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Removal of reactive azo dyes from water by zero-valent iron reduction followed by peroxidase-catalyzed polymerization.

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REMOVAL OF REACTIVE AZO DYES FROM WATER BY FE^0 REDUCTION FOLLOWED BY PEROXIDASE-CATALYZED POLYMERIZATION

By

Mousumi Mani Biswas

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Faculty of Graduate Studies and Research through the

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ABSTRACT

Removing reactive azo dyes from textile wastewater is a significant challenge due to their color, non-biodegradability and toxicity. Although various treatment methods are available, it was hypothesized that reduction by zero-valent Fe followed by enzymatic treatment could be an environmentally friendly and cost effective approach. Zero-valent Fe cleaves the azo bond, reducing the dyes to aromatic amines, which are then oxidized and polymerized by enzymatic treatment. Finally, these polymers are removed by coagulant (PEI) aided sedimentation and filtration. The effectiveness of zero-valent Fe and *Arthromyces ramosus* peroxidase (ARP) treatment in the proposed process were studied on two representative reactive azo dyes, Reactive Red 2 (RR2) and Reactive Black 5 (RB5). Over 97 % of the dyes and about 80% (for RB5) to 89% (for RR2) of dye breakdown products were removed in 5.5 hours by these three steps. Optimum reaction conditions for the enzymatic treatment were determined to be neutral pH for RR2, pH 5.5 to 6.5 for RB5, enzyme to substrate ratio of 9 U/mL : 1 mM for both the dyes, and H_2O_2 to substrate ratio of 6 : 1 for RR2 and 9 : 1 for RB5. The optimum coagulation conditions were: pH 6.0 to 7.0 for RR2, pH 4.8 to 6.0 for RB5 and 200 to 250 mg/L PEI concentration with 100 mg/L alum. The final products were colourless, transparent solutions having low residual UV-vis absorbance. To gain insight into ARP action on these two dyes, two model compounds, diphenylamine (DPA) and 2-amino-8-naphthol-3,6-disulfonic acid (ANDSA) were studied. Fe^0 treatment was ineffective in breaking – NH- bond present in DPA, while ARP could oxidise the substituted napthol amine with – OH, and $-NH_2$ functional groups and secondary amine with an –NH- bond in DPA. The optimum enzyme concentration was 4 U/mL for 1 mM of ANDSA and 2 U/mL for 1 mM

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of DPA. H_2O_2 to substrate ratio for ANDSA was 2.4 : 1 and 1 : 1 for DPA. A comparison with other treatment methods asserted the superior advantage of the proposed process in terms of actual pollutant and colour removal.

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DEDICATION

To my parents

Suryakanta & Renuka

for their blessings

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CONTENTS

LIST OF NOMENCLATURE

Abbreviations

4-AAP	4- aminoantipyrine
ANDSA	2-amino-8-naphthol-3,6 disulfonic acid
AOP	advanced oxidation process
AQDS	anthraquinone 2-6 disulfonate
ARP	*Arthromyces ramosus* peroxidase
BOD	biochemical oxygen demand
CiP	*Coprinus cinerus* peroxidase
CMP	*Coprinus macrorhizus* peroxidase
COD	chemical oxygen demand
DPA	diphenylamine
EGSB	Expanded Granular Sludge Blanket Reactor
HRP	horseradish peroxidase
Lip	lignin peroxidase
MnP	manganese peroxidase
PBR	Packed Bed Reactor
PEI	polyethyleneimine
RB5	Reactive Black 5
RR2	Reactive Red 2
SBBR	Sequential Batch Biofilm Reactor
SBP	soybean peroxidase
TNBS	trinitrobenzenesulfonic acid
TOC	Total Organic Carbon
UASB	Upflow Anaerobic Sludge Blanket Reactor
UV	Ultra-Violet

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CHAPTER 1: INTRODUCTION

1.1   Background

Removal of reactive azo dyes from textile industry effluents is a significant challenge in industrial wastewater treatment. Even as low as 1 mg/L of dye renders visible color in the discharged water which is unacceptable for aesthetic reasons (O'Mahony et al., 2002). Colour also affects the photosynthetic activity and gas solubility of the aquatic ecosystem (Robinson et al., 2002). Dyes are toxic to the aquatic environment, and dye degradation products, the aromatic amines, are carcinogenic and mutagenic to human beings (Van der Zee et al., 2002; Gottlieb et al., 2003). According to the Canadian Environmental Protection Act (CEPA), 1999, textile mill effluents are classified as toxic under "Second Priority Substance List ", for which "risk management strategies have to be developed and implemented". Environmental regulations of other developed countries also require removal of color and dye compounds from industrial effluents (EU directive 91/271) (Robinson et al., 2002).

Around 7 x 10^5 metric tons of synthetic dyes are being produced every year worldwide out of which 5-10 % are discharged with the effluents (Yu, 2001). About 60-70% of all commercial dyes are azo dyes containing the azo (-N=N-) bond (O'Neill et al., 2000). The textile industry is the largest consumer of dyes and reactive azo dyes constitute 45% of the total textile dye consumption (Arslan-Alaton, 2003). With the increasing use of cotton fiber (50% of total world fiber consumption), reactive azo dyes have become the fastest growing class of cellulose dyestuff. Their bright color, good fastness property, simple and less energy intensive application make them very popular

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(O'Mahony et al., 2002). However, these dyes require 10 times more water during the application process and are readily hydrolyzed without complete fixation. The fixation rate of reactive azo dyes is the lowest (less than 75 %) among the dyestuffs (Table A.2.4) (Arslan-Alaton, 2003). Consequently, a large quantity of reactive azo dyes is discharged with effluents.

1.2   Current Color Removal Methods

The most commonly used color removal methods are physical (adsorption, filtration, flotation), chemical (coagulation, oxidation, reduction, electrolysis) and biological (aerobic, anaerobic) (Banat et al., 1996). These methods are not entirely satisfactory in terms of cost, efficiency and environmental impact (Robinson et al., 2001). A summary of these processes is presented in Tables A.1.1, A.1.2 and A.1.3.

Physical methods used in removing color are not satisfactory. Adsorption by activated carbon, a commonly used physical method, is expecnsive due to high cost of the regeneration of the media (Robinson et al., 2002). Nanofiltration and reverse osmosis can only be used as pretreatment of textile wastewater, whereas ultrafiltration and microfiltration can be used as selective pretreatment (Van der Zee, 2002). All these processes require post treatment of the effluent and the sludge and therefore, are not cost effective.

Chemical methods like coagulation and flocculation are often used for removing color (Nemerow, 1978). However, the large quantity of sludge generated by this process is a serious drawback (Van der Zee, 2002). Though the color is removed from water, the unchanged dye molecule can still be present in the sludge. Organic polymers (cationic,

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anionic) can remove color effectively with lower amount of sludge. However, these polymers present in the sludge are toxic to aquatic life even at very low concentrations (Van der Zee, 2002). Thus, disposal of the sludge remains an environmental problem. Therefore, these chemical methods are not very environmentally friendly.

Advanced oxidation processes (AOP) also have some limitations. Ozonation leads to complete decolorization; however, the reaction products, especially aldehydes, are toxic (Aarslan-Alaton, 2003). Fenton's process requires low pH (2-5), which is not suitable for highly alkaline textile effluent (Van der Zee, 2002). Photocatalytic processes using UV are not successful for highly colored wastewater (Van der Zee, 2002). Photocatalytic processes using ZnO and TiO_2 result in total decolorization and mineralization (Peralta-Zamora et al., 1999). All, these AOP require high initial investment and input energy.

Biological treatment under aerobic conditions is not suitable to degrade stable and long lasting reactive azo dyes (Van der Zee et al., 2002; Robinson et al., 2001). Azo dyes are inherently resistant to aerobic treatment because oxygen is a more effective electron acceptor than azo dyes (Stolz, 2001).

However, azo bonds in these dyes are susceptible to reductive fission under anaerobic conditions (Beydilli et al., 1998). Such breakdown generates aromatic amines, which are carcinogenic; therefore, disposal of these by-products still remains an environmental problem. These amines can be degraded aerobically. Therefore a two-step process constituting anaerobic treatment of the dyes followed by an aerobic degradation of amines can be a potential treatment process for azo dyes. Studies demonstrated that anaerobic treatment could remove up to 97 % of color and 60 % of COD and a

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subsequent aerobic treatment can remove an additional 30 % COD by removing aromatic amines (Delee et al., 1998).

Zero-valent iron, Fe^0, is an effective reducing agent for azo dyes (Nam and Tratnyek, 2000; Cao et al., 1999). It is abundant and inexpensive, making the treatment process cost effective and environmentally friendly. Zero-valent iron reduction is used widely for dechlorination of contaminated groundwater. It has also been successfully used to initiate remediation of more complex chemicals like pesticides (DDT, DDD, Alachlor, Metolachlor, etc) (Sayles et al., 1997).
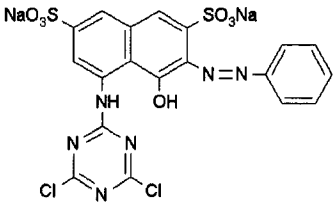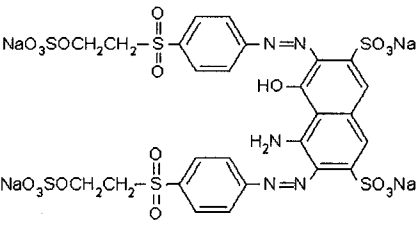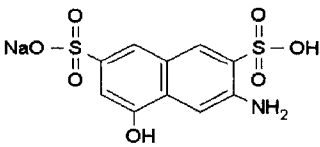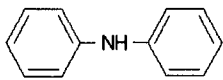
Peroxidase enzymes can successfully catalyze removal of toxic compounds like phenols and anilines from wastewater (Klibanov et al., 1980; Karam and Nicell, 1997; Duran and Esposito, 2000). Horseradish peroxidase (HRP) in the presence of hydrogen peroxide can remove 90 % of various phenols, naphthols and aromatic amines from synthetic wastewater (Klibanov et al., 1980). In the presence of hydrogen peroxide and enzyme, phenols and amines form phenoxyl and anilinium cation radicals, respectively, which further dimerise and polymerize and precipitate. Several other peroxidases like *Arthromyces ramosus* peroxidase (ARP) (Ibrahim et al., 2001), *Coprinus macrorhizus* peroxidase (CMP) (Al-Kassim, et al., 1994), Soybean peroxidase (SBP) (Caza et al., 1999; Mantha, 2001), *Coprinus cinerus* peroxidase (CiP), (Masuda et al., 2001) have shown good potential in removing different pollutants from real and synthetic wastewater. However, substituted phenols and anilines, which give colored products, may be difficult to remove from water by enzymatic treatment alone. Different coagulant aids, polycationic coagulant like chitosan and PEI, have been used for removal of these products from water after enzymatic reaction (Wada et al., 1995).

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1.3 Proposed Treatment Process

A treatment process constituting anaerobic reduction of azo dyes using zero-valent Fe followed by aerobic treatment of aromatic amines using peroxidase enzyme was studied. The enzymatic reaction byproducts were removed by coagulation and filtration using a coagulant aid.

This work investigated the effectiveness of *Arthromyces ramosus* peroxidase (ARP) in the proposed process. Two representative reactive azo dyes, Reactive Red 2 (RR2) and Reactive Black 5 (RB5) were considered for this study. These two dyes are widely consumed in the textile industry and have been studied for various degradation processes (Table A1.2 and A1.3). The molecular structures of these two dyes are presented in Table 1.1 and their physical properties are presented in Table A 3.1

Table 1.1 : Structures of Reactive Azo Dyes and Model Compounds

Reactive Red 2	
Reactive Black 5	
Model Compound : 2-amino-8-naphthol-3, 6-disulfonic acid	
Model Compound : Diphenylamine	

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1.4   Research Objectives

The primary objectives of this research were, to:

- Establish the effectiveness of zero-valent Fe in reducing two representative reactive azo dyes, Reactive Red 2 (RR2) and Reactive Black 5 (RB5).

- Evaluate the efficacy of *Arthromyces ramosus* peroxidase (ARP) to remove the Fe^0 reduction products, the aromatic amines.

- Remove the enzymatic treatment end products by using a suitable coagulant.

- Determine the optimum process parameters (pH, enzyme concentration, hydrogen peroxide to substrate ratio and reaction time) at room temperature, for these steps.

1.5   Supporting Studies

Some additional studies were carried out to gain insight on ARP reactive properties on the zero-valent Fe breakdown products of RR2 and RB5, which are substituted napthol amines. An exploratory study was conducted with two model compounds: (1)   2-amino-8-naphthol-3,6-disulfonic   acid   (ANDSA)   and   (2) diphenylamine (DPA) (refer Table 1.1), to get a better understanding on how ARP catalyzes the oxidation of RR2 and RB5 breakdown products. ANDSA has a naphthalene ring with all three functional groups present, whereas DPA is a secondary amine with the –NH- bond as in RR2.

This investigation included the following studies to:

- Ascertain whether zero-valent Fe reduces the –NH- bond in RR2 to create smaller molecules where naphthalene and triazene rings get separated out. The effect of

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Fe on –NH- bond in diphenylamine (a model compound) was studied. This indicated that in the RR2 breakdown product, the triazine ring is still attached to the napthol group (by the –NH- bond) making it indeed a big molecule.

- Appraise the effect of ARP to oxidize the –NH- bond in the RR2 breakdown product.

- Determine whether substituted napthol amines with all three functional groups (–OH, -NH$_2$ and –SO$_3^-$) can be removed by ARP. The oxidation kinetics of ANDSA, having similar structure as the RR2, RB5 breakdown products, in presence of ARP were studied.

1.6 Scope of Study

The scope of study was:

- Preliminary evaluation of the proposed two-step process on two dyes, Reactive Red 2 and Reactive Black 5 in laboratory scale batch reactors in synthetic wastewater. The synthetic wastewater constitutes of the dye solution only.

1.7 Organization of the Thesis

This thesis is organized as follows:

Chapter 2, *Literature review* provides the theoretical understanding of zero-valent Fe reduction, peroxidase enzymatic action and the critical process parameters that are involved. This chapter also anticipates some experimental results.

Chapter 3, *Materials and Methods* documents the materials used and analytical techniques adopted during various experimental studies.

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Chapter 4, *Results and Discussion* collates the observations, discusses the research findings.

Chapter 5, *Conclusions and Recommendations* summarizes the research findings indicates avenues for further explorations.

Chapter 6, *Engineering Implications, Comparative Study and Contributions* discuss application related issues and the competitiveness of the proposed process compared to other processes.

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CHAPTER 2 : LITERATURE REVIEW

The present work proposes dye removal from water in three steps. The first step involves reduction of the dye molecules by anaerobic zero-valent iron treatment, the second step utilizes a peroxidase to treat the products formed during the iron treatment and the last step precipitates and separates out the enzymatic reaction products from water. This chapter presents the literature review on reaction mechanisms and process parameters for all three treatment steps involved. The discussion attempts to derive some insights on the expected outcomes of these treatment processes for the two selected dyes, RR2 and RB5.

2.1 Zero-valent Iron Treatment

Zero-valent metals are strong reducing agents. They have been used for synthesis of organic amines from nitroaromatic compounds. Application of zero-valent iron (Fe^0) in the environmental remediation area started with organic dechlorination of contaminated groundwater under anaerobic conditions (Johnson et al., 1998). With increasing understanding of the process mechanism, more and more recalcitrant organic compounds such as, DDT, DDE, various pesticides and herbicides like alachlor, atrazine, etc, and azo dyes are being treated by zero-valent iron under anaerobic conditions (Sayles et al., 1997; Eykholt and Davenport, 1998; Nam and Tratnyek, 2000). Successful reduction of a wide variety of compounds by Fe^0 under anaerobic conditions has proved its effectiveness for wastewater treatment.

2.1.1 Mechanism

Fe^0 reduction under anaerobic conditions is a surface-mediated "electrochemical corrosion process, which takes place in several steps" (Choe et al., 2001; Weber, 1996).

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In the first step (mass transfer reaction) the target organic compound comes in contact with the Fe metal surface and gets adsorbed on it. In the next step (chemical reaction), the electron transfer takes place to oxidize the iron metal and reduce the substrate organic compound. The second step is followed by another mass transfer reaction where the reduced product gets desorbed from the metal surface. The anodic and cathodic half reactions takes the following pathway :

$$Fe^0 \rightarrow Fe^{2+} + 2e^- \dots\dots\dots\dots\dots\dots\dots\dots\dots (2.1)$$

$$H_2O \rightarrow H^+ + OH^- \dots\dots\dots\dots\dots\dots\dots\dots (2.2)$$

$$RX + 2e^- + H^+ \rightarrow RH + X^- \dots\dots\dots\dots\dots\dots (2.3)$$
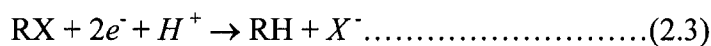
Where "R" represents the aromatic group, and "X" represents halogen group.

The associated cathodic reaction may vary depending on the reactivity of the electron acceptor and their redox potential. The oxidation potential of $Fe^0 \rightarrow Fe^{2+}$ system is -0.44 V. The aqueous chemistry may affect the actual potential. If the reduction potential of an organic compound is greater than -0.44 V, it will get reduced by the Fe metal (Davenport, 1996).

The following alternate reaction path is also possible:

$$2H_2O + 2e^- \rightarrow OH^- + H_2 \dots\dots\dots\dots\dots\dots\dots (2.4)$$
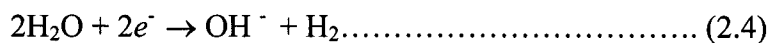
where the H_2 reduces the adsorbed substrate under catalytic presence of Fe surface in the Fe- H_2O system (Choe et al., 2001).

Since Fe^0 oxidation is a surface-mediated process, it is suitable for reduction of water-soluble compounds. However, for the hydrophobic compounds like pesticides, herbicides, etc., electron mediators such as quinones, natural organic matter (NOM) and

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porphyrins can be used to mediate the reduction process (Weber, 1996; Tratnyek et al., 2001).

The iron surface contains both reactive and non-reactive sites. Even for reactions with higher intrinsic rate, the observed rate may be lower because only a portion of the organic substrate molecules will get a chance to bind with the reactive sites (Gotpagar et al., 1999). Thus the rate of reaction depends on fractional reactive site (ratio of reactive sites to total sites) available for the reaction. A freshly prepared and cleaned iron surface can reduce the reaction induction period (reaction time delay) (Lavine et al., 2001). Reactive sites and reduction rate can be increased by using ultra-fine, nano-scale metal particles (Choe et al., 2000), continuous surface cleaning and by acid pretreatment. Pretreatment using hydrochloric acid increases the iron surface by a factor of 7.6; however, there can be a loss of 4.9 % of Fe during acid wash. Continuous mixing also showed better reaction rate (Agrawal and Tratnyek, 1996). Ultrasonic cleaning increased surface by 169 % (Geiger et al., 2002).

In the past, when Fe^0 was applied to treat contaminated ground water, it was observed that anaerobic conditions favored the reduction process. Anaerobic conditions can be maintained in the laboratory by degassing or by using oxygen scavengers like Na_2SO_3 or $FeSO_4$ (Mantha, 2001). Under anaerobic conditions, zero-valent iron (Fe^0) reduces nitroaromatic compounds to the corresponding amines. Formation of other intermediate compounds like nitroso or hydroxylamine is insignificant (Agrawal and Tratnyek, 1996). The overall reaction mechanism for nitroaromatic compound degradation takes place according to the following equation:

$$3Fe^0 + ArNO_2 + 6H^+ \rightarrow 3Fe^{2+} + ArNH_2 + 2H_2O \dots\dots\dots\dots\dots (2.5)$$

where Ar represents the aromatic group.

Theoretically, 3 moles of Fe are required for every mole of aromatic compound since the reaction stoichiometry is 3:1. Oxidation of Fe is the major side reaction. Re-oxidation of intermediates can cause more Fe to be consumed. Hence 5 to 6 atoms of Fe may be required to reduce a nitro-group instead of the stoichiometric requirement of 3 atoms (Lavine et al., 2001). Thus, to compensate for the iron loss due to acid wash and other side reactions, an excess of iron over the stoichiometric amount is required for the completion of reaction.

Fe degradation of azo dyes by cleaving of azo bonds takes places in two steps, of which the first step is reversible (Cao et al., 1999). Hence, some of the intermediate products (hydrazo, -NH-NH-) from the first step may return to the original compound.

2.1.2 Effect of pH and Choice of Buffer

The pH affects the reduction efficiency. A lower pH improves Fe reduction efficiency (higher yield and lower reaction time) as more H^+ are available for the reaction (Deng et al., 2000). Reduction product (corresponding amines) recovery also depends on the pH. Some of these reduction products (amines) are carcinogenic, requiring recovery followed by an appropriate removal / treatment process. When Fe^0 reduction was used to reduce nitrobenzene, complete conversion of nitrobenzene to aniline was observed at pH greater than 5.0. However below pH 5.0, no aniline was detected in the solution. This may be due to the protonation of aniline (pKa = 4.6) which prevented desorption of the product from metal surface (Agrawal and Tratnyek, 1996; Mantha et al., 2001). Therefore aniline adsorption must be avoided by operating at a higher pH range if possible.

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Carbonate and acetate anions present in the carbonate or acetate buffers compete with the substrate in a Fe^0 reduction process. Carbonate anions bind more strongly to the iron surface compared to acetate anions, therefore carbonate buffers slow down the substrate reduction when used to control the pH (Lavine et al., 2001). Therefore appropriate choice of buffer is important for efficient reduction.

2.1.3 Effect of Substrate Structure

Structure of the compound and the presence of various substituent groups affect the redox potential of the compound, which determines the intrinsic Fe^0 reduction rate. Groups with higher electron affinity pose a greater barrier for the electron transfer to take place (Davenport, 1996). For example, though dechlorination of substrate is expected during Fe treatment, not all compounds get dechlorinated. Such reaction depends on the structure of the compound. In s-chlorotriazine herbicides, the reduction potential of the C-X bond (X is halogen group) is in the order of -1 to -3, which is far too negative for Fe to affect. As a result, these herbicides could not be dechlorinated by iron metal (Davenport, 1996). In case the Cl^- ions are released due to dechlorination, these halide ions clean and enhance the pitting of the Fe surface by breaking the Fe-oxide layers. This cleaning and pitting increases the number of Fe active sites and autocatalyses the reduction (Gotpagar et al., 1999). A similar situation might happen with RR2 dye. No good correlation was obtained between decolorization by Fe and the dye molecular structure (Nam and Tratnyek, 2000).

Sulfonated compounds when treated with Fe^0, may get adsorbed on Fe^0 surface via the sulfonic group by forming a bridged bidentate complex (Bandara et al., 1999; Roy et al., 2003). The presence of a large number of bulky sulfonate groups may adversely

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affect the reduction potential of the azo bond due to steric hindrance, thus preventing the dye molecule from approaching the Fe^0 surface closely. However larger amount of Fe^0 with more active sites can compensate for this hindrance (Bandara et al., 1999). This means, higher amount of Fe^0 may be required for dye molecules with more sulfonate groups.

2.1.4 Reaction Products and Yield

The azo bond is cleaved when azo dyes are degraded by Fe^0. Aromatic amine and amino-naphthol compounds are formed along with hydrazo (-NH-NH-) as an intermediate. Stoichiometric amounts of aromatic amines were detected for small dye molecules like Orange II (Nam and Tratnyek, 2000). Some dye molecules may also be adsorbed on the Fe^0 surface, which may result in an apparently incomplete mass balance. Product yield increases with the rate of mixing (Nam and Tratnyek, 2000). This is also consistent with the observation that the rate of dye degradation was proportional to the available Fe surface area (Cao et al., 1999). Similar results were also obtained when nitroaromatic compounds were treated with Fe^0 (Westerhoff and James, 2003). It was postulated that incomplete mass balance of nitroaromatic compounds to aniline might be due to sorption of nitroaromatic compounds and ammonium ion on the iron surface, production of unmonitored nitrogen oxide gas species, like NO_2, N_2O and formation of unstable intermediates which may not be present in the solution phase.

2.1.5 Corrosion Products

Iron hydroxides are formed due to anodic reaction. The resulting Fe^{2+} and Fe^{3+} resides in three states – (i) hydrated or complexed in solution, (ii) precipitated as solid or (iii) adsorbed in oxide layer. The green brown precipitate which is formed is a mixture of

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Fe $(OH)_2$ and $Fe(OH)_3$ but gradually they evolve to form Fe_3O_4 and γ-Fe_2O_3 (Johnson et al., 1998). The presence of iron oxides and other oxides passivate the iron surfaces and slow down the reduction process.

2.1.6 Reaction Inhibitors

Inhibition of the iron surface is a common hindrance during Fe^0 treatment. Other than the Fe-oxides (anodic side reaction products), there are different factors which may also inhibit the Fe surface. Non-reactive adsorbates commonly found in soil, such as catechol, ascorbate, acetate and EDTA, were observed to compete with the substrate for Fe reactive sites (Johnson et al., 1998). The presence of such compounds may slow down the reduction process.

Ethanol is often used to dissolve water insoluble compounds, during their remediation. The presence of ethanol reduces the sorption of organic substrates on to metal surface and hence reduces the rate of Fe^0 reduction process (Clark II et al., 2002).

2.1.7 Zero-valent Iron Treatment Discussion Summary

Based on the above discussion, the following inferences can be drawn:

- Fe^0 treatment is suitable to reduce water soluble compounds; however, to reduce hydrophobic compounds, electron mediators will be required.

- Fe^0 reduction can cleave the azo bonds in anaerobic conditions giving rise to aromatic amines.

- Fe^0 pre-treatment, proper mixing, and solution pH are important for the reduction. Iron in excess iron over the stoichiometric requirement may be needed. More iron will be required to reduce compounds with more sulphonate groups.

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- Stoichiometric production of aromatic amines may not be observed in the reaction mixture due to sorption on the Fe surface.

- Reaction mixtures may contain Fe^0 corrosion products along with aromatic amines.

- The aromatic amines may re-oxidize to produce colored substances, which will require further treatment.

2.2   Enzymatic Treatment

Enzymes are the key components used by microorganisms to degrade various chemical compounds when they are used to treat wastewater (biological processes). In enzymatic treatment, an isolated enzyme is used instead of the whole microorganism. Enzymes are highly specific biological catalysts. In recent years, considerable research has been carried out to apply enzymes for industrial wastewater treatment. The motivations behind this growing importance of enzymatic treatment are: (i) conventional chemical and biological treatment processes are not successful in achieving the required degree of pollutant removal; (ii) enzymes can remove specific pollutants; (iii) cheaper enzymes are available due to advances in biotechnology and cheaper purification and extraction processes (Karam and Nicell, 1997).

Significant advantages of enzymatic treatment over conventional physical, chemical and biological treatment processes are (Taylor et al., 1998) :

- Minimum environmental impact
- Application to a broad range (but specific type) of compounds
- Operation under wider pH and temperature range
- No delay associated with biomass acclimatization

- Reduced sludge volume
- Simple process control
- Small footprint
- Less reaction time

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- Operation under mild and less corrosive conditions
- Treatability of very dilute concentration
- Low capital cost
- Less energy consumption

The usual disadvantage associated with enzymes is inactivation but this can be improved by using the immobilized enzyme. Though enzymes are costly due to their production costs, however, bulk production of enzymes from a cheap source will certainly overcome this cost factor (Karam and Nicell, 1997).

2.2.1 Choice of Peroxidase Enzyme

Peroxidases are oxidoreductases. They catalyse the oxidation of a wide range of electron donors in the presence of hydrogen peroxide. Peroxidase was chosen for the study because : (i) their success in oxidising large complex molecules has been well established; (ii) they have been researched extensively, hence their mechanisms and application processes are well understood and documented; (iii) there is a possibility of economy of scale in production, because they have variety of applications such as, pulp and paper bleaching, soil remediation, on-site waste destruction, wastewater treatment, biocatalysis, etc., and their wide presence among living organisms. Enzymatic treatment, using heme peroxidases, such as, HRP (horseradish peroxidase) ARP (*Arthromyces ramosus* peroxidase), SBP (soybean peroxidase) have been successful in removing toxic aromatic compounds such as, phenol, aniline, substituted phenols and anilines, naphthols, benzidines, biphenols and related heteroaromatic compounds from wastewater (Klibanov et al., 1980; Taylor et al., 1998).

Application of horseradish peroxidase (HRP) in wastewater treatment has been very well researched. *Arthromyces ramosus* Peroxidase (ARP) was selected for

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enzymatic treatment in the present work because of certain advantages over other peroxidases. Ease of production of ARP makes economic bulk production a possibility. Hypomycete *Arthromyces ramosus* (fungi imperfecti) produces large quantity of this extra-cellular enzyme. Also, treatment cost with ARP was found to be aboutone fourth of that with HRP for removal of 2 mM phenol from refinery wastewater (Ibrahim et al., 2001). Other significant advantages are : higher turnover capacity similar to HRP while having broad substrate specificity for hydrogen donors as HRP (Villalobos and Buchanan, 2002). The structure of ARP also allows aromatic amines and naphthol amines to suitably bind to its active site. Aromatic amines and naphthol amines are expected Fe breakdown products of azo dyes.

ARP is a monomeric glycoprotein with molecular mass of 41 kilodalton. It shows the highest sequence similarities (43 % and 41%) with lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymes from *P. chrysosporium*. The heme group occupies a crevice between two large domains very similar to LiP and MnP (Nakayama and Amachi, 1999). ARP has an exposed heme edge (Smith and Veitch, 1998; Tsukamoto et al., 1999), that makes it suitable for bigger molecules. Other properties of ARP are presented in Table A.4.

2.2.2 Reaction Mechanism

The peroxidase reaction mechanism is given below (Nakayama and Amachi, 1999):

$$E\ [Fe(III)] + H_2O_2 \xrightarrow{k_1} Compound\ I\ [Fe\ (IV)^{\bullet}\ ] + H_2O\ ...(2.6)$$

$$Compound\ I\ [Fe\ (IV)^{\bullet}\ ] + AH_2 \xrightarrow{k_2} Compound\ II\ [Fe\ (IV)] + AH^{\bullet}\ ....(2.7)$$

$$Compound\ II\ [Fe\ (IV)] + AH_2 \xrightarrow{k_3} E\ [Fe\ (III)] + AH^{\bullet} + H_2O\ ....(2.8)$$

$$2\ AH^{\bullet} \xrightarrow{k_4} A + AH_2\ (or\ A_2H_2)\ ....(2.9)$$
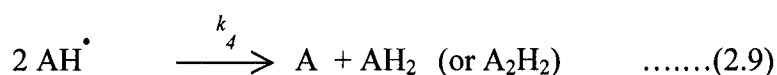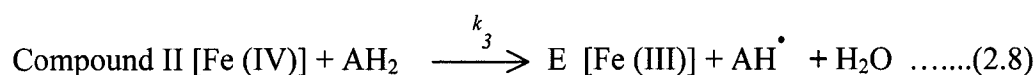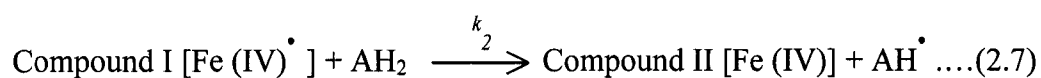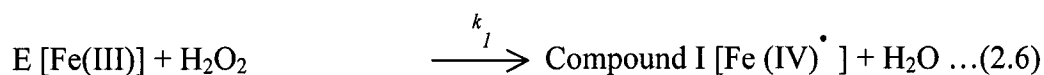
where "A" represents an aromatic compound and $^{\bullet}$ indicates a radical

This shows a generally accepted mechanism of peroxidase-catalyzed oxidation. Hydrogen peroxide removes two electrons from peroxidase, yielding a highly oxidized state, Compound I. Compound I then undergoes two successive one-electron reductions by AH_2. First, it gets reduced to Compound II and a free radical after reacting with one aromatic molecule, AH_2. Compound II and I differ only by one electron on the porphyrin ring. Another AH_2 molecule subsequently reacts with Compound II by adding a second electron to it and yielding native peroxidase. The free radicals then combine to form AH_2 and an oxidation product A, or dimerisation of the free radicals leads to formation of A_2H_2 (Nakayama and Amachi, 1999).

When an amine phenol mixture is the substrate, along with the peroxidase catalysed co-oxidation, a non-enzymatic exchange reaction between phenoxy and aminyl radical also takes place, as:

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$$PhO^\bullet + AmNH_2 \Leftrightarrow PhOH + AmNH^\bullet \quad \dots\dots\dots\dots\dots (2.10)$$

Where PhOH & $PhO^\bullet$ are phenol compound and phenoxyl radical, $AmNH_2$ and

$AmNH^\bullet$ are amine compound and amine radical.

Depending on the reactivity of phenol and amine with enzyme, the forward or backward reaction may be favoured and amine or phenol, the initial substrate, may be regenerated (Karasyova et al., 2001).

2.2.3 Effect of pH

Enzymes carry out the best substrate conversion at a certain pH. ARP showed better performance in the pH range 5.0 to 8.0 depending on the type of hydrogen donor used. k_1 and k_2, the rate constants for formation of Compound I and Compound II, depend on pH. k_1 remains constant in neutral and basic pH but decreases in acidic range. k_2 and k_3, the rates of reduction of Compounds I and II are maximum at pH 8.0 and decrease with higher pH. Usually, for peroxidases, k_2 is at least 10 times bigger than k_3 but for ARP they are equal at pH 5.0 to 6.0. This may be because of unusually high reduction potential of Compound II. This indicates that ARP can be active even at lower pH as 5.0 (Nakayama and Amachi, 1999).

2.2.4 Reaction Stoichiometry

The enzyme gives better substrate removal performance at a certain optimum concentration of hydrogen peroxide and enzyme. At higher enzyme concentration, catalase effect may be predominant and will stop the reaction by decomposing hydrogen peroxide. On the other hand, a relatively higher hydrogen peroxide concentration when

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compared to enzyme may inactivate the enzyme (Nicell et al., 1995). Hence an optimum concentration for both the enzyme and hydrogen peroxide is required. According to the mechanism presented earlier, Eq.2.6-2.9, for every mole of peroxidase consumed, two moles of aromatic compound are oxidized giving a stoichiometry of 1:2. However, the stoichiometry of hydrogen peroxide to substrate reported in the literature is almost 1:1 (Nicell, 1991; Al-Kassim et al., 1994; Masuda et al., 2001; Ibrahim et al., 2001; Villalobos and Buchanan, 2002). It is assumed that the peroxide is consumed in the side reaction for forming dimers and polymers and hence raises the hydrogen peroxide demand (Villalobos and Buchanan, 2002; Nicell, 1991). Similarly, in the case of enzyme to substrate ratio, 1:1 stoichiometry has been found to be optimum though the mechanism shows a figure of 1:2 (Masuda et al., 2001; Mantha, 2001). Both hydrogen peroxide to enzyme and enzyme to substrate ratios may depend on the enzyme preparation, polymerization and precipitation mechanisms (Masuda et al., 2001).

2.2.5 Reaction Temperature

In case of phenol removal by HRP, the catalytic turnover was found to increase with the lowering of reaction temperature (Nicell, 1991). Similar results have also been observed with CiP (Masuda et al., 2001). A possible explanation is that certain amount of enzyme gets adsorbed on the polymeric end product, which reduces the catalytic lifetime and turnover of the enzyme. These polymers are less soluble at lower temperature, thus they precipitate without adsorbing the enzyme and thereby increasing the catalytic lifetime (Masuda et al., 2001, 2002). For ARP, the optimum temperature is reported to be 40^0 C. Its thermal stability is 30 minutes up to 50^0 C at pH 7.0 (Nakayama and Amachi,

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1999). This means that enzymatic reaction should be carried out at temperature below 40^0 C. In the current study, all the experiments were carried out at room temperature.

2.2.6 Reaction Time

Two to three hours reaction time was sufficient to get 90% substrate removal for most of the phenols and aromatic amines by enzymatic treatment in batch operation (Nicell, 1991, Al-Kassim et al., 1994; Masuda et al., 2001). Similar order of reaction time was used in the present study.

2.2.7 Reaction byproducts

RR2 and RB5 dye molecules have phenolic, amino and sulphonic groups attached to benzene and napthalene ring, which are expected to play critical roles to influence the type of enzymatic end products formed.

Fe reduction products for RR2 and RB5 are aromatic amines and amino-naphthol compounds. When phenol and aniline are the substrates, polyphenols and polyanilines are the major products formed after oxidative reaction by peroxidase. Variables such as: type of enzyme, pH, substrate, etc. may significantly influence the type of reaction byproducts (Nicell et al., 1995). For phenol as a substrate, the main reaction products are phenolic polymers and soluble dimers. When HRP reacts on phenol substrate, quinone is formed in addition to the polymers (Wagner and Nicell, 2002 b). Whereas, when aniline is the substrate, peroxidase forms quinone imines as the intermediate products which finally transform to polyanilies (Liu et al., 1999; Mantha, 2001). Substituted phenols and anilines seem to form colored end product on peroxidase treatment. Removal of these colored products required chitosan or other coagulant aids (Wada et al., 1995).

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Sulfonated substrate can give two different results. If the ortho position with respect to the phenolic or amine group is substituted, de-sulfonation is favored, as observed in case of 3,5-dimethyl-4-hydroxy and 3,5-dimethyl-4-amino benzene sulfonic oxidation by HRP, LiP and MnP. On the other hand, if ortho position is not substituted, then oxidation of this ortho position is favored compared to desulfonation, which was also observed when the same peroxidase enzymes oxidised 4-amino and 4-hydroxy benzenesulfonic acid as substrate (Muralikrishna and Renganathan, 1993).

Oxidation of amino-phenol compounds may take place through -OH or $-NH_2$ depending on their position in the aromatic ring. Polymerisation of ortho- and para-aminophenols takes place through $-NH_2$ groups while $-OH$ is conserved. But for meta-aminophanols both -OH and $-NH_2$ seem to take part in polymerisation, as observed in case of 2,3- and 4- aminophenol polymerisation by HRP (Shan et al., 2003). Oxidation of naphthol compounds with HRP seems to form more hydrophobic polymers as compared to phenol and amines (Klibanov et al., 1980). They seem to produce oligomers with coupling at various positions in the aromatic ring, for example - HRP catalyzed polymerization of 2-naphthol (Premachran et al., 1996).

Though some researchers have attempted to co-relate substrate structure to the final end products, RR2 and RB5 structures are so complex that it is difficult to predict the enzymatic end products. Thus all the dynamics as discussed above may have a role to play.

2.2.8 Enzyme Inhibition

Different side reactions that take place during enzymatic oxidation of aromatic compounds can render the enzyme inactive and thus limit its lifetime. Enzyme inhibition

can take place by different ways (Masuda et al., 2002; Nicell and Wright, 1997): (i) temporary inhibition can take place in an excess of peroxide, when the intermediates, Compound I and II react with hydrogen peroxide forming Compound III; (ii) terminal inactivation, where the free radicals irreversibly bind with the enzyme; (iii) permanent inactivation by adsorption, where the polymers formed during the enzymatic reaction adsorb the enzyme, thereby blocking the access to its active site.

The temporarily inactive form, Compound III, may come back to the native form depending on the reaction conditions. Recovery of initial enzyme activity or the return of Compound III to the native enzyme form depends on the initial concentration of hydrogen peroxide. For lower hydrogen peroxide concentration, such as 0.25 mM, ARP acting on phenol completely regained its activity. However, for higher hydrogen peroxide concentration, such as 4 mM, almost 20 % of the enzyme activity was lost permanently (Villalobos & Buchanan, 2002). The presence of excess hydrogen peroxide or absence of aromatic substrate is known to inactivate ARP more when compared to other peroxidase enzymes. Hence the amount and timing of hydrogen peroxide addition is critical for maintaining the ARP in active form. Keeping the instantaneous enzyme concentration low can control the second form of enzyme inhibition. This will lower the amount of free radicals and therefore minimise enzyme inactivation by these free radicals. The third form of inactivation can also be minimised. Higher substrate and hydrogen peroxide concentration in the presence of lower enzyme concentration will tend to favor the formation of dimers rather than larger polymer molecules (Villalobos & Buchanan, 2002). Unlike polymers, dimers do not inhibit enzymes. Hence suitable timing for

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addition, concentration of enzyme and hydrogen peroxide are the key control variables to overcome enzyme inactivation.

2.2.9 Reactor Operation

Different methods to overcome enzyme inactivation by hydrogen peroxide are to (i) keep the free radical concentration low at any time by step addition of enzyme and also hydrogen peroxide; (ii) reduce the amount of enzyme available for inactivation by step addition of enzyme; (iii) reduce the amount of hydrogen peroxide available at any time by step addition (Al-Kassim et al., 1994; Ibrahim et al., 2001; Villalobos and Buchanan, 2002). Step addition of hydrogen peroxide also improves phenol removal with ARP, as observed by some researchers (Ibrahim et al., 2001; Villalobos and Buchanan, 2002). This may be because when sufficient reaction time is provided, Compound III, the temporary inactive form returns to its native enzyme form which removes more substrate.

Reactors can be operated in batch, semi-batch or continuous manner. Discontinuous semi-batch operation with CMP could remove more phenol as compared to batch operation (Al-Kassim et al., 1994). A continuous-flow reactor with HRP can improve the phenol removal as compared to batch reactor (Nicell, 1991). This is because instantaneous hydrogen peroxide and enzyme concentration remain low in continuous-flow reactors. In the present study, batch process reactors were used along with step addition of hydrogen peroxide to minimize enzyme inactivation.

2.2.10 Enzyme Turnover

The catalytic lifetime of an enzyme is often expressed in terms of catalytic turnovers (Klibanov et al., 1980). Catalytic turnovers are defined as the number of times the enzyme can perform its catalytic cycle before becoming inactive. In other words, it is

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the number of substrate molecules converted by a single molecule of enzyme before it becomes permanently inactive. The presence of certain additives, such as polyethylene glycol (PEG), gelatin and polyelectrolytes, can improve the life of the enzyme (Wu et al., 1997, 1998; Caza et al., 1999; Buchanan and Nicell, 1998). PEG is reported to improve the turnover of ARP for phenol removal by almost 40 % (Ibrahim et al., 2001). ARP was successful in removing 90-93% of phenol from both synthetic and refinery wastewater with a peak turnover capacity between 76,000 and 79,000 (Ibrahim et al., 2001; Villalobos and Buchanan, 2002).

2.2.11 Enzyme Treatment Discussion Summary

The following inferences can be made based on the above discussion:

- ARP can oxidize the aromatic compounds formed after the Fe^0 treatment of the azo dyes.

- Optimization of H_2O_2, enzyme concentration and pH is required for getting maximum yield.

- Enzymatic treatment should be carried out at temperatures below 40 ^{0} C for at least 2 to 3 hours.

- Resulting product could be a polymer or dimer, which may be water-soluble. In case it is water-soluble, a coagulant aid or any alternative process may be required to precipitate this product and finally remove it from water.

2.3   Expected Results

2.3.1: Reactive Red 2 (Trade name Procion Red MX 5B)

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This is a monoazo dye with chlorotriazine as reactive group. It is highly water-soluble. The dye is expected to be stable in water within pH range of 4.0 to 10.0. Hydrolysis of this dye does not occur by simple interaction with water but can occur if heated at high pH followed by returning the pH to neutral (dos Santos et al., 2003). Therefore it can be expected that only change of pH will not result in its hydrolysis. Zero-valent iron powder reduction of this dye followed by photo-oxidation resulted in hydrogenated structure (Ar-NH-NH-Ar), substituted benzene and naphthalene ring compounds (Feng et al., 1999). It was hypothesized that the ring structure of substituted triazine might depart from dye molecule after Fe^0 reduction, however, no clear evidence was available. Hence the question about whether the triazine ring remains attached to the dye molecule or not after Fe^0 reduction is still open. The molecular structure of the Fe^0 treatment product will remain complex if the triazine ring remains appended. Such a complex structure might pose a hindrance to subsequent enzymatic treatment in Step 2. Therefore, this information about triazine ring is relevant to develop insights about ARP action on complex molecules. In RR2, the triazine ring remains attached to the naphthol moiety of the dye molecule by a –NH– bond (Table 1.1). A model compound, diphenylamine has a similar secondary amine bond. Therefore, Fe treatment on this model compound, diphenylamine, was carried out to get an indication whether this –NH– bond gets cleaved under anaerobic Fe^0 treatment. This is discussed further in section 2.4.5.

2.3.2 Reactive Black 5 (Trade name Ramazol Black B)

This is a bisazo dye containing sulfatoethylsulfone reactive groups. The Fe^0 treatment of this dye was proposed to be carried out within the pH range 4.0 to 10.0. This

dye is reasonably stable in water solution within pH range of 4.0 to 10.0. Basic pH above 10.0 changes its absorbance spectrum, which indicates formation of some temporary intermediate compound in this basic pH (Alaton and Balcioglu, 2001). However the absorbance pattern returns to original one once the pH is brought back to normal, which indicates that the intermediate returns to original dye form (Alaton and Balcioglu, 2001). Auto-oxidation of anaerobic reaction (UASB reactor) products of RB5 resulted in partial re-colorization (Sponza et al., 2002; Van der Zee et al., 2000). Since the present study was carried out under anaerobic conditions, re-colorization of the Fe treatment products was expected upon exposure to aerobic conditions.

2.3.3 Intermediate Compounds from Zero-valent Iron Treatment

The reaction conditions proposed in the anaerobic stage of the present work were the same as for the study by Mantha (2001), where nitrobenzene was reduced by Fe^0. One mM sodium sulfite was found to be optimum concentration for deoxygenation in the above study. Therefore the similar condition was maintained in the current study. This was expected to reduce the azo bond in the dye molecule.

Anaerobic reduction of the azo bonds results in multi-substituted benzene and naphthalene compounds (aromatic amines). The chemical structure of dyestuffs and reaction condition determines the quantity of aromatic amines formed. The degree to which the azo group is reduced, depends on the electron density around the $-N=N-$ bond. Electron donating groups, such as $-NH_2$ and $-OH$, increases electron density around the bond and facilitate the reduction and formation of aromatic amines. However, electron withdrawing groups such as, $-SO_3$ and $-COO^-$, may cause reduction by simply introducing hydrogen atoms in the azo group (Pielesz et al., 2002).

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Based on the RR2 and RB5 dye structures, the following compounds were expected to be formed after zero-valent iron reduction:

RR2 degradation

1. Aniline

2. Di-sulfonated amino-naphthol compound

3. Hydrogenated azo bond without bond breakage

4. Triazine group

5. Corrosion product and impurities such as, salts

RB5 Degradation

1. Aromatic amines

2. Di-sulfonated amino-naphthol compound

3. Hydrogenated azo bond without bond breakage

4. Corrosion products and impurities such as, salts

Since the Fe reaction product may be a mixture of different compounds as mentioned above, an analysis was required about the suitability of peroxidase to treat this mixture in the subsequent step. Aniline and naphthol compounds are good substrates of peroxidase (Klibanov et al., 1980; Mantha, 2001). As other Fe degradation products are substituted benzene and naphthalene compounds with –OH and –NH$_2$ groups, their reaction behaviours with peroxidases were expected to be similar to that of aniline and naphthol compounds. Therefore it was expected that these compounds could also be treated by peroxidases. All these evidences suggested that peroxidases may be suitable for removing aromatic amines from the Fe reduction product obtained in the first step.

2.3.4 Impact of Impurities and other Substances

Wastewater may contain sodium salts of sulfite (SO$_3^{2-}$), iodide and nitrite (Wagner and Nicell, 2002a). Textile wastewater contains sodium salts because dyes and

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auxiliary chemicals usually carry sodium (Arslan and Balcioglu, 2000). Sodium salts of sulfite, iodide and nitrite are substrates of peroxidase, hence, they can compete with the aromatic amines for the enzyme. However the presence of inorganic anions, such as sulfite, has no negative impact on phenol removal by HRP (Wagner and Nicell, 2002a). In the presence of sodium chloride and ammonium chloride etc., the enzyme lost its catalytic stability faster. As the dye used in this study was impure (purity of ~50%), hence such side reaction was expected.

The presence of metal ions, such as Fe(II), Zn, Ni, Cu, may have some effect on the enzymatic reaction. These metals can react with the oxygen containing ligands, such as carboxylic groups in the protein molecule, causing inactivation of the enzyme (Wagner and Nicell, 2002a). However, filtration after Fe treatment should eliminate presence of any metal from the solution. The enzymatic reaction for treatment of phenol from foundry and craft pulping wastewater showed that peroxidase was able to selectively remove phenol even in the presence of different dissolved substances in wastewater (Cooper and Nicell, 1996; Wagner and Nicell, 2001).

2.3.5 Diphenylamine

This compound has a –NH- bond. Fe treatment of this compound is expected to indicate whether such –NH- bond also gets cleaved in case of RR2. Besides this, the degradation of this compound is also interesting as it is an identified pollutant according to EU and US EPA (Drzyzga, 2003; TRI data, 1995). It is used as a stabilizing agent in nitrocellulose-based explosives and as an anti-oxidant preservative for harvested apple and pear crops. The redox potential of diphenylamine is 0.78V (Pankratov and Shchavlev, 2001). Since it is higher than the Fe^0 redox potential (-0.44V), it is probable

that this compound can be degraded by breaking the –NH- bond, which is the only likely candidate. The degradation products would then be aniline and benzene.

2.3.6 2-amino-8-naphthol-3, 6-disulfonic acid

2-amino-8-naphthol-3,6-disulfonic acid (ANDSA) is a sulfonated amino-naphthol compound. Action of ARP on this model compound was studied to gain insights about suitability of ARP to degrade complex sulfonated amino-naphthol compounds. The Fe treatment products of RR2 and RB5 in Step 1 was a mixture of similar sulfonated amino-naphthol compounds. Besides this, ANDSA is a potential pollutant from the dye industry (Zhu et al., 2002; Stolz, 1999). ANDSA, which is commercially known as H-acid, is widely used as a dye intermediate. Since the compound has both –OH and -NH$_2$ groups, it was considered to be a possible substrate of enzyme.

From the above discussions it was concluded that Fe reduction of the two dyes followed by enzymatic removal of aromatic amines held promise to remove the dye compounds from water. It was expected that the study on diphenylamine would give better insight into the Fe degradation process of RR2 and RB5, whereas the study on ANDSA would yield insights into enzymatic removal of substituted naphthalene compounds.

2.4 Coagulation and Precipitation

Coagulants are used for removing color and COD from wastewater (Nemerow, 1978). Alum is a common coagulant used in enzymatic wastewater treatment to remove the reaction byproducts (Mantha, 2001, Al-Kassim et al., 1994). For substituted phenol and anilines, cationic polymers, such as PEI (synthetic cationic coagulant aid), chitosan (natural cationic polymer) were effective (Wada et al., 1995). Presumably, because

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substituted phenols and anilines formed quinone type compounds, which were highly negatively charged, and alum was not sufficient to neutralise these charges. PEI and chitosan could remove more than 80% of color and total organic carbon (TOC) from paper and pulp industry wastewater (Ganjidoust et al., 1996, 1997). PEI and alum were used together in this present work.

2.4.1 Mechanism

PEI is a cationic coagulant. It has primary, secondary and tertiary amine groups and it is highly branched with a repeating chemical unit as $-(CH2-CH2-NH)-$. It becomes charged at low pH and weakly dissociated at higher pH range of 8.0 to 9.0, which is the pKa of primary amine group (Andersson and Bergstrom, 2002). In presence of adequate amount of H^+, the amine groups undergo protonation to form positively charge NH_4^+, which effectively binds with negatively charged substrate molecules to form large molecular complexes. Hence, at a pH level below 8.0, it performs better (Andersson and Bergstrom, 2002). However at lower pH the substrate molecules themselves may alter their charges in the presence of H^+ that may work against the performance of the coagulant aid. Therefore, an optimum pH may be observed when the coagulation phenomenon is best manifested. Alum in water forms a gel which entraps these substrate-PEI complexes and precipitate them.

Total nitrogen content of PEI is 32.5%. One manufacturer claimed PEI to be toxicologically benign and approved by FDA for indirect food contact application (www.polymerenterprise.com, Jan 2004). It has many interesting applications, such as: i) purification of protein from feed stock; ii) immobilization of biocatalyst; iii) soluble carrier for enzymes and affinity legands; iv) retention of inorganic pigments on papers,

etc; v) color removal from wastewater (Andersson and Hatti-Kaul, 1999). The success of PEI to remove color has attracted researchers to explore its application in enzymatic wastewater treatment. The enzymatic reaction product of the substituted phenols and anilines form negatively charged quinones. These quinones easily react with the nitrogen from the amino groups of the PEI polymer by cross coupling and the resulting product precipitate easily from water (Wada et al., 1995).

The structure of the PEI can be expressed as (Anderson and Bergstrom, 2002)
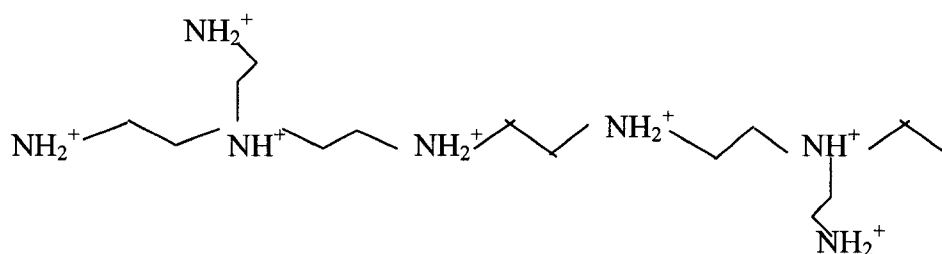
The coagulant concentration is important. For anilines and p-chloroanilines (concentration 0.5 mM along with 1 mM phenol as co-substrate), optimum concentration of PEI was found to be 40-90 mg/L resulting in 100 % substrate removal (Wada et al., 1995). For removing color from paper and pulp industry wastewater, with pollutant content of 300 mg/L, a PEI concentration of 100-200 mg/L could achieve 80 % color and TOC removal (Ganjidoust et al., 1996, 1997). PEI also settled down along with the pollutant, therefore its addition did not increase TOC of the treated water.

Therefore it was inferred that:

- PEI and alum can remove colored products after the enzymatic reaction.

- Higher concentrations of PEI may be required depending on the substrate structure.

- TOC content of the treated water may not rise due to PEI addition.

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CHAPTER 3 : MATERIALS AND METHODS

This chapter describes the experimental procedures and analytical techniques used in the study.

Experimental Studies

To achieve the objectives of the research, following experiments were carried out:

- Effect of pH on Fe^0 treatment of two dyes – RR2 and RB5.

- Reaction rate for Fe^0 treatment of RR2 and RB5.

- Effect of pH on ARP activity with RR2 and RB5 Fe^0 reduction products.

- Effect of H_2O_2 concentration on ARP activity with RR2 and RB5 Fe^0 reduction products.

- Effect of ARP concentration on ARP activity with RR2 and RB5 Fe^0 reduction products.

- Reaction rate for ARP action on RR2 and RB5 Fe^0 reduction products.

- Effect of pH on coagulation of RR2 and RB5 enzymatic reaction products by PEI.

- Effect of PEI concentration on coagulation of RR2 and RB5 enzymatic reaction products.

- Fe^0 treatment of model compound - DPA.

- Effect of pH on ARP activity with model compounds - DPA and ANDSA.

- Effect of H_2O_2 concentration on ARP activity with DPA and ANDSA.

- Effect of ARP concentration on ARP activity with DPA and ANDSA.

- Reaction rate for ARP action on DPA and ANDSA.

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3.1 Zero-Valent Iron Treatment of RR2 and RB5

3.1.1 Materials

Two dyes, Reactive Red 2 (dye content~ 50%), lot no. 12623DQ and Reactive Black 5 (dye content~ 55%), lot no. 04906CR were purchased from Sigma Aldrich Chemicals, Milwaukee, WI. They were used as delivered. Iron metal was purchased from Fisher Chemicals, NJ, (FL-04-1102: lot no. 028418) as iron filings having size of 40 mesh. Purity of the metal was 99.98 % with major impurities being phosphorous, (16 ppm), cobalt (14 ppm), nickel and manganese (10 ppm), as stated by the supplier. ACS grade cobaltous chloride and sodium sulphite were obtained from Fisher Scientific, Pittsburg, PA. Analytical grade trinitrobenzenesulfonic acid (TNBS) (picryl sulfonic acid) was obtained from Sigma Aldrich Chemicals, St Louis, MO. All acetate, phosphate and carbonate buffers were prepared as per Gomori (1955). Plastic Syringes (6 mL) were purchased from Becton Dickinson & Co, Clifton, NJ. Syringe filters (bulk, non-sterile, size 0.2 micrometer) were obtained from Pall Gelman Laboratories, Mississauga, ON. Disposable polystyrene semi-micro cuvettes were used to measure the absorbance of the samples. They were purchased from Bio-RAD Laboratories, Mississauga, ON, Canada. Quartz cuvettes with path length 10 mm was purchased from Hellma (Canada) Limited, ON. Whatman No 42 filter papers were used along with vacuum system for filtering the Fe reaction products.

3.1.2 Equipment

Absorbance of the solutions was measured by using a Hewlett-Packard Diode Array Spectrophotometer, Model 8452A, with wavelength range between 190 to 820 nm and 2 mm resolution. The spectrophotometer was interfaced with a HP Vectra ES/12

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Computer. The batch reactor vials were shaken at maximum setting on a Burrel Model 75 wrist action shaker by Burrel, Pitsburgh, PA. pH was measured by IQ 200 pH meter fitted with ISFET probe from IQ Scientific, London, ON.

3.1.3 Experimental Procedure

All reactions were conducted at room temperature, 18-22 ^{0} C. All solutions were prepared in de-ionized water. All acetate, phosphate and carbonate buffers were prepared as per Gomori (1955).

Iron Pretreatment: Zero-valent iron was pretreated with HCl as recommended by Agrawal and Tratnyek (1996). This was done to remove any metal oxide present on the surface. The measured quantity of Fe was taken in a glass vial and kept soaked in 10 mL of 10 % HCl for 20 minutes. Then, the iron particles were washed twice with de-ionized and de-oxygenated water to remove the metal oxides. The particles were then washed four times with 15 mM carbonate buffer (pH 9.5) to ensure removal of all chlorides from the surface. The buffer was previously made anaerobic by using sodium sulphite. This was followed by rinsing and maintaining in sodium sulphite solution to prevent any contact with oxygen. Sodium sulphite solution was prepared fresh each time the Fe treatment was done.

Batch Reactors: Batch reactor experiments were carried out in 30 mL vials sealed by screw caps. The vials were shaken on a wrist shaker on maximum setting of 10 for required period of time. This ensured proper mixing and sufficient contact with Fe particles. Solutions were made anaerobic by using sodium sulphite and 1% (w/w) cobaltous chloride. In general, all the vials received 1 g Fe, 1 mM dye solution, 1mM sodium sulphite solution and 40 mM buffer. After the reaction, the batch reactors were

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kept on a magnet to allow the iron particles to settle at the bottom. The solutions were filtered afterwards by using filter paper and vacuum.

pH Optimisation: Different buffers, such as acetate (pH 3.2 to 5.6), phosphate (pH 6.5-7.5) and carbonate (pH 8.0-10.0), were used to determine the optimum pH for the Fe^0 reduction process.

3.1.4 Analytical Techniques

Estimating reduction of Azo bond: Percentage reduction of azo bond was determined by monitoring absorbance at λ_{max} by spectrophotometer. Percent reduction at any point was calculated as :

$$\%reduction = \frac{A_{in} - A_{fin}}{A_{in}} x100 \qquad \ldots\ldots\ldots(3.1)$$

where A_{in} is the absorbance before Fe treatment and A_{fin} is the absorbance after adding Fe.

Precipitating Fe corrosion products : Aqueous corrosion products - $Fe(OH)_2$ and magnetite Fe_2O_3 were formed for both the dyes. The corrosion products were precipitated by bringing the pH from 5.6 to normal pH of 7.0 by adding phosphate buffer (0.5 M, pH 7.4).

TNBS Test: Aromatic amines, the Fe reduction products were detected by using the TNBS test (Mantha, 2001). A reaction mixture of 1 mL was made from 100 µL of 10 mM of TNBS, 100 µL of 0.5 M phosphate buffer of pH 7.4 and 800 µL of sample and water. Reaction was allowed to proceed for the required time and then the absorbance was measured at maximum absorbance wavelength (λ_{max}) for the corresponding aromatic amine-TNBS compound against a reagent blank. Aniline was one of the Fe reduction

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products for RR2. A calibration curve was prepared for aniline with absorbance and concentration, which showed a linear relationship. Concentration was determined using a molar extinction coefficient of 13200 $M^{-1}.cm^{-1}$. Sodium sulphite formed an adduct with aniline; hence, a new calibration curve was prepared by using aniline solution doped with sodium sulphite. The absorbance was measured at 398 nm (λ_{max}) and a slightly higher extinction coefficient of 13400 M^{-1} cm^{-1} was obtained (Appendix B-3). In case of RB5, similar calibration curve could not be made, as the aromatic amines, which were the Fe reduction products of RB5, were not available as standards. Hence, the percentage removal were estimated from the ratio of absorbance values measured before and after enzymatic treatment.

Table 3.1 : Parameters for analysis of aromatic amines by TNBS test

Substrate	Peak wavelength, nm	Peak time	pH	Extinction coefficient $M^{-1}. cm^{-1}$
aniline	398	30 min	7.4	13400
ANDSA	410	1 h	7.4	1100
RR2 Fe breakdown product	398	1 h	7.4	~8000-9000 based on aniline
RB5 Fe breakdown product	400	1 h	7.4	~5400-6000 based on aniline

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3.2 Enzymatic Reaction

3.2.1 Materials

Arthromyces ramosus peroxidase was a gift from Biotech Environmental Inc. and is a developmental preparation of Novo Nordisk, Denmark. It has Rz value (optical purity index) of ~0.5. The specific activity of the ARP stock solution was 2000 U/mL based on an assay using 4-aminoantipyrine (4-AAP) phenol and hydrogen peroxide as substrate (Ibrahim et al., 2001). One unit of activity is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and at 25 ^{0}C temperature. The enzyme was stored at 4 ^{0}C. A sub-stock was prepared with activity 400 U/mL, which was used for the experiments. Hydrogen peroxide (30 % w/v), analytical grade monobasic and dibasic sodium phosphate was purchased from BDH, Toronto, ON. Peroxide solutions for the experiment was prepared weekly. Catalase (EC 1.11.1.6), lot no: 81H7146, was purchased from Sigma Aldrich Chemical Co, St Louis, MO. The normal activity of catalase was 1500 U/mg dry solid and 2000 U/mg protein. Polyethyleneimine (PEI), lot no:14520PR, was obtained from Sigma Aldrich, Milwaukee, WI. Alum, as aluminum sulphate ($Al_2(SO_4)_3,16\ H_2O$), lot no: 14238, was obtained from BDH, Toronto, ON.

3.2.2 Experimental Procedure

Preparation of Fe reduction products: The reaction products after Fe^0 reduction were allowed to aerate for 30 minutes. During this time the pH was adjusted to neutral range (7.0) by adding phosphate buffer (pH 7.4, 0.5 M). The colloidal particles formed by this process were separated by filtration.

Batch reactors: Batch experiments were set up to study the various parameters like pH, H_2O_2 to substrate ratio, enzyme concentration, and reaction time. All batch experiments

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were conducted in 30 mL glass vials. Reaction mixture volume for RR2 and RB5 enzymatic treatment was 10 mL, while for ANDSA and DPA it was 20 mL. Each batch reactor received specific amount of Fe^0 reaction product, various concentrations of H_2O_2 and enzyme and required buffer. Since the reaction compounds contained different amounts of –OH and -NH_2 groups, the stoichiometric amount of H_2O_2 and proportional amount of enzyme were added. For all the experiments, H_2O_2 was added stepwise to avoid instant inactivation of the enzyme. The contents of the vials were mixed thoroughly and continuously with teflon coated magnetic stirrer. The reactions were stopped after 3 h by adding catalase, which broke down H_2O_2 to water and oxygen. At the end of the reaction, the products were filtered and samples were withdrawn for colorimetric and absorbance test.

Coagulation: After enzymatic treatment, PEI was added to the reaction mixture followed by rapid and then slow mixing to form colloids with the reaction products. Finally, alum was added and pH was adjusted to neutral to help settle the colloidal particles. These particles were subsequently removed by filtration.

3.2.3 Analytical Techniques

Enzyme Activity Assay: Enzymes were measured by their catalytic activity. 4-aminoantipyrine (4-AAP)-phenol was used as color generating mixture, which generated colour when peroxidase and peroxide were added to it. The rate of color generation was proportional to the enzyme activity. The assay mixture contained phenol, 4-AAP, and hydrogen peroxide where enzyme was used in limited quantity. The rate of reaction was measured by measuring the rate of formation of colored products that absorbed light at a peak wavelength of 510 nm with an extinction coefficient of 6000 M^{-1} cm $^{-1}$ based on

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peroxide. One unit of activity is defined as number of micromoles of H_2O_2 utilized in one minute at pH 7.4 and at temperature 25 ^0C in an assay mixture containing 10 mM phenol, 2.4 mM 4-AAP and 0.2 mM H_2O_2. The detailed description of this assay is presented in Appendix B-1.

Hydrogen Peroxide Assay: This colorimetric end point assay achieves the measurement of hydrogen peroxide concentration using *Arthromyces ramosus* peroxidase (ARP) as catalyst and phenol and 4-AAP as color-generating substances. This assay was set up with hydrogen peroxide as the limiting substrate in the mixture. Thus, the intensity of color generated was proportional to the amount of peroxide present in the sample. The assay volume was kept as 1 mL. The detailed procedure is presented in Appendix B.2.

TNBS Test for Aromatic Amines: Remaining aromatic amines in a mixture were measured by the TNBS test as mentioned in Section 3.1.4. In the case of RR2, one of the products was aniline so the standard curve was used for measuring the concentration of the remaining aromatic amine. For RB5, the reaction products positively responded the TNBS test indicating formation of aromatic amines. However, no exact standards for these aromatic amines were available. Hence, the difference between the UV absorbance values for the mixture, before and after the enzymatic reaction, gave the estimates of percentage aromatic amine removal.

Optimum Concentration of PEI: Optimum PEI concentration was determined by comparing reduction in absorbance of the reaction products before and after enzymatic treatment.

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3.3 Model Compounds

3.3.1 Materials

2-amino-8-naphthol-3,6-disulfonic acid, monosodium salt, (purity 80-90 %) was purchased from Sigma Aldrich rare chemical library, Milwaukee, WI. Diphenylamine (purity 99%), lot No: 07325EO was purchased from Sigma Aldrich Chemicals, Milwaukee, WI. Aniline was purchased from Fisher Scientific, NJ.

3.3.2 Equipment

HPLC was purchased from Waters Co, Milford, MA, USA. The Waters System had a model 2487 dual λ absorbance detector, Waters Model 1525 Binary HPLC pump and Waters Model 717 Autosampler. The column was C18 (5 μM, 4.6x150 mm) operated by Breeze software. The elution was isocratic using 80/20 % methanol and water. The UV-VIS detector was set in dual mode at 280 nm diphenylamine and aniline and at 254 nm for benzene.

3.3.3 Experimental Procedure

The enzymatic treatment of both DPA and ANDSA was carried out as discussed in Section 3.2. ANDSA also responded to the TNBS test. A reaction mixture of 1 mL was made from 100 μL of 10 mM of TNBS, 100 μL of 0.5 M phosphate buffer of pH 7.4 and 800 μL of ANDSA sample and water. Reaction was allowed to proceed for 60 minutes and then the absorbance was measured at 410 nm against a reagent blank. A calibration curve was prepared for ANDSA. Difference of concentration between the before and after enzymatic treatment gave the % removal of ANDSA by enzymatic treatment. DPA did not respond to the TNBS test under the present conditions, hence, absorbance and HPLC was used for measuring percentage removal.

Fe^0 treatment of DPA was done by using the same method as described in Section 3.1. The breakdown products were identified by HPLC method. Standard curves for DPA and aniline are given in Appendix C.

3.4 Estimation and Minimization of Errors :

There could be two types of error in any analytical study - determinate and (systemic) and indeterminate (random) error. Determinate errors are introduced due to improper experimental design and they are inherent to a particular method. Quite often a significant portion of these determinate errors can be controlled or minimised. These types of errors can be minimized by strictly following the experimental protocols like - time, amount of reactant, order or steps of addition of the compounds and recalibrating the instruments on regular basis. Systemic errors can also be minimised by appropriate experimental design. For example, systemic error can occur when a very low reactant concentration is used in colorimetric assay; therefore, preferably all observations should be taken at a recommended concentration range.

Indeterminate errors are variations in experimental conditions, which cannot be controlled directly. However, impact of these random errors can be estimated. Uncontrollable errors may be introduced due to equipment inaccuracies or due to human factors like - measurement errors, sampling errors etc. Variations in electronic equipment have strong correlation with time, due to drift and aging. Therefore, variation in observations noted within a short period, say within few hours, are predominantly due to human and other factor, whereas observations noted across a wider span of time, say across the whole week or month, is due to both equipment variations and human factors. The following experiments can give estimates of variations due to these two factors, equipment variation and human factor in this atudy.

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3.4.1 Experiment design

Experiment 1 : This was designed to estimate variance introduced due to human and miscellaneous factor, other than equipment variations. All observations were taken consecutively on the same day within a few hours, without resetting or re-calibrating the equipment (spectrophotometer is allowed 1 hour warm up time), with the same experimental batch reactors. The following template was used to record the sample observation data against the same parameter (e.g. UV-vis absorbance).

Equipment and experimental details: TNBS test for ANDSA (aromatic amine) done by spectrophotometer. For process details refer to Appendix B.3. 1mM solution of ANDSA was prepared by diluting 5 mM stock. 100 μL of 10 mM of TNBS reagent was added to 100 μL of 0.5 M phosphate buffer (pH 7.4), 100 μL of 1mM ANDSA and 700 μL of distilled water. This mixture was allowed for 1 hour of reaction time. Eight test tubes of this mixture were prepared. UV-vis absorbance for these 8 reactors were noted at 410 nm with the spectrophotometer. The spectrophotometer was set against a reagent blank containing 100 μL reagent, 100 μL buffer, 800 μL of distilled water.

Time, location of observation: Lab -B79, Dept. Chemistry and Biochemistry, University of Windsor.

Data Collected

Table 3.2 : Error Estimation due to Human Effect.

Sample No.	1	2	3	4	5	6	7	8
Time								
Absorbance observed.	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8

Estimation: The estimation of the average and variance is given by (Caulcutt and Boddy, 1983)

$$\mu_x = \Sigma_{1\,to\,n}\; x_i\,/\,n \;=\; \text{average of } x_1 \text{ to } x_8, \text{ where } n = 8$$

where as, the estimated variance due to human and other factors is given by

$$\sigma_x = (n \,\Sigma\, x_i^2 - (\Sigma\, x_i)^2)\,/\,n\,(n\text{-}1)$$

$$s = \sqrt{\sigma_x}$$

$s =$ standard deviation for samples x_1 to x_8, where $n = 8$

Presentation of data: The absorbance data can be presented in two alternate formats

<u>As confidence interval (say at 95 % confidence):</u> $\quad \mu_x \pm 2.364 * \dfrac{s}{\sqrt{n}}$

1) <u>As average and standard deviation:</u> μ_x and s

Experiment 2 : To estimate variance introduced due to spectrophotometer. All sample observations were taken on different days. Each sample reactor was prepared each day afresh before each observation,. Similar procedure as in Experiment 1 was followed, except each sample was a new batch prepared on the day of observation. The following template was used to record the sample observation data against the same parameter (e.g. UV-vis absorbance).

Time, location of observation: Jan to Mar 2004, Lab -B79, Dept. Chemistry and Biochemistry, University of Windsor

Equipment and experimental details: TNBS test for ANDSA (aromatic amine) by spectrophotometer.

Data Collected

Table 3.3 : Error Estimation due to Equipment Effect

Sample No.	1	2	3	4	5	6	7	8
Date								
Parameter observed	y_1	y_2	y_3	y_4	y_5	y_6	y_7	y_8

Estimation: The estimation of the parameter and its standard deviation due to all factors (equipment, human and other factors) are given by:

μ_y = average of y_1 to y_8

s = standard deviation for samples y_1 to y_8, and n = 8

The "t" statistics with degree of freedom = 7 was used to estimate the confidence intervals. For 95 % confidence interval, t = 2.364.

CHAPTER 4: RESULTS AND DISCUSSION

This chapter presents the experimental observations and discussions.

4.1 Properties of the Dyes

4.1.1 UV-vis absorbance characteristics

The absorbance characteristics uniquely identify the presence and concentration of the dye in a solution. Figures 4.1 and 4.2 present the absorbance spectra of the two dyes. The observed absorbance characteristics are:

Pure dye solution	Peak wavelength (λ_{max})
Reactive Red 2, 25 μM	538 nm (extinction coefficient 15477 M^{-1}. cm^{-1})
Reactive Black 5, 25 μM	596 nm (extinction coefficient 25742 M^{-1}. cm^{-1})
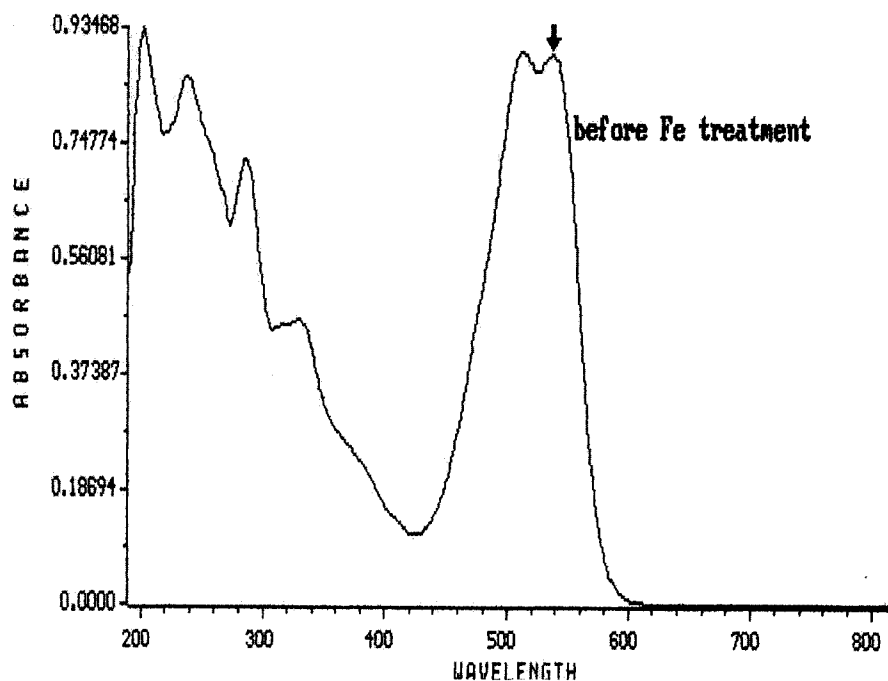
Figure 4.1: UV-vis absorbance spectra of Reactive Red 2, 25 μM solution, pH 7.0, room temp.

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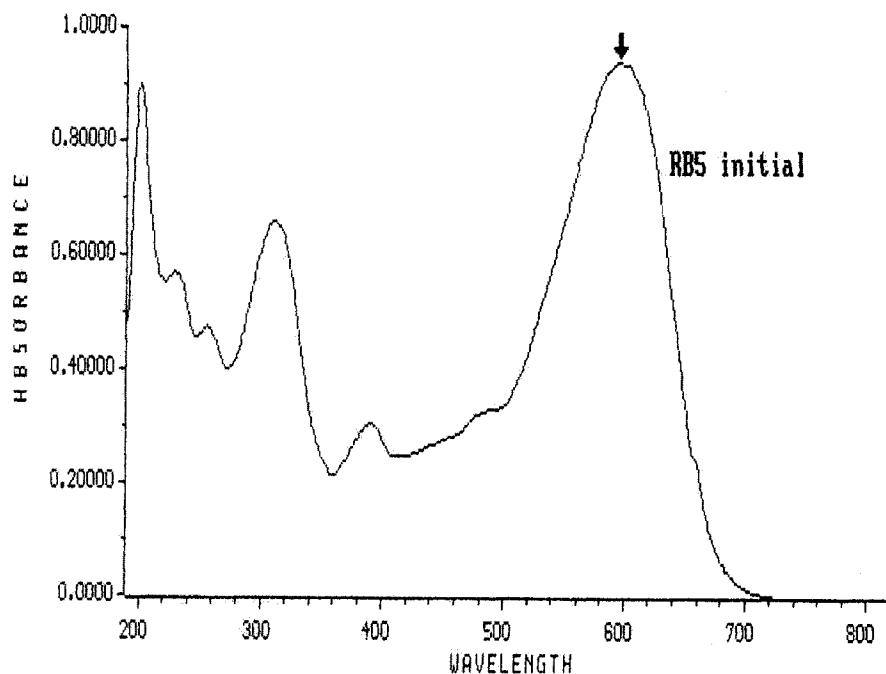
Figure 4.2: UV-vis absorbance spectra of Reactive Black 5, 25 μM solution, pH 7.0, room temp.

Figures 4.3 and 4.4 present the visual observations for the two dyes during the entire treatment process.
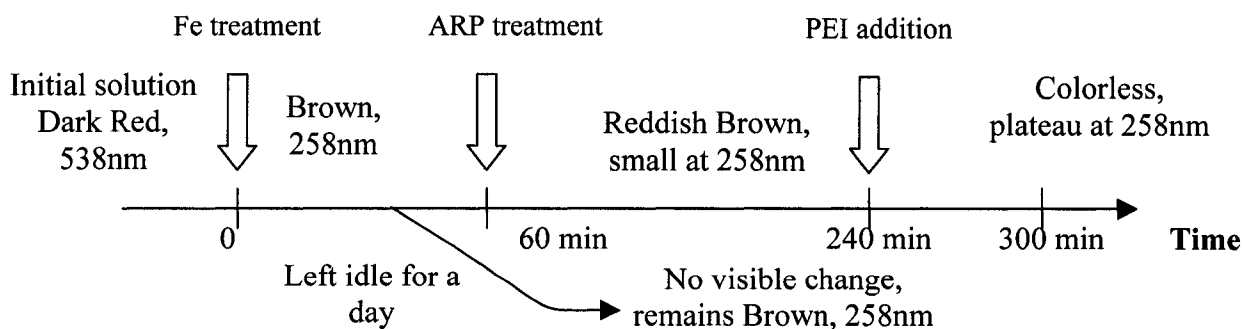
Figure 4.3: Visual observations and absorbance characteristics during RR2 treatment

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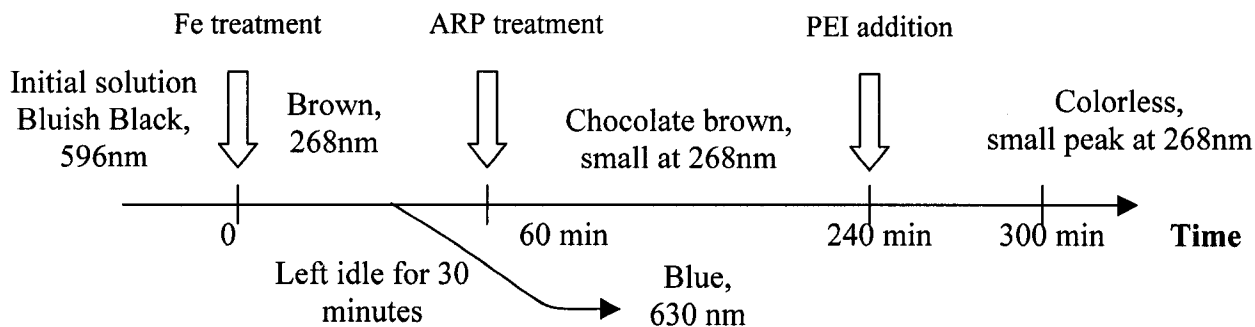
Figure 4.4 : Visual observations and absorbance characteristics during RB5 treatment

Certain control experiments, as described below, were carried out to identify whether the reagents had any effects on the dyes.

4.1.2 Stability of the Dyes within pH Range of 4.0 to 10.0

Both dyes were found to be stable within the pH range of 4.0 to 10.0 in 40 mM buffer concentration. No significant changes were observed either in visible color, or the UV-vis absorbance characteristics for these dyes. All the subsequent stages of the proposed treatment process were carried out within this pH range. Textile effluents had basic pH, while a lower pH (around 4.0) favored the dye breakdown by Fe^0 reduction and enzymatic treatment was most effective around neutral pH.

4.1.3 Inadequate Impact of Coagulant (PEI) on Dye Color and Solution

For both the dyes, no significant effect was observed after addition of the coagulant polyethyleneimine (PEI), at a concentration of 200 mg/L in 1 mM dye solution. The dye solutions lost their transparency and formed suspended particles. However, after filtering, the filtrate displayed absorbance characteristics similar to that of the respective dyes.

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Only the peak absorbance at λ_{max} were reduced by 30 %. This established that PEI alone was inadequate to remove the dye colour. Since RR2 and RB5 contain SO_3^- groups, they were expected to bind with a cationic coagulant, but depending on pH, the presence of cationic groups in the dye molecules, like $-NH_2$ will hinder this binding. Hence, it was observed that PEI was not effective to remove the dyes.

4.1.4 Effect of Na_2SO_3 on Dye

Less than 5 % reduction in peak absorbance (at λ_{max}) was observed for both the dyes at 1 mM concentration when treated with 1 mM sodium sulphite (Na_2SO_3). Also the absorbance characteristics remained the same as that of the dye solution.

4.1.5 Effect of H_2O_2 and ARP on Dye

H_2O_2 and ARP, either individually or together at 1 mM and 1 U/mL concentration respectively, had no effect on both the dyes at 1 mM concentration. The peak and absorbance characteristics after addition of these reagents remained the same as that of the dye solution. Further, the enzyme activity test and H_2O_2 color test showed the presence of entire amounts of enzyme and H_2O_2. This proved that the colour of the dye could not be removed under this condition.

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4.2 Zero Valent Iron Treatment

4.2.1 Effect of pH

The Fe^0 reduction process was studied within a pH range of 2.5 to 10.0. One g Fe was used for 1 mM dye solutions with 40 mM buffer and 1mM Na_2SO_3 in 30 mL batch reactors. Figure 4.5 presents the percent of peak absorbances at λ_{max}, remaining after 1 hour under various pH conditions. The absorbance characteristics after Fe^0 reduction for both dyes are presented in Figures 4.6 and 4.7.
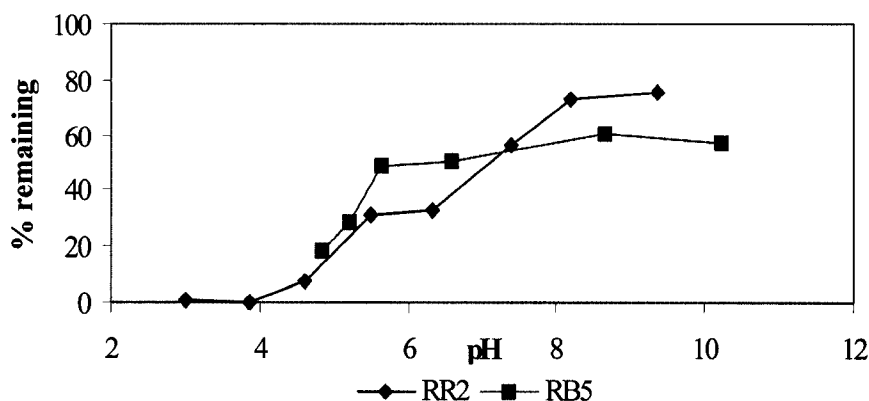
Figure 4.5: Effect of pH on dye reduction, after 1 hour with 1 mM dye concentration and 1 g Fe^0

Acidic pH range favors reduction for both dyes, as observed by previous researchers (Cao et al., 1999; Section 2.1). However, aniline adsorption on Fe surface takes place at lower pH below 5. Therefore to avoid problems related to adsorbtion of aniline to Fe surface a pH of 4.0-5.0 was considered suitable. For all subsequent studies, Fe^0 treatments were carried out at pH 4.8, which is also recommended as the optimum Fe treatment condition. Acetate buffer gave better removal compared to phosphate and carbonate buffers. This observation is consistent with the earlier findings (Lavine et al., 2001).

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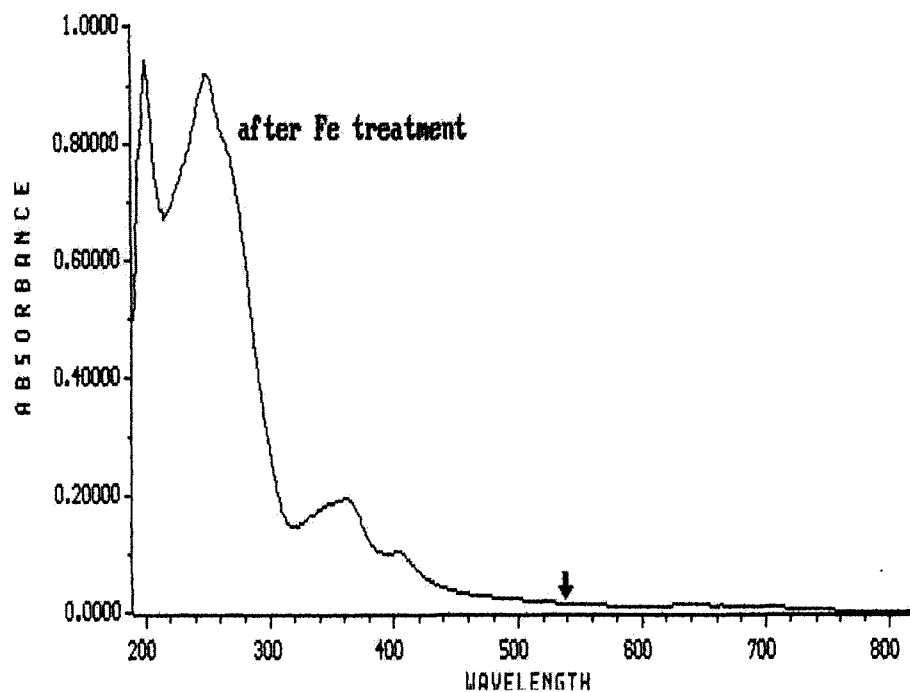
Figure 4.6: UV-vis absorbance spectra for RR2 after Fe^0 reduction, with 1 mM dye solution, 1 mM Na_2SO_3, 40 mM acetate buffer at pH 4.8, 1 g Fe^0, room temp, 1 h reaction time, final pH 5.6.
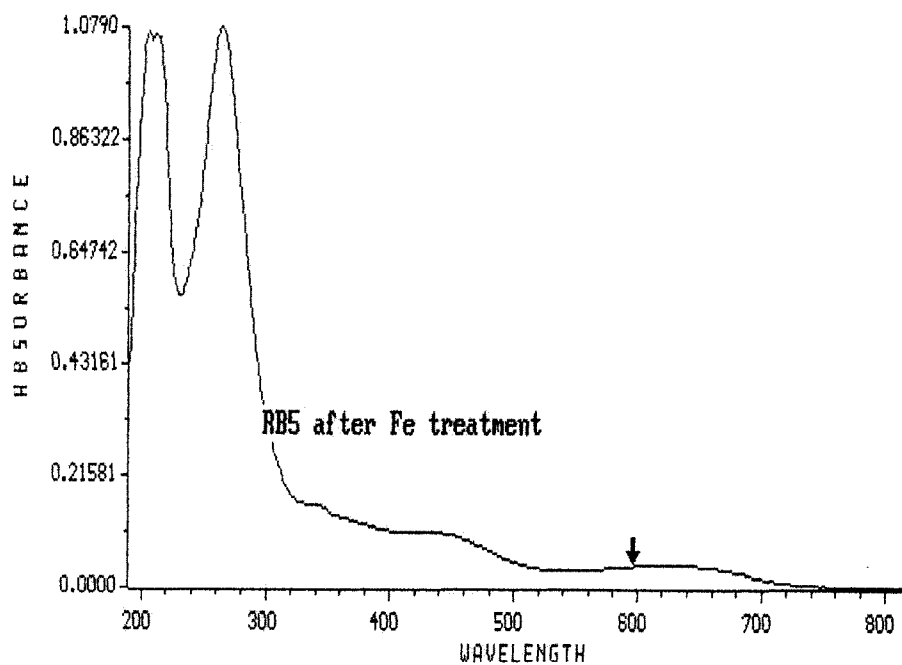
Figure 4.7: UV-vis absorbance for RB5, after Fe^0 reduction with 1 mM dye solution, 3 mM Na_2SO_3, 60 mM acetate buffer at pH 4.8, 3 g Fe^0, room temp, 1 h reaction time, final pH 6.0

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4.2.2 Reaction Time

The rate of Fe^0 reduction with time for both dyes was studied and the results are presented in Figures 4.8 and 4.9.
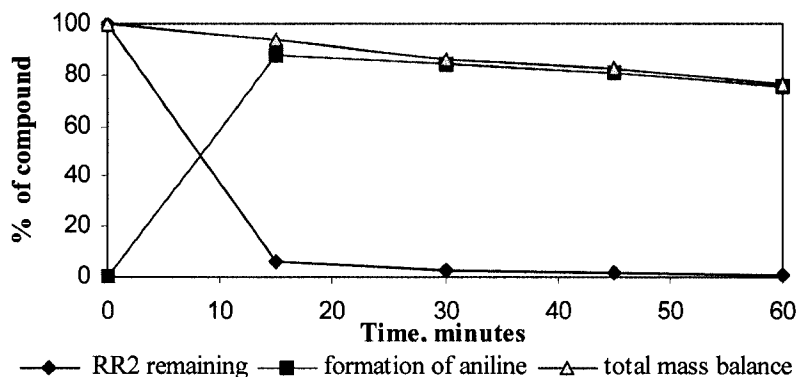
**Figure 4.8: Fe^0 reduction rate for RR2
at pH 4.8, 1mM dye solution, 1 g Fe^0 and 1mM Na_2SO_3.**

RR2 reduction by Fe^0 was quite rapid and almost 95 % of dye was reduced in 30 minutes. One of the RR2 breakdown products was aniline, which was confirmed by TNBS test. The stoichiometric amount of aniline was not recovered, which was expected as some aniline was adsorbed on the iron surface as discussed in Section 2.1.4. However under similar conditions, RB5 required longer time to get reduced (Figure 4.9).
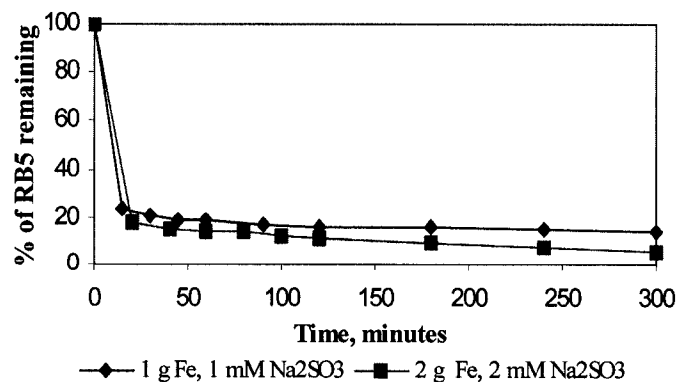
**Figure 4.9: Fe^0 reduction rate for RB5
at pH 4.8, 1 mM dye solution.**

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Only 80 % of RB5 was removed in 1 hour with 1 g Fe^0 with 1 g Fe^0 and it increased to 85% reduction after 2 hour reaction time. Over 90 % removal was observed when the reaction was allowed to run for 2 hours with a higher amount of Fe (2 g) and Na_2SO_3 (2 mM). Allowing the process to continue for longer time to 5 hours improved the removal efficiency by another 5 %. When the reaction was run with 3 g Fe^0 and 3 mM sodium sulfite, the reduction rate was even better (95 % in 1 h). But high sodium sulfite required more enzyme and hydrogen peroxide in the next step and still the final color removal was not satisfactory. Hence, 2 g Fe^0 and 2 hours reaction time was considered for studying ARP and coagulation processes for RB5. Formation of the aromatic amines could not be quantified as no ready standard was available for calibration by TNBS test.

The significant difference in reaction rates between RR2 and RB5 may be due to a complex interplay of many factors. Both the dyes were impure (purity ~50%) and some impurities might have either accelerated or passivated the Fe^0 active sites and the reaction in case of RB5. It might be also possible that 1 mM RB5 requires 3 gm Fe compared to 1 gm Fe requirement in case of RR2. However the reaction mechanism as discussed below indicate that only 2 g Fe should be sufficient for 1 mM RB5.

Fe^0 reduction of reactive dye ($R^1N{=}NR^2$), which yields aromatic amines, can be expressed as follows :

$$4H_2O \quad = \quad 4H^+ \quad + \quad 4OH^-$$

$$2Fe^0 \quad = \quad 2Fe^{2+} \quad + \quad 4e^-$$

$$R^1N{=}NR^2 \; + \; 4e^- \; + \; 4H^+ \quad = \quad R^1NH_2 \; + \quad R^2NH_2$$

$$4H_2O \; + \; 2Fe^0 \; + \; R^1N{=}NR^2 \quad = \quad 4OH^- \; + \; 2Fe^{2+} \; + \; R^1NH_2 \; + \; R^2NH_2 \; \text{.......(2.11)}.$$
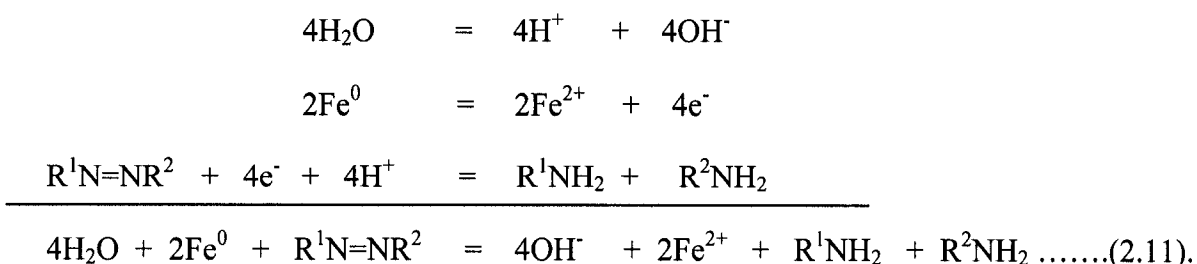
Hence, 2 moles of Fe^0 are required to reduce every mole of dye.

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For bisazo dye, these equations can be expressed as:

$$8H_2O + 4Fe^0 + R^1N=NR^2-R^3N=NR^4 = 8OH^- + 4Fe^{2+} + R^1NH_2 + R^2NH_2 + R^3NH_2 + R^4NH_2$$

$$\ldots\ldots\ldots (2.12)$$
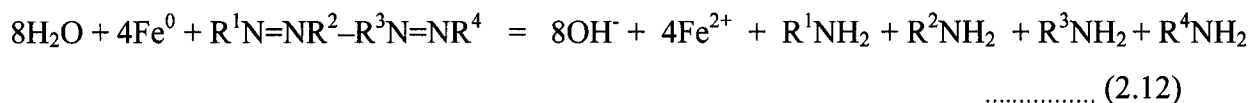
Where R^1, R^2, R^3 and R^4 are the different aromatic groups.

According to the above equation, twice the amount of Fe is required for bisazo dyes, as compared to monoazo dyes. RB5 is a bisazo dye; hence, it needs double the amount of Fe^0 as compared to RR2. The hypothesis behind this explanation is that a reactive site (pits and cracks) of Fe^0 surface can take part in the reduction reaction only once. This is consistent with the fact that, once two electrons are released from an active site, the site is oxidized to Fe^{2+} and an oxide layer is formed. The redox potential of Fe^{2+} is quite low; hence, the oxide layer acts as an effective barrier to further electron transfer and blocks subsequent reactions. Almost the entire 1 g Fe^0 was passivated after reacting with 1 mM dye solution in the first 1 hour when only 1 g Fe^0 was allowed to react with 1mM of RB5 (Figure 4.8). This was evident as little improvement in reduction (5%) was observed in the next 4 hours. The redox potential of azo bonds in RR2 and RB5 are not significantly different. In both the cases, the azo bond is between benzene and a napthalene ring with sulphonate and hydroxyl groups in ortho positions (Table 1.1).

Therefore, it is expected that the lower rate of reaction for RB5 was due to the relative inaccessibility to Fe^0 active sites, rather than due to difference in inherent reactivity. Such a difference in access to Fe sites may be due to: (i) steric hindrance, as molecular structures are significantly different; (ii) competition for sites with other impurity molecules or (iii) formation of complex reversible composites with impurities or intermediates. Two experiments were conducted to establish which of these three

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possibilities were predominant. To check whether impurities in RB5 was competing with the dye molecules for Fe^0 sites, a mixture of RR2 and RB5 was treated with 3 g Fe^0 (stoichiometric amount for 1 mM RR2 and RB5 as established earlier) and also with 5 g Fe^0 (higher amount). The results are presented in Figures 4.10 and 4.11 and compared with the results obtained separately for RR2 and RB5 solutions.
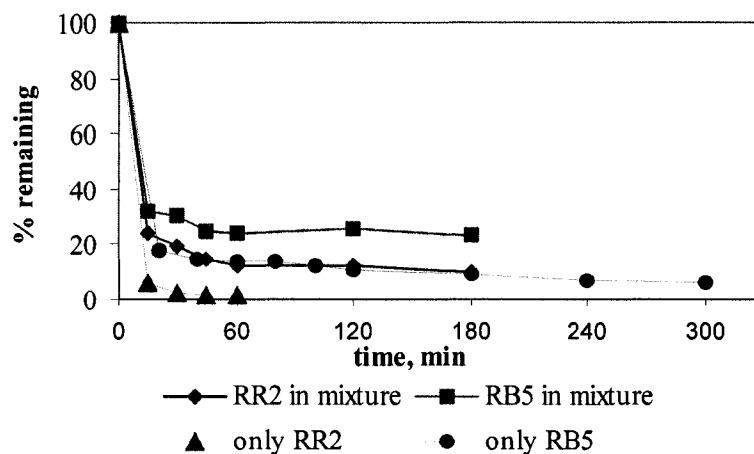
Figure 4.10: Fe^0 reduction rate for RR2 and RB5 mixture at 4.8 pH, 1 mM RR2, 1 mM RB5 with 3 g Fe

The comparison between the reaction rates of RR2 and RB5 in mixture and separate treatment in Figure 4.10 indicates that:

- The initial rate of reaction and the over all pattern for all the 4 lines were same, which indicates that the rate of reaction and mechanism remained unchanged even though RR2 and RB5 were mixed for Fe^0 treatment.

- In case of both the dyes, the percentage reduction decreased when treated in the mixture. The relative difference between the two trend lines for RR2 and RB5 reduction remained the same and the rate of reduction of RR2 was faster than RB5. This indicated that a third compound present in RB5 as impurity was competing for the Fe^0 surface with both the dyes. This third compound was

reacting with the Fe^0 at a faster rate than the two dyes and there was not enough Fe^0 surface area remaining for RR2 and RB5 to complete the reduction.

- The percent RB5 remaining in mixture was around 25 %, which indicates that it was starved of Fe surface by an amount of 25 % or less.

In order to confirm this hypothesis about presence of an impurity compound in RB5, which consumed some Fe^0, a similar experiment was carried out with excess of Fe so that the dyes were not starved of Fe^0. 5 g of Fe was used which was enough to cover the additional 25 % requirement.
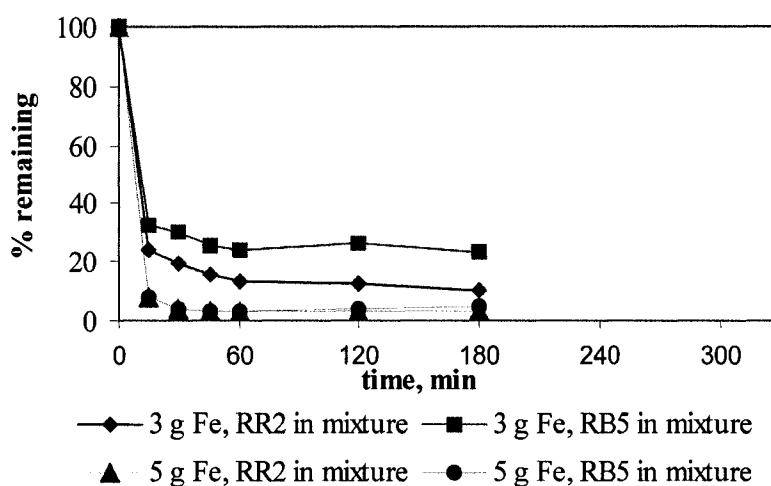
Figure 4.11: Fe reduction rate for RR2 and RB5 mixture
at pH 4.8, 1 mM RR2, 1 mM RB5 with 5 g Fe^0

The results, as presented in Figure 4.11, confirm the hypothesis that there were impurities with RB5, which were consuming Fe^0 surface. With excess Fe^0, a complete reduction of RR2 and RB5 could take place and resulted a dye conversion efficiency of around 96 to 98% for both RR2 and RB5. In real life situations, effluents will contain unknown quantities of compounds other than the dyes, which will compete for Fe^0 surface. Hence excess amount of Fe^0 will have to be used at the Fe^0 treatment stage.

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4.2.3 Comparison between RR2 and RB5 Fe^0 Reduction

There is a insignificant difference in the conversion efficiencies between RR2 and RB5 under similar conditions. Both dyes were 1 mM each are in the same mixture and were treated with 5 g Fe^0. The experimental data are presented in Table 4.1.

Table 4.1: Difference in RR2 and RB5 conversion efficiencies.

Time in min.	% of RR2 remaining (a)	% of RB5 remaining (b)	Difference (a-b)
15	7.66	7.48	Not considered
30	3.96	3.97	Not considered
45	2.96	3.37	-0.41
60	2.85	3.41	-0.56
120	2.98	3.65	-0.67
180	3.04	4.76	-1.72

The difference between RR2 and RB5 is considered once the reaction was completed after 45 minutes, and the % remaining values stabilizes. It can be concluded that the efficiency of conversion in the case of RR2 is slightly better than RB5. This might be due to the lower steric hindrance for RR2 to access the active Fe^0 sites compared to that for RB5. It may be noted that molecular diffusivity of RR2 (3.28 X 10^{-6} cm^2 /sec) is higher than that of RB5 (2.77 X 10^{-6} cm^2 /sec) (Table A.3.1). Therefore all the above discussion indicated that RR2 reduction rate was intrinsically better than RB5 reduction.

RR2 has a triazine reactive group. On reduction, organic dechlorination could take place and the Cl of the triazine group might be released in the solution as chloride (Monson et al., 1998). These Cl acted as Fe corrosion enhancer, which improved the availability of active Fe sites for reaction. Thus chloride had a catalytic effect on the reduction reaction (Johnson et al., 1998; Gotpagar et al., 1999). Therefore the reduction

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was faster in case of RR2 as compared to RB5. It may be noted that chloride pretreatment can improve availability of Fe^0 surface active sites by a factor of 7.6 (Section 2.1.1). However RB5 will also benefit from this effect if it is in the same mixture.

Previous researchers did not observe this difference for a bisazo dye (Naphthol Blue Black), which may be because they had started with a high Fe^0 quantity, 144 mg/mL (Nam and Tratnyek, 2000). The present study provided only 33.3 mg/mL Fe for RR2 and 66.6 mg/mL Fe for RB5. Moreover, the surface area concentration, mixing rate, iron source and type were different for these two studies.

4.2.4 Reaction Products

It appears that Fe reduction did not reduce the –NH- bond in RR2 under the studied reaction conditions within 1 hour and the breakdown product possibly had both napthalene and triazene ring, held together by the –NH- bond. This was indicated by the results obtained with the study on diphenylamine (a model compound) reaction with Fe (Section 4.2.5).

On exposure to air for 1 hour after Fe^0 reduction, the RB5 reaction product turned into a deep blue colored solution and developed a new small absorbance peak at 630 nm. In case of RR2, on exposure to air, the Fe^0 reaction product did not change its color or the absorbance characteristics. For both the dyes, if the reaction products were stored for a day, they responded poorly to the TNBS test for aromatic amines (20 % less response in a day). As shown later the performance in ARP treatment along with PEI aided coagulation also decreased. A possible explanation is that the di-sulfonated naphthol-amines, which were formed due to Fe^0 reduction (Section 2.3.3), were auto-oxidized in the presence of air to form some compounds, which did not respond to TNBS test. Di-sulfonated amino

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naphthol compounds are sensitive to oxygen and decompose readily in aerobic condition (Kudlich et al., 1999). Tri-aminohydroxy naphthalene di-sulfonic acid, a di-sulfonated amino- naphthol compound whose structure is very similar to the Fe^0 reduction product of RB5, gets auto oxidized in presence of oxygen to a naphthoquinoneimine which has blue colour (Kudlich et al., 1999).

This implies that storage time and exposure to air are critical for the Fe reaction products. However a maximum exposure of 30 minutes can be tolerated before the enzymatic treatment, which is sufficient to carry out pH neutralization and filtration of the Fe reduction products.

4.2.5 Fe^0 Reduction of Diphenylamine

DPA reduction was carried out under the similar condition as the dyes. One of the Fe^0 treatment breakdown products of DPA is aniline. Fe^0 treatment of DPA and resulting aniline was monitored by HPLC at two different channels, Channel 1 at 254 nm and channel 2 at 280 nm. Generation of Aniline in the solution was also monitored by TNBS test. The HPLC chromatograms for DPA reduction are given in Appendix C.4, Figures C.4.1 to C 4.4. Chromatograms for Channel 1 are only presented here because it shows the presence of all three compounds. Fe^0 reduction results of diphenylamine (DPA) with time are presented in Figure 4.12.

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% remaining vs Time, h

Legend:
—◆— DPA, absorb. at 254 nm   —■— DPA by absorb. at 280 nm
—▲— Aniline by absorb. at 254 nm   —━— Aniline by absorb. at 280 nm
—✳— DPA extract at 254 nm   —●— DPA extract at 280 nm
—+— Aniline by TNBS
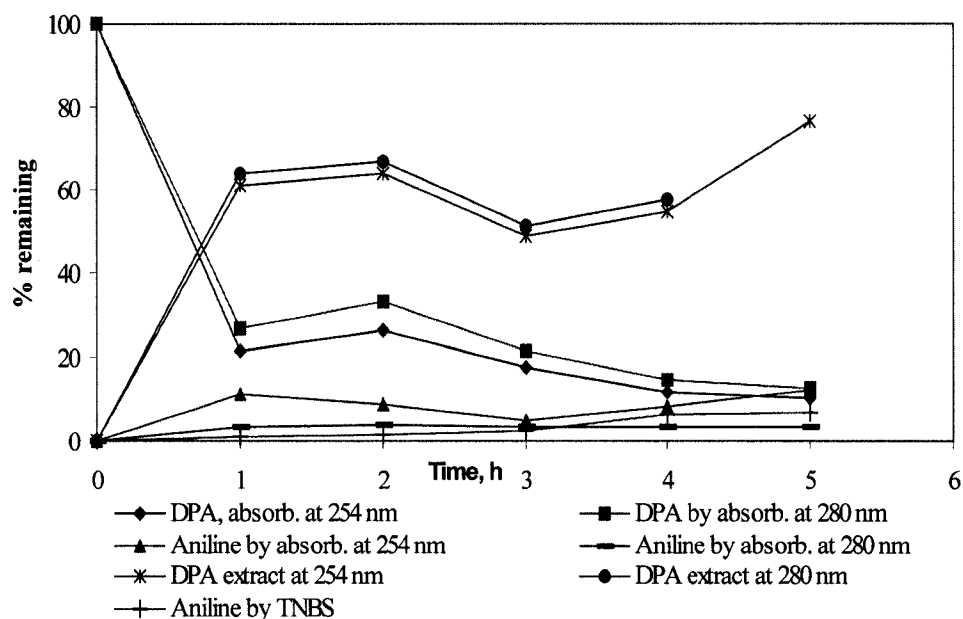
Figure 4.12: Fe^0 reduction for DPA
0.2 mM solution, at pH 5.1, 1 g Fe, 1 mM Na$_2$SO$_3$

From the results it is apparent that around 80% of the original DPA concentration was adsorbed on the Fe^0 surface. Only 12% DPA was reduced to aniline during 5 hours reaction time and no other compound was detected. It seems that the cleavage of the – NH- bond in DPA by Fe^0 reduction is a slow process. It is hypothesized that the mass transport to the iron surface was faster than the chemical reaction of DPA reduction to aniline. It is also presumed that DPA formed irreversible binding on the metal surface and, therefore, was not subjected to reduction reaction.

Based on these observations, it is expected that the similar –NH – bond of RR2 was less likely to break during Fe^0 treatment under similar reaction conditions within 1 hour. Moreover, the presence of the naphthol ring and bulky sulfonate groups in RR2 likely prevented the -NH- bond to bind with the Fe^0 reactive sites.

4.3 Enzymatic Treatment

The results about enzymatic action on two model compounds, 2-amino-8-naphthol-3,6-disulfonic acid (ANDSA) and diphenylamine (DPA), are presented and discussed first to gain insights into the enzymatic reaction on the Fe^0 reduction products of the dyes.

4.3.1 Amino-naphthol di-sulfonic acid (ANDSA)

Control experiments demonstrated that ARP or H_2O_2 separately had no effect on ANDSA, whereas, ARP and H_2O_2 together effectively converted this naphthol amine to a brownish red colored transparent solution without any visible precipitate. It had new UV-Vis absorbance peak at 428 nm. Hydrogen peroxide concentration and enzyme activity were monitored to confirm that the reaction was indeed an enzymatic reaction. The TNBS test indicated more than 90% conversion of aromatic amine. Perhaps the dimers or polymers formed were water-soluble due to the presence of sulfonate groups. The optimum reaction parameters are presented below.

4.3.1.1 Effect of pH: The effect of pH on enzymatic reaction of ANDSA was studied in the range of 3.0-9.0 under stringent conditions with 1 U/mL ARP, 1 mM substrate and 2 mM hydrogen peroxide.
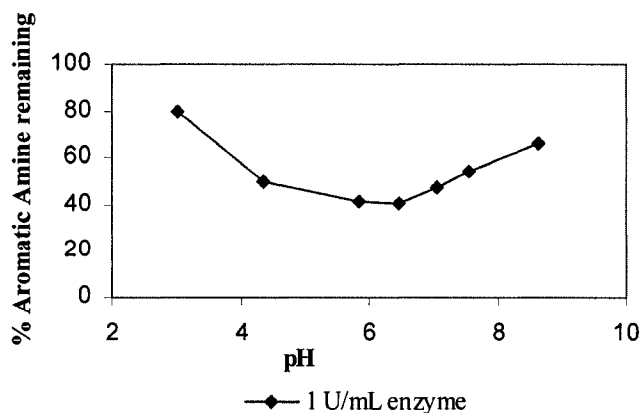
Figure 4.13 : Effect of pH,
for 1 mM ANDSA, 2 mM H_2O_2 concentration, observed after 3 h.

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The results are presented in Figure 4.13. The optimum pH was between pH 5.8 and 7.0. At this lower enzyme concentration (1U/mL), 50 % of the aromatic amine was still remaining in the optimum pH range.

4.3.1.2 Effect of H_2O_2 to Substrate Ratio: The effect of different concentrations of H_2O_2 on ARP treatment of ANDSA was studied in the range between 0.5 to 2.5. This range was selected based on the fact that ANDSA has two functional groups that can react with ARP (Table 1.1). The results are presented in Figures 4.14 and 4.15.
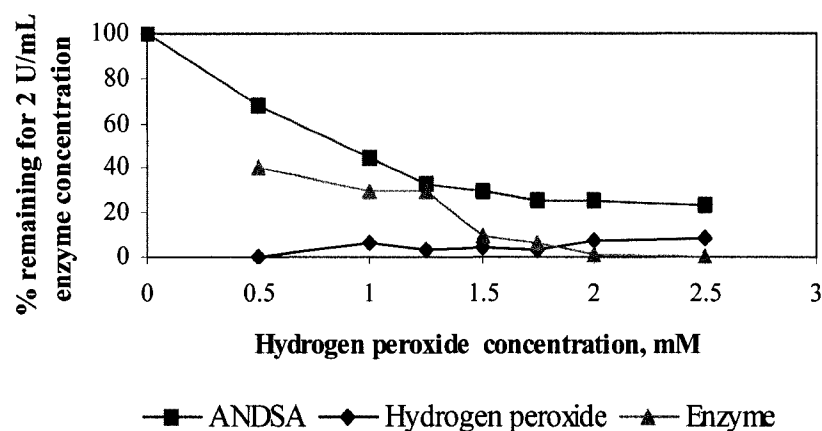
Figure 4.14: Effect of H_2O_2 concentration, at pH 7.0 , for 1 mM ANDSA, 2 U/mL ARP, observed after 3 h.
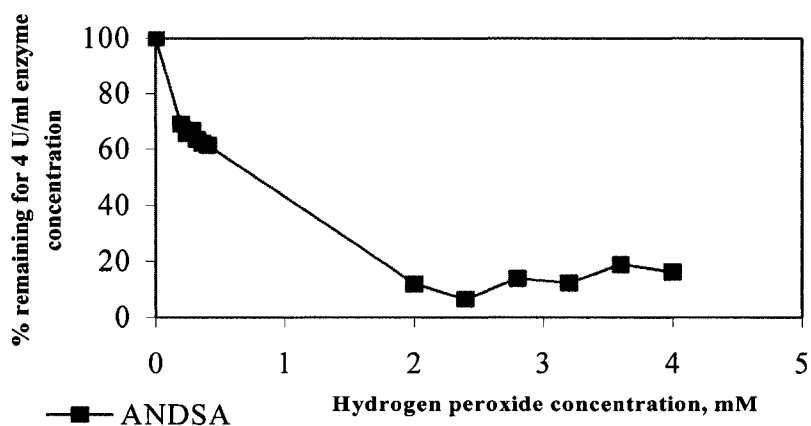
Figure 4.15: Effect of H_2O_2 concentration, at pH 7.0, for 1 mM ANDSA, 4 U/mL ARP, observed after 3 h.

ANDSA has two active functional groups, -NH₂- and –OH per molecule, which react with ARP. Therefore, it was anticipated that, for every mM of ANDSA 2 mM of H_2O_2 and 2 U/mL ARP might be required. On conducting an experiment with 2 U/mL ARP, it was observed that the ANDSA removal was still monotonically decreasing with H_2O_2 concentration (Figure 4.14) and there was no remaining ARP at higher H_2O_2 concentrations. On conducting another experiment with higher amount of ARP (4 U/mL) and H_2O_2 (2-4 mM), the ANDSA removal improved from 80% to 94% (compare Figures 4.14 and 4.15). For this amount of ARP (4 U/mL), the optimum H_2O_2 concentration was observed to be 2.4, so the optimum H_2O_2 to substrate (per functional group) ratio was around 1.2. The observed H_2O_2 to substrate ratio is consistent with previous studies on phenol (between 0.9 and 1.0 : 1) and aniline with SBP (1.5 :1) (Villalobos and Buchanan, 2002; Ibrahim et al., 2001; Mantha, 2001).

4.3.1.3 Effect of enzyme concentration : The effect of enzyme concentration for ANDSA was conducted at the pH of 7.0 and with 2.4 mM hydrogen peroxide and the results are presented in Figure 4.16. The optimum enzyme concentration was 4 U/mL, which
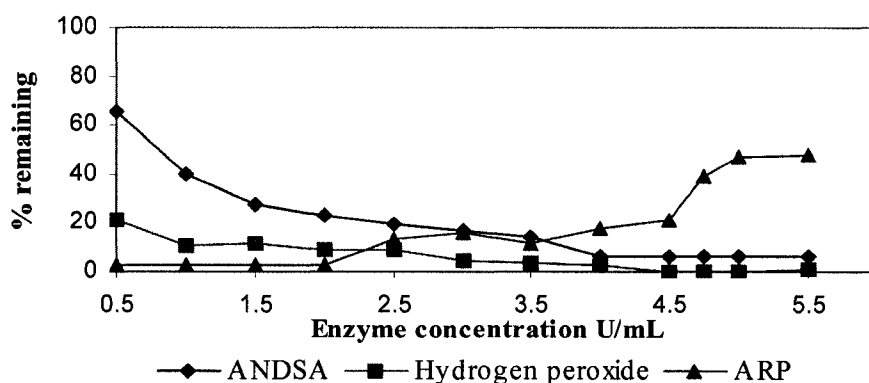
Figure 4.16: Effect of enzyme concentration,
at pH 7.0 , for 1 mM ANDSA, 2.4 mM H₂O₂, observed after 3 h.

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resulted in over 94 % conversion of ANDSA. No further improvement in percent removal of aromatic was observed at higher enzyme concentrations. At these higher concentrations of enzyme, there was no remaining hydrogen peroxide in the reaction mixture, which was the main reason for no further improvement in the removal rate. Previous studies reported the enzyme concentration to be 8 U/mL for aniline, 2 U/mL for p-chloroaniline and 30 U/mL for o-chloroaniline (Taylor et al., 1998) and around 1 for phenol (Villalobos and Buchanan, 2002; Ibrahim et al., 2001). For aniline, the position of substituents on benzene or naphthalene ring with respect to amine group determined how good was the substrate for ARP. Meta-substitution gave better results compared to para, ortho or un-substituted compounds. Since ANDSA has a group in meta position so it proved to be a good substrate for ARP.

4.3.1.4 Reaction Time: The ARP action on ANDSA against time was studied and the results are presented in Figures 4.17, 4.18, 4.19 and 4.20. The same experiment was carried out under two different conditions; in one, the entire amount of H_2O_2 was added at the beginning and in the other, H_2O_2 was added in three equal amounts at 0 minute, after 30 and 60 minutes. It can be observed that 3 hours time was required to remove 94% of aromatic amines. A new product was formed which had peak absorbance at 428 nm.
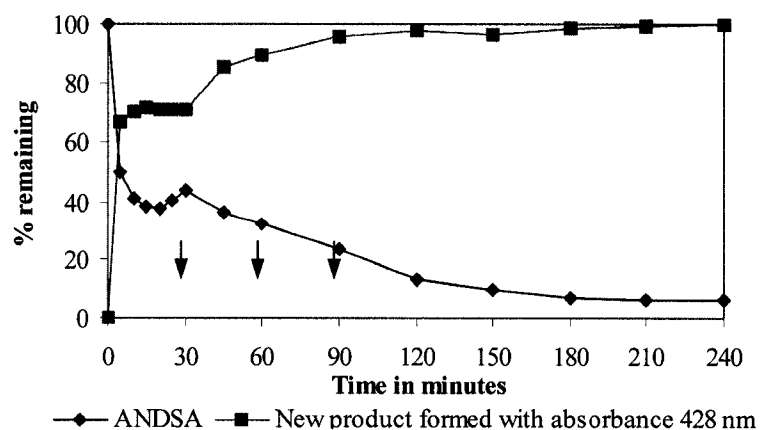
Figure 4.17 : Aromatic remaining and new product formation against time, at pH 7.0, for 1 mM ANDSA, 2.4 mM H_2O_2 added in steps at 0, 30, 60 min, 4 U/mL ARP

Figure 4.18 compares the percentage of aromatic removal by both step and single addition of hydrogen peroxide. When hydrogen peroxide was added once in the beginning, of the percentage of aromatic removal was almost 10 % more as compared
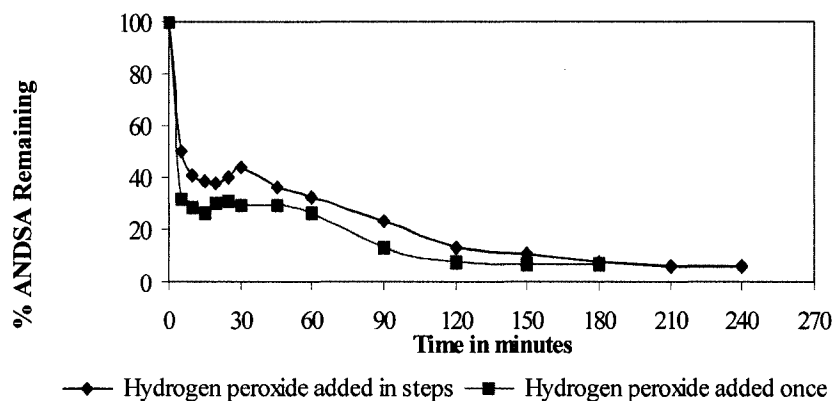
Figure 4.18: Aromatic amine remaining against time,
at pH 7.0 , for 1 mM ANDSA, 2.4 mM H_2O_2, 4 U/mL ARP

to when it was added in steps. This may be because of formation of more radicals at this step, which were oxidized faster in the first case. But when the reactions were continued for longer time, say for 3 hours, the total aromatic removal was same for both the cases. The amount of hydrogen peroxide and enzyme remaining in the two modes of operation are given in Figures 4.19 and 4.20. It is observed that more enzyme was getting inactivated within the initial 60 minutes when the entire amount of H_2O_2 was added at the beginning (Figure 4.20). During this period, the ANDSA conversion under these two conditions was not as high as the difference between the enzyme remaining. It was expected so because an excess hydrogen peroxide inactivated ARP. However, given sufficient time, ARP got activated again and returns back to the original form (Section 2.2.9). This is consistent with other works (Villalobos and Buchanan, 2002). But the entire amount of inactivated ARP did not return to active state as a significant portion of

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ARP was lost when H_2O_2 is added at the beginning. When all the hydrogen peroxide was added at the beginning, there was no hydrogen peroxide remained after 90 minutes and thus there was very little improvement in aromatic removal after this point. Although, it may seem that both these operations had the same effect after sometime. But there can be at least 10-15 % savings in both enzyme and hydrogen peroxide concentration in step addition of hydrogen peroxide (Figures 4.19 and 4.20).

—♦— Hydrogen peroxide added in steps —■— Hydrogen peroxide added once
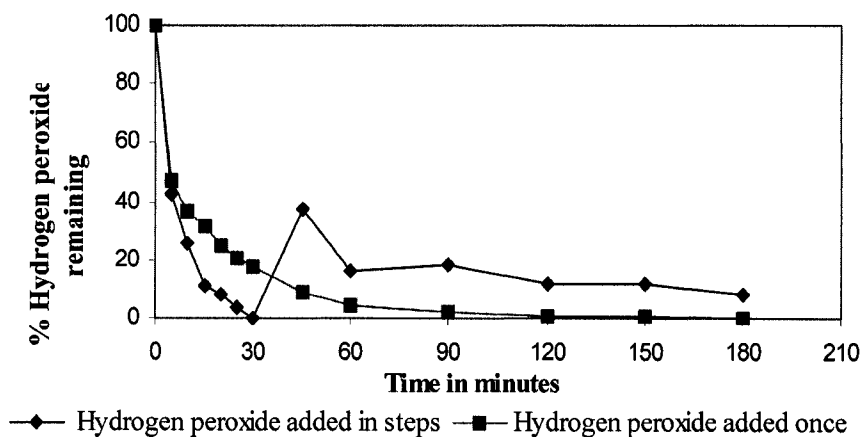
Figure 4.19: H_2O_2 remaining against time,
at pH 7.0 , for 1 mM ANDSA, 2.4 mM H_2O_2, 4 U/mL ARP

—♦— Hydrogen peroxide added in steps —■— Hydrogen peroxide added once
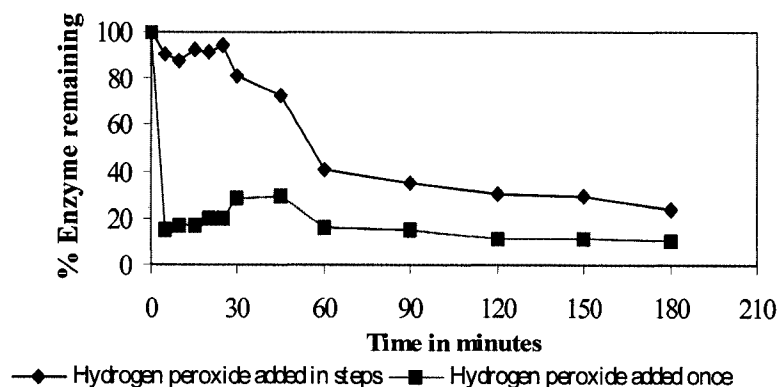
Figure 4.20: Enzyme remaining against time,
at pH 7.0 , for 1 mM ANDSA, 2.4 mM H_2O_2, 4 U/mL ARP

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Taylor et al., 1998 reported similar enzyme savings to be around 15 to 20 %.

After 30 minutes, the TNBS response for aromatic amine displayed a small peak (Figures 4.17 & 4.18). This may be due to some intermediates, which responded to TNBS test. This may be explained as follows by the following reaction equations where ANDSA is represented as $PhOH-SA-AmNH_2$; E, E1 and E2 are active enzyme, Compound 1 and Compound 2 respectively (Section 2.2.2) and P1 and P2 are the reaction products.

$$E \ + \ H_2O_2 \ \rightarrow \ E1 \ + \ H_2O \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4.1)$$

$$PhOH-SA-AmNH_2 + \ E1 \ \rightarrow E2 \ + PhO^\bullet-SA-AmNH_2 \ \ldots\ldots\ldots\ldots\ldots (4.2)$$

$$PhOH-SA-AmNH_2 + \ E1 \ \rightarrow \ E2 \ + PhOH-SA-AmNH^\bullet \ \ldots\ldots\ldots\ldots (4.3)$$

$$PhOH-SA-AmNH_2 + \ E2 \ \rightarrow \ E \ + PhO^\bullet-SA-AmNH_2 \ \ldots\ldots\ldots\ldots (4.4)$$

$$PhOH-SA-AmNH_2 \ + \ E2 \ \rightarrow \ E \ + PhOH-SA-AmNH^\bullet \ \ldots\ldots\ldots\ldots (4.5)$$

$$PhO^\bullet-SA-AmNH_2 \ + \ E1 \ \rightarrow \ E2 \ + PhO^\bullet-SA-AmNH^\bullet \ \ldots\ldots\ldots\ldots (4.6)$$

$$PhOH-SA-AmNH^\bullet + \ E2 \ \rightarrow \ E \ + PhO^\bullet-SA-AmNH^\bullet \ \ldots\ldots\ldots\ldots (4.7)$$
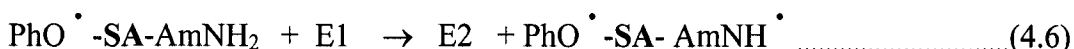
Non-enzymatic reactions can be expressed as :

$$2 \ PhO^\bullet-SA-AmNH_2 \ = \ P1 \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4.8)$$

$$2 \ PhOH-SA-AmNH^\bullet \ = \ P2 \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4.9)$$

$$PhOH-SA-AmNH_2 + PhO^\bullet-SA-AmNH_2 \Leftrightarrow PhOH-SA-AmNH_2 + PhOH-SA-AmNH^\bullet$$

$$\ldots\ldots\ldots\ldots (4.10)$$
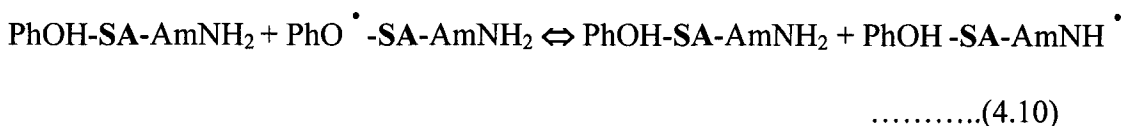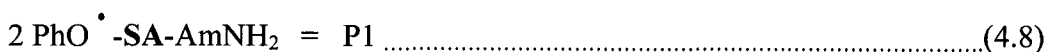
Double radicals are relatively unstable and hence Equations 4.6 and 4.7 can be ignored compared to other reactions.

It is known that a phenolic group tends to react better with ARP compared to amino group. (Optimum enzyme concentration for 1 mM phenol is 1 U/mL while that is 8 U/mL for 1 mM aniline as reported by Ibrahim et al., 2001; Taylor et al., 1998). The phenolic group in ANDSA is in the meta position with respect to amino group, therefore the phenolic group bound faster with ARP when compared to amino group to form phenoxyl radicals. This means, initially there were more phenoxyl radicals compared to aminyl radicals. Hence the forward direction was preferred and according to the equation (4.10), aminyl radicals were formed and the concentration of amine decreased. After some time when phenoxyl radical concentration decreased, the backward reaction proceeded to form products by radical transfer and there was again a rise in amine concentration. At the same time the reaction (4.8) continued forming the products. These reactions proceeded until complete conversion of phenol occurred by forming product P1, after which gradual oxidation of amine group occurred.

4.3.1.5 Possible Mechanism: The proposed mechanism for enzymatic oxidation of ANDSA is presented in Figure 4.21. In presence of ARP, free radicals are formed in the first step. Subsequently, there could be a different reaction depending on the reactivity of the free radicals and the reaction condition (Stiborava, 1996; Spadaro et al., 1994). These radicals can (Scheme I) polymerize by –OH group or by –NH– groups (also refer section 2.2.7). There can be nucleophilic attack by water on the free radicals and release of a second electron to form quinone, which can further polymerize (Scheme II). The free radicals can also form quinone imines (Scheme III). Desulfonation can be another

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possibility (Scheme IV), where one electron oxidation of ANDSA may produce anilinium cation radicals, which can be attacked by water resulting in elimination of ammonium ion. This can get oxidized by another electron and subsequently again attack by water. As a result, partial desulfonation may take place resulting in quinone formation.
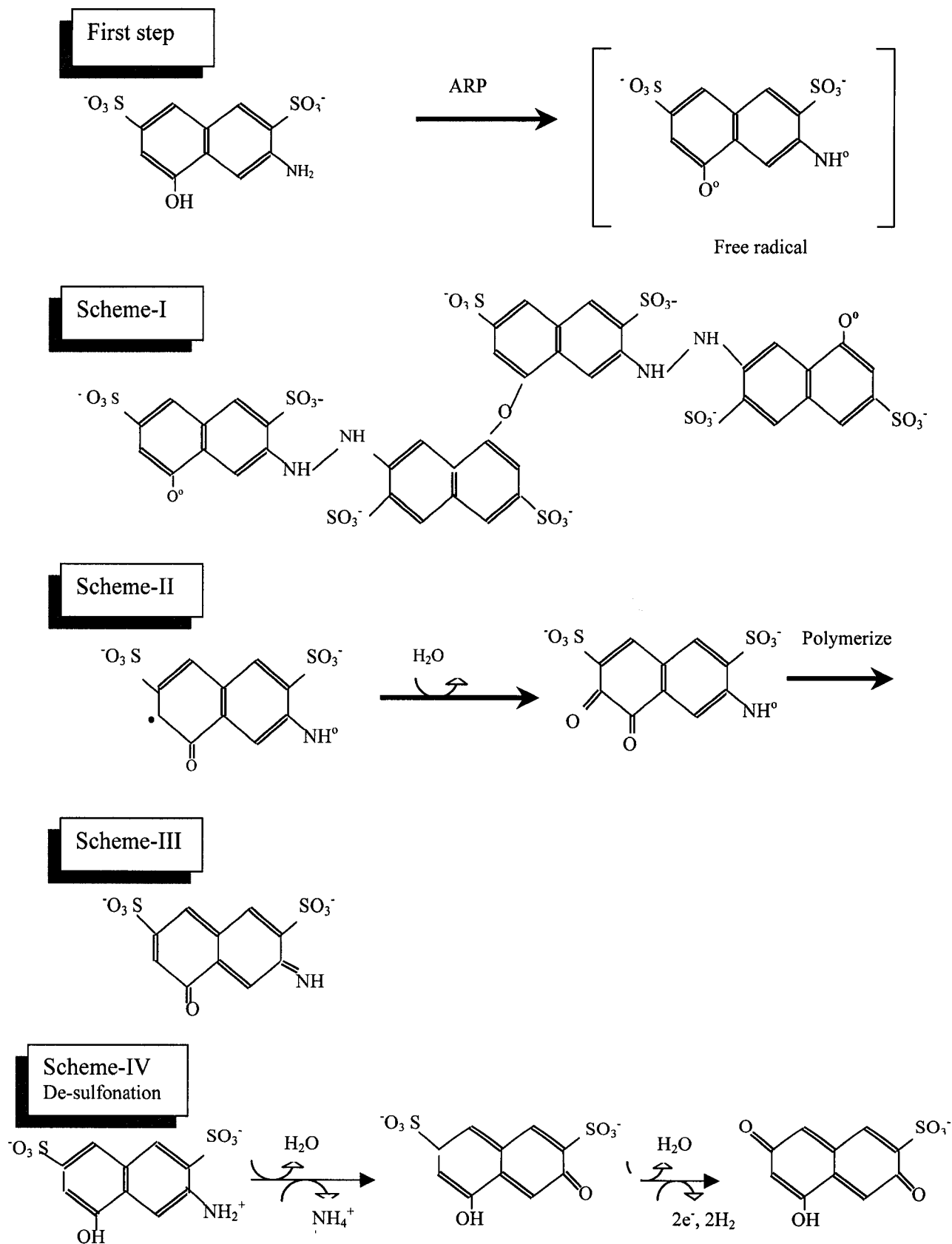
Figure 4.21: Proposed mechanism for enzymatic oxidation of ANDSA

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4.3.1.6 Reaction Kinetics: The rate of ARP action on ANDSA was studied and the results are presented in Figure 4.22. Initial velocities were plotted against substrate concentrations along with the best fit curve. The procedure for estimating these figures is presented in Appendix D.
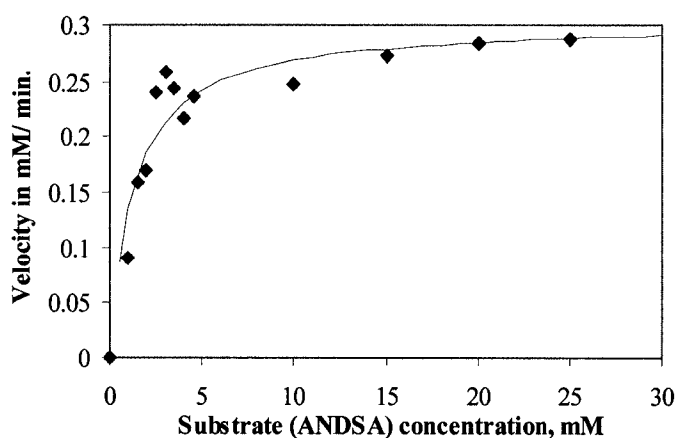
Figure 4.22: ARP reaction kinetics for ANDSA at pH 7.2, for 2.4 mM H_2O_2 , 4 U/mL ARP

The Michaelis-Menten constant, K_m is 1.25 ± 0.28 mM and the maximum velocity, V_{max} is 0.302 ± 0.016 mM / min.

Both the above values show the 95 % confidence interval.

The ARP reaction kinetics is given by :

$$V = \frac{0.302\ S}{1.25 + S} \qquad \dots\dots\dots\dots\dots\dots\dots\dots 4.11$$

k_{cat}, the turnover number which is defined as the maximum number of substrate molecule that can be converted per molecule of enzyme in unit time is given by (Palmer, 1995) :

$$k_{cat} = \frac{V_{max}}{E_0} \qquad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(4.12)$$

where E_0 is enzyme concentration.

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The conversion factor between mM and activity (C_a) for ARP is $5.15 * 10^{-5}$ mM /(U/mL) (Ibrahim et al., 2001). The kinetics was studied with 4U/mL ARP; therefore the ARP concentration is given by

$$E_0 = U * C_a = 4 * 5.15 * 10^{-5} = 20.6 * 10^{-5} \text{ mM}$$

Therefore,

$$k_{ca} = \frac{0.302}{20.6 * 10^{-5} * 60} = 24.5 / s$$

This can be compared with the k_{cat} for ARP action on phenol, which is 977 /s and has a corresponding Michaelis-Menten constant, K_m, equal to 9.45 mM. For o-phenylenediamine oxidation by ARP, k_{cat} was found to be 910/s and K_m was 0.5 mM (Kaniya and Nagamune, 2002). This shows that ANDSA is a less reactive substrate for ARP when compared to phenol and o-phenylenediamine. Also the catalytic efficiency (the relative rate of reaction at low substrate concentration) k_{cat}/K_m in $mM^{-1}.s^{-1}$, can be calculated from above values, which are ~20 for ANDSA, ~103 for phenol and ~1800 for o-phenylenediamine.

4.3.2 Diphenylamine

The Fe reduction process (Section 4.2.5) did not affect DPA. Therefore, it was decided to find out if DPA was a substrate of ARP. This helped in understanding whether the similar secondary amine –NH- bond present in RR2 was affected by the enzymatic treatment. DPA was found to be a good substrate of ARP. More than 90 % of DPA was removed under neutral pH, when the solution turned cloudy with faint yellow colour and a new UV-vis absorbance peak appeared at 440 nm. DPA did not respond in TNBS test, hence, the peak absorbance at 280 nm was monitored by both UV-Vis absorbance and HPLC for determining optimum reaction conditions. Control experiments with enzyme and hydrogen peroxide alone did not change the UV-absorbance spectra showing that these reagents had no affect on DPA when added individually. H_2O_2 color test confirmed that only 10% H_2O_2 was consumed after 3 h when H_2O_2 alone was added. The enzyme activity test confirmed that none of the added enzyme was consumed in 3 h in the control experiment with only enzyme. Hence it was concluded that the oxidation of DPA was a combined action of both H_2O_2 and enzyme. The optimum parameters for the DPA oxidation by ARP are discussed below.

4.3.2.1 Effect of pH: The effect of pH on enzymatic action of diphenylamine (DPA) is presented in Figure 4.23. The optimum pH range for ARP action on DPA was found to be between 5.5 and 7.4, which is a very broad range. At lower pH around 40 % of the enzyme was still active since ARP can have some lower pH activity, though there was little hydrogen peroxide remaining (5-10 %) at this pH. For pH higher than 7.5, the entire amount of enzyme was inactivated and a large amount of DPA and hydrogen peroxide were still remaining.

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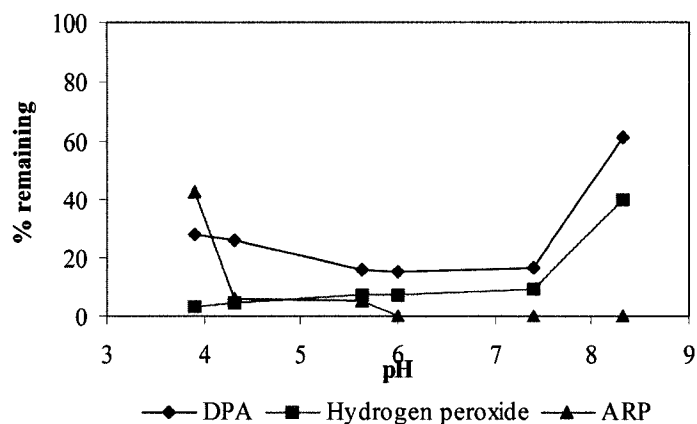
Figure 4.23: Effect of pH,
for 0.2 mM DPA, 0.2 mM H₂O₂, 0.1 U/ml ARP, observed after 3 h.

4.3.2.2 Effect of H₂O₂ to substrate ratio: The effect of H_2O_2 to substrate ratio is presented in Figure 4.24.
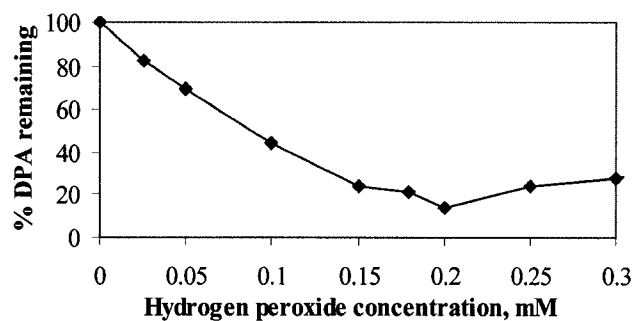
Figure 4.24: Effect of H₂O₂ concentration,
at pH 7.3 , for 0.2 mM DPA, 0.2 U/ml ARP, observed after 3 h.

The optimum H_2O_2 concentration was found to be 0.2 mM for 0.2 mM DPA. At higher hydrogen peroxide concentration, the removal efficiency decreased and the solution turned light yellow, perhaps due to formation of quinones. It is presumed that at lower peroxide concentration dimers were formed, while at higher concentration, these are further oxidized to produce quinones.

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4.3.2.3 Effect of enzyme concentration : The effect of enzyme concentration was studied

at a pH of 7.3 and the results are presented in Figure 4.25.
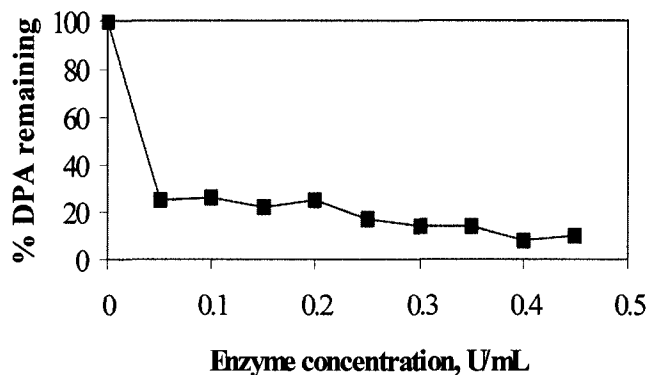
**Figure 4.25: Effect of enzyme concentration,
at pH 7.3, for 0.2 mM DPA, 0.2 mM H₂O₂, observed after 3 h.**

It may be noted that even at very low enzyme concentration there was around 70 % DPA

removal. This shows that DPA is a very reactive compound. Presence of small amount of

enzyme and hydrogen peroxide was enough to form the radicals and to subsequently

form the dimers. However, at this low enzyme concentration (also at low hydrogen

peroxide concentration) the reaction mixture remained colorless, indicating that there was

no quinone formation at this stage. Almost 90 % DPA was removed in three hours at a

enzyme concentration of 0.4 U/mL. Hence, this was considered to be optimum enzyme

concentration for 90 % DPA removal.

4.3.2.4 Reaction Time: The optimum reaction time for DPA oxidation was studied under

optimum reaction conditions such as pH of 7.3, hydrogen peroxide concentration of 0.2

mM and enzyme concentration of 0.4 U/mL. The results are presented in Figure 4.26.

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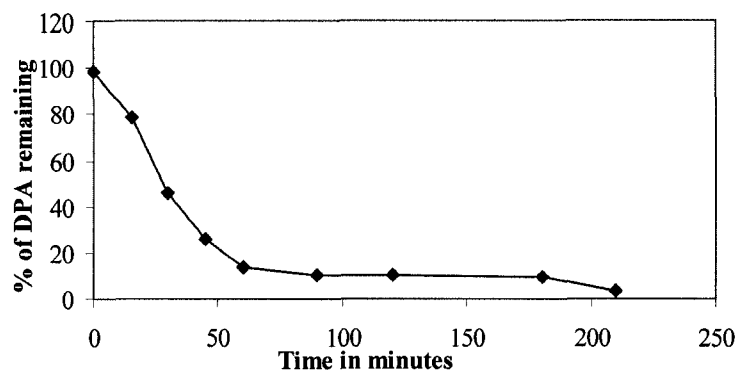
Figure 4.26: DPA remaining with time,
at pH 7.3, for 0.2 mM DPA, 0.2 mM H_2O_2, observed after 3 h.

The above figure shows that around 90 % of DPA was oxidised in one hour. Allowing the experiment to run for another 2 hours did not improve much in the removal rate. But after around 4 hours, 98 % removal was obtained. The above results show that DPA is a good substrate of ARP. Therefore, it can be concluded that the -NH- bond present in RR2 is also susceptible to enzymatic oxidation in the present study. This also indicates that in case of RR2, Fe reduction product 3 hours time was sufficient to oxidise the –NH- bond present in the RR2 molecule. The HPLC result (Figure 4.27) shows a new peak with retention time at 2.445 min, which elutes before DPA (retention time 3.669 min). This shows that DPA was oxidized to a product, which may be more polar compound than DPA. Oxidation of DPA in acid (non enzymatic reaction) produced a diphenoquinone-diimine (Pankratov, 2001). The similar type of product also may be expected in this case.

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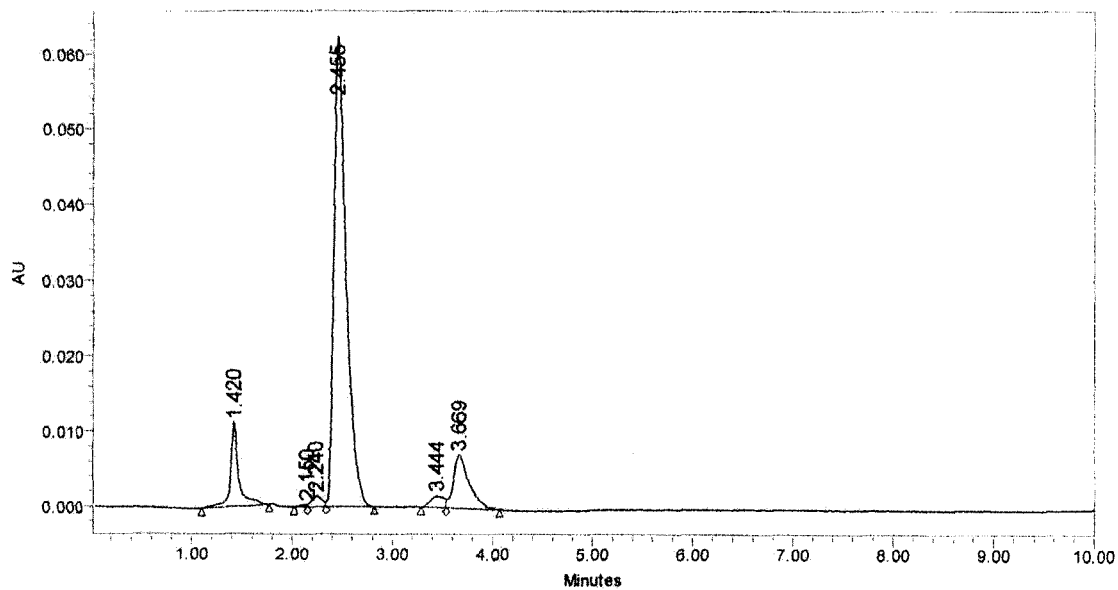
	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	1.420	63980	9.20	10828	12.90
2	2.150	1584	0.23	365	0.43
3	2.240	10528	1.51	1417	1.69
4	2.455	526227	75.63	62660	74.63
5	3.444	14659	2.11	1554	1.85
6	3.669	78775	11.32	7140	8.50

Figure 4.27: HPLC chromatogram of DPA oxidation at 254 nm, DPA concentration 0.2 mM, hydrogen peroxide 0.2 mM, ARP 0.4 U/mL, 3 h reaction time , pH 7.3, new product peak at 2.455 min, DPA at 3.669 min.

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4.3.3 Reactive Red 2 Fe^0 Reduction Product

Fe^0 treatment reduced 98 % of RR2 to aromatic amines. The reaction products were aniline and a naphthol amine attached to a triazine ring by a –NH- bond. ANDSA, a naphthol amine and diphenylamine containing a similar –NH- bond proved to be substrates of ARP (Sections 4.2 and 4.3). Therefore, the Fe reduction products of RR2 are also expected as substrates of the enzyme. Since PEI was used finally to remove the enzymatic reaction products, a control experiment was done on Fe^0 reduction product of RR2 with PEI. The UV absorbance was reduced by 80 % (compare Figure 4.28 with Figure 4.6) but TNBS test detected 60% aromatic amine in the solution after PEI treatment. This established the requirement for enzymatic reaction. Enzymatic reaction was carried out with 0.5 mM RR2 Fe reduction product. There are four active functional groups in 1 molecule of RR2 breakdown products hence 0.5 mM contains half of the functional groups present in 1 mM RR2 that can react with ARP.
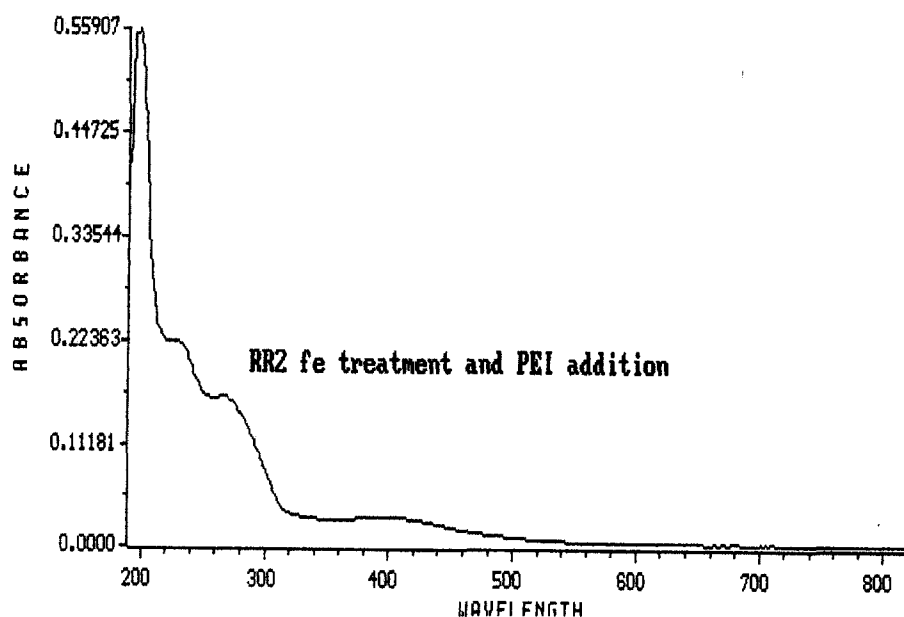
Figure 4.28: UV-vis absorbance spectra of 1 mM RR2 Fe reduction product in control experiment after PEI addition, pH 7.0, 3 h reaction time, PEI conc. 200 mg/L, 100 mg/L alum.

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On enzymatic treatment of Fe reduction products of RR2, no precipitate were observed except for formation of slightly turbid reddish brown solution having color similar to that of ANDSA enzymatic reaction product. On filtration the optical transparency of the reaction mixture improved. The terms "RR2" and "RB5" are used synonymously in place of Fe^0 reduction products of RR2 and RB5 respectively.

4.3.3.1 Effect of pH: The effect of pH on enzymatic action on Fe reduction product of RR2 was studied in the range 3.8 to 9.5 and the results are presented in Fig 4.29. The reaction was carried out under stringent condition with 1 U/mL ARP and 1.5 mM H_2O_2.
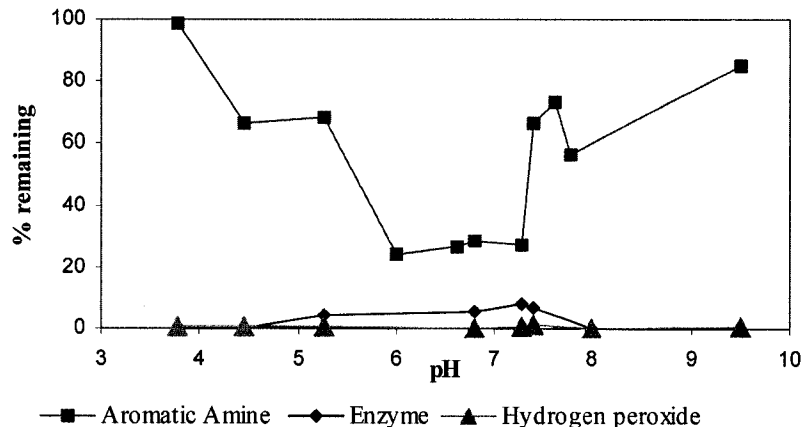
Figure 4.29: Effect of pH, for 0.5 mM RR2, 1.5 mM H_2O_2 , 1 U/mL ARP, observed after 3 h.

The optimum pH range was between 6.0 and 7.4. This optimum range was found to be similar to both ANDSA and DPA. There was no remaining hydrogen peroxide in the whole range of pH while some enzyme was remaining in the range between 5.5 and 7.5. From this figure, it seems that even though the enzyme was inactivated in slightly acidic and basic pH, hydrogen peroxide was consumed by some other reaction. Also 1.5 mM hydrogen peroxide was not enough to carry out more than 75 % removal of aromatic in presence of 1 U/mL enzyme. The first point may be possible as the RR2 Fe reduction

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products may contain some Fe_2^+ which can consume H_2O_2. Therefore, higher amounts of enzyme and hydrogen peroxide were required for further reaction.

4.3.3.2 Effect of H_2O_2 to substrate ratio: The effect of different H_2O_2 concentrations on aromatic removal was studied at optimum pH of 7.2 and the results are presented in Figure 4.30 and 4.31.

Figure 4.30: Effect of H_2O_2 concentration, at pH 7.2, for 0.5 mM RR2, observed after 3 h.

Figure 4.31: Effect of H_2O_2 concentration, at pH 7.2, for 0.5 mM RR2, 4 U/mL ARP, observed after 3 h.

Figure 4.30 indicates that when the reaction was conducted with 2 U/mL enzyme, the optimum ratio was between 1 and 1.5. But the aromatic removal was only 80 %. There was no hydrogen peroxide remaining after 3 h. This indicated that some more enzyme and hydrogen peroxide were required to further carry reaction. Experiments with a higher amount of enzyme (4 U/mL) shifted the optimum range for hydrogen peroxide to 3 mM. In this range, 90 % aromatic removal was achieved. It may be noted that the remaining hydrogen peroxide was still low, 2% at 2 mM and 4 % at 3 mM. The enzyme remaining was also very low. Figures 4.30 and 4.31 indicate that the optimum H_2O_2 concentration was 3. Fe reduction products from 0.5 mM of RR2 contain 2 functional groups, so the optimum H_2O_2 to substrate ratio is 1.5.

4.3.3.3 Effect of enzyme concentration : This experiment was conducted at the optimum pH and at the optimum hydrogen peroxide concentration of 3 mM. The effect of enzyme concentration is presented in Figure 4.32. The observed optimum enzyme concentration was 4.5 U/mL, which resulted in 94 % removal of aromatic. At 4 U/mL enzyme concentration, 90 % removal of aromatic was obtained, but increasing the concentration by another 0.5 U/mL, 4 % improvement in aromatic removal was achieved.
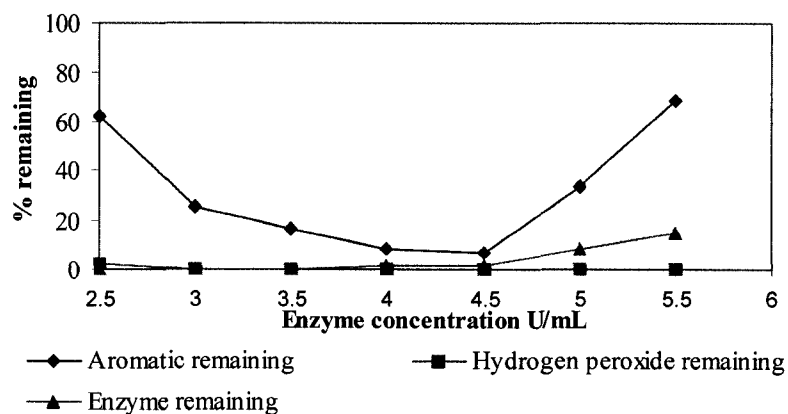
Figure 4.32: Effect of enzyme concentration,
at pH 7.2, for 0.5 mM RR2, 3 mM H_2O_2, observed after 3 h

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At concentration higher than this the aromatic removal decreased. This was probably due to the dissociation of hydrogen peroxide to water due to catalase action of the enzyme.

4.3.3.4 Reaction Time: The action of ARP on RR2 Fe reduction product against time was studied and the results are presented in Figure 4.33. This reaction was carried out under optimum condition i,e pH 7.2, 3 mM of hydrogen peroxide, and 4.5 U/mL of enzyme. After 2 hours 94 % removal of aromatic amine was observed. Continuing the reaction for another one hour did not improve the removal rate. Hence, two hours reaction time was considered to be sufficient for 94 % aromatic removal.

Figure 4.33: Aromatic amine conversion against time, at pH 7.2, for 0.5 mM RR2, 3 mM H$_2$O$_2$, 4.5 U/mL ARP

4.3.3.5 Possible Mechanism: The mechanism of Fe reduction and subsequent ARP facilitated oxidation of RR2 is presented in Figure 4.34.

In case of RR2, the visual observations after ARP treatment was very similar to that for ANDSA. Hence, it can be presumed that their reaction mechanism with ARP was be similar to some extent. Figure 4.21, for the proposed reaction mechanism for ANDSA, may be referred to understand the RR2 mechanism. The Fe treatment breakdown products for RR2 are multisubstituted naphthol (Compound A) and aniline (Compound

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B). In presence of ARP, aniline (Compound B) will form anilinium radical. Substituted naphthol (Compound A) will undergo one electron oxidation to form naphthalinium cation radical. These free radicals might behave in various manners depending on the reaction environment (Stiborova, 1996). They may either form dimers and polymers, oxidize further to give two electron oxidation product or combine with other compounds depending on reactivity.

Substituted naphthol (compound A) may undergo one electron oxidation to form naphthalinium cation radical. This radical is then attacked by water resulting in quinone and elimination of amine group as ammonium ion. Quinone will further undergo another one electron oxidation to form a cation which is subsequently attacked by water at the carbon, bearing the sulfonic group and resulting in eliminating of the sulfonic group as sulfite (Muralikrishna and Renganathan, 1993). Finally naphthoquinone (Compound C) may be formed.

The free anilinium and naphthalinium radicals may combine at possible locations of the aromatic ring to form polymers. These radicals may combine with radicals of similar type or may combine with each other (Stiborova, 1996). Anilinium radicals can combine with the naphthol moiety at two possible locations: at the -NH- bond between the triazine ring and naphthol moiety connection and at $-NH_2$ group in the naphthol moiety.

Fe reduction products of RR2 were proven to be good substrates of ARP and after enzymatic reaction they are expected to produce naphthoquinone and polymers. However identification of the end products should be carried out to elucidate the mechanism more accurately.

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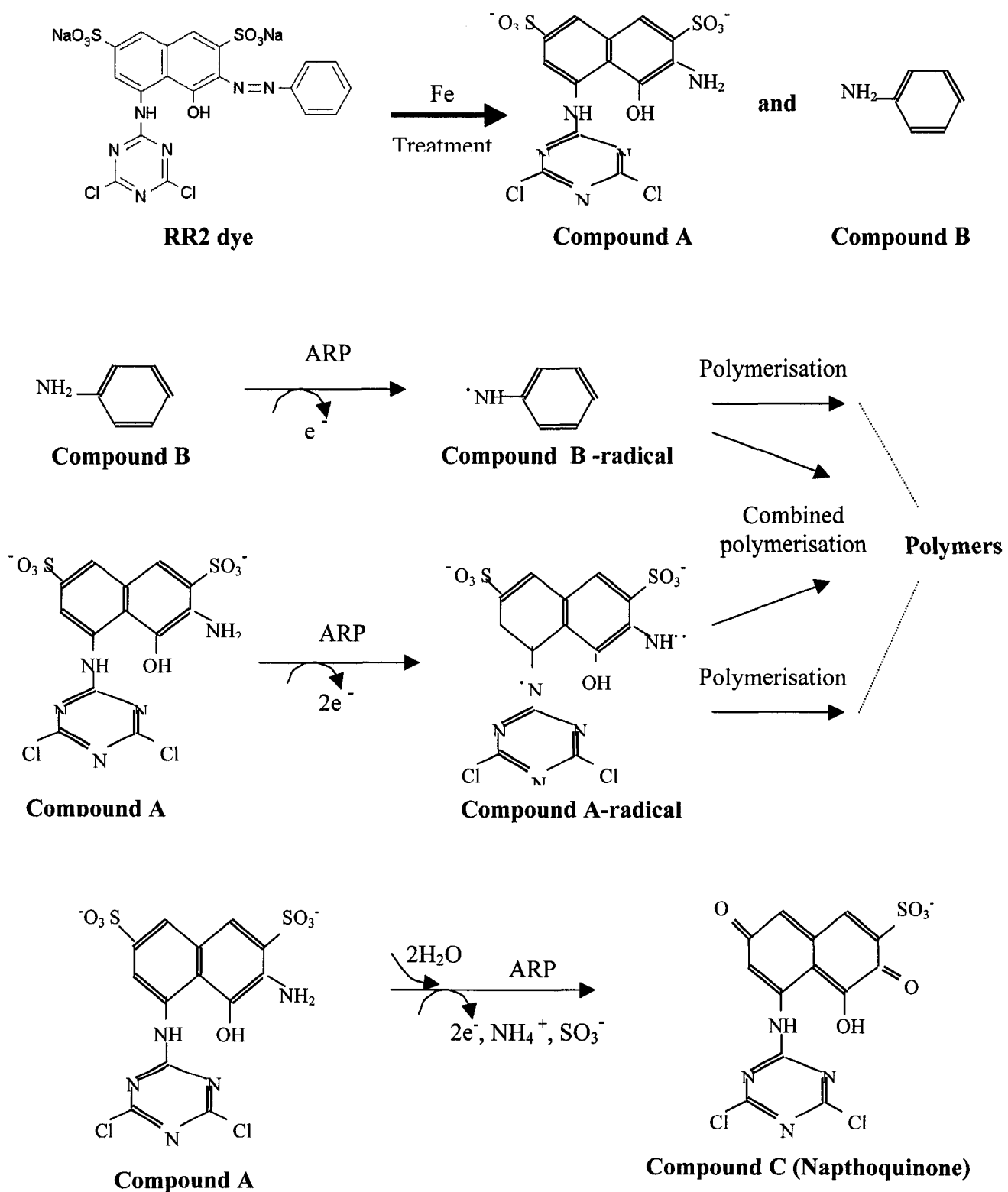
Fig 4.34 : Proposed mechanism for RR2 Fe reduction and oxidation by ARP

4.3.4 Reactive Black 5 Fe^0 Reduction Product

After Fe reduction of RB5, multi-substituted aromatic and naphthol-amines were formed. On enzymatic treatment of Fe reduction product of RB5, no visible precipitate was observed except the formation of a transparent chocolate brown color. The Fe reduction products of RB5 have 6 functional groups, which can react with ARP. The enzymatic treatment was done with 0.5 mM RB5 Fe reduction product. Hence, there were half the number of functional groups were present in 0.5 mM as compared to 1 mM of Fe reduction products. Control experiments were conducted with ARP and enzyme separately. Enzyme had no effect on RB5. However, around 20 % of hydrogen peroxide was consumed when it was added separately. This may be due to the presence of naphtholamines, which are prone to auto-oxidation. Hence some H_2O_2 was consumed for this auto-oxidation reaction. Similarly, control experiment was conducted with PEI to see if PEI could remove the Fe reduction products. Even 200 mg/L PEI and slightly acidic pH of 5.6 was not enough to remove the aromatic amines from water, which was evident from the unaltered absorbance spectrum after PEI addition (compare Figure 4.35 with Figure 4.7
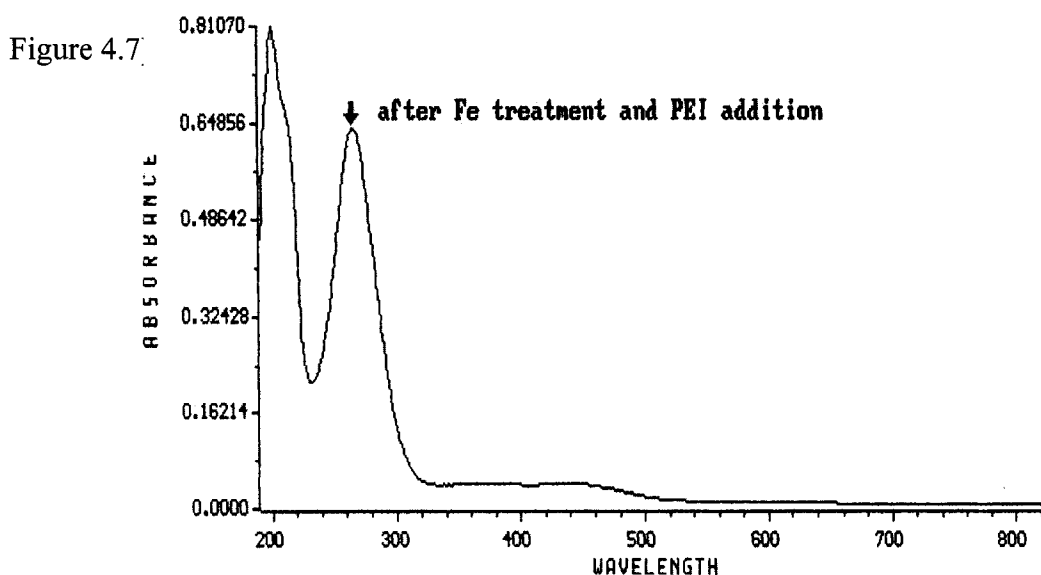
Figure 4.35: UV-vis absorbance spectra for 1 mM RB5 Fe reduction product in control experiment after 200 mg/L PEI and 100mg/L alum addition, at pH 5.6, 3 h reaction time.

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4.3.4.1 Effect of pH: The ARP oxidation of RB5 was conducted in a pH range of 3.5 to 9.3 under two different enzyme concentrations: 2 U/mL which is less than the requirement of 3 U/mL and 4 U/mL which is more than the requirement. The effect of pH on enzymatic action on RB5 Fe reduction product is presented in Figure 4.36.
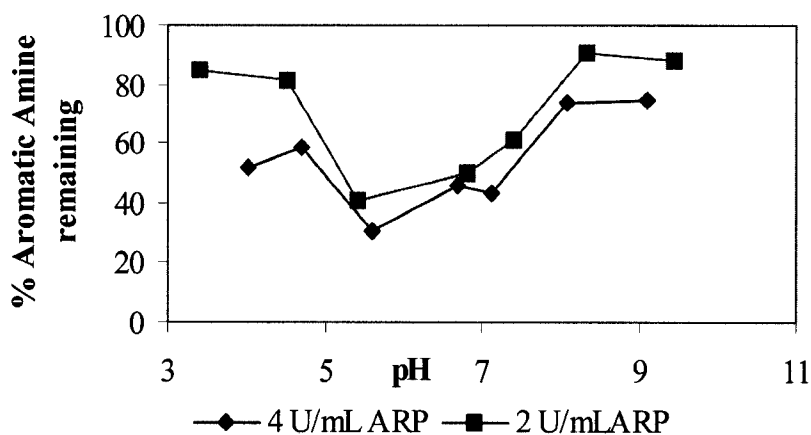
Figure 4.36: Effect of pH, for 0.5 mM RB5, 3 mM H_2O_2, observed after 3 h.

The optimum pH range was between 5.4 and 6.0. One mM RB5 Fe reduction product has 5 amino groups. At acidic pH, these amino groups may get protonated and help in the enzymatic reaction. It may also be noted that when sufficient amount of enzyme was added, around 20 % more removal of aromatic was obtained outside this optimum range.

4.3.4.2 H_2O_2 to Substrate Ratio: The effect of different H_2O_2 concentrations was studied in the range of 2 and 5 mM because the stoichiometric H_2O_2 requirement was 3 mM. The pH was maintained at 5.4. The results are presented in Figures 4.37 and 4.38.
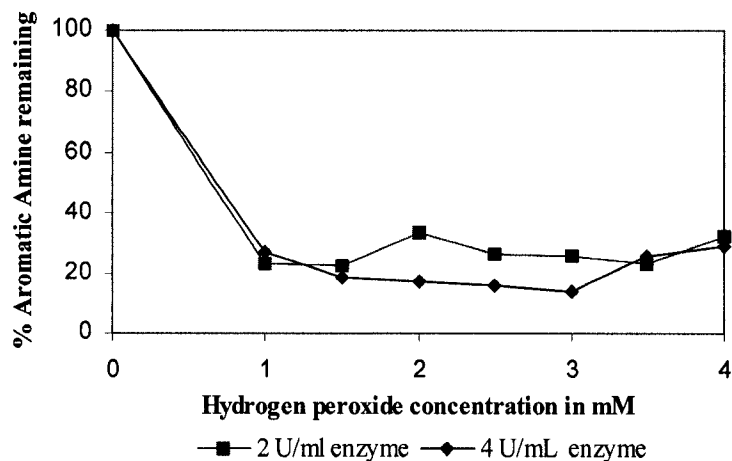
88
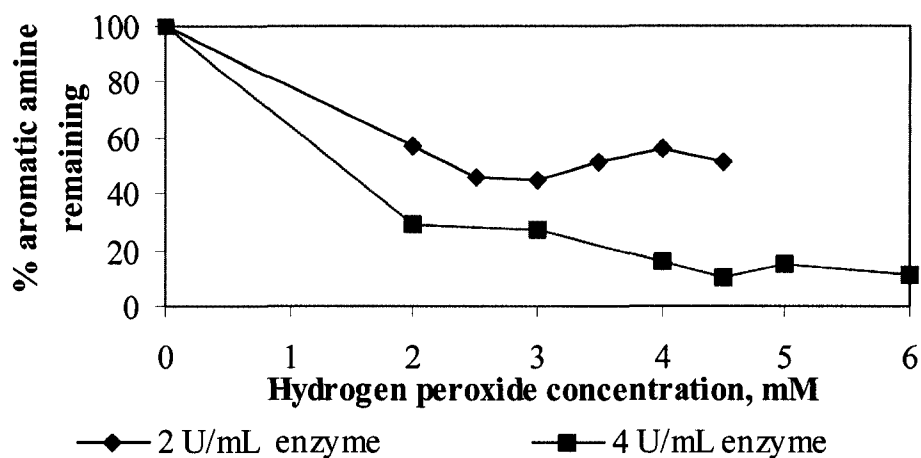
**Figure 4.37: Effect of H_2O_2 concentration,
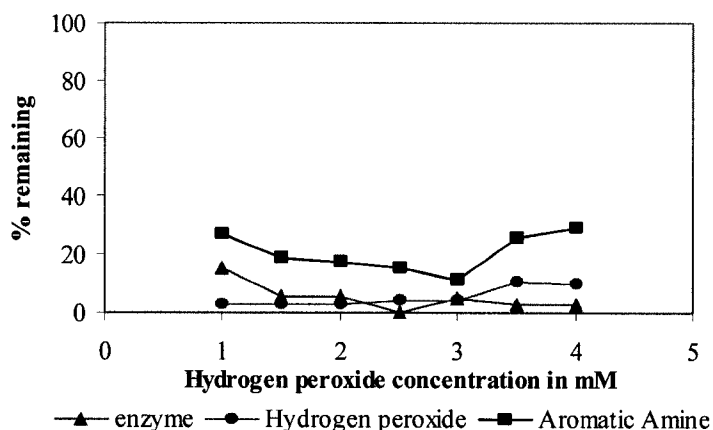at pH 5.4 , for 0.5 mM RB5, observed after 3 h.**
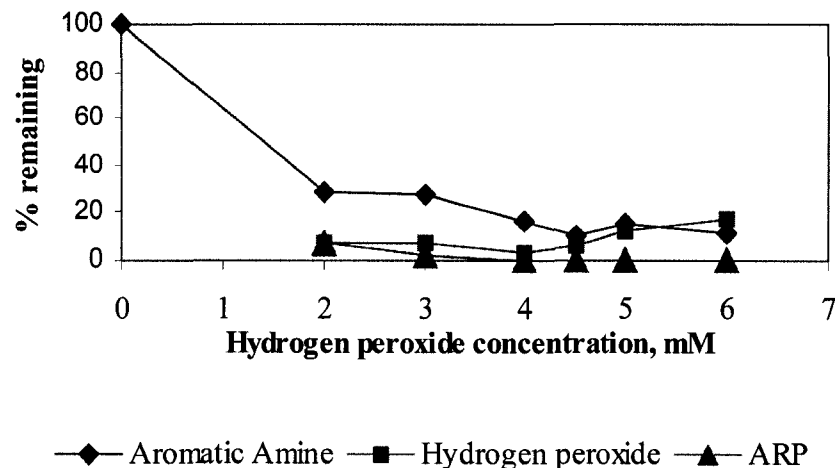
**Figure 4.38: Effect of H_2O_2 concentration,
at pH 5.4, for 0.5 mM RB5, 4U/mL ARP, observed after 3 h.**

It may be noticed that at lower enzyme concentration only 50 % aromatic was removed. However, at higher enzyme concentration (4 U/mL), almost 91 % aromatic removal was achieved with 4.5 mM hydrogen peroxide concentration. Figure 4.37 shows that the optimum H_2O_2 concentration is 4.5 mM. Therefore the optimum hydrogen peroxide ratio per functional group is 1.5. At this optimum concentration only 10% hydrogen peroxide

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remained after 3 h, while there was no remaining enzyme (Figure 4.38). This shows that if more enzyme was provided higher aromatic removal may have been achieved.

4.3.4.3 Effect of enzyme concentration : The effect of enzyme concentration was studied at pH 5.4 , hydrogen peroxide concentration of 4.5 mM and enzyme concentration in a range of
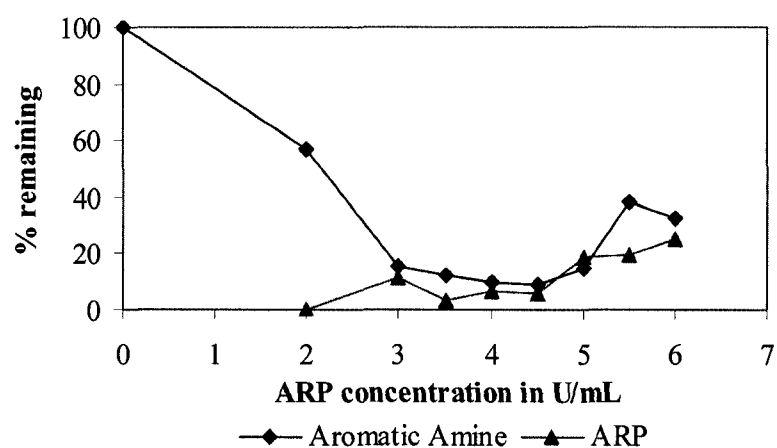
Figure 4.39: Effect of enzyme concentration,
at pH 5.4 , for 0.5 mM RB5, 4.5 mM H₂O₂, observed after 3 h.

2 to 6 U/mL. The results are presented in Figure 4.39. At enzyme concentration of 4.5 U/mL, 92 % conversion of aromatic was observed. This shows around 1% improvement in aromatic removal at 4.5 U/mL as compared to the same at 4 U/mL. The observed optimum enzyme concentration was in the range of 4 - 4.5 U/mL. There was less than 5 % of enzyme and hydrogen peroxide remaining for this optimum range after 3 h of reaction time, showing maximum utilization of both the reagents in this optimum range. Hence the optimum enzyme concentration was between 4 and 4.5 U/mL and the optimum enzyme concentration per functional group is 1.5 U/mL. This is similar to the optimum ratio in case of ANDSA.

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4.3.4.4 Reaction Time: ARP action on RB5 Fe reduction product against time was studied under the optimum pH, enzyme and hydrogen peroxide concentrations and the results are presented in Figure 4.40. After 2 hours, around 92 % removal of RB5 was observed and by continuing the reaction for another one hour the aromatic removal improves by only 1%, hence 2 hours reaction time was sufficient for 92 % removal. After 15 minutes, the TNBS response for aromatic amine went up (Figure 4.40) as was noticed for RR2 and ANDSA also possibly due to formation of an intermediate, which responded to TNBS test.
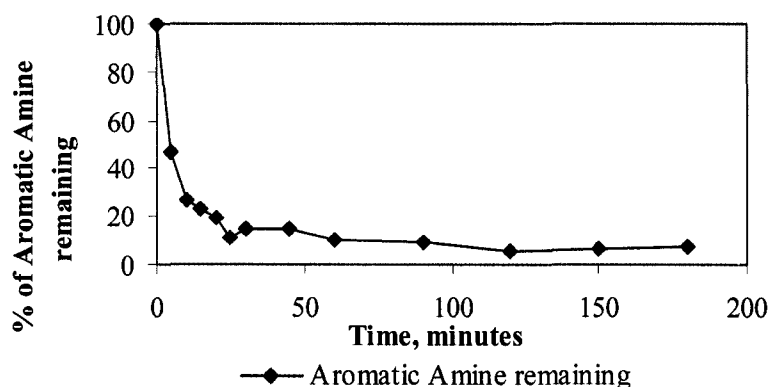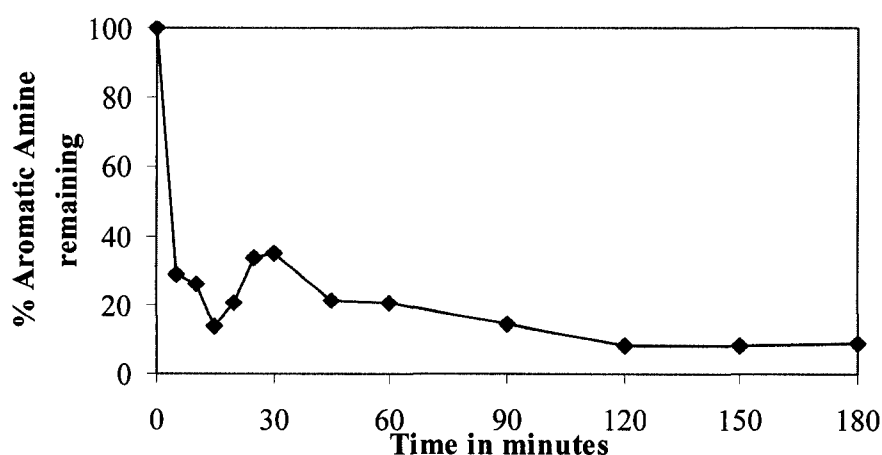

Figure 4.40: Aromatic amine conversion against time, at pH 5.4 , for 0.5 mM RB5, 4.5 mM H₂O₂, 4.5 U/mL ARP.

4.3.4.5 Proposed mechanism: The mechanism of Fe reduction and subsequent ARP facilitated oxidation of RB5 is presented in Figure 4.41. This is similar to RR2.

For RB5, substituted amine (Compound X) and naphthalamine (Compound Y) are formed after Fe treatment. In presence of ARP and H_2O_2, these compounds will form anilinium and naphthalinium radicals. The amine (Compound X) will undergo two-electron oxidation to form a quinone (Compound X2). The naphthalamine (Compound Y) will similarly be oxidised to form naphthoquinone (Compound Y2).

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The anilinium and naphthalinium free radicals may also combine with each other at possible locations to form complex polymers. Due to presence of sulfonic groups, the polymers are water-soluble as discussed earlier. So the Fe treatment end products of RB5 proved to be good substrates of ARP and enzymatic reaction produced colored products, which probably were quinone and naphthoquinone. These colored products were removed from water by further treatment.

Figure 4.41: Proposed mechanism for RB5 Fe reduction and oxidation by ARP.

Compound Y

Fe reduction

Compound X

$^-O_3SOCH_2CH_2O_2S$ —NH$_2$

$^-O_3SOCH_2CH_2O_2S$ —NH$_2$ **Compound X**   ARP   e$^-$ $^-O_3SOCH_2CH_2O_2S$ —NH$^\cdot$ **Compound X radical**   Polymerisation   **Polymers**

$^-O_3SOCH_2CH_2O_2S$ —NH$^\cdot$ **Compound X radical**   2H$_2$O   ARP   e$^-$, NH$_4^+$ $^-O_3SOCH_2CH_2O_2S$ —O   **Compound X2 (Quinone)**

Compound Y   ARP   4e$^-$ **Compound Y- radical**   Polymerisation   **Polymers**

Compound Y- radical   2H$_2$O   ARP   2e$^-$, NH$_4^+$ **Compound Y2 (quinone - imine)**

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4.3.5 ARP Treatment Reaction Products

No precipitation was observed after ARP treatment on RB5 Fe reduction products. However in case RR2 there might be some precipitate, as the solution was slightly turbid. The transparency improved on filtration. In general it is expected that the ARP treatment will result some polymers, which may precipitate.

In case of RR2 these anilinium-naphthol polymers inherit the sulfonate groups from the naphthol moiety, which makes them water soluble. On the other hand, when anilinium radicals combine with each other to form polymers/ dimers, a little visible precipitate may be observed. Perhaps this is happening in case of RR2. Naphthoquinone, the other RR2 end product, has a sulphonate group, hence it is water soluble. In case of RB5 all the Fe reduction and enzymatic treatment products contain sulfonate groups, which makes them water soluble. Hence no precipitation is also observed after ARP treatment. These colored products were removed by adding a coagulant aid, PEI along with alum, in the final stage.

Figures 4.42 and 4.43 show the absorbance spectra after enzymatic treatment on 0.5 mM Fe treated RR2 and RB5. These may be compared with the Figures 4.6 and 4.7, which are the Fe^0 reduction products of 1 mM RR2 and RB5. After enzymatic treatment, UV absorbance reduction for RR2 was 22 % (at 258 nm) and 33 % (at 266 nm) for RB5 were observed. The absorbance peaks only reduced but stayed at the same λ_{max} as for the parent dyes.

94
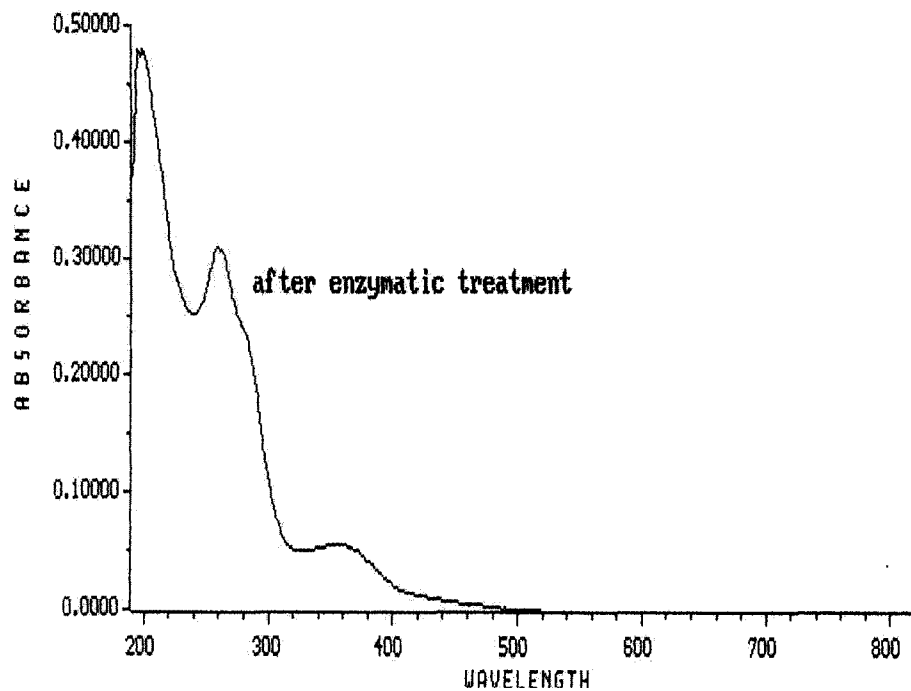
Figure 4.42: UV-vis absorbance of enzymatic reaction product of 0.5 mM
Fe treated RR2, 3 mM H_2O_2, 4.5 U/mL ARP, pH 7.0, 3 h reaction time.
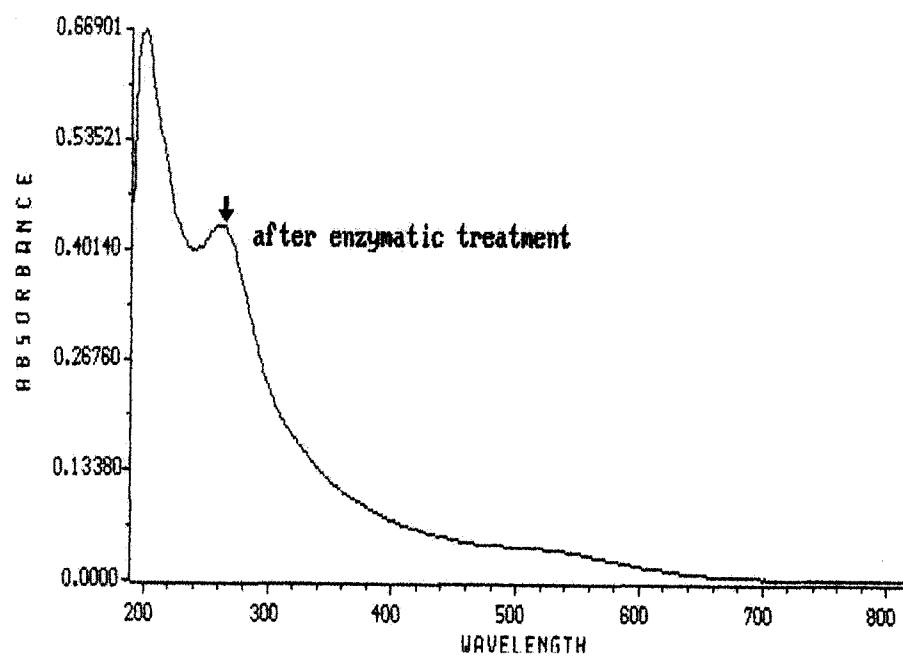
Figure 4.43: UV-vis absorbance of enzymatic reaction product of 0.5 mM
Fe treated RB5, 4.5 mM H_2O_2, 4.5 U/mL ARP, pH 5.4 , 3 h reaction time.

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4.4 Coagulation

Since the products of the enzymatic treatment still rendered colour, a coagulant aid, polyethyleneimine (PEI), was used to precipitate the products. Alum alone was not effective to remove the colored products. In all the cases after adding PEI, alum was added at a concentration of 100 mg/L and pH was adjusted to neutral range to help settle the flocs. Alum made the flocs settle faster. After this PEI-aided coagulation, there was no residual color in the water except some smaller absorbance in the UV region. Around 2 h reaction time was sufficient for effective color removal. It is assumed that some chemical reaction is also involved (Schiff's base formation) along with the physical coagulation process. Hence, PEI may have acted as a "chemical complexing agent" rather than just a coagulant. Experiments were carried out to identify the optimum parameters for effective removal of the colored products. The results are presented in the following sub-sections.

4.4.1 Reactive Red 2 Reaction Product

4.4.1.1 Optimum pH: The effect of pH on coagulation process is presented in Figure 4.44.
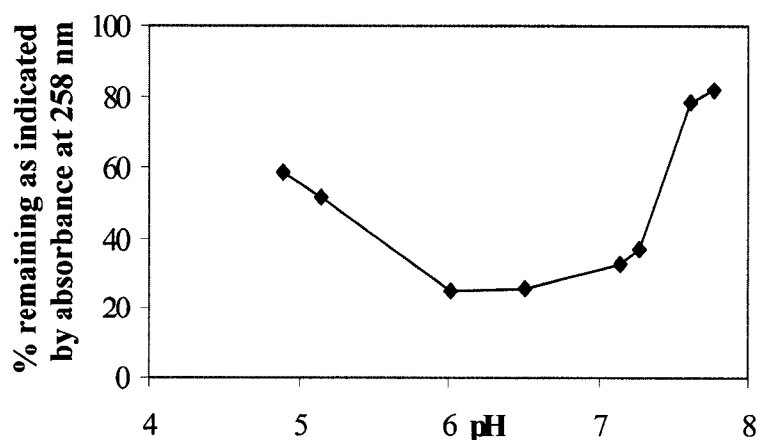
**Figure 4.44 : Removal efficiency at different pH,
for 0.5 mM RR2, in presence of 100 mg/L PEI, 100 mg/L alum.**

The optimum pH was determined under stringent condition where the coagulant concentration was kept 100 mg/L so that only pH had the effect on the removal. The peak absorbance at 258 nm was monitored for determining the removal efficiency. The coagulation process was found to be most effective within a pH range of 6.0 to 7.4 for RR2. This is consistent with the fact that PEI is more effective at pH less than 8 which helps in protonation of the amine groups. These positively charged ammonium ions bind with the negatively charged quinones, the enzymatic reaction products, and remove them from water (Section 2.4).

4.4.1.2 Optimum Coagulant Concentration: The effect of coagulant concentration at the optimum pH (neutral) is presented in Figure 4.45. The observed optimum PEI concentration was between 200 to 250 mg/L for RR2, which resulted in 91 % compound removal. The resulting water was colorless. Previous researchers have found an optimum concentration of 100 mg/L for removal of enzymatic reaction product of chloroanilines with tyrosinase (another enzyme) (Wada et al., 1995). Hence, further work is needed to find a suitable coagulant with lower concentration.
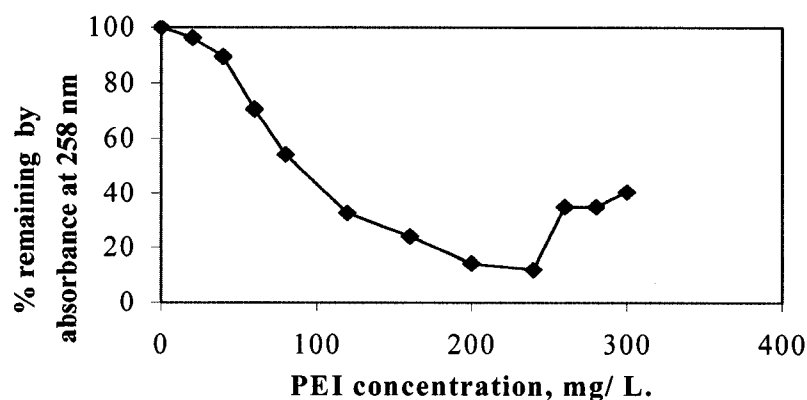
Figure 4.45 : Removal efficiency at different PEI concentration,
at pH 7.0, 100 mg/L alum, for 0.5 mM RR2.

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4.4.2 Reactive Black 5 Reaction Product

4.4.2.1 Optimum pH: Similar experiments were conducted with RB5 enzymatic treatment product with 100 mg/L PEI. The UV absorbance at 266 nm was monitored for measuring removal efficiency. Acidic pH, around 5.0 was found to give better result for RB5 coagulation process (Figure 4.46).
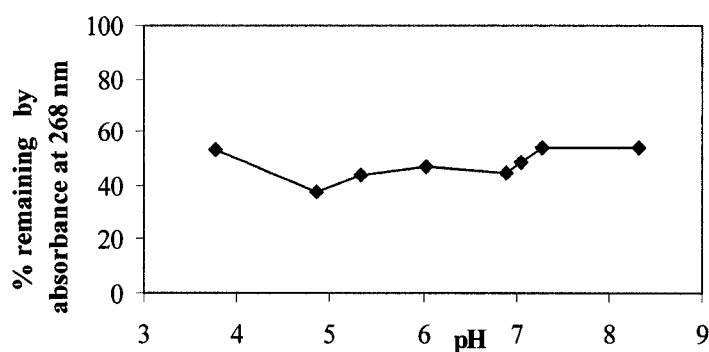
Figure 4.46 : Removal efficiency at different pH, for 0.5 mM RB5, 100 mg/L PEI, 100 mg/L alum.

This is lower than the optimum pH observed in case of RR2 (optimum at neutral pH). Such difference can be attributed to the presence of more amino groups in RB5 breakdown products, which introduced an additional factor in the coagulation mechanism.

4.4.2.2 Optimum Coagulant Concentration : The effect of coagulant concentration under optimum pH (5.4) is presented in Figure 4.47. The observed optimum PEI concentration was around 200-250 mg/L for RB5. It can be observed that even with the optimum PEI concentration, 18% of RB5 enzymatic reaction product was still remained in solution. Since PEI has amine groups in the molecule, it may have hindered the binding of RB5 enzymatic reaction product with PEI.

98
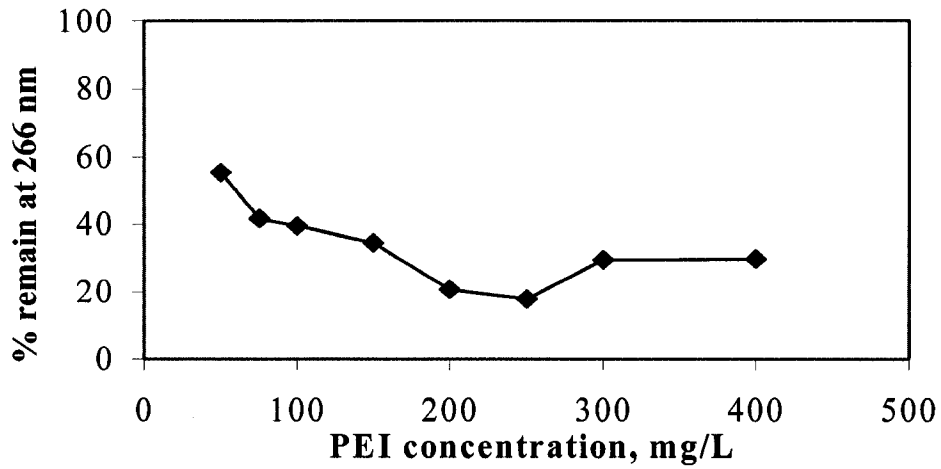
Figure 4.47 : Removal effciency at different PEI concentration, at pH 5.6, 100 mg/L alum, for 0.5 mM RB5

4.4.3 ANDSA Reaction Product

4.4.3.1 Optimum pH : The effect of pH on coagulation process is presented in Figure 4.48

The coagulation process was found to be most effective within a pH range of 6.0 to 7.0

for ANDSA.
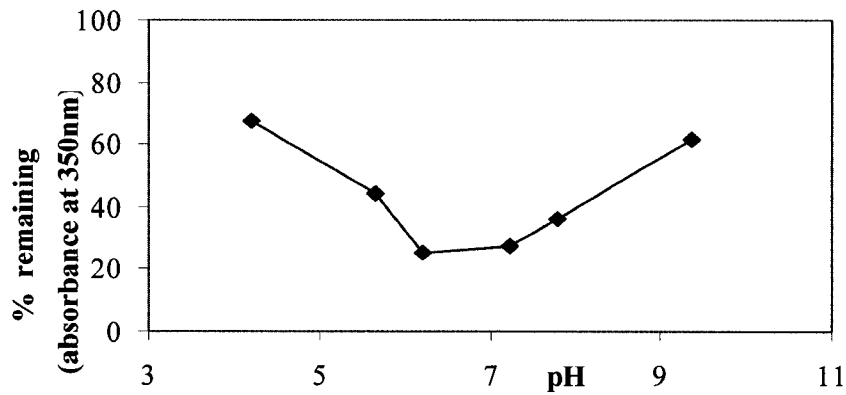
Figure 4.48 : Removal efficiency at different pH, for 1 mM ANDSA, 100 mg/L PEI, 100 mg/L alum.

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4.4.3.2 Optimum Coagulant Concentration: The effect of coagulant concentration at the optimum pH of 6.8 is presented in Figure 4.49. The observed optimum PEI concentration is at 250 mg/L for ANDSA which gave 91 % color removal. Therefore the real cause behind its apparent poor performance in case of RB5 needs to be investigated.
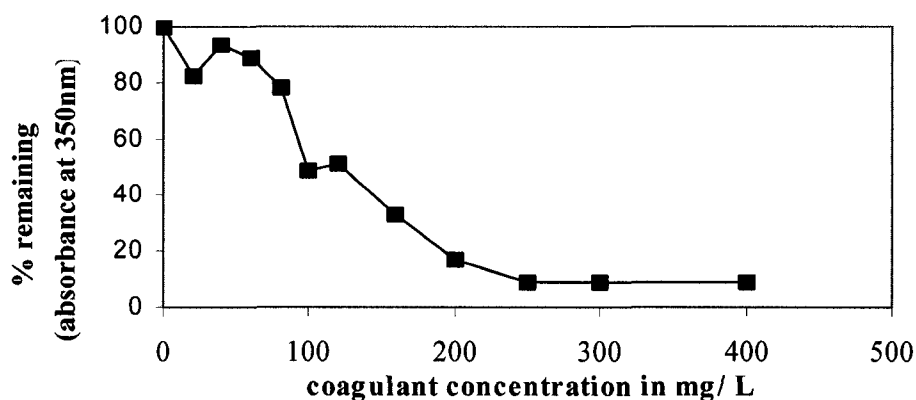
Figure 4.49 : Removal efficiency at different PEI concentration, at pH 6.8, 100 mg/L alum, for 1 mM ANDSA.

The extent of actual removal of the ARP oxidation products by the coagulation process is indicated by the absorbance spectra of the solution after PEI aided coagulation and precipitation. Figures 4.50 and 4.51 present absorbance spectra after PEI-aided coagulation and precipitation. This may be compared with the absorbance spectra of the initial dye solutions, before and after Fe and ARP treatment (Figure 4.1, 4.2, 4.6, 4.7, 4.42 and 4.43. The comparison shows that the both the UV and Vis absorbance are removed after the final treatment stage and hence both the dye and the compounds are actually removed from water.

100
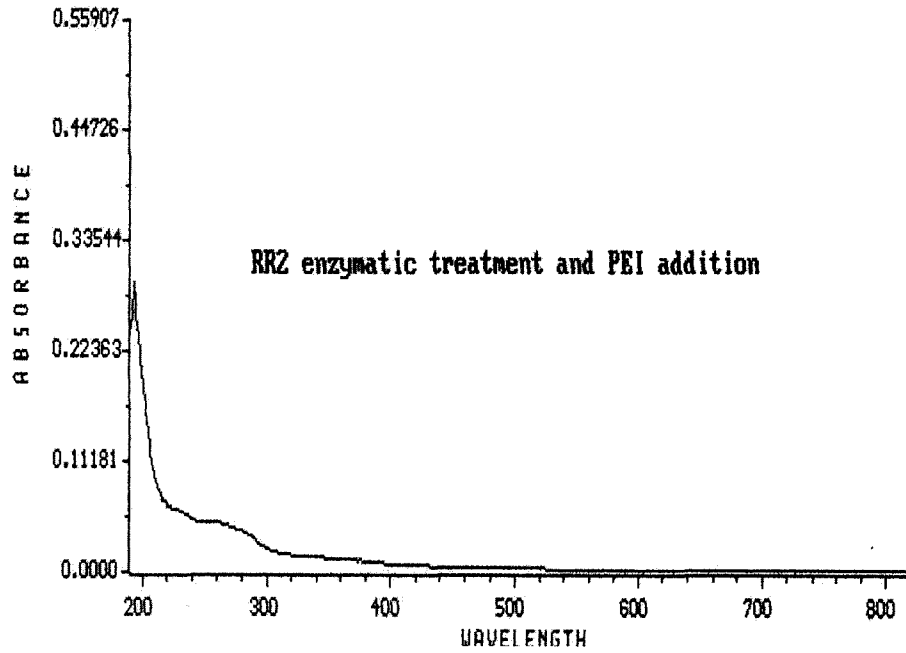
Figure 4.50 : UV-vis absorbance spectra after PEI treatment for Reactive Red 2, 240 mg/L PEI, 100 mg/L alum, pH 7.0, 2 h reaction time.
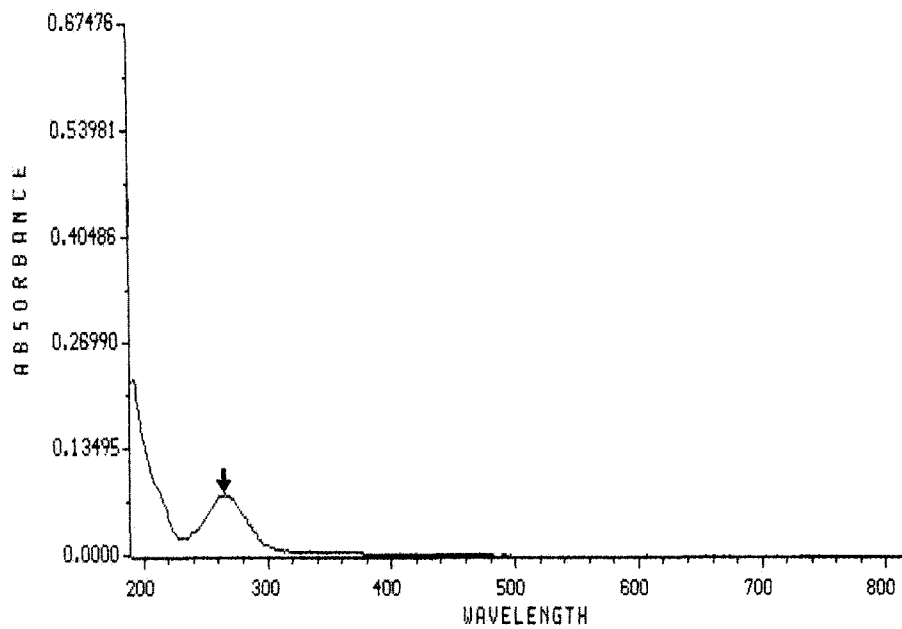
Figure 4.51 : UV-vis absorbance spectra after PEI treatment for Reactive Black 5, 240 mg/L PEI, 100 mg/L alum, pH 5.6 , 2 h reaction time.

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4.4.4 Effective Removal of Dye and Color

To determine the maximum possible removal of the dye breakdown products, a set of experiments were carried out with RR2 and RB5 with excess Fe, followed by ARP treatment and subsequently PEI aided coagulation and precipitation. A higher amount of hydrogen peroxide and enzyme were used to compensate for the consumption of peroxide by sodium sulfite. The Table 4.2 presents the observations and experimental conditions.

Table 4.2: Maximum possible removal.

Treatment steps		Fe treatment	ARP treatment	PEI-aided coagulation
RR2	Reaction Condition	1 mM dye conc., 1.5 g Fe, 1.5 mM of Na_2SO_3, pH 4.8, 60 mM acetate buffer for 1 h.	5 U/ml ARP, 5 mM H_2O_2 added in steps, pH 7.0, for 2 h.	200 mg/L PEI, 100 mg/L alum, pH 7.0 for 2 h
	Results	98 % dye breakdown, estimated from reduction of absorbance at 538 nm.	94 % amine removal (by TNBS test)	91 % reduction in absorbance of peak at 258nm
RB5	Reaction Condition	1 mM dye conc., 5 g Fe, 5 mM of Na_2SO_3, pH 4.8, 60 mM acetate buffer for 1 h.	5 U/ml ARP, 5.5mM H_2O_2 added in steps, pH 5.64, 2 h	250 mg/L PEI 100 mg/L alum, pH 5.4 for 2 h
	Results	97 % dye breakdown, estimated from reduction of absorbance at 596 nm.	92 % amine removal (by TNBS test)	82 % reduction in absorbance of single peak at 268nm

The results show that Fe treatment efficiency improves for RB5, but the enzymatic removal efficiency and PEI treatment efficiency remained the same.

Fe treatment products of both dyes are aromatic amines. The ARP oxidation product of these aromatic amine are quinones. In similar situations, phenol was oxidised by HRP to

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form quinones which could be removed by a natural cationic coagulant, Chitosan (Wagner and Nicell, 2002b). PEI is a very similar cationic coagulant as Chitosan (Wada et al, 1995). Though quinones are toxic (Wagner and Nicell, 2002b), but they are similarly removed by PEI. PEI also removes the polymers, the other enzymatic treatment end products. Hence it can be presumed that a large portion of dye breakdown products are removed by PEI-alum and the treated water has lower level of toxicity.

Experimental observations also confirm the same. On Fe treatment the primary peak absorbances at λ_{max} for the RR2 and RB5 (538 and 596 nm) were reduced by 97 to 98%. However, new absorbance peaks appeared (primary ones at 258 nm for RR2 and 268 nm for RB5, refer Figures 4.6, 4.7) due to formation of new molecules. ARP treatment followed by PEI aided coagulation removed all these new peaks. This indicates that the breakdown products were indeed removed.

From Figures 4.50 and 4.51, it is evident that in both the cases, only a single small peak or plateau remained, therefore the extent of removal of these compounds can be estimated from the ratio of peak absorbance before and after ARP and PEI treatment. It is estimated that around 91 % (for RR2) and 82 % (for RB5) removal of the dye breakdown products have been achieved by the ARP and PEI treatment. Therefore it can be concluded that this three-step process is quite effective in removing not only the color but also the end products from water. However some background absorbance remained at around 190 nm. Spectra presented by other researchers also had similar residual absorbance (Deng et al.,1996; Vinodgopal et al.,1998)

4.5 Error Estimation

Two statistical experiments (refer Section 3.4.1) were carried out to estimate errors due to human, equipment and other factors. The results are presented below in Table 4.3 (**Experiment 1**) and Table 4.4 (**Experiment 2**).

Table 4.3 : Errors due to human and other factors for ANDSA in TNBS test.

Sample no.	1	2	3	4	5	6	7	8
Time	9:40	9:41	9:42	9:43	9:44	9:45	9:46	9:47
Absorbance	0.11432	0.10901	0.10709	0.10718	0.11136	0.11513	0.11101	0.11227

The data in Table 4.3 were collected within short interval of time and therefore the variations due to spectrophotometer were negligible. The standard deviation due to human and other factors (other than spectrophotometer) was estimated as 0.003015, which is a small 2.71% of the observed mean value. The 95% confidence interval is 0.110921 ± 0.00252 ("t" statistics with degree of freedom =7) of the observed value.

Table 4.4 : Errors due to spectrophotometer for ANDSA in TNBS test..

Sample no.	1	2	3	4	5	6	7	8
Date	Jan	Jan	Jan	Feb	Feb	Mar	Mar	Mar
Absorbance	0.11428	0.11266	0.11853	0.11003	0.11343	0.10954	0.12816	0.10954

The data in Table 4.4, collected over a large span of time, included the variations due to spectrophotometer also. Standard deviation due to all factors, including spectrophotometer was estimated as 0.006283, which is, 5.49% of the observed mean value of 0.114525. The 95% confidence interval is within 0.114525 ± 0.00526 ("t" statistics with degree of freedom =7) of the observed value.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The study results established the followings:

- Zero-valent Fe effectively reduced the two dyes – RR2 (removal efficiency 98 %) and RB5 (removal efficiency 95-97 %) to aromatic amines under anaerobic conditions at acidic pH 4.8, which could be further treated.

- Fe breakdown products for these dyes were substituted anilines and naphthol amines. RR2 was reduced to an aromatic naphthol amine, which had both the naphthalene and triazine rings attached together.

- The aromatic amines generated by Fe^0 reduction was removed by ARP under optimum pH, H_2O_2 to substrate ratio, enzyme concentration and reaction time at room temperature. The removal efficiency obtained and the optimum parameters are presented in Table 5.1.

Table 5.1 : Removal efficiency and optimum parameters for ARP treatment.

	For 0.5 mM RR2 breakdown products	For 0.5 mM RB5 breakdown products
Removal efficiency	94 % as amine removal.	92 % as amine removal.
Optimum pH	6.0 to 7.5	5.5 to 6.5
H_2O_2 to substrate ratio	3 : 1	4.5 : 1
Enzyme concentration	4.5 U/mL : 1 mM	4.5 U/mL : 1 mM
Reaction time	2 h	2 h

- The aromatic amines with functional groups (-OH, -NH₂, -SO₃⁻) in the naphthalene ring proved to be substrates of ARP.

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- Michelis-Menten constant (K_m) and the turnover number (k_{cat}) for ARP reaction on a model substrate (ANDSA) indicated that ANDSA which is an aromatic amine with naphthalene ring and -OH, -NH$_2$, -SO$_3^-$ groups, was a less reactive substrate for ARP as compared to phenol. This may indicate that substituted anilines bound with ARP before naphthol amines when present in a mixture.

- The –NH- bond between benzene rings in DPA was not affected by Fe treatment but it was readily oxidised by ARP treatment.

- Polyethyleneimine (PEI) was required as a chemical complexing agent to remove the colored enzyme treatment products. An optimum PEI concentration of 200 to 250 mg/L with 100 mg/L of alum was effective to remove most of the color and pollutants (82% for RB5, 91% for RR2) from the water. The optimum pH for RR2 was between 6.0 and 7.0, and it was between 4.8 and 6.0 for RB5.

5.2 Recommendations

Any further work in this area will have to address the three key concerns: (i) cost, (ii) effectiveness and (iii) feasibility of logistics in treating real effluents. This will require further study and analysis of real effluent contents and their impact on the proposed process, followed by a study to identify possible best processes, enzymes, reagents, process conditions and operations that can complement, modify and improve the three stage process to make it suitable for real textile/ dye manufacturing plant effluents. Typical composition of textile industry effluents is presented in Appendix- A.2. However before this, the effectiveness of Fe and ARP treatment needs to be studied and established on a wide variety of reactive azo dyes. It may be worthwhile to explore why PEI was not

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as effective in case of RB5, whether this can be improved upon, or whether alternative coagulants have to be used for RB5. The final reaction products may be identified to get a better understanding about the reaction mechanisms. The study may be extended to find any useful application of the final polymeric end products.

CHAPTER 6: ENGINEERING IMPLICATION, COMPARATIVE STUDY AND CONTRIBUTIONS

6.1 Engineering Implications

The research findings have the following possible implications in treatment plant :

Fe Treatment

- Zero-valent iron or enzyme treatment alone cannot remove both color and the two reactive dyes – RR2, RB5. A three stage process comprising of anaerobic Fe treatment, ARP treatment and PEI aided coagulation are required.

- Fe treatment efficiency improves in acidic pH (below 5.0) and pH 4.8 is recommended. However, textile effluents have higher pH (9.0 to 11.0) (Delee et al., 1998). This implies the need for pretreatment of the effluents to bring down the pH.

- The Fe treatment process removal efficiency is sensitive to iron surface area, surface cleaning, mixing rate, presence of impurities. An excess quantity of iron, finer mesh size iron particles, rapid mixing, frequent cleaning, may be essential for complete degradation of azo bond.

- Some compounds compete with dye molecules for the Fe active sites or hinder the surface-mediated reaction mechanism and thus deteriorate the dye degradation efficiency and reaction rate. Potential sources of such compounds are - buffers, additives used in the reaction and various compounds present in the textile effluent itself. Hence, in real situation, the dye effluent may be treated at the point of source before they get mixed with other effluents.

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- The Fe reduction products of the azo dyes rapidly get oxidized in presence of air. This phenomenon decreases ARP treatment efficiency. Therefore, the Fe reduction products need to be promptly fed to the next stage for enzymatic treatment. Excess oxygen scavengers may slow down such oxidation, but they are likely to increase the hydrogen peroxide and enzyme demand in the subsequent ARP treatment.

ARP Treatment

- Fe^0 breakdown products of other similar reactive azo dyes having –OH, -NH_2 groups and –NH- bond may be similarly oxidised by ARP.

- The ARP treatment efficiency of the RR2 and RB5 Fe^0 reduction products were observed to be optimum at neutral pH range of 6.0 to 7.4 for RR2 and pH 5.0-6.0 for RB5. This implies that after Fe^0 treatment, an additional step may be required to adjust the pH before ARP treatment. A normal room temperature around 22-25^0 C is sufficient to have better performance of the enzymatic treatment.

- Step addition of hydrogen peroxide can prolong the catalytic life of the enzyme. Such incremental addition can bring economy in enzyme consumption of 10 to 20%.

- Two to three hour reaction time is required to get over 90% removal efficiency Sufficient reaction time allows all the temporarily inactivated ARP to return to the active form and take part in catalytic activity.

- Specific salts and metals can inhibit the enzyme. Such contaminants can come via water, buffers, additives or the effluent itself. These can increase or decrease the enzyme and peroxide demand. Appropriate measures should be taken to minimize their adverse impact, to achieve economy on enzyme consumption.

- The enzyme reaction products for RR2 and RB5 are colored and water-soluble Therefore, coagulant aids are required to remove them.

Coagulation and Precipitation

- PEI with alum was effective in removing colored products from the solution in case of RR2 but not as effective in case of RB5. Therefore, an alternative or an additional coagulant such as chitosan may be used to remove RB5.

- PEI is non-toxic and gets removed along with the precipitates. Therefore, its addition is not hazardous. However, the effect of its high nitrogen content on the environment needs to be studied.

- PEI performs better at pH below 8.0, hence can be directly used after enzymatic treatment, which is carried out at the neutral range between for RR2 and slightly acidic range for RB5.

6.2 Comparison with other Treatment Processes

Table 6.1 presents a comparison between the results obtained in the present work and the best of the class results obtained by various other removal techniques. The best of class processes are selected on the basis of reported dye colour removal percentage alone, which were based on the UV-vis peak absorbance reduction at λ_{max} for these dyes. Since reduction of λ_{max} does not necessarily mean removal of the whole compound; therefore, the removal figures reported for these best of class processes do not represent the actual pollutant removal effectiveness. These best of class processes are selected from Tables A.1.1 to A 1.3, which present the studies on different color removal methods and studies on the same two reactive dyes – RR2 and RB5.

Table 6.1 : A comparison with other best of class processes on the same two dyes.

Dye	Dye removal in Fe treatment	Amine removal in ARP treatment	Absorbance reduction after PEI aided coagulation	End to end effective removal	Removal by best of the class process	
					Best removals	**Best times**
Reactive Red 2	98% [1] in 1 h	94% [1] in 2 h	91% [1] in 2 h	89 % in 5.5 h	100% [2] in 20 min 100% [3] in 90 min 100% [4] in 1 h 100% [5] 95% [6] in 10 h 87.3% [7] in 83 h	In 20min, 100%[2] In 1 h, 100%[4] In 2 h, 100%[3] In 2.5 h, 64.6%[8]
Reactive Black 5	97% [1] in 1 h	92% [1] in 2 h	82% [1] in 2 h	80 % in 5.5 h	100 % [9] in 90 min 100 % [10] in 2 h 99% [5] 98% [11] in 1 h 96% [12] in 6-26 days 94.6 % [13] in 48 h 84.1-87.6% [14] in 205days 77.8% [7] in 83 h	In 1 h, 98% [11] In 90min,100 %[9] In 2 h, 100 % [10] In 6 h, 40% [15]

Reference (Table A.1.1-A.1.3 for details)

(1) The present study, from Table 4.2.

(2) Total decolorisation in 20 min by photocatalytic UV/TiO$_2$ & H$_2$O$_2$ by So et al., 2002.

(3) Total decolorisation in 90 min, by photodegradation on aqueous Fe (III) by Deng et al., 1996.

(4) Total disappearance of absorbance at 538 nm in 1 hour by Feng et al., 1999.

(5) 100 % colour for RR2, 99% colour for RB5 by activated Sludge UASB by Van der zee et al., 2002. (Time not mentioned for RB5).

(6) 95 % decolorisation by thermophilic EGSB reactor by dos Santos et al., 2003.

(7) 87.3 % decolorisation for RR2, 77.8% for RB5 by biological, anaerobic suspended growth culture by Beydilli et al.,1998.

(8) 64.6 % decolorisation in 2.5 hour by UV / Fe^0 system by Deng et al., 2000.

(9) 100 % decolorisation, 60 % mineralisation by ultrasonic treatment by Vinodgopal et al., 1998.

(10) 100 % color, 75 % TOC removal by photocatalytic H$_2$O$_2$/UV by Alaton and Balcioglu, 2001.

(11) 97 to 98 % reduction in absorbance in visible region by photocatalysis by TiO$_2$ by Arslan et al., 2000.

(12) 96 % COD removal in 6 to 26 days by Sponza and Isik, 2002.

(13) 94.6 % decolorisation by biological process by Yu et al., 2001.

(14) 84.1 to 87.6 % color removal by biological process by Gottleib et al., 2003.

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(15) 40 % COD removal in 6 hour by Wang et al., 2003.

The observed dye conversions in the three stages of the proposed process were observed under optimum conditions and minimum treatment time. The data is taken from Table 4.2.

End to end effective removal is calculated as

= removal in Fe treatment stage * removal recorded after coagulation.

Total treatment time include the treatment times for three stages and the filtration time of 30 minutes, done between Fe and ARP treatment.

The removal percentages indicated for the Fe treatment stage are based on the peak absorbance reduction at λ_{max} for RR2 and RB5 at 538 and 596 nm respectively. The removal after PEI-aided coagulation is based on peak UV-vis absorbance reductions at 258 nm for RR2 and 268 nm for RB5, where as the amine removal percentage after ARP treatment is based on the TNBS assay.

The high dye breakdown percentage (98, 97%) as a result of Fe treatment is comparable with similar figures reported by other researchers (presented under the column -"Removal by best of class processes" in Table 6.1). Amine removal percentage (92 to 94 %) and the absorbance characteristics (as presented in Figures 4.50 and 4.51) after coagulation stage indicate the extent of removal of end products, and are also significant. On the other hand the "end to end effective removal" figures indicate the extent of effective visible color and also breakdown product removal. The end to end conversion removal efficiency is lower in case of RB5. The lower conversion for RB5 is predominantly due to relatively poor performance of PEI and lower conversion by ARP.

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Table 6.2 exhibits a comparison between the results from present work and those from other multi stage treatment processes selected from Table A.1.1 of Appendix A.1.

Table 6.2 : A comparison with other multi stage processes.

Treatment process	Dye	Removal %	Reference
Anaerobic - aerobic biotreatment. UASB and Aerobic reactor	Reactive procion dyes, 150 –750 mg/L conc.	77% color removal, 88% COD removal in 19 h	O'Neill et al., 1999
Biological process. SBR (anerobic followed by aerobic)	*Reactive red 3.1,* 40-400 mg/L dye conc.	93% dye removal, 92% COD removal, in 24 + 27 h	Bromley-Challenor et al., 2000
Biological process. SBBR (anaerobic followed by aerobic)	Same as above	90% dye removal, 85% COD removal, in 24 + 27 h	
Biological process. Activated Sludge STR (aerobic followed by aerobic)	Same as above	5% dye removal, 90% COD removal, in total time 42 h	
Proposed three stage process (Fe, ARP, PEI)	*Reactive Red 2,* 1230 mg/L (50% pure)	98 % dye removal (reduction of peak absorbance), 94 % amine removal, 89% end product removal, in total time 5.5 h	Present work
Same as above	*Reactive Black 5,* 1876 mg/L (55% pure)	97 % dye removal (reduction of peak absorbance), 92 % amine removal, 80% end product removal, in total time 5.5 h	Same as above

On analysis of the other studies the following observations were made :

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- Other researchers have reported the decolorization / removal percentage based on reduction in absorbance at λ_{max}. Thus these reported removal figures indicates the extent of dye breakdown, not the extent of actual pollutant removal from water. The end product sometimes have colour, even though there is no absorbance at the corresponding λ_{max}. Van der zee et al., 2002 reported a yellow coloured end product for RR2, though at 538 nm there was no absorbance.

Even though the absorbance at λ_{max} for the dyes were reduced to a great extent (between 77 to 100%) after treatment, many researchers (Deng et al., 1996; Feng et al., 1999; Van der zee et al., 2001; So et al., 2002; dos Santos et al., 2003; Beydilli et al., 1998; Vionodgopal et al. 1998; Yu et al., 2001) have acknowledged that either some absorbance was present in the UV-vis range or some end products remained in the treated water.

Again, most of the above mentioned work were conducted with very dilute dye solutions (5 to 300 mg/ L). It is known that the colour removal by any process decreases with the increase in dye concentration (Deng et al., 1996; Van der zee et al., 2002; dos Santos et al., 2003). Dye concentration in an actual dye bath can reach 800 mg/L for reactive dyes (O'Neill et al., 1999). Researchers who created synthetic effluents also used higher dye concentration (76 to 583 mg/ L) (Arslan et al., 2000). RB5 concentration is usually in the range of 0.8-2.6 g/L in real exhausted dye bath effluent (Arslan and Balcioglu, 2000). In general, dye concentration in real effluent varies between 10-250 mg/L and in simulated wastewater is between 10- 7000 mg/L (O'Neill et al., 1999). As these studies reported work with lower dye concentration, so the real performance of

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these processes in worst case and actual treatment conditions cannot be judged from these figures alone.

In contrast, in addition of being environmentally friendly, the proposed process in the present work demonstrates the following positive points:

- Clear, transparent solution without any colour by visual observation at the end of the third step.

- Good dye removal effectiveness (97%, 98%), satisfactory colour and end product removal effectiveness (80 % for RB5, 89 % for RR2) in 5.5 hours.

- Significant reduction of the entire absorbance spectra.

- Satisfactory removal under high dye concentration (1230 mg/L for RR2 and 1876 mg/L for RB5). In this aspect the proposed process is sufficiently robust and therefore, it has a good potential in real wastewater treatment.

COD, TOC removal in the present study were not explicitly measured, as the comparison of the absorbance spectra before and after the treatment gave the required estimates about pollutant removal. After considering all these dimensions, it can be asserted that the end-to-end removal efficiency of the proposed three-stage process is quite competitive when effectiveness, cost, time and other logistics are considered in totality.

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6.3 Contributions

This thesis proposed a novel three-stage treatment process to remove both color and dye from water. A combination of three stages: (i) Zero- valent iron treatment under anaerobic condition, (ii) followed by a enzymatic treatment and (iii) finally coagulant-aided precipitation was able to achieve more effective removal of coloured dye compound. *Arthromyces ramosus* peroxidase (ARP) was proven to be an effective enzyme for this process.

Experimental data were presented to establish the effectiveness of the proposed process for two common reactive azo dyes : Reactive Red 2 (RR2) and Reactive Black 5 (RB5). The key optimum process parameters like pH, reagent quantity, reagent preparation, operation temperature, reactor operations, etc. for the proposed process were identified for these two dyes. The process shows an optimism that this three step process has a potential to treat other reactive azo dyes as well.

A comparative study of the available literature related to textile wastewater treatment was summarized. The conversion effectiveness and time performance of the proposed process was compared against the same metrics from the best processes. The proposed process was found to be competitive on basis of its ability to remove both visible colour and pollutants.

To gain insight about the ARP mechanism, the action of ARP on two model compounds: diphenylamine (DPA) and 2-amino-8-naphthol-3, 6-disulfonic acid (ANDSA) was also studied. These two compounds are known water and soil pollutants. ARP treatment on these two additional pollutants was studied for the first time.

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APPENDICES

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APPENDIX A.1: Comparative Study of Decolorisation /Removal Techniques.

Table A.1.1: Comparison of various water treatment processes for dye removal (Azo dyes are in italics)

Process	Process condition	Type of dyes	Colour removal (%)	Remark	Reference
Biological by whole culture having HRP, LiP, MnP.	0.8 µM dye conc. in 48 h	*Disperse yellow 3, Naphthol analog of Disperse yellow 3*	100%	Quinone and dimersideatified by HPLC	Spadaro and Renganathan, 1994
Enzymatic, by Laccase from *P. cinnabarinus* pure culture	25 mg/L dye conc. in 25 h	Chicago sky blue	100 %	Color disappeared but new intermediate formed.	Schliephake et al., 2000
Biological , by Pseudomonous strain	100 mg/L dye conc. Anoxic, in 48 h	Acid Violet 7	99.6	Only reported decolorisation in terms of reduction in absorbance in visible spectra. Nitrate, an ubiquitous salt was observed as an inhibitor.	Yu et al., 2001
		Acid red 151	98.9		
		Reactive black 5	94.6		
		Acid yellow 34	89.5		
		Indigo carmine	87.9		
		Acid green 27	60.5		
		Acid red 183	26.7		
		Reactive blue 2	24.3		
Biological , by Pseudomonous strain in municipal sludge	100 mg/L dye conc. Anoxic, in 48 h	*Acid Violet 7*	96.5	Negligible color removal (less than 5%) by municipal sludge alone,	Yu et al., 2001
		Reactive blue 2	77.2		
		Acid green 27	80.5		
		Acid red 183	57.3		
		Indigo carmine	73.1		

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Process	Process condition	Type of dyes	Colour removal (%)	Remark	Reference
Enzymatic, by MnP from *Bjerkendera sp.*	100 mg/L dye conc. pH 4.8 in 1 h	Orange II	95%	Absorbance in the visible range disappears. But absorbance in UV range remains.	Mielgo et al., 2003
Biological process. Activated Sludge UASB	100-300 mg/L dye conc. 30^0 C	*20 different azo dyes including RR2, RB5*	73-100%	Recolorisation on exposure to air. Triazine dye had slowest rate of degradation	Van der zee et al., 2001
	RR2 in 6 days, time not mentioned for other dyes.	*Reactive red 2*	99%		
		Reactive black 5	100%		
Biological process. PBR (anerobic followed by aerobic)	40-400 mg/L dye conc. 24 & 27 h	*Reactive red 3.1*	93% of dye 92 % of COD	Recolorisation of end product takes place after anaerobic stage	Bromley-Challenor et al., 2000.
Biological process. SBBR (anaerobic followed by aerobic)	40-400 mg/L dye conc. 24 & 27 h		90% of dye 85 % of COD		
Biological process. Activated Sludge STR (aerobic followed by aerobic)	40-400 mg/L dye conc. in total time of 42 h		5 % of dye. 90 % of COD		

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Process	Process condition	Type of dyes	Colour removal (%)	Remark	Reference
Enzymatic, by pure HRP	0.15 mM dye conc. pH 4.7	*4-amino azobenzene*	90.2		Stiborave et al., 1996
	0.15 mM dye conc. pH 8.4	*Sudan 1*	85%		
	0.15 mM dye conc. pH 4.7-8.4	*Sudan II*	0		
	0.15 mM dye conc. pH 4.7-8.4	*Sudan III*	0		
	0.15 mM dye conc. pH 4.7-8.4	*Orange II*	0		
Biological , by anaerobic suspended growth culture	50-2000 mg/L dye conc. 35⁰ C, neutral pH, methanogenic condition, in 83 h	*Reactive red 2*	87.3%	Aromatic amines are formed. Higher dye concentration of 500-2000 mg/L is toxic for methanogenic bacteria	Beydilli et al., 1998
		Reactive black 5	77.8%		
Biosorption on wheat straw and apple pomac	Mixture of 5 dyes 10-200 mg/L dye conc. Particle size 600 μm	*Ramazol and Cibacron dyes*	81-91% color		Robinson et al., 2001
Biological by White rot fungi Laccase, LiP, MnP	199 mg/L dye conc. in 16-20 h	*Cibacron and Ramazol mixture (Ramazol black B)*	73.9-85.7 %	Nitrogen rich media is better. Organisms requires more nutrient to functions better.	Robinson et al., 2001

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Process	Process condition	Type of dyes	Colour removal (%)	Remark	Reference
Anaerobic / aerobic biotreatment UASB and Aerobic reactor	150 –750 mg/L dye concentration in 19 h	Reactive procion dyes	77% color removal., 88% COD removal	Maximum removal was observed when dye concentration was low.	O'Neill et al.1999
Enzymatic by pure HRP, LiP, MnP.	0.8 μM dye conc. in 2 h	*Naphthol analog of Disperse yellow 3*	70% by HRP 60% by LiP 10% by MnP	From HPLC and GC MS the products were identified as acetanilide and naphthoquinone.	Spadaro and Renganatha n, 1994
Biological , by Laccase from T.versicolor Fungal culture	100 mg/L dye conc. in 16 days	*Acid Violet 7*	160 mg dye/l/h	Azo dyes are not substrate Anthraquinone dye is a good substrate.	Yu et al., 1999
		Acid green 27	40 mg dye/l/h		
		Indigo carmine	40 mg dye/l/h		
Enzymatic, by crude HRP	15 mg/L dye conc. pH 2.5, 25⁰ C, in 9 h	Crystal violet	0.02 mM /L/min	Dye degradation was 17 times slower compared to phenol as the substrate	Bhunia et al., 2001
		Ramazol blue	0.017 mM /L/min		
		Cibacron red	0.0028 mM /L/min		
		Cibacron Blue	0.0018 mM /L/min		
		Ramazol violet	0.0006 mM/L/min		
		Ramazol Black	nil		
Biosorption by Fungi *Rhizopus arrhinus*	0-400 mg/L dye conc. pH 2, Particle size > 600 μm, 1 g biomass per L in 20 h of contact time	Reactive Dye mixture	Max. 200 mg/g adsorbent	Low pH requirement may be problem	O' Mahony et al., 2002

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Table A.1.2 : A Comparative Study of Various Treatment Processes to Remove Reactive Red 2

Process	Process condition	Extent of removal & remark	Reference
Advanced oxidation process (AOP), Photodegradation on aqueous Fe (III)	5-25 mg/L dye conc. UV / Fe 3+ pH 3 in 0-90 min	Total decolorisation by reduction of absorbance measured at 539 nm. Absorbance in UV range remains after decolorisation . Degradation rate decreases with increasing dye concentration.	Deng et al., 1996
AOP, Fe reduction and photooxidation	100 mg/L dye conc. High temp Hg lamp pH 2 30 min Fe reduction followed by 30 min irradiation	Decolorisation measured by total disappearance of absorbance at 538 nm after Fe reduction. Subsequent photooxidation causes only 0.01% mineralisation. Formation of substituted benzene and naphthalene rings which stays in the solution	Feng et al., 2000
Biological process. Activated Sludge UASB reactor	100-300 mg/L dye conc. 30^0 C, in 6 days	100 % removal of absorbance measured at λ_{max} 539 nm. But yellow colour reaction product remains, decolorisation rate is slower compared to other azo dyes. Absorbance spectra in the UV region remains as aromatic amines remains, which requires further aerobic treatment	Van der zee et al., 2001
AOP, Photocatalytic UV/TiO$_2$, & H$_2$O$_2$	40 mg/L dye conc. Dye in suspension with TiO2. pH 4.5, in 80 min	Total decolorisation in 20 min, 90 % mineralization in 80 min (measured by TOC loss), measured by absorbance Alkaline condition favors, cyanuric acid may be end product.	So et al., 2002

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Process	Process condition	Extent of removal & remark	Reference
Biological thermophilic EGSB reactor with redox mediator, AQDS	Hydrolysed dye, 100-170 mg/L dye conc. 55^0C Hydraulic retention time in reactor- 10 h	95% decolorisation with AQDS, 91% decolorisation without AQDS. Aniline is formed in the process, no mention about further treatment.	dos Santos et al., 2003
Biological., by Anaerobic suspended growth culture from municipal sewage sludge	300 mg/L dye conc. 35^0C, neutral pH, methanogenic condition, in 83 h	77.8% decolorisation breakdown products are Aromatic amines.	Beydilli et al., 1998
AOP, UV / Fe^0 system	10-50 mg/L dye conc. pH 3.4 Fe^0 less than 100 mesh Fe conc. 0.5-5 g/L, in 2.5 h	64.6% decolorisation absorbance is reduced in the whole UV-Vis range.	Deng et al., 1999

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Table A.1.3 : A Comparative Study of Various Treatment Processes to Remove Reactive Black 5.

Process	Process condition	Extent of removal & remark	Reference
Physical treatment, by Ultrasonic waves	33 µM dye solution 640 kHz, in 90 min	100 % decolorisation measured by disappearance of visible absorbance peak 60 % mineralisation , formation of oxalate, sulfate and nitrate ions.	Vinodgopal et al., 1998
AOP, Photocatalytic H_2O_2/UV	Hydrolysed dye 75 mg/L dye conc., in 2 h	100 % color removal , 62% COD removal , 75% removal	Alaton et al., 2001
Biological process, Activated Sludge UASB	100-300 mg/L dye conc. 30^0 C, Time not mentioned	99% colour removal Absorbance in UV region remains as aromatic amines remains, which requires further aerobic treatment.	Van der zee et al., 2002
AOP, Photocatalysis by TiO2	583 mg/L dye conc. Simulated dye bath, in 1 h	97-98 % reduction in absorbance in visible region. 30-37 % TOC removal , removal decreases with increase in effluent concentration	Arslan et al., 2000
Biological , Anaerobic /aerobic sequencial batch reactor UASB/CSTR	100 mg/L dye conc. in 6 to 26 days	96 % total COD removal , 84 % color removal in 6 days	Sponza and Isik, 2002
Biological , by *Pseudomonous* strain	100mg/L dye conc. Anoxic, in 48 h	94.6 % decolorisation, no minerilization or disappearance of absorbance in UV range will take place, because biological process do not work well for stable azo dyes. Nitrate, an ubiquitous salt in textile waste has severe inhibitory effect on decolorisation. Extent of decolorisation depends on dye concentration and biomass concentration. Higher dye concentration will give lower removal.	Yu et al., 2001

Process	Process condition	Extent of removal & remark	Reference
Biological., Anaerobic suspended growth culture	300 mg/L dye conc. 35^0 C, neutral pH methanogenic ondition in 10 h	87.3% color removal	Beydilli et al., 1998
Biological., by *E. faecalis and C.butyricum* strain. Laboratory baffled reactor, sequential anaerobic and aerobic	500mg/L dye conc. in 205 days	84.1-87.6% color removal Intermediate produced after anaerobic stage is toxic but final effluent product after aerobic stage is non toxic.	Gottleib et al., 2002
AOP, Ozonation	2 g/L dye in 6 h	40 % COD removal, 25 % TOC removal.	Wang et al., 2003

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A.2 Typical Composition of Textile Effluents

The chemical content for a simulated exhausted dye bath is presented below to give an idea of typical composition of textile dye effluent. The recipe of the simulated effluent was made to match the actual textile industry effluent.

Table A.2.1: Simulation recipe for effluent from an integrated plant.

Dye	Concentration	Function	Reference
Ramazol Black B (Reactive Black 5)	538 mg/L	Dye	Arslan et al., 2000
Other dyes	92-84 mg/L	Dye	
Assisting chemicals			
Urea	3 g/L	Increase solubility of the dyestuff	
NaCl	70 g/L	Transfer dyestuffs to fabric	
Na_2CO_3	5 g/L	(pH buffer) Produces covalent bond between dyestuff and fabric	
NaOH	4 g/L	Produces covalent bond between dyestuff and fabric	

Table A.2.2: Simulation recipe for effluent from a dyehouse mill.

Dye	Concentration	Function	Reference
Procion dyes	6-86 mg/L	Dye	Alaton et al., 2002
Assisting chemicals			
Aacetic acid	0.79 g/L	Neutralizes wash water	
NaCl	41 g/L	Transfer dyestuffs to fabric	
Na_2CO_3	13 g/L	(pH buffer) Produces covalent bond between dyestuff and fabric	
NaOH	0.51 g/L	Produces covalent bond between dyestuff and fabric	
Polyether based co-polymer, micro dispersion	1.20 g/L	Anti-creasing agent	
Acryl-co-polymer-phosphor mixture	0.85 g/L	Sequestering agent	
Alcyl phenol poly glycol ether	0.50 g/L	Detergent	

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Table A.2.3: Characteristics of a 15-fold diluted reactive dyebath effluent

Parameter	Value
TOC (mg/L)	46.8
BOD $_5$ (mg/L)	Below detection limit
AOX (Halogenated compound) (mg/L)	0.102
CO_3^- (mg/L)	490.6
Cl $^-$ (mg/L)	1659.0
pH	10.9

(Source: Alaton et al., 2002)

Table A.2.4: Fixation rate of different dyestuffs

Dye application class	Fibre	Degree of fixation (%)	Loss to effluent (%)
Reactive	Cellulose	50-90	10-50
Sulfur	Cellulose	60-90	10-40
Direct	Cellulose	70-95	5-30
Vat	Cellulose	80-95	5-20
Acid	Polyamide	89-95	5-20
Metal Complex	Wool	90-98	2-10
Disperse	Polyester	90-100	0-10
Basic	Acrylic	95-100	0-5

(Source: O'Neill et al., 1999)

Reactive azo dyes like RR2 and RB5 have lower fixation rate therefore a larger portion of those are discharged as effluents.

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A.3: Properties of RR2, RB5 and model compounds

Table A.3.1: Chemical properties of the dyes and model compounds

Compound	Reactive Red 2	Reactive Black 5	2-amino-8-naphthol-3, 6-disulfonic acid	Diphenylamine
CAS number	17804-49-8	17095-24-8	-	122-39-4
Chemical formula	$C_{19}H_{13}Cl_2N_6NaO_7S_2$	$C_{26}H_{26}N_5NaO_{19}S_6$	$C_{10}H_9NO_7S_2$	$C_{12}H_{11}N$
Molecular weight	595.4	927.9		169.2
λ_{max}	538 nm	596 nm	-	-
Melting Point, ^0C	>300 °C	>300 °C	-	52.5-54 °C
Purity	50%	55%	80-90 %	99%
Molecular *Diffusivity	3.28 X 10-6 cm^2/ sec	2.77 X 10-6 cm^2/sec.	-	-

(*Calculated by reference Tucker and Nelkhen, 1982)

Table A.3.2: Toxicity of reactive azo dyes and some of their degradation products

Dye	Toxicity (EC$_{50}$), mg/L	Biodegradability	Reference
Reactive Black 5	Parent dye : 27.5 ± 4.01 Hydrolyzed dye : 11.4 ± 3.68 Bacteria reduced form : 0.7 ± 0.09	Not totally biodegradable (BOD$_{28}$/ COD$_t$ = 1.4%)	Wang et al, 2003
Procion Crimson (Similar to RR2)	Parent: 34.7 ± 0.27 Hydrolyzed: 37.7 ± 1.72	-	Gottlieb et al., 2003
Procion Navy (Similar to RR2)	Parent: 18.9 ± 5.65 Hydrolyzed: 27.9 ± 3.28	-	
ANDSA (H-Acid)	48.6 ± 2.68	-	Gottlieb et al., 2003
Sulfanilic acid (Similar to RB5 breakdown products)	21.5 ± 6.72	-	Gottlieb et al., 2003

EC$_{50}$ is the sample concentration that inhibits 50 % of the light output after a 5 min exposure period.

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A.4 Properties of *Arthromyces ramosus* Peroxidase (ARP)

Property	Value
Molecular wt.	41,000 KD
Nuumber of subunits	1
Amino acid per sub unit	344
Number of disulfide bond	4
Optimum pH	5-9
Optimum Temperature , ° C	40
pH stability	5-9 at 30 ° C for 16 h
Thermal stability	Upto 50 ° C (at pH 7 for 30 min)
Co factor	1 protoheme IX per enzyme
RZ value	2.7
Cellular localization	Extra-cellular
Metal ion requirement	2 endogenous Ca+ ion

(Source: Nakayama and Aamachi, 1999)

B.1 ARP Activity Assay

B.1.1 General

ARP enzyme activity assay is carried out to determine the amount of active enzyme present in a sample. This assay uses saturation concentrations of phenol, 4-aminoantipyrine (4-AAP) and an appropriate concentration of hydrogen peroxide such that the initial reaction rate is proportional to the enzyme activity. The rate is measured by observing the rate of color formation in a reacting solution, in which ARP catalyses the reaction between phenol and H_2O_2. The end products formed react with 4-AAP to form a pink color solution which shows peak absorbance at 510 nm and has extinction coefficient of 6000 M^{-1} cm $^{-1}$, based on peroxide.

One unit of activity is defined as number of micromoles of H_2O_2 utilized in one minute at pH 7.4 and at 25 ^0C in an assay mixture containing 10 mM phenol, 2.4 mM 4-AAP and 0.2 mM H_2O_2.

B.1.2 Preparation of Reagents

B.1.2.1 Phosphate Buffer (0.5 M, pH 7.4)

In a 1000 mL volumetric flask, add the following reagents:

13.796 g monobasic sodium phosphate (NaH_2PO_4, H_2O)

56.78 g of dibasic sodium phosphate (Na_2HPO_4)

Distilled water to make a 1000 mL solution.

---

[1] Source: Lab Manual, Enzymology lab, Room B79 and Mantha, 2001.

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B.1.2.2 Phenol (0.1 M) in Phosphate Buffer (0.5 M, pH 7.4)

Dissolve 9.411 g of phenol in 1000 mL of 0.5 M phosphate buffer solutions.

B.1.2.3 Hydrogen Peroxide (100 mM)

Dilute 567 μL of 30 % (w/v) hydrogen peroxide to 50 mL with distilled water. This is to be freshly prepared each time an activity assay is carried out.

B.1.2.4 Assay Mixture

In a beaker add the following in the given order:

25 mg AAP

42.4 mL water

100 μL of 100 mM H_2O_2

5 mL of 0.1 M phenol in 0.5 M phosphate buffer

B.1.3 Procedure

The assay volume is 1 mL and the assay should be conducted before the substrate depletion becomes significant. In a semi-micro cuvette, place the followings according to the given order:

950 μL of assay mixture

50 μL of solution containing ARP enzyme.

B.1.4 Estimation of ARP activity

One unit of activity is defined as number of micromoles of H_2O_2 utilized in one minute at pH 7.4 and at temperature 25 ^{0}C in an assay mixture containing 10 mM phenol, 2.4 mM 4-AAP and 0.2 mM H_2O_2. The activity of ARP in the cuvette is obtained from

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the average slope of the curve (absorbance per minute) within the linear range. Therefore, the activity within the cuvette, in units of U/mL is calculated as:

$$\text{Activity (U/mL)} = \frac{\text{Slope (AU/ minute)}}{6000 \text{ AU } \text{L/mol}} \times \frac{10^6 \mu\text{mol}}{\text{mol}} \times \frac{1\text{L}}{1000 \text{ mL}}$$

Here AU represents absorbance units and 6000 AU L/mol is the factor that relates color development to peroxide consumption.

Activity of the sample is estimated as:

$$\text{Sample activity (U/mL)} = \text{Activity in cuvette (U/mL)} \times \frac{1000}{\text{Sample volume}}$$

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B.2   Hydrogen Peroxide Assay

B2.1   General

This endpoint colorimetric assay is used to determine the concentration of hydrogen peroxide in a sample. The assay uses *Arthromyces ramosus* peroxidase as a catalyst and 4-aminoantipyrine and phenol as a color-generating co-substrates. In this assay the amount of H_2O_2 introduced into the assay sample is the only limiting reactant; therefore, the degree of the color development in the reaction is proportional to the amount of peroxide in the sample. Once the maximum amount of color has been developed, the absorbance (at 510 nm) is measured in UV-VIS spectrometer and the concentration of H_2O_2 in the cuvette is measured by means of a calibration curve. H_2O_2 concentration in the sample is calculated by taking into consideration of the dilution factor of the sample in the cuvette.

B.2.2   Preparation of Reagent

B.2.2.1 Phosphate Buffer (0.5 M, pH 7.4)

In a 1000 mL volumetric flask, add the following reagents:

13.796 g monobasic sodium phosphate (NaH_2PO_4, H_2O)

56.78 g of dibasic sodium phosphate (Na_2HPO_4)

Distilled water added to make a 1000 mL solution.

B.2.2.2 Phenol (0.1 M) in Phosphate Buffer (0.5 M, pH 7.4)

Dissolve 9.411 g of phenol in 1000 mL of 0.5 M phosphate buffer solutions.

B.2.2.3 Assay Mixture

In a beaker add the following in the given order:

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41 mg AAP

10 mL of phenol (0.1 M) in phosphate buffer (0.5 M, pH 7.4)

200 μL of ARP stock solution

9.8 mL distilled water

The final total volume of the assay reagent is made to 20 mL.

B.2.3 Calibration Procedure

Make a stock solution of H_2O_2 with a concentration of 1.0 mM. From this stock solution prepare a standard ranging from 0 to 1.0 mM. In a test tube place the following solutions in the given order:

200 μL of assay reagent

750 μL of distilled water

50 μL of standard sample

The total volume of the assay mixture must be 1 mL and hydrogen peroxide concentration in the assay mixture must be below 50 μM. Immediately after the adding the H_2O_2 standard, shake the tube and then wait until the color is fully developed (approx.10 minutes). Put the assay in a semi-micro cuvette and read the peak absorbance at 510 nm. Repeat the procedure for all the standards, taking three measurements for each. Make a plot of absorbance *vs.* H_2O_2 concentration in the cuvette, and determine the slope of the line using a linear regression. A typical calibration curve is presented in the figure B.2.1.

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B.2.4 Estimation of Hydrogen Peroxide concentration

In a semi-micro cuvette, place the following reactants in the given order:

200 μL of assay reagent

0-750 μL of distilled water

50-800 μL of sample

The total volume in the cuvette must be 1 mL.

Immediately after addition of the sample, shake the cuvette and then wait for 10 minutes. Read the absorbance at the peak wavelength of 510 nm. Determine the cuvette H_2O_2 concentration from the calibration curve presented in Fig. B.2.1. From this cuvette H_2O_2 concentration, determine the sample hydrogen peroxide concentration of the sample as :

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \times \frac{1000\ \mu L}{Sample\ volume,\ \mu L}$$
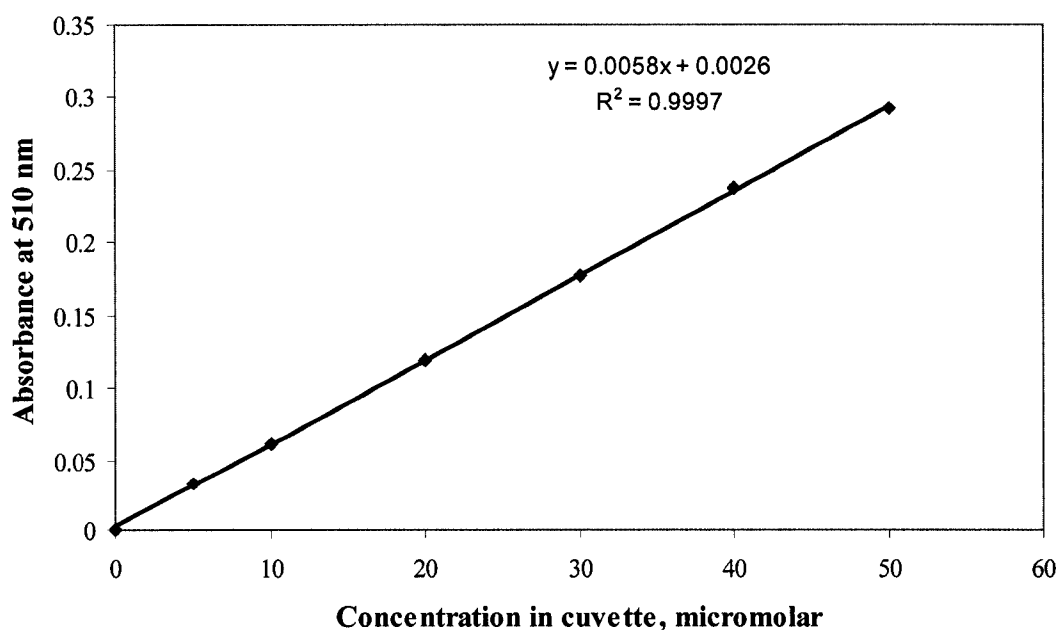

Figure B.2.1 Calibration curve for hydrogen peroxide at 510 nm

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B.3   TNBS Assay for Aromatic Amines

B.3.1   General

The purpose of this assay is to determine aromatic amines present in a sample. The assay employs 2,4,6 –trinitrobenzenesulfonic acid (TNBS) to produce color after reacting with amines present in the aqueous solutions. The sample solutions are buffered at pH 7.5 and the amount of aromatic amine is the only limiting substance in the solution. Therefore the intensity of color generated is proportional to the amount of aromatic amine present in the sample. Each colored solution of amine absorbs light at a distinct wavelength with a unique extinction coefficient. Standard calibration curve for only aniline is generally available.

B.3.2   Preparation of Reagent

B.3.2.1 Phosphate Buffer (0.5 M, pH 7.4)

In a 1000 mL volumetric flask, add the following reagents :

13.796 g monobasic sodium phosphate (NaH_2PO_4, H_2O)

56.78 g of dibasic sodium phosphate (Na_2HPO_4)

Add distilled water to make a 1000 mL solution.

B.3.2.2 TNBS Solution (10 mM)

In a 10 mL flask, add 29 mg of TNBS.

Add distilled water to make a 10 mL solution.

Fresh solutions are to be prepared each time the test is carried out.

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B.3.3 Calibration procedure

Make a stock solution of aromatic amine with a concentration of 1 mM. From the stock solution, make sub-stock solutions of concentrations ranging from 0-500 μM. In a final volume of 1.0 mL, add the solutions in the following order:

100 μL of assay reagent

100 μL of phosphate Buffer (0.5 M, pH 7.4)

0-800 μL of sample plus water

Keep the aromatic amine concentration in the cuvette below 50 μM. Samples are allowed to stand for 30 minutes for color development and then absorbance is measured at the peak wavelength against a reagent blank. Repeat the procedure for all the standards, taking three measurements for each. Make a plot of absorbance vs. aromatic amine concentration in the cuvette, and determine the slope of the line using a linear regression. A typical calibration curve for aniline is presented in the figure B.3.1.

B.3.4 Estimation of Aromatic Amines

In a semi-micro cuvette place the following in the order given:

100 μL of assay reagent

100 μL of phosphate buffer (0.5 M, pH 7.4)

50-800 μL of sample plus water

Shake the cuvette, allow some time for colour formation (1 h for ANDSA), then note the absorbance, and estimate the cuvette aromatic amine concentration from the calibration curve. For aniline use the calibration curve as presented in Fig. B.3.1.

Calculate the aromatic amine concentration in the sample from the following equation :

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$$[\text{Amine}]_{\text{sample}} = [\text{Amine}]_{\text{cuvette}} \times \frac{1000\ \mu L}{\text{Sample volume},\ \mu L}$$

Where, $[\text{Amine}]_{\text{cuvette}}$ is determined from the calibration curve.

y = 0.013x + 0.0127
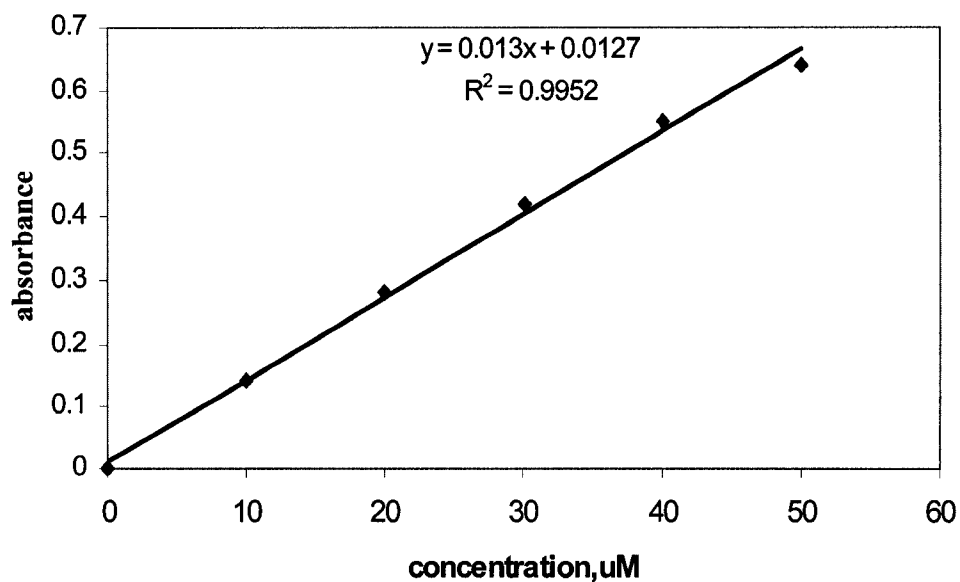R^2 = 0.9952

Figure B.3.1: Calibration curve for Aniline (with 1mM sodium sulphite) for TNBS test at 398 nm
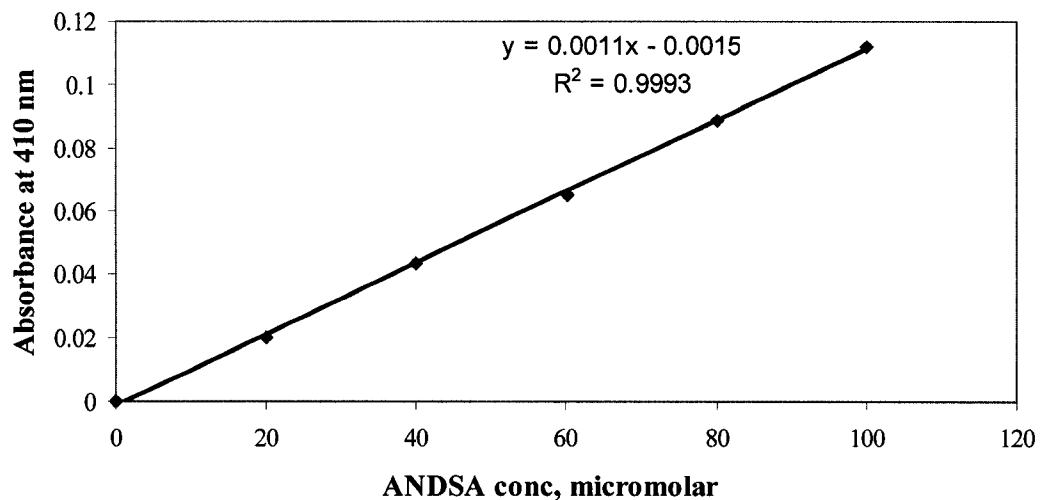

y = 0.0011x - 0.0015
R^2 = 0.9993

Figure B.3.2: Calibration curve for ANDSA (H-Acid) for TNBS test at 410 nm

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APPENDIX C: HPLC STANDARD CURVES

HPLC was used to identify and quantify the Fe reduction products in case of DPA. The products were speculated to be benzene and aniline. First a standard curve was prepared to which can be used to determine the concentration of DPA, Aniline or Benzene in the Fe reduction product.

<u>Preparation of HPLC standard curves</u> : Different known concentration of DPA, aniline and benzene varying from 0.05 to 0.2 mM was prepared after proper dilution. Since the Fe reduction was done with sodium sulfite as scavanger, these standard solutions were doped with 1 mM sodium sulfite. The samples were run in HPLC for some period (~10 min). The peak area vs. concentration was plotted to get the standard curve for DPA, aniline and benzene. Methanol was used for extracting DPA from Fe particles after reaction. DPA in methanol shows different peak area in HPLC. So another standard curve was prepared for DPA in methanol. A similar standard curve for aniline was also prepared. Since no benzene could be detected after the Fe treatment of DPA, a standard curve for benzene is not presented.

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C.1 DPA

$$y = 1E{+}07x - 19861$$
$$R^2 = 0.9976$$

$$y = 3E{+}06x - 6445.9$$
$$R^2 = 0.9974$$

♦ channel 1- 254 nm   ■ channel 2- 280 nm

—— Linear (channel 1- 254 nm)   —— Linear (channel 2- 280 nm)
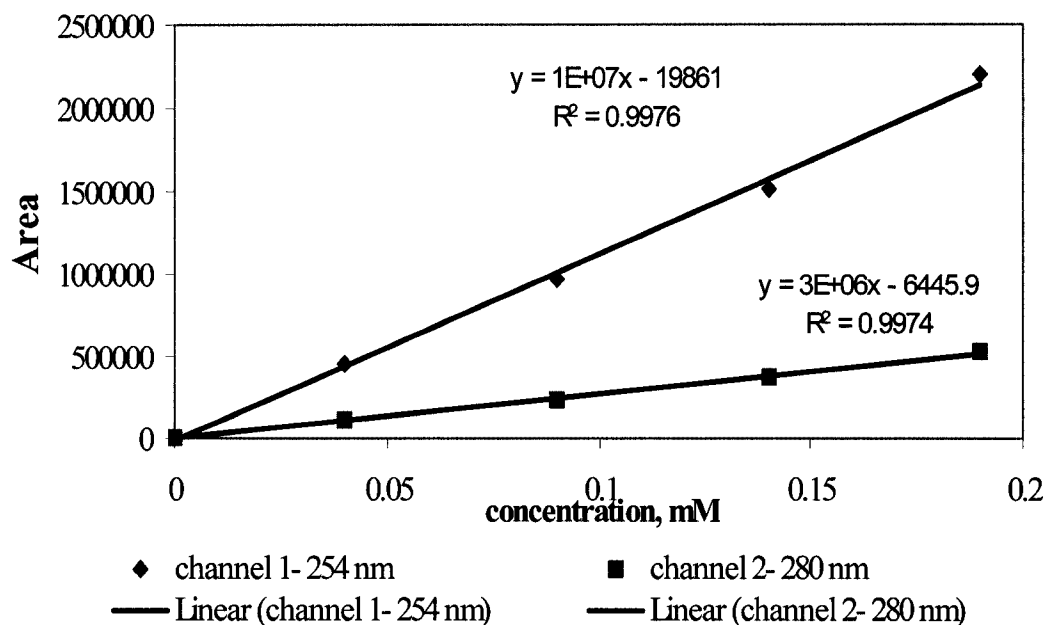
Figure C.1.1 : HPLC Standard curve for DPA with sodium sulphite in water

C.2 DPA in Methanol

$$y = 2E{+}07x + 136883$$
$$R^2 = 0.9982$$

$$y = 5E{+}06x + 39374$$
$$R^2 = 0.9977$$

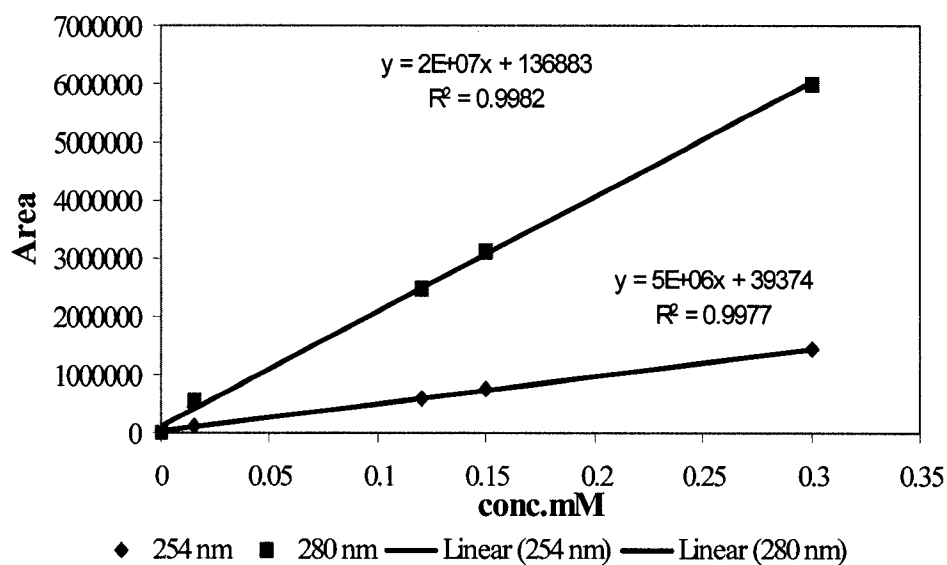♦ 254 nm   ■ 280 nm —— Linear (254 nm) —— Linear (280 nm)

Figure C.2.1 : HPLC standard curve for DPA with methanol

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C.3 Aniline

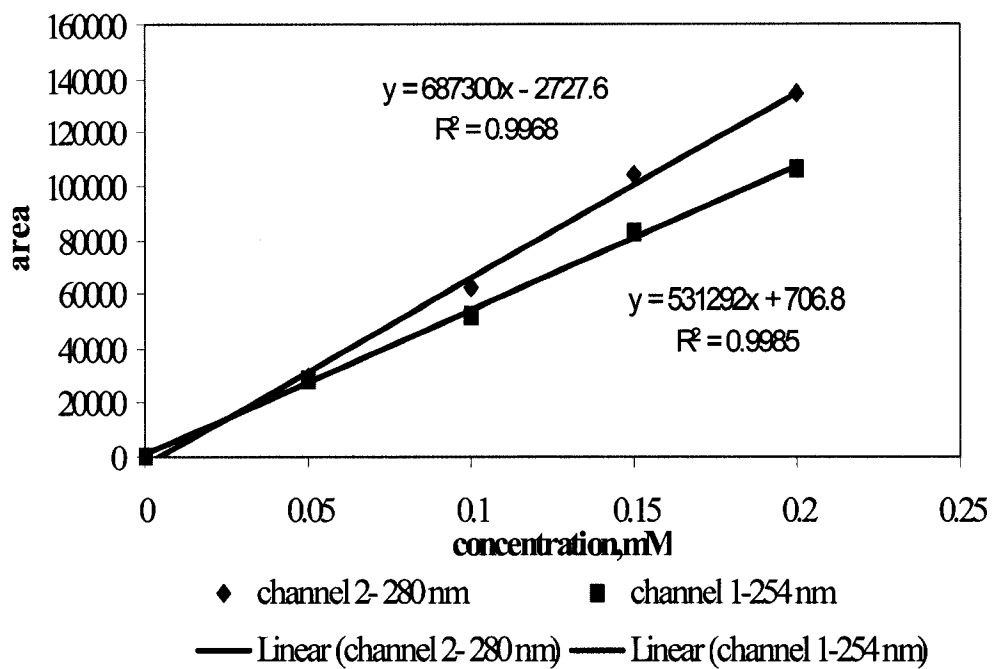
Figure C.3.1 : HPLC standard curve for anilne with sodium sulphite

C.4 HPLC Chromatogram for DPA Fe treatment

SAMPLE INFORMATION	
Sample Name: 1s	Acquired By: System
Sample Type: Unknown	Date Acquired: 5/8/04 5:23:03 PM
Vial: 2	Acq. Method: 80%A 20%B DUAL
Injection #: 1	Date Processed: 5/8/04 8:46:35 PM
Injection Volume: 10.00 ul	Channel Name: 2487Channel 1
Run Time: 10.00 Minutes	Sample Set Name: m
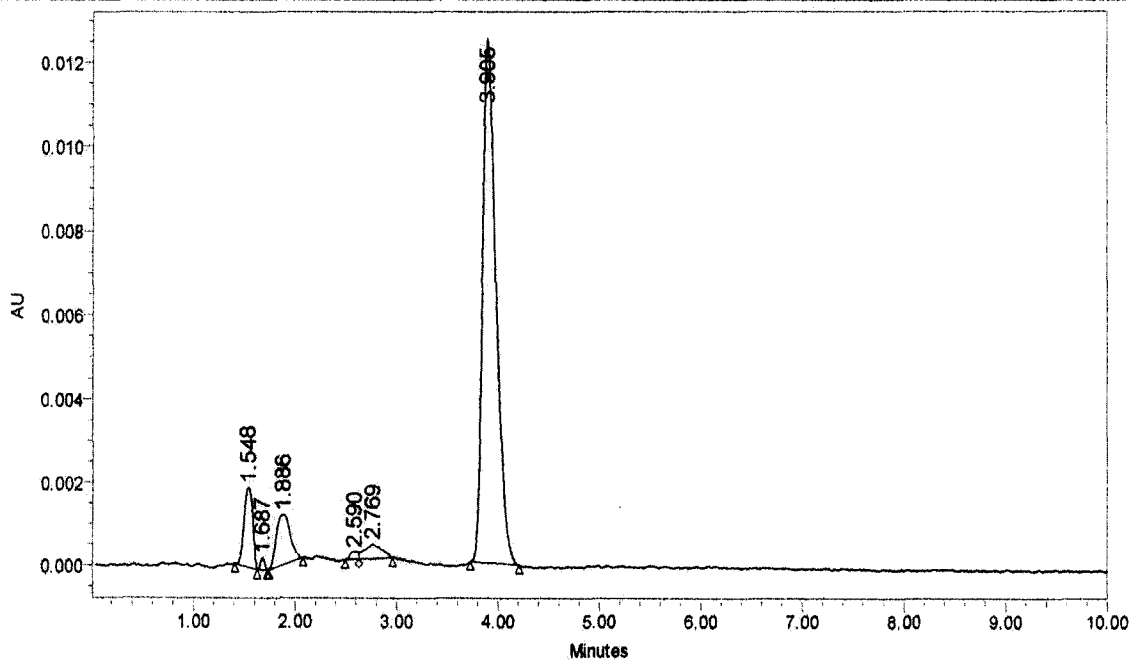
	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	1.548	11462	7.95	1963	11.89
2	1.687	831	0.58	278	1.68
3	1.886	11429	7.93	1220	7.39
4	2.590	947	0.66	180	1.09
5	2.769	4063	2.82	339	2.05
6	3.905	115400	80.07	12526	75.89

Figure C.4.1: Chromatogram of DPA observed at 254 nm after being treated with 1 g Fe, 1 g Na$_2$SO$_3$, at 7.0 pH, after 1 hour reaction time.

SAMPLE INFORMATION

Sample Name:	5s
Sample Type:	Unknown
Vial:	6
Injection #:	1
Injection Volume:	10.00 ul
Run Time:	10.00 Minutes

Acquired By:	System
Date Acquired:	5/8/04 6:30:18 PM
Acq. Method:	80%A 20%B DUAL
Date Processed:	5/8/04 8:55:02 PM
Channel Name:	2487Channel 1
Sample Set Name:	m
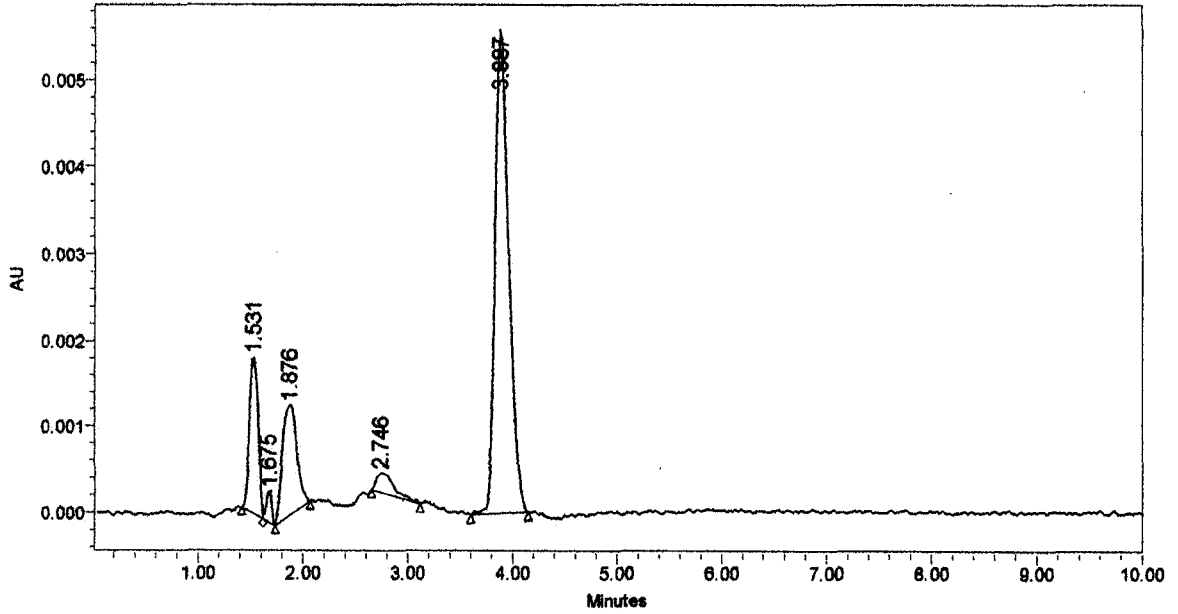
	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	1.531	10552	13.52	1830	19.72
2	1.675	1270	1.63	359	3.87
3	1.876	12247	15.70	1286	13.85
4	2.746	2661	3.41	219	2.36
5	3.887	51289	65.74	5588	60.20

Figure C.4.2: Chromatogram of DPA observed at 254 nm after being treated with 1 g Fe, 1 g Na_2SO_3, at 7.0 pH and 4 hour reaction time.

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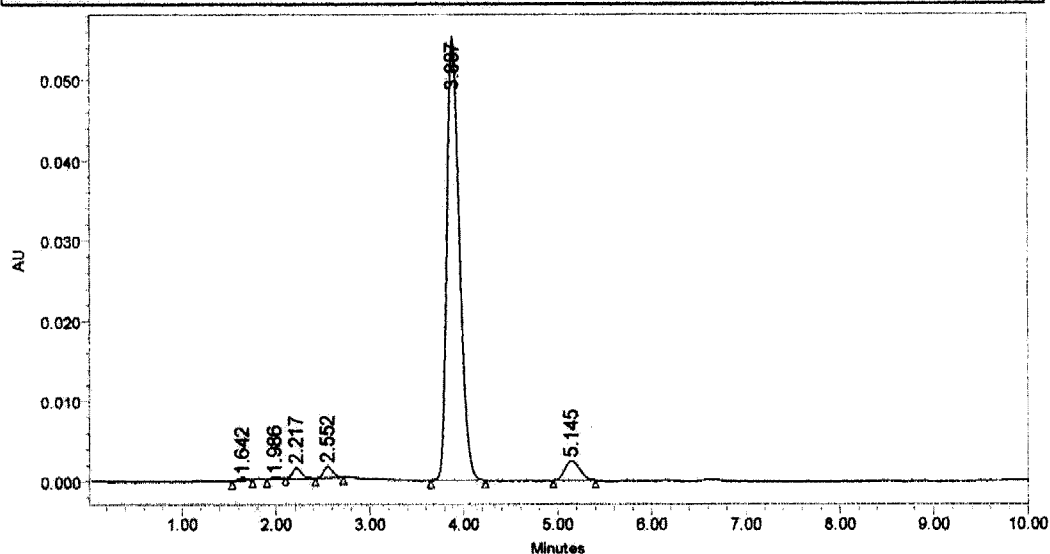## SAMPLE INFORMATION

Sample Name:	e4	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/8/04 7:54:25 PM
Vial:	11	Acq. Method:	80%A 20%B DUAL
Injection #:	1	Date Processed:	5/8/04 8:58:32 PM
Injection Volume:	10.00 ul	Channel Name:	2487Channel 1
Run Time:	10.00 Minutes	Sample Set Name:	m

	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	1.642	2053	0.36	319	0.52
2	1.966	2136	0.37	291	0.47
3	2.217	10006	1.76	1418	2.31
4	2.552	10050	1.76	1449	2.36
5	3.887	518393	90.99	55381	90.37
6	5.145	27070	4.75	2424	3.96

Figure C.4.3: Chromatogram of DPA extracted from iron surface, after Fe treatment with 1 g Fe, 4 hour run, at pH 7.0, observed at 254 nm.

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SAMPLE INFORMATION

Sample Name:	Unk.	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/6/04 8:36:04 PM
Vial:	1	Acq. Method:	80%A 20%B DUAL
Injection #:	1	Date Processed:	5/6/04 8:59:29 PM
Injection Volume:	10.00 ul	Channel Name:	2487Channel 1
Run Time:	10.00 Minutes	Sample Set Name:	m
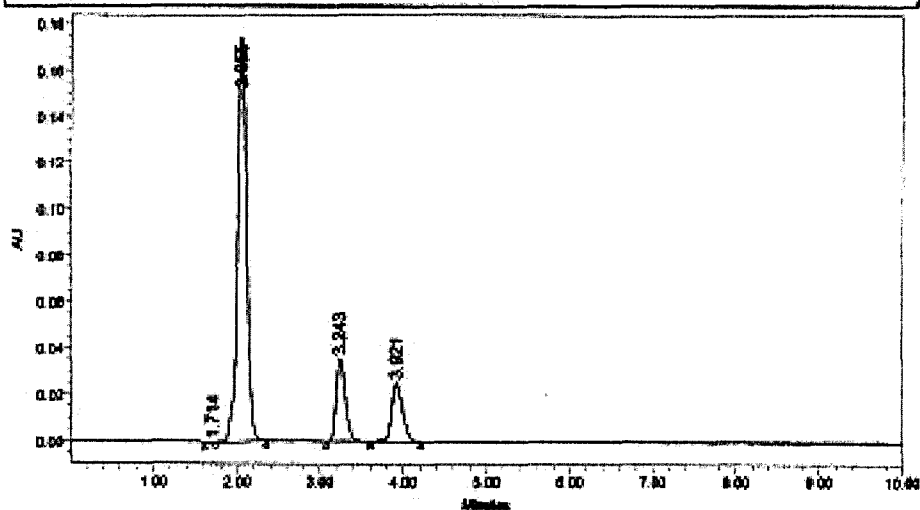
	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	1.714	1685	0.09	305	0.13
2	2.055	1328178	72.64	178027	74.07
3	3.243	267295	14.65	36025	15.16
4	3.921	230124	12.61	25268	10.64

Figure C.4.4: Chromatogram of DPA (3.821 min), aniline (2.055 min) and benzene (3.243 min) in mixture observed at 254 nm

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APPENDIX D:

Estimation of Kinetics Rate Constants for ANDSA reaction with ARP

D.1 Procedure

Determination of initial velocity: ARP reaction on ANDSA was carried out for different substrate concentration (1- 25 mM) with 4U/mL ARP, 2.4 mM hydrogen peroxide concentration at pH 7.02 and at room temperature. The amount of aromatic amine (ANDSA) remaining was measured by TNBS test at 2 minutes interval for the first 10 minutes. Remaining ANDSA concentration vs. time was plotted and a polynomial curve was fitted to this data sets for each ANDSA concentration. The order of polynomial was chosen to obtain the best fitting curve (maximise R^2 to nearly 1) whose Y intercept is equal to the absorbance value for TNBS test at initial ANDSA concentration. The first order coefficient of each best-fit curve was taken as the initial velocity for the particular substrate concentration. This generated the initial velocity data set for various substrate concentrations as presented in Table D.1.

Determination of rate constants: Initial velocities were plotted against substrate concentrations. A non linear regression of this data was carried out for the Michaelis – Menten equation using software application : NLREG ver 6.1, evaluation copy available from the internet.

Michaelis-Menten equation: $V = \dfrac{V_{max} * [S]}{K_m + [S]}$

Where S is substrate concentration, V = velocity, V_{max} = maximum velocity, K_m = Michaelis-Menten constant (Palmer, 1995).

On feeding the data and script the application computed the rate constants: V_{max} and K_m.

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D.2 Observed Data

Table D.1 Initial rate of reaction vs. substrate concentration for ANDSA

ANDSA conc., mM	1	1.5	2	2.5	3	3.5
Initial rate of reaction, mM / min	0.090426	0.15824	0.17056	0.24014	0.25771	0.24351

ANDSA conc., mM	4	4.5	10	15	20	25
Initial rate of reaction, mM / min	0.21612	0.23748	0.24728	0.27257	0.28289	0.28662

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D.3 A software program written to estimate non linear regression parameters to fit first order kinetics equation.

Software used : Evaluation copy, NLREG version 6.1
Copyright (c) 1992-2004 Phillip H. Sherrod.

```
Title "Kinetics";
Variables x,y;
Parameters p,q;
Function y = (p*x)/(q+x);
plot;
data;
0       0
1       0.090426
1.5     0.15824
2       0.17056
2.5     0.24014
3       0.25771
3.5     0.24351
4       0.21612
4.5     0.23748
10      0.24728
15      0.27257
20      0.28289
25      0.28662
```

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D.4 Nonlinear regression results obtained using the software

---- Final Results ----

NLREG version 6.1
Copyright (c) 1992-2004 Phillip H. Sherrod.

Kinetics
Number of observations = 13
Maximum allowed number of iterations = 500
Convergence tolerance factor = 1.000000E-010
Stopped due to: Relative function convergence.
Number of iterations performed = 6
Final sum of squared deviations = 6.8068337E-003
Final sum of deviations = -9.0025657E-003
Standard error of estimate = 0.0248758
Average deviation = 0.0166315
Maximum deviation for any observation = 0.0442197
Proportion of variance explained (R^2) = 0.9191 (91.91%)
Adjusted coefficient of multiple determination (Ra^2) = 0.9118 (91.18%)
Durbin-Watson test for autocorrelation = 1.341
Analysis completed 6-Jun-2004 23:48. Runtime = 0.16 seconds.

---- Descriptive Statistics for Variables ----

Variable	Minimum value	Maximum value	Mean value	Standard dev.
x	0	25	7.076923	8.01001
y	0	0.28662	0.2079651	0.08374927

---- Calculated Parameter Values ----

Parameter	Initial guess	Final estimate	Standard error	t	Prob(t)
p	1	**0.302336009**	0.01627929	18.57	0.00001
q	1	**1.24541882**	0.2842399	4.38	0.00110

---- Analysis of Variance ----

Source	DF	Sum of Squares	Mean Square	F value	Prob(F)
Regression	1	0.07736046	0.07736046	125.02	0.00001
Error	11	0.006806834	0.0006188031		
Total	12	0.08416729			

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VITA AUCTORIS

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- Assistant Manager (Technical services), 1999- 2002.
  Indian Oil Corporation Ltd. India

- Engineer, 1995-1999, Indian Oil Corporation Ltd., India,

Awards:

Graduate Tuition Scholarship, University of Windsor, Summer, 2004