of Fe-EDTA (0.12 mmol L^{-1}) at pH 6.0 and 9.0. However, at pH 3.0, the activity of peroxidase in the Fe-EDTA containing mixture was significantly lower than the control. Hence in the subsequent experiments, the concentration of Fe-EDTA was varied at pH 6.0 and 9.0.

Table 2: Effect of Ferric-EDTA on the activity of crude peroxidase

Mean Guaiacol Activity (U mL^{-1})		
pH	**Control**	**With Fe-EDTA**
3.0	0.102±0.001[c]	0.072±0.002[d]
6.0	0.592±0.002[a]	0.580±0.008[a]
9.0	0.177±0.006[b]	0.195±0.001[b]

FeEDTA = Ferric-EDTA
Note: Means that have been assigned the same letter(s) are not significantly different at *p=0.05*

The crude enzyme had maximum guaiacol activity at pH 6.0. At this pH, all the Fe-EDTA treated sets (except 0.56 mmol L^{-1} Fe-EDTA) showed higher activity than the untreated control (0.17 U mL^{-1}). In the set treated with 0.23 mmol L^{-1} (0.19 U mL^{-1}) and 0.35 mmol L^{-1} (0.18 U mL^{-1}) Fe-EDTA, the activity was significantly higher than with the other treatments (see **Figure c**).

At pH 9.0 the activity of crude peroxidase increased linearly with an increase in the tested concentrations of Fe-EDTA (r = + 0.986) as seen in **Figure d**. The activity was highest (0.24 U mL^{-1}) at 0.56 mmol L^{-1} Fe-EDTA. The enhancement of the activity of crude peroxidase with the addition of Fe-EDTA lies between 9.35 to 38.01%for the tested concentrations of Fe-EDTA.

As seen in **Figure e**, the decolourisation of MO (20 mg L^{-1}) increased from 26.98% in the absence of Ferric EDTA to 37.31% in its presence. Similarly, the decolourisation of BB (30 mg L^{-1}) increased from 3.96% to 17.37% when Ferric EDTA is included in the reaction mixture. The Fe-EDTA treated set showed 10.16% decolourisation of Tartrazine (10 mg L^{-1}) over 24 h, while the untreated showed 8.58% decolourisation at pH 9.0, as seen in **Figure e.**

The Fenton process and its modification, which uses ferrous ammonium sulphate (FAS) instead of ferrous sulphate, decolourise a majority of azo dyes optimally in the pH range 3.0-4.0 (Abo-Farah, 2010; Habib *et al.*, 2012). 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 2.0-4.0). These radicals can oxidise a wide variety of organic compounds, including azo dyes (Habib *et al.*, 2012). The sequence of reactions of the Fenton-like oxidation wherein a ferric salt reacts with hydrogen peroxide leading to the formation of hydroxyl radicals is as described by Abo-Farah (2010).

Equations 1 to 6

$$Fe^{3+} + H_2O_2 \rightarrow Fe - OOH^{2+} + H^+ \quad \ldots\ldots (1)$$
$$Fe - OOH^{2+} \rightarrow Fe^{2+} + {}^{\cdot}OOH \quad \ldots\ldots (2)$$
$$Fe^{2+} + {}^{\cdot}OOH \rightarrow Fe^{3+} + {}^{-}OOH \quad \ldots\ldots (3)$$
$$Fe^{3+} + {}^{\cdot}OOH \rightarrow Fe^{2+} + O_2 + H^+ \quad \ldots\ldots (4)$$
$$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + {}^{\cdot}OOH \quad \ldots\ldots (5)$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\cdot}OH \quad \ldots\ldots (6)$$

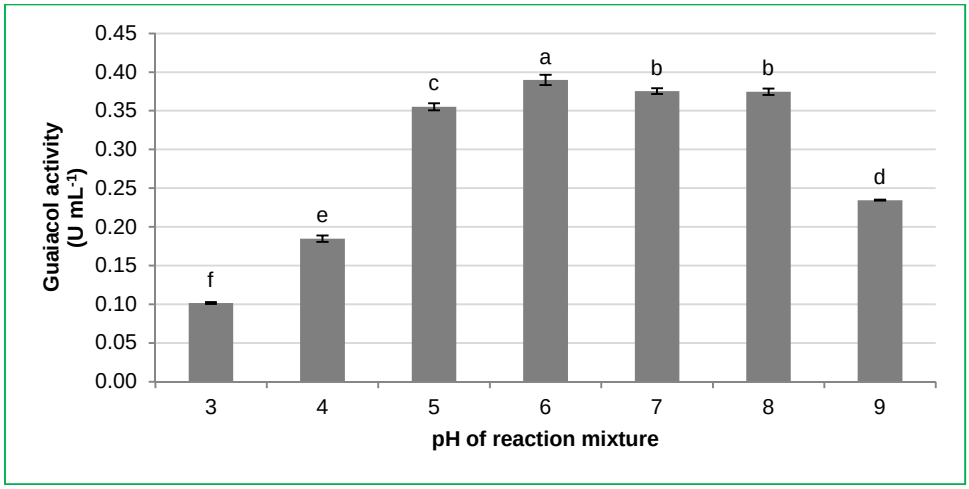

Figure b: Activity of crude peroxidase at different pH

Means that have been assigned the same letter(s) are not significantly different at *p=0.05*

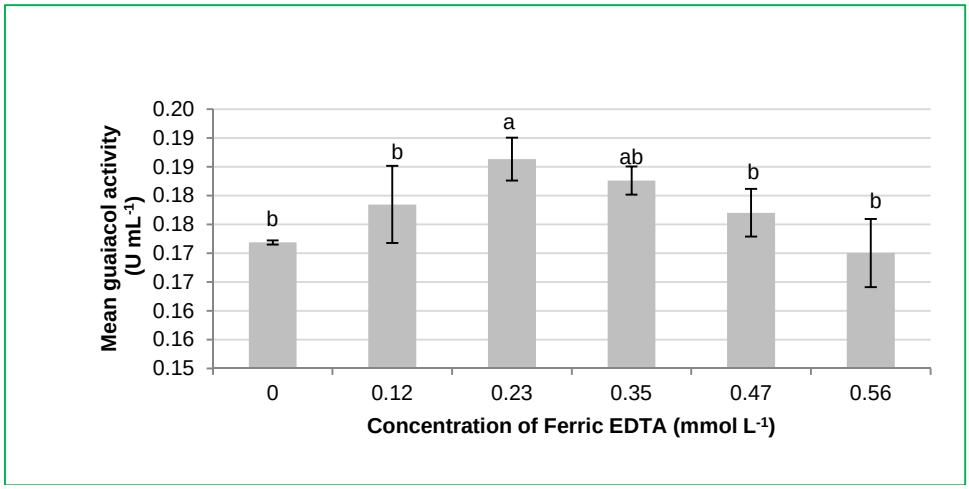

Figure c: Effect of concentrations of Ferric-EDTA on the activity of crude peroxidase at pH 6.0

Means that have been assigned the same letter(s) are not significantly different at *p=0.05*

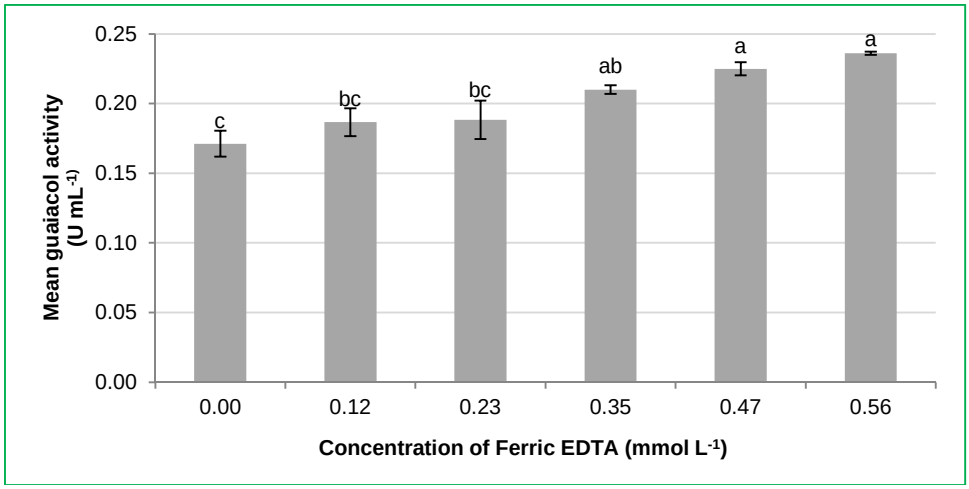

Figure d: Effect of concentration of Ferric-EDTA on the activity of crude peroxidase at pH 9.0

Means that have been assigned the same letter(s) are not significantly different at *p=0.05*

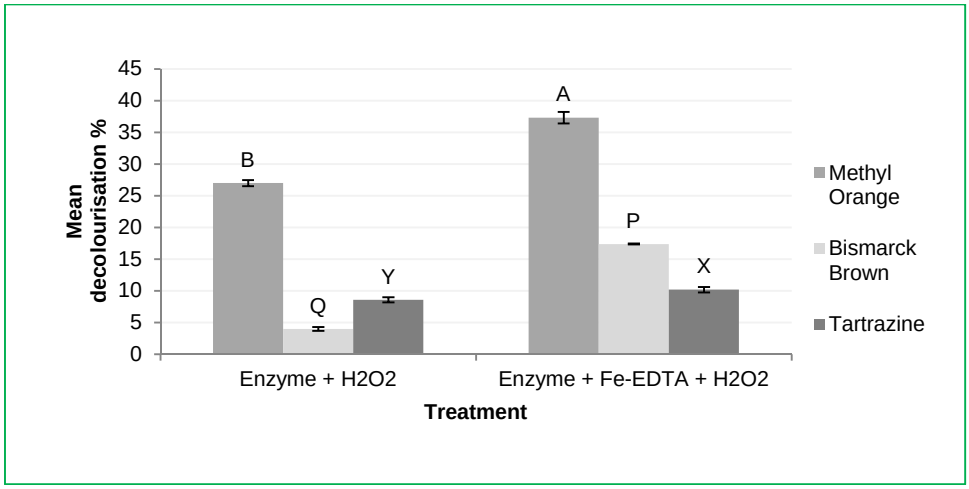

Figure e: Effect of Ferric-EDTA on enzymatic decolourisation of three azo dyes by crude peroxidase
(FeEDTA = Ferric-EDTA), In a given series (Methyl Orange, Bismarck Brown or Tartrazine) means that have been assigned the same letter(s) are not significantly different at *p =0.05*

The Fenton process is known to occur in a highly acidic environment. During our preliminary experiments, when the optimum pH for decolourisation of azo dyes by Fenton reaction (pH 3.0-4.0) was achieved by acidifying an aqueous solution with HCl, the oxidation reaction proceeded very rapidly and up to 90% decolourisation could be obtained within 2 hours. It has been reported that during the reaction, hydrogen peroxide (H_2O_2) is rapidly consumed due to which replenishment of H_2O_2 becomes necessary (Li, 2007). This need for dosing is often seen as a disadvantage which limits the application of the Fenton process. However, if the pH 3.0 of the reaction mixture was maintained using an appropriate citrate buffer, the same decolourisation was achieved after 12-16 hours. Experimental evidence indicates that the presence of a chelating agent like citrate can lower the oxidation rate by limiting the availability of the iron ions (Li, 2007). This observation led to the question whether a greater availability of Fe ions could increase the extent of oxidation of azo dyes by a heme containing enzyme like horseradish peroxidase (HRP). Adding Fe-EDTA to the reaction mixture, provided additional Fe^{3+} for oxidation of substrate in a controlled way without causing precipitation of Fe $(OH)_3$ even at pH 9.0. Thus, the rapid consumption of H_2O_2 was prevented and the decolourisation reaction could be performed at relatively higher pH, without precipitate formation.

The mechanism by which horseradish peroxidase can reduce H_2O_2 to •OH, and ultimately to H_2O in the presence of a suitable reducing agent (Chen and Schopfer, 1999) is similar to the mechanism for free or chelated Fe^{3+} ions in the Fenton-like process (Abo-Farah, 2010).The apparent similarity between the Fenton reaction and the catalytic cycle of horseradish peroxidase (HRP) was the starting point for the experiments in the present work. The Fenton reaction uses an iron salt along with hydrogen peroxide to bring about the oxidation of a variety of electron rich molecules while HRP, being a heme containing oxidative enzyme, uses the heme centre as the site of catalysis for similar reactions. On examining the structure of the enzyme HRP, it is seen that in the resting state, the Fe^{3+} of the heme catalytic centre, is essentially chelated by the tetradentate Protoporphyrin IX (Veitch, 2004). Ferric-EDTA (Fe-EDTA) is an easily available chelated form of Fe^{3+} that is similar to the heme Fe^{3+} in the peroxidase. It has been shown that a complex formed by the interaction of hydrogen peroxide and ferric EDTA can oxidise organic substrates like the enzyme peroxidase (Walling *et al.*, 1975). The oxidation of azo dyes using a combination of Ferric-EDTA and hydrogen peroxidase has also been reported (Nam *et al.*, 2001). Increasing the amount of enzyme in the reaction mixture would increase the decolourisation up to a certain extent, after which the availability of substrate (dye) or co-substrate (H_2O_2) would become a limiting factor (Ambatkar and Mukundan, 2014). Since, Fe-EDTA seems to mimic the action of HRP, adding it to the reaction mixture, is similar to increasing the amount of enzyme without actually adding more enzyme to the reaction mixture. As a consequence of adding Fe-EDTA, a greater number of sites of oxidation are available, resulting in greater decolourisation until the availability of either dye or co-substrate limited the progress of the reaction.

Effluent treatment using crude enzymes is gaining importance, since the use of pure enzymes is prohibitively expensive for large scale reactions. Crude enzymes may sometimes suffer from the drawback that the desired enzyme is present in a consortium of enzymes, and at a relatively lower concentration. A combination of crude HRP and Fe-EDTA can overcome the limitations of low concentration of enzyme. The addition of a complex iron salt like Ferric EDTA was found to enhance the decolourisation of Methyl Orange, Bismarck Brown at pH 6.0 and Tartrazine at pH 9.0 by 8.69%, 14.17% and 1.58% respectively. The greater availability of Fe (III) ions probably explains the improved activity of crude peroxidase in the presence of Fe-EDTA and the increased decolourisation of MO, BB and TZ by crude peroxidase in the presence of Ferric-EDTA (as compared to the non-Fe-EDTA controls). At pH 6.0, the activity of the enzyme (towards guaiacol) is highest (**Figure b**). The activity of the enzyme increases with the addition of Fe-EDTA up to 0.23 mmol L^{-1} (**Figure c**) after which, it decreases. It is likely that at concentrations of Fe-EDTA higher than 0.23 mmol L^{-1}, the peroxidase activity decreases due to the increase in the Fe/H_2O_2 ratio which can adversely affect the progress of the oxidation reaction in acidic conditions.The relative concentrations of Fe ions and H_2O_2 in the Fenton reaction are important (Li, 2007; Barbusński and Filipek, 2009).

As mentioned earlier, the activity of crude peroxidase is relatively lower at pH 9.0 than within the range of pH 5.0 to 8.0. Some pilot studies with a recalcitrant azo dye, Tartrazine however showed that the decolourisation obtained at pH 9.0 was greater than at lower pH. The effect of Fe-EDTA on the decolourisation of Tartrazine was observed. After 24 hours of the reaction, the decolourisation of Tartrazine obtained by the set containing Fe-EDTA was significantly higher than that of the control set. The results show that the addition of Fe-EDTA enhances the activity of the crude enzyme even outside the optimum pH range for azo dye decolourisation. The complex formed by Fe-EDTA and H_2O_2 has been found to show high peroxidase activity near pH 9.0 (Walling *et al*. 1975). This probably explains the linear increase in activity with the increasing concentration of Fe-EDTA at pH 9.0 (**Figure d**).The enzyme control set, i.e. the one which contained only Fe-EDTA and H_2O_2 initially showed lower decolourisation than the set with Fe-EDTA, enzyme and H_2O_2. Eventually, the enzyme control set, showed decolourisation comparable to that of the Fe-EDTA, enzyme and H_2O_2 set. This can be explained by the fact that after about 18 hours, the enzyme tends to get deactivated or even denatured causing no further decolourisation of the substrate dye.

The decolourisation of azo dyes by Fenton and Fenton-like reaction occurs optimally in the range pH 3.0-4.0. Azo dyes have been reported (Maddhinni *et al*., 2006) to be decolourised effectively by HPR in the pH range 3.0-4.0. The effluent treatment research is being directed towards being able to modify existing methods to work in neutral and near-neutral conditions (Li, 2007). Hence, the decolourisation-enhancing effect of Ferric-EDTA on crude peroxidase at pH 6.0 is a significant finding. In the course of surveying scientific literature related to the present work, it appears that the present work is the first report of augmentation of peroxidase activity using a chelated Ferric salt. The use of Fe-EDTA as an additive in the enzymatic decolourisation of azo dyes can reduce the amount of enzyme required to achieve decolourisation. The findings from the present work have application in the enzymatic decolourisation of azo dye containing effluents, where the amount of oxidative enzyme utilised, needs to be reduced to make the treatment more cost effective. Further work on optimising reaction conditions and reducing the reaction time for the peroxidase mediated decolourisation of azo dyes using Fe-EDTA as an additive can provide a relatively inexpensive effluent treatment method that is environmentally more acceptable than conventional methods.

4.0 Conclusion:

From the findings of the present study, we can conclude that the additive Fe-EDTA mimics the oxidative action of the enzyme Horseradish Peroxidase. The use of Fe-EDTA enhances the HRP mediated decolourisation of the tested azo dyes – Methyl Orange, Bismarck Brown and Tartrazine. Fe-EDTA enables the enzyme to decolourise the dyes outside the optimum pH range reported for enzymatic decolourisation of azo dyes. The use of Fe-EDTA can help to reduce the amount of enzyme required for a large scale application like wastewater treatment and has the potential to make it a more cost effective process.

5.0 Acknowledgement:

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