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Color reduction of azo dyes and removal of 1,4-phenylenediamine from synthetic wastewater using Arthromyces ramosus peroxidase.

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COLOR REDUCTION OF AZO DYES AND REMOVAL OF 1,4-PHENYLENEDIAMINE FROM SYNTHETIC WASTEWATER USING *ARTHROMYCES RAMOSUS* PEROXIDASE

BY

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ABSTRACT

Arthromyces ramosus peroxidase (ARP) has been studied extensively to catalyze the oxidation of various phenolic compounds in the presence of hydrogen peroxide to form polymers which are readily precipitated from solution. The objective of this thesis was to extend research to other industrial wastewaters. Azo dyes and one representative dye breakdown product: 1,4-phenylenediamine (p-PD) were chosen.

Experiments were conducted to investigate the feasibility and efficiency of using ARP and hydrogen peroxide to reduce the color of the azo dyes and to remove 1,4-phenylenediamine (p-PD). All tests were carried out in continuously-stirred batch reactors. The dyes selected included Naphthol Blue Black, Crocein Orange G, Acid Red 4, and Disperse Orange 3. The optimum conditions to achieve highest efficiency of dye color reduction or p-PD removal were determined for the following parameters: pH, ARP concentration, hydrogen peroxide concentration and reaction time.

ARP efficiently reduced the color of the selected dyes except Disperse Orange 3 from synthetic wastewater in the presence of hydrogen peroxide. The optimum pH for different dyes was 7.0-8.0. The optimum ARP concentration range was from 0.1 to 0.2 U/mL, the optimum H_2O_2 range was from 100-200 μM for 50 μM dyes and the reaction time needed was less than 1 hour.

Experiments were also conducted to reduce the color of the Disperse Orange 3 using zero-valent iron. Results showed that more than 98% conversion efficiency could be achieved in 1 hour.

For p-PD removal, the optimum pH was 7.6 and the optimum ARP concentration, hydrogen peroxide concentration, and reaction time were 0.02 U/mL, 120 μM, 4 h for 100 μM p-PD and 0.05 U/mL, 400 μM and 4 h for 300 μM p-PD.

A simple and convenient colorimetric method was developed to measure 1,2-phenylenediamine (o-PD), 1,3-phenylenediamine (m-PD), 1,4-phenylenediamine (p-PD) and aniline based on their reaction with trinitrobezenesulfonic acid (TNBS) in the presence of sodium sulfite.

DEDICATION

This thesis is dedicated to my parents, Yuling Wang and Genji Wang, for the support and encouragement they have always given to me

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LIST OF NOMENCLATURES

AR4	Acid Red 4
ARP	*Arthromyces ramosus* peroxidase
COG	Crocein Orange G
DO3	Disperse Orange 3
HPLC	High Performance Liquid Chromatography
HRP	horseradish peroxidase
m-PD	1,3-phenylenediamine
NBB	Naphthol blue black
o-PD	1,2-phenylenediamine
p-PD	1,4-phenylenediamine
TNBS	trinitrobenzenesulfonate

1. INTRODUCTION

Environmental regulations in many countries have now made it mandatory to reduce the color of dye wastewater prior to discharge. Increasing concerns about color in effluent are leading to worldwide efforts to develop more effective color removal processes (Cooper, 1995; Nawar and Doma, 1989). Commonly used dye wastewater treatments include chlorination, ozone treatment, adsorption, precipitation, flocculation, electrochemical treatment and ion-pair extraction. Dye color reduction has also been studied by using zero-valent iron (Nam and Tratnyek 2000). Azo dyes are seldom subjected to biological treatment because they are resistant to degradation by bacterial oxidation while fermentation reduction reactions may yield toxic products (Shaul et al., 1991). Enzymatic treatment introduced by Klibanov et al. (1980) is considered to be a feasible alternative to the conventional treatment methods. Some studies have already been conducted using various enzymes to reduce the color of dyes (Wong and Yu, 1999, Pasczynski et al., 1992). Among them, laccases and peroxidases (such as horseradish peroxidase) are the most commonly used. Furthermore, since the enzymes have been extensively studied to remove anilines in wastewater, the enzymatic treatment has the potential not only to reduce the color of the dyes directly but also to remove the secondary products of dyes reduced by other methods.

1.1 Enzyme background

With more and more stringent laws and regulations, there is an increasing emphasis on the removal of specific pollutants from waste mixtures and on the multimedia (air, land and water) effects of treatment processes. Existing waste treatment processes have various limitations associated with the removal of specific chemicals to regulated levels.

Enzymes represent one means by which selective removal of pollutants can be accomplished in wastewaters. Enzymes catalyze chemical reactions that would otherwise be too slow at ambient temperatures to be of interest and, therefore, can achieve chemical transformations that may be difficult to achieve efficiently with conventional chemical and biological treatment processes.

The enzyme used in this study was *Arthromyces ramosus* peroxidase (ARP), a 41 kD monomeric glycoprotein containing one mole of protoheme IX per mole of enzyme as a prosthetic group (Lokman et al., 2001). It is isolated from the hypomycete *Arthromyces ramosus* (fungi imperfecti), which produces large amounts of peroxidase extracellularly and is viewed as being better suited to large-scale production because it is produced extracellularly by its parent organism (Taylor et al., 1996; Villalobos and Buchanan, 2002). ARP has a broad specificity for phenolic and aniline hydrogen donors, which makes the enzyme interesting for industrial applications.

1.2 Enzymatic wastewater treatment

1.2.1 Source of dye waste

Azo dyes, the largest and most versatile class of dyes, account for about 70% of the annually produced amount of dyes (estimated worldwide as 1 million tons in 1994) (Stolz, 2001). More than 2,000 different dyes are currently used to dye various materials. The most important characteristic of azo dyes is that they have the azo chromophore (-N=N-) connecting two carbon systems, at least one of which is aromatic.

Textile, tannery, pulp and paper, paint and electroplating industries are the major consumers of dyes. It has been reported (Zollinger, 1987) that 10% of dye is lost to wastewater as a result of inefficient dyeing processes when the dyestuff does not bind to

the fibers. Azo dyes constitute an important class of xenobiotics, exhibiting toxic and carcinogenic effects. The recalcitrance of the azo dyes to biological degradative processes results in severe contamination of the rivers and ground water in areas of the world with a high concentration of dyeing industries (Maguire and Tkacz, 1991)

All the azo dyes and azoic dyes are combinations of benzene and naphthalene derivatives, with the groups such as amino, sulfo, nitro, hydroxyl, or alkyl (usually methyl). When the azo bond is reductively cleaved in such a structure, two aromatic amines are produced and the color of the dye is reduced. Lots of the dyes or their decomposition products (mainly benzene and naphthalene with different substituent groups) are potential or proved carcinogens, toxic and recalcitrant to treatment. Therefore, beside color reduction of dyes, removal of the remaining decomposition products must also be considered and studied.

1,4-phenylenediamine (p-PD) is an important dye intermediate and decomposition product arising from reductive methods. Therefore, it was selected for study by the enzymatic treatment with ARP in the presence of hydrogen peroxide.

1.2.2 Current methods for dye wastewater treatment

In textile processing industry, the aim is to reduce the unrecyclable residues and also the amount of wastewater to be discharged. Usually, the reduction in wastewater pollutant loads is achieved by the following production-integrated measures as shown in Table 1.1.1 (Rott and Minke, 1995):

Table 1.1.1 Available unit processes applicable to azo dyes (Rott and Minke, 1995)

Biodegradation	Physico-chemical	Separation processes
Activated sludge process	Chemical oxidation	Filtration
Fixed film processes	Electrochemical	Membrane filtration
Fixed bed	processes	Precipitation/flocculation
Fluidized bed	Reductive	Evaporation
Granular fixed	transformation	Adsorption
Rotating biol. contactor		Ion-exchange
Trickling filters		Complex formation

All of these have serious restrictions as economically feasible methods for decolorizing textile wastewaters, because of high cost, formation of hazardous by-products or intensive energy requirements.

All biological techniques can be taken as material destructive processes that show obvious differences concerning efficiency, particularly, in elimination of poorly biodegradable substances such as the dyes. The color reduction effect is so small with aerobic activated sludge processes that normally a further physico-chemical step, a transition to fixed film processes or a preliminary anaerobic step, is necessary. Under anaerobic conditions, an extensive color reduction and digestion of aerobic-refractory substances can be achieved. With the physico-chemical material destructive processes an extensive oxidation of substances is achieved by use of strong oxidizing agents like O_3 or H_2O_2, partially in combination with UV-radiation and/or under high pressure or through electrolytic processes. Color reduction can also be achieved under reducing conditions by using low valent metals or other methods. Separation processes produce concentrates for which reuse is not possible, hence a subsequent disposal step must be included (Rott and Minke, 1999).

For biological wastewater treatment, a 40-50% color removal can be anticipated due to biodegradation and adsorption of the dyes on flocculated sludge. The main drawback of biological processes is the long period needed for biological organisms to become acclimated and to the low biodegradability of dyes (Marmagne and Coste, 1996).

Physical-chemical processes alone can not provide satisfactory results, even with the addition of specific coagulants that can enhance color removal considerably. The color removal of insoluble dyes is insufficient. Color removal efficiency of coagulation-flocculation processes is strongly dependent on the dye types. Cationic dyes do not coagulate at all, making their removal by physical-chemical (coagulation-flocculation) process impossible. Acid, direct, vat, mordant and reactive dyes usually coagulate, but the resulting floc is of poor quality and does not settle well even after introduction of a flocculant. Thus, a coagulation-flocculation treatment yields mediocre results. Only sulfur and disperse dyes coagulate well, settle easily and have excellent removal efficiency (Marmagne and Coste, 1996).

Microfiltration membranes can only remove disperse and vat dyes efficiently and almost have no removal for other dyes. On the other hand, ultrafiltration achieves complete color removal for all classes of dye, but care is needed to avoid membrane clogging, which appears to occur rapidly. Naturally, nanofiltration will allow complete color removal, but with less membrane fouling. High removal rates (over 90%) can be achieved using activated carbon for cationic, mordant, and acid dyes. For direct, sulfur, dispersed, and reactive dyes, efficiency is moderate (over 40%) and for vat dyes, color removal is very low (under 20%) (Marmagne and Coste, 1996).

Generally, the color removal is effective and fairly rapid when using ozone; but, it is the class of dye that is most significant in determining the behavior of the dyes. Ozone

has very efficient color removal for acid, mordant, cationic direct, reactive, and sulfur dyes. However, dispersed and vat dyes are generally difficult to remove, even at high ozone concentration (Marmagne and Coste, 1996).

None of the traditional physical-chemical and biological treatment methods have been found to be very suitable as they produce large quantities of sludge (Stephen, 1995). All these methods are significantly different in terms of color removal, volume capability, operating speed and capital cost. Many synthetic dyes are resistant to conventional wastewater treatment systems (Seshadri et al., 1994. Shaul et al., 1991).

On the other hand, enzymatic methods have many advantages over physical and chemical treatments such as degradation of dye molecules to carbon dioxide and water, formation of less sludge and environmental compatibility (Azmi et al., 1998).

1.2.3 Use of enzymes

The major characteristic of enzymes is that they react with their substrates with specificity. Such specificity allows enzymes to remove target pollutants selectively, while utilizing any required chemical reactants with high stoichiometric efficiency, thus making enzymatic treatment an important means in waste treatment. Enzymes also have the potential to combine the advantages of selectivity with the simplicity, reliability and predictability of conventional chemical treatment systems.

The following potential advantages of an enzyme-based treatment over conventional biological treatment were noted by Nicell et al. (1993):

ñ application to a broad range of compounds,

ñ action on, or in the presence of, many substances which are toxic to microbes,

ñ operation at both high and low concentrations of contaminants,

ñ operation over wide temperature, pH and salinity ranges,

- no shock loading effects,
- no delays associated with acclimatization of biomass,
- reduction in sludge volume(no biomass generation), and
- better defined system with simpler process control.

They also mentioned the following potential advantages of an enzyme-based treatment over chemical/physical processes:

- operation under milder, less corrosive conditions,
- operation in a catalytic manner,
- operation on trace level organic compounds and organic not removed by existing chemical/physical processes,
- reduced consumption of oxidants, and
- reduced amounts of adsorbent materials for disposal.

1.3 Objective

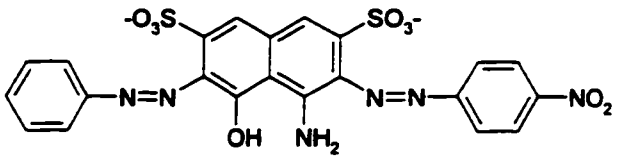
The objectives of this study were to:

1. optimize the reaction parameters, in buffered deionized water, to achieve the highest removal of selected azo dyes and 1,4-phenylenediamine by *Arthromyces ramosus* peroxidase (ARP) and hydrogen peroxide;
2. reduce the color and determine the product (s) of Disperse Orange 3 using zero-valent iron; and
3. develop a simple method for the p-PD examination by using trinitrobenzenesulfonate (TNBS).

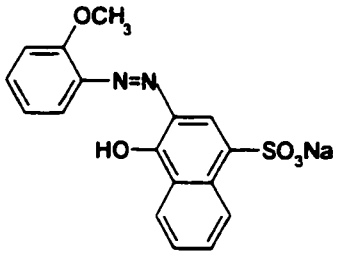
1.4 Scope

The scope of this study included:

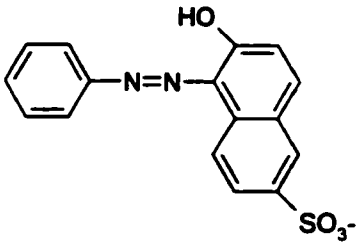
1. Azo dyes studied were Naphthol Blue Black, Crocein Orange G, Acid Red 4, and Disperse Orange 3. The dye intermediate, 1,4-phenylenediamine (p-PD) was also studied. The chemical structures of these compounds are shown in Figure 1.1.1.
2. Parameters optimized included pH, ARP concentration, hydrogen peroxide concentration (or molar ratio of hydrogen peroxide to substrate), and reaction time.
3. Study the color reduction efficiency of Disperse Orange 3 and use the methods of UV-spectrum, a colorimetric test and HPLC to test the product (s) of the reduction of DO3 by zero-valent iron.
4. The effect of pH, sodium sulfite concentration, reaction time on the phenylenediamines and aniline in a colorimetric test for anilines based on reaction with trinitrobenzensulfonate (TNBS) in the presence of sodium sulfite.



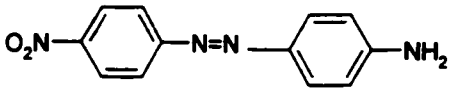
Naphthol Blue Black



Acid Red 4



Crocein Orange G



Disperse Orange 3

Figure 1.1.1 Azo dyes selected for study

2. LITERATURE REVIEW

2.1 Enzymatic decoloriztion of azo dyes

2.1.1 Peroxidase background

The use of individual enzymes, as from distinct from whole microbes, in the removal of toxic organic compounds from industrial wastewater was exemplified by Klibanov and coworkers (Klibanov et al., 1983). The mechanism of enzyme-catalyzed reactions is of interest to many researchers. Dunford (1991) reviewed the two-electron reaction mechanism of peroxidases to explain this kind of reactions. The reactions involved in the peroxidase catalytic cycle are illustrated in Figure 2.1.1. During the cycle, the ground state enzyme (E) undergoes a two-electron oxidation by hydrogen peroxide forming an intermediate state called Compound I (E_i). Compound I accepts an aromatic compound (AH_2) in its active site and carries out its one-electron oxidation, liberating a free radical (•AH) that is released back into the solution, and converting Compound I to Compound II (E_{ii}). A second aromatic compound is accepted in the active site of compound II and is oxidized, resulting in the release of a second free radical and the return of the enzyme to its resting state, completing the catalytic cycle. The free radicals diffuse from the active center of the enzyme into the solution and then non-enzymatically react to form dimers, trimers, and so forth, eventually resulting in higher oligomers and polymers, which are of low solubility and can be easily removed from wastewater by coagulation and sedimentation or filtration (Klibanov et al., 1983, and Nicell et al., 1992).

The peroxidatic reaction mechanism is shown below (Dunford, 1991):

$$E + H_2O_2 \rightarrow E_i + H_2O \qquad \text{Eq. 2.1}$$

$$E_i + AH_2 \rightarrow E_{ii} + \bullet AH \qquad \text{Eq. 2.2}$$

$$E_{ii} + AH_2 \longrightarrow E + \bullet AH + H_2O \qquad \text{Eq. 2.3}$$

The overall enzymatic reaction is as follows:

$$H_2O_2 + 2AH_2 \xrightarrow{E} 2 \bullet AH + 2H_2O \qquad \text{Eq. 2.4}$$

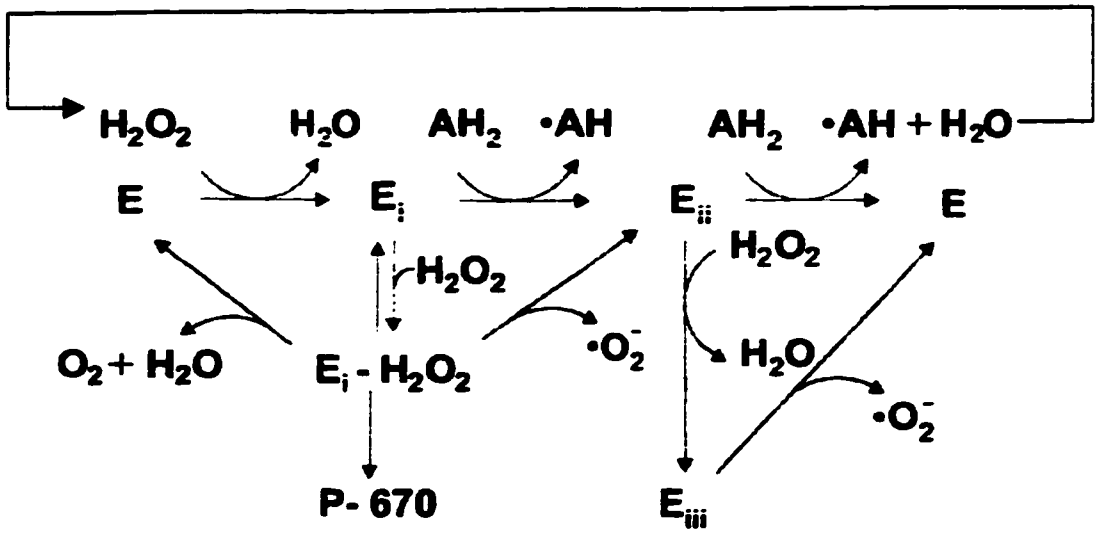


Figure 2.1.1 Complete peroxidase catalytic cycle with proposed side reactions

(E, E_i, E_{ii}, and E_{iii} represent the ground state enzyme, Compound I, Compound II, and Compound III, respectively; AH_2 and •AH represent an aromatic substrate and aromatic radical, respectively; P-670 represents the inactive peroxidase form called verdohaemoprotein. Dunford, (1991)).

Based on the catalytic cycle shown in Fig 2.1.1, two moles of aromatic compound are oxidized for every mole of peroxide consumed, yielding a stoichiometry of 1:2 or 0.5. However, the observed stoichiometry reported in the literature is based on moles of peroxide consumed per mole of aromatic removed from solution (Yu et al., 1994; Nicell et al., 1992; Nicell, 1994). Therefore, the value of the stoichiometry that used to produce the polymers that eventually precipitate from solution is 1. The observed stoichiometry becomes very close to 1 as the size of precipitating polymer increases (Nicell, 1994).

Various side reactions that take place during the removal process are responsible for enzyme inactivation or inhibition, causing a limited catalytic lifetime (Ibrahim et al., 2001; Nicell et al., 1993), and increasing treatment costs. As shown by Eq. 2.5, Enzyme inactivation or inhibition by hydrogen peroxide (with limited or no aromatic substrate present) results in the formation of Compound III (E_{iii}), which is a catalytically inactive form of the enzyme (Ibrahim et al., 1997, Buchanan and Han 2000), but does not represent a terminal inactivation, since Compound III decomposes back to the resting state of peroxidase (Arnao et al., 1990).

$$E_{ii} + H_2O_2 \text{---> } E_{iii} + H_2O \qquad \text{Eq. 2.5}$$

In the case of HRP, formation of a permanently inactive enzyme form, designated P-670, has been observed in the presence of excess hydrogen peroxide. In addition to these side reactions, other inactivation mechanisms have been proposed. Several studies have indicated that inactivation of HRP occurs during phenol polymerization, where the end-product polymer adheres to HRP molecules, thereby enclosing them and hindering the access of substrate to the enzyme's active site (Nicell et al., 1993). Klibanov et al. (1983) speculated that a free radical might return to the active site of the enzyme and bind to the heme edge, causing permanent enzyme inactivation by blocking the access to the active site or upsetting the geometric configuration of the enzyme, thus eliminating its catalytic ability. It is likely that both of these mechanisms contribute to permanent HRP inactivation during the removal process. Buchanan and Han (2000) reported the inactivation behavior of ARP to differ from that of HRP; the ARP seemed to be more sensitive to the presence of excess hydrogen peroxide.

Some researchers have suggested other mechanisms to explain the enzyme-catalyzed reactions (Eling et al., 1990); but the core part of these mechanisms is also the

free radical formation by the peroxidase. Peroxidases catalyze the one-electron oxidation of aromatic amines, giving rise to nitrogen- and /or carbon-centered radicals (Eling et al., 1990). Although their detection by electron spin resonance is difficult, aromatic amine free radicals can be detected indirectly based on their reaction with glutathione giving the parent compound and a glutathionyl free radical. Stiborova et al. (1996) have observed that no oxygen consumption took place during the incubation of peroxidase, N,N-dimethyl-4-aminoazobenzene (DAB) and H_2O_2 but it was observed after addition of glutathione to the reaction mixture. Such observations indicate that peroxidase oxidizes DAB to a free radical metabolite(s). DAB is oxidized by peroxidase to N-methyl-4-aminoazobenzene (MBA), aminobenzene (AB), and four unknown products. The formation of MBA and AB by N-demethylation reactions is supposed to be the one-electron radical reaction.

2.1.2 Use of peroxidase in wastewater treatment

Over the past two decades, the application of enzyme-catalyzed treatment of these organic compounds has been studied widely and deeply (Nicell et al., 1992). The Windsor group has been actively investigating the conditions under which this method can be applied to a given type of wastewater (Taylor et al., 1996). The enzymes and the target organic compounds that have been tested so far under different conditions are:

- Enzymes: horseradish peroxidase (HRP), *Arthromyces ramosus* peroxidase (ARP), soybean peroxidase (SBP), laccase and *Coprinus macrohizus* peroxidase (CMP)
- Substrates: phenol, o-cresol, m-cresol, p-cresol, o-chlorophenol, m- chlorophenol, p-chlorophenol, 2,4-dichlorophenol, pentachlorophenol, aniline, nitrobenzene,

nitro- and dinitro-toluenes, p-phenoxyphenol, o,o'- and p,p'-biphenol, bisphenol A (known as 4,4'-isopropylidenediphenol), 1- and 2-naphthols, nitrophenols, chloroanilines, nitroanilines, nitrotoluidines

- Oxidants: hydrogen peroxide, oxygen
- Parameters; pH, temperature, type and concentration of enzyme, type and concentration of substrate, concentration of hydrogen peroxide or oxygen, use of additive, process layout and reactor design.

Kinetic models have also been developed and tested both for the removal of phenol with HRP and ARP under different conditions (Yu et al., 1994; Wu et al., 1998)

In the enzyme-catalyzed reaction, an oxidative enzyme (plant or microbial) catalyzes the oxidation of phenols and aromatic amines with an appropriate oxidant, generating phenoxyl and anilinium cation radicals, respectively. According to the reaction mechanism proposed, polymerization has been assumed to occur by step-wise coupling of radicals to form dimers, trimers, and eventually oligomers and polymers which have low solubility and can be removed easily by settling from the wastewaters (Taylor et al., 1998).

Selectivity for the removal of certain compounds is important in order to meet increasingly strict regulatory criteria or to facilitate further treatment. The traditional activated sludge method is commonly used to reduce organic load in municipal and industrial wastewater; however, it has difficulty in removing toxic pollutants to low levels. Physicochemical treatment processes are not very selective in terms of the number of pollutants removed during treatment; therefore, such processes are more economically feasible for the treatment of dilute wastewaters. If toxic compounds are removed selectively, the bulk of the organic material in wastewater may be treated biologically,

thereby reducing overall treatment costs. Enzymatic treatment represents one method by which selective removal of pollutants may be accomplished (Aitken, 1993).

So far, several dyes have been studied for color reduction using various enzymes, as shown in Table 2.1.1

The relatively short catalytic lifetime and high cost of the enzyme are the main significant disadvantages. The short catalytic lifetime has been attributed to the inactivation of the peroxidase. Klibanov et al. (1983) have suggested that, with phenols, this inactivation occurs during the enzymatic reaction of phenols due to the interactions of the phenoxyl radicals with the active site of the enzyme. The high cost of enzyme is due to its complex manufacturing processes and lower amounts produced. This disadvantage can be partially overcome when large amounts of enzymes are produced by industrialized processes.

Table 2.1.1 Dyes studied for color reduction by enzymes

Dye	Enzyme	pH	Eff.(%)	Source
Sudan I	HRP	8.4	85	1
4'-OH-Sudan I	HRP	8.4	60	1
6-OH-Sudan I	HRP	8.4	93	1
4',6-Di(OH)-Sudan I	HRP	8.4	94	1
4-Aminoazobenzene	HRP	4.7	90	1
4-Methylaminoazobenzene	HRP	4.7	87	1
4-Dimethylaminoazobenzene	HRP	4.7	85	1
3-Methoxy-4-aminoazobenzene	HRP	4.7	53	1
Acid violet	Pseudomonas	7.0-8.0	99	2
Acid red 151	Pseudomonas	7.0-8.0	99	2
Reactive black 5	Pseudomonas	7.0-8.0	95	2
Aid yellow 34	Pseudomonas	7.0-8.0	92	2
Reactive blue 2	Pseudomonas	7.0-8.0	24	2
Acid green 27	Pseudomonas	7.0-8.0	76	2
Acid red 183	Pseudomonas	7.0-8.0	27	2
Indigo carmine	Pseudomonas	7.0-8.0	88	2
Bromophenol blue	Lignin peroxidase[a]	4.0	93	3
Congo red	Lignin peroxidase	4.0	54	3
Methylene blue	Lignin peroxidase	2.5	>75	3
Methyl green	Lignin peroxidase	3.0	>75	3
Methyl orange	Lignin peroxidase	2.5	>75	3
RBBR[b]	Lignin peroxidase	2.5	>75	3
Toluidine blue O	Lignin peroxidase	2.5	>75	3
Poly R-478	Lignin peroxidase	3.5	46	3
Poly S-119	Lignin peroxidase	4.0	79	3
Poly T-128	Lignin peroxidase	4.0	48	3

Note: Source: 1--Stiborova et al. (1996); 2--Yu et al. (2001); 3--Ollikka et al. (1993)

a: Lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. b: Remazol brilliant R.

The selectivity of enzymes sometimes turns itself to be a disadvantage. The removal efficiency strongly depends on the structure of compounds to be removed. Stiborova et al. (1996), while using horseradish peroxidase (HRP) to remove azo dyes, found that the aminoazo dyes (DBA, MAB and aminoazobenzene (AB)), as well as the non-aminoazo dyes Sudan I and its C-hydroxy derivatives, were efficiently oxidized. However, compounds structurally similar to Sudan I, namely Sudan II, Sudan III and Orange II, did not act as peroxidase substrates. If the benzene ring in the molecule of Sudan I was substituted by a methyl, phenylazo, or sulfo group, the resulting compound was not oxidized by peroxidase. The presence of a free hydroxy group in the Sudan I molecule was of key importance for its oxidation by peroxidase; however, the derivative of Sudan I in which the hydroxyl group was acetylated (O-acetyl-Sudan I) was not oxidized by peroxidase.

Wong and Yu (1999) proposed a mediated dye degradation mechanism and made it possible to reduce the color of dyes which were not substrates of enzymes and could not be oxidized directly by enzymes. This mechanism is shown in Figure 2.1.2. A dye molecule (M) which is the substrate of laccase is oxidized by the enzyme forming a cation radical (M^{+}). The radical is an active and unstable substance which may decompose automatically to the product of enzymatic oxidation (P_M) or oxidize other nonsubstrate dye molecules (D) returning to its original state (M). The oxidized molecule of non-substrate dye is unstable and decomposes into final product (P_D). The electrons taken by laccase finally go to dioxygen forming water. By this reaction mechanism, the dye molecules which are laccase substrates play a mediating role and promote the decomposition of those dyes which are not the substrate of laccase per se. Finally, dyes of

Finally, dyes of different chemical structures can be decomposed by this enzyme. This fact implies that the laccase-substrate dyes in an industrial effluent can promote the color reduction of those non-substrate dyes.

Soares et al. (2002) suggested that disazo dye color reduction proceeded via one-electon oxidation which led to the formation of a phenoxy radical which in turn was oxidised by laccase to produce a carbonium ion in which the charge was localized on the phenolic ring carbon with the azo linkage.

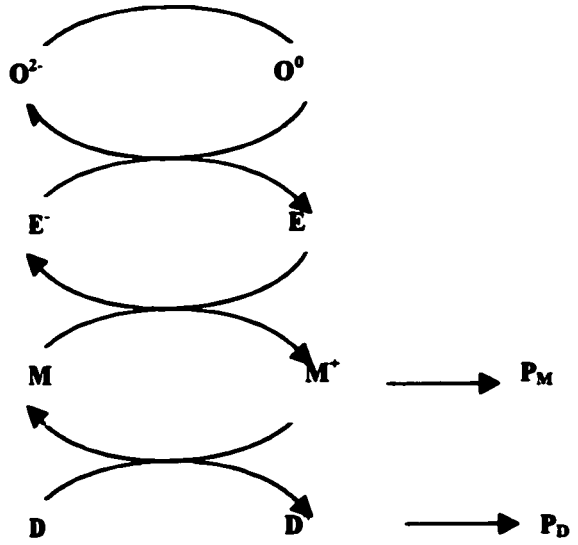


Figure 2.1.2 A mediator-involved dye degradation mechanism proposed by Wong and Yu. This mehanism involving oxygen in dioxygen (O^0) and water (O^{2-}): laccase in rest (E) and reduced (E^-) forms; dye mediator in the original (M) and cation radical (M^+) forms. P_M and P_D are the decomposition products from the oxidized mediator and nonsubstrate dyes (Wong and Yu, 1999)

Spadaro and Renganathan (1994) have studied the oxidation of a non-sulfonated azo dye by the lignin peroxidase from *P. chrysosporium.* and proposed a reaction mechanism (Figure 2.1.3). The non-sulfonated azo dye they studied was 1-(4'-acetamidophenylazo)-2-naphthol. Their results showed the formation of

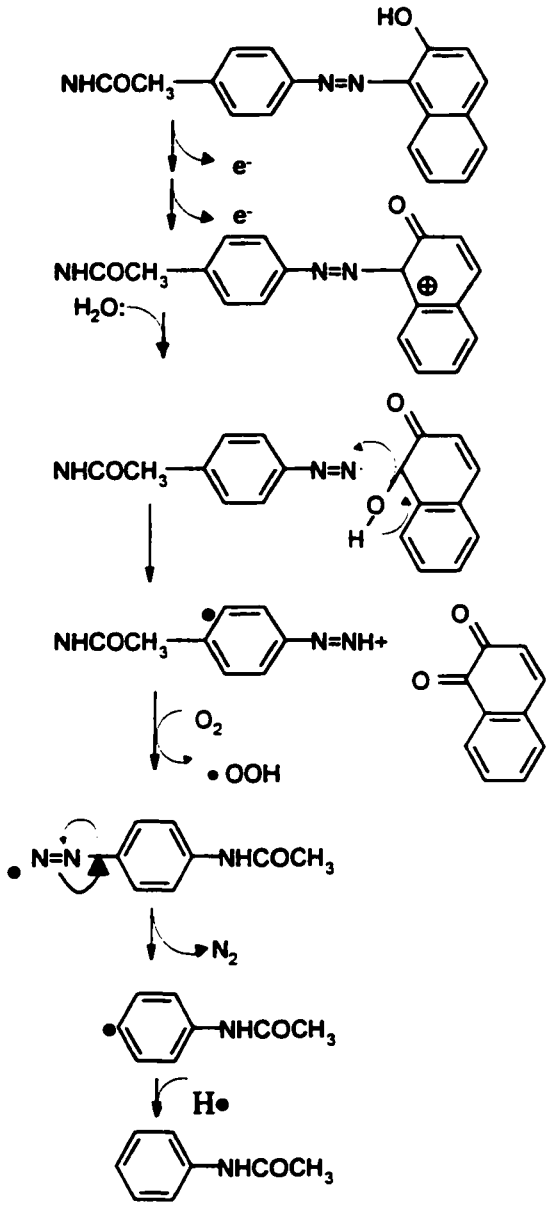


Figure 2.1.3 Proposed reaction mechanism for the oxidation of 1-(4'-acetamidophenylazo)-2-naphthol by the lignin peroxidase from Phanerochaete chrysosporium (Sparao and Renganathan 1994)

1,2-naphtoquinone and acetanilide. This suggested that the oxidized form of the lignin peroxidase abstracted two electrons from the phenolic ring of the dye, which resulted in formation of the corresponding carbonium ion on the C-1 carbon of the naphthol ring. This carbonium ion was then hydrated by a nucleophilic attack of water to an intermediate that broke down to naphthoquinone and an unstable phenyldiazene. It was suggested that this phenyldiazene could be oxidized by molecular oxygen to the corresponding radical, which would finally split off molecular nitrogen under formation of the phenyl radical, which was stablilized by the abstraction of a hydrogen radical from its surroundings (Spadaro and Renganathan 1994).

The enzymatic mechanism (Shown in Figure 2.1.4) for the oxidation of sulfonated azo dyes by fungal peroxidases has been studied independently by Goszczynski et al. (1994) who incubated 3,5-dimethyl-4-hydroxyazobenzene-4'-sulfonic acid with a crude peroxidase preparation from *P. chrysosporium* and analyzed the products formed. From the identified products, they also suggested an initial oxidative activation of the dye with the formation of a carbonium ion followed by a nucleophilic attack of water on this cationic species. From the metabolites observed, it was suggested that this unstable tetrahedral intermediate could either break down by a symmetric cleavage of the azo group which would produce a quinone imine and a nitroso compound or an asymmetric cleavage resulting in a quinone and a phenyldiazene. These direct oxidation products would finally undergo various spontaneous reactions and result in the formation of various secondary products.

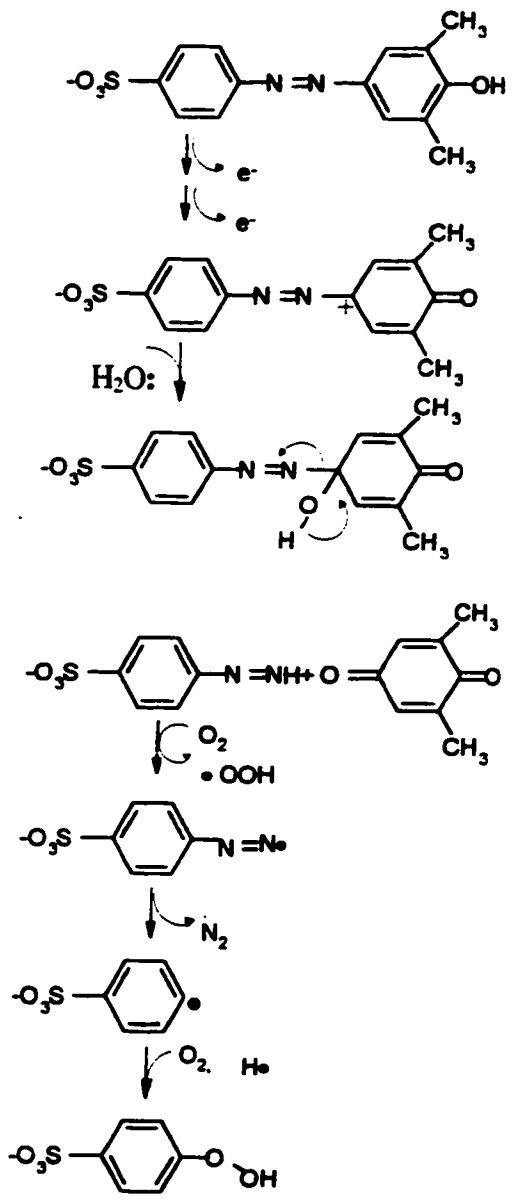


Figure 2.1.4 Proposed reaction mechanism for the oxidation of 3,5-dimethyl-4-hydroxyazobenzene-4'-sulfonic acid by the lignin peroxidase from Phanerochaete chrysosporium (Goszczynski, et al. 1994)

2.2 Color reduction of azo dyes with zero-valent iron

Textile preparation, dyeing and finishing plants are currently being forced to treat their effluents at least partially prior to discharge to publicly owned treatment works (POTWs) due to the high organic load, strong and resistant color as well as high dissolved solids (Easton, 1995). Since commercial dyestuffs are intentionally designed to be recalcitrant under typical usage conditions, a marked resistance of these dyes to aerobic biological treatment is not unusual (Gurnham, 1995). Although the majority of commercial textile dyes are water-soluble (hydrophilic), the hydrophobic disperse dyes are also used heavily (Baugham and Weber, 1994). The disperse dyes are widely employed for dyeing polyester fabric. The process of dyeing synthetic fabrics like alkantara, polyester, cellulose acetate, acrylic and polyamide materials is mostly accomplished by the application of disperse dyes (Szpyrkowicz et al., 2001). In plants that use dyestuffs, physical, chemical, or biological methods are used to treat wastewater containing these dyes. However, because most disperse dyes are not readily degraded even by biological treatment, large amounts of them are released into the environment. As disperse dyes are insoluble in water, they are dispersed with detergents, which may be a reason for their insusceptibility to biological degradation (Sugimori et al., 1999). Rather poor treatment efficiencies were reported for the biological treatment of disperse dyes and contradictory results were obtained via chemical oxidation methods such as ozonation and Fenton's reaction (Chu and Ma, 2000).

Although some treatment processes, such as coagulation and carbon absorption, may remove certain categories of dyes to about 90% (Poon and Vittimberga, 1981), the main drawback of these processes is the generation of a large amount of sludge or solid waste, resulting in high operational costs for sludge treatment and disposal.

Zero-valent iron, Fe^0, in the form of powder, is a strong reducer, and it is cheap and easy to get. Application of reduction by zero-valent iron has been studied to remove nitroaromatic compounds (Mantha et al., 2001, Agrawal and Tratnyek, 1996). It has also been extensively investigated for remediation of groundwater contaminated with chlorinated solvents (Gillham and O'Hannesin, 1994; Matheson and Tratnyek, 1994), nitrate (Cheng et al., 1997) and various metals (Cantrell et al., 1995). By reduction of critical functional groups, Fe^0 can be used to initiate remediation of more complex anthropogenic chemicals, such as pesticides (Eykholt and Davenport, 1998; Sayles et al., 1997). Dyes are among the complex chemicals that can be reduced by zero-valent iron (Nam and Tratnyek, 2000; Cao et al., 1999).

Pretreatment of concentrated streams of wastewater before their combination and subsequent introduction to the wastewater treatment plant can lead to a significant decrease in organic load and color and is thus has become a common practice (Szpyrkowicz et al., 2001).

The reduction of azo dyes by Fe^0 is of interest, not only for its potential application in the color reduction of wastewaters from dye use and manufacturing, but also because it provides a abiotic reduction of azo linkages and the kinetics of fast reactions with granular iron metal (Weber, 1996; Nam and Tratnyek, 2000). The abiotic reduction of azo linkages in environmental media has been inferred from experiments done with anaerobic sediments (Weber and Wolfe, 1987; Yen et al., 1991; Weber and Adams, 1995).

Cao et al. (1999) have suggested a two-step mechanism to explain the results of Acid orange II dye reduced by zero-valent iron (Figure 2.2.1). They found that the first step was reversible. Degradation solution taken out of the reaction bottle was composed of four components, Acid orange II, two degradation products and intermediate

compound, in certain proportions. Some of the unstable intermediate compound decomposed into the products, and meanwhile, some of it returned to Acid orange II.

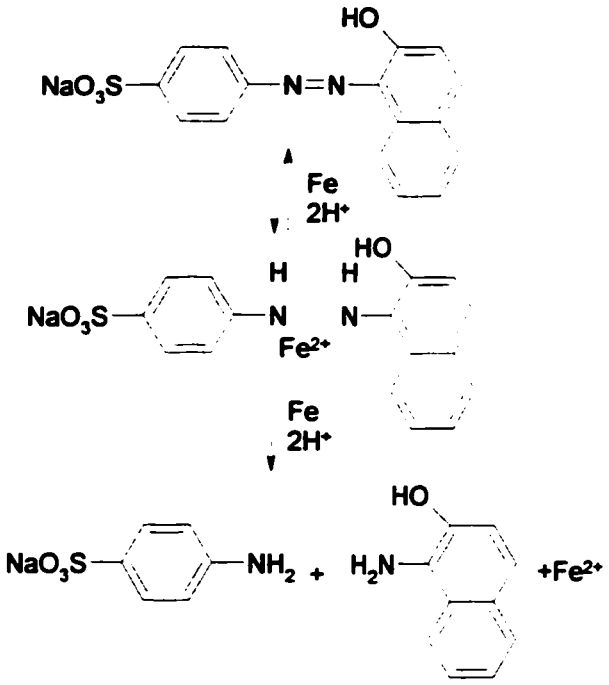


Figure 2.2.1 Degradation mechanism of Acid Orange II in Fe^0-H_2O system (Cao et al., 1999)

2.3 p-phenylenediamine removal by *Arthromyces ramosus* peroxidase and hydrogen peroxide

p-Phenylenediamine (p-PD) is the main aromatic amine used in hair dye formulations (Ames et al., 1975). Many azo dyes used by industry also contain fragments related to the p-phenylenediamine moiety (Chung and Stevens, 1993). Upon azo reduction of these compounds by environmental or intestinal microorganisms, p-PD is released (Chung and Stevens, 1993; Chung et al., 1992). The azo dyes undergo breakdown reactions and lead to the release of aromatic amines especially under anaerobic conditions. The p-PD has been detected in the biodegradation of azo dyes under both anaerobic (Donlon et al., 1997; Razo-Flores et al., 1997) and anoxic conditions (Zissi and Lyberatos, 1996); however, no further degradation of p-PD has been observed (Meyer, 1981; Razo-Flores et al., 1997) under these conditions.

p-PD has been reported to increase the formation of liver tumors in mice (Sontag, J.M. 1981), although it is considered to be a non-carcinogen (Miller and Baumann, 1945). Many of the p-PD derivatives have been found to be mutagenic (Shahin, 1989). Chung et al. (1992) recently reported that p-PD is the moiety responsible for the mutagenic activity of many azo dyes. p-PD also produces contact anaphylaxis, occupational allergy, contact secondary leukoderma, breathing difficulties, myotoxic activity and tumor necrosis. (Mederos et al., 1999). Therefore, disposal of p-PD and p-PD containing compounds is becoming an important environmental issue (Chung et al., 1995).

Since polyaniline, an organic conducting polymer, is being successfully used as an additive for plastics, such as nylon and PVC, to increase their conductivity and hence to confer antistatic properties to these materials, polymerization of p-PD has attracted the

attention of many investigators (Cataldo, 1996). Ichinohe et al. (1998) studied polymerization of p-PD using horseradish peroxidase and hydrogen peroxide. Future industrial usage of p-PD is expected be quite high; hence, the consideration of its removal from the wastewater is an important environmental issue.

It is reported that certain chemical oxidants, such as potassium persulfate, have been used as oxidants to polymerize p-PD (Cataldo, 1996). Under this condition, the p-PD was found to yield polyquinoxaline, a material having a ladder structure and fully oxidized state, somewhat similar to that shown by pernigraniline.

Ichinohe et al. (1998) described the mechanism of oxidative polymerization of p-PD catalyzed by horseradish peroxidase as shown in Figure 2.3.1. The initial step of HRP–catalyzed oxidation with hydrogen peroxide as an oxidant is the formation of oxidized HRP (Compound I). This subsequently reacts with a substrate to form a radical as illustrated in Figure 2.3.1 for p-PD. They suggested that p-PD polymer retained disubstituted benzene nuclei and the polymerization proceeded mainly via N-N coupling and formed the linear structure polymer (see Figure 2.3.1). They also suggested a reversible equilibrium between two chromophores and there were changes of electron conjugation systems by protonation at nitrogen atoms of azo and quinoneimine structure (Figure 2.3.2) and this would result in the development of a new absorption band at a longer wavelength with concomitant decrease of absorption bands due to azo and quinoneimine groups.

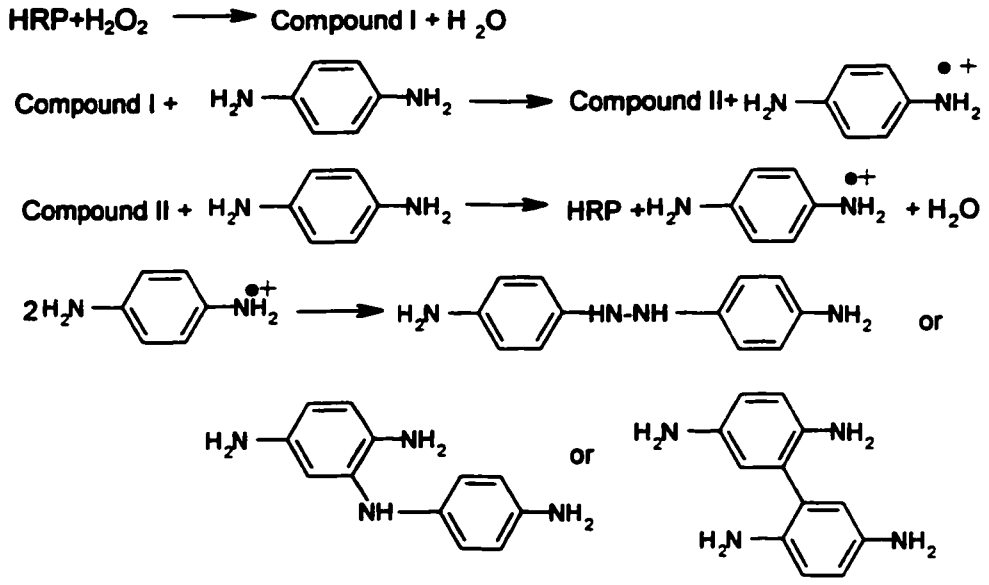


Figure 2.3.1 Mechanism of p-PD polymerization (1) (Ichinohe et al., 1998)

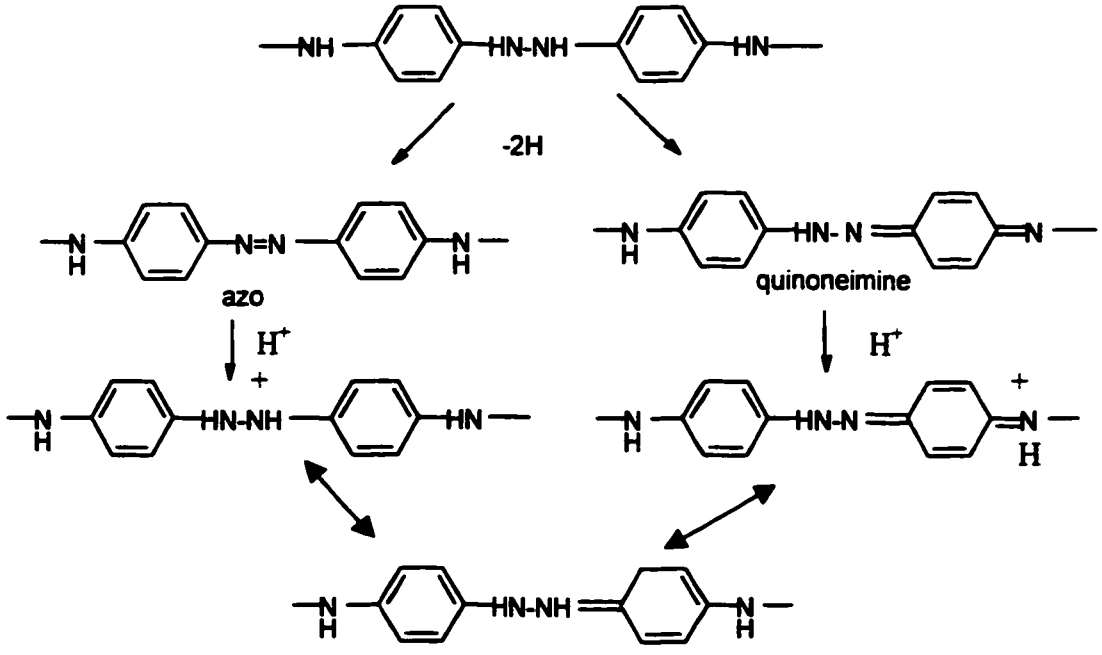


Figure 2.3.2 Mechanism of p-PD polymerization (2) (Ichinohe et al., 1998)

Cataldo (1996) proposed the initial formation of a polyaniline with quinonediimine pendant groups or with pendant diamino groups and the final step was the formation of a ladder polymer composed alternating units of benzenoid and quinoid rings as happened with pernigraniline (Figure 2.3.3).

2.4 TNBS test for phenylenediamines and aniline

The standard method to test these chemicals is EPA Method 8270 by GC-MS. The OSHA has a complex method using HPLC to test the filtered and extracted samples from air. Reddy et al. (1993) used a reversed-phase high-performance liquid chromatographic method for separation and quantitation of a mixture solution containing p-PD. All these methods need expensive equipment and complex procedures for sample preparation. Because of the instability of these chemicals, a simple site (field) test is needed to obtain reliable results. Since trinitrobenzenesulfonate (TNBS) has been used to estimate aliphatic amino groups (Fields, 1972), it is possible to be used as a material to examine the p-PD.

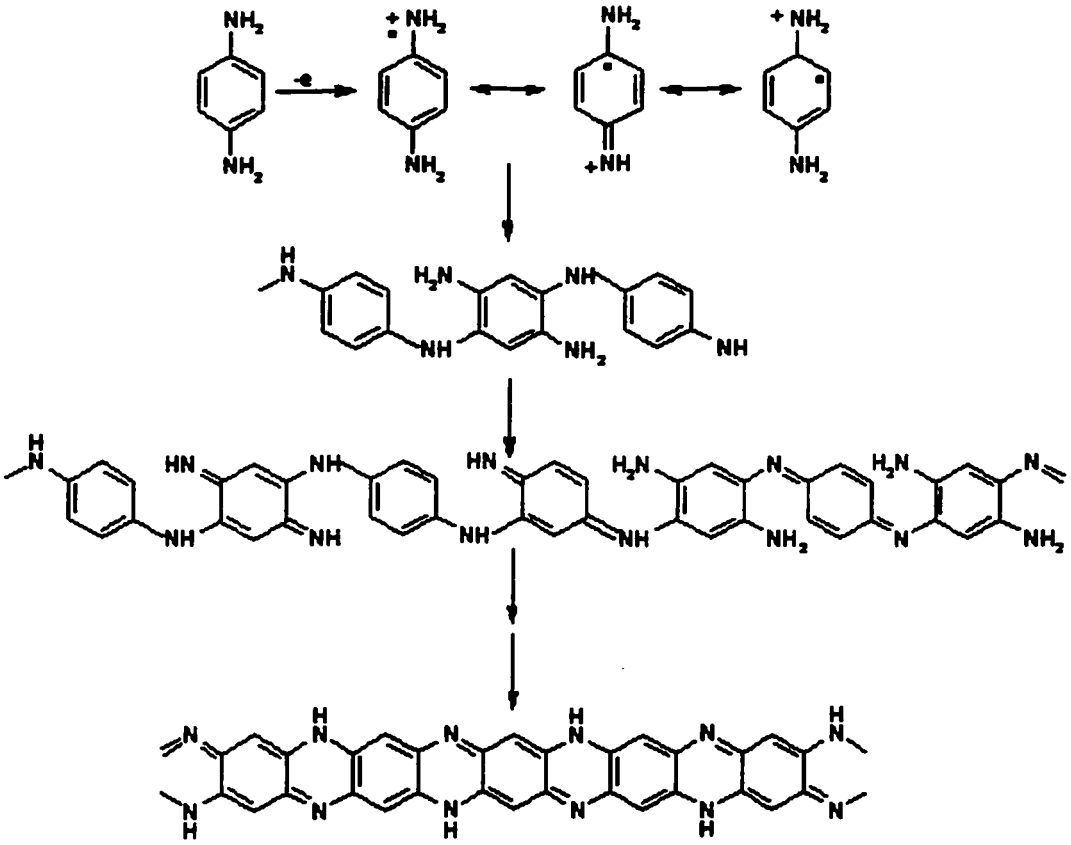


Figure 2.3.3 Mechanism of p-PD polymerization (3) (Cataldo, 1996)

3. MATERIALS AND METHODS

3.1 Materials

Arthromyces ramosus peroxidase (ARP) with an RZ≈0.5 was a gift from Biotech Environmental Inc. of a developmental preparation of Novo Nordisk, Bagsvaerd, DK. The specific activity of *Arthromyces ramosus* peroxidase stock solution was 2200 U/mL based on the assay which used phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide as substrates (Ibrahim et al., 2001). One unit of activity was defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 25 °C. The enzyme was stored as a liquid at 4°C until required. The activity of the stock solution was measured at least once a week to monitor the changes in activity, if any.

Catalase enzyme (EC 1.11.1.6) was purchased from Sigma Aldrich Chemicals Co., St. Louis, MO. The nominal activity of the catalase was 15,000 Units/mg dry solid and 23,000 Units/mg protein. Aqueous stock solutions of catalase (1 mg/mL) were stored at 4°C.

Hydrogen peroxide (30% w/v), ACS grade potassium ferricyanide, sodium bicarbonate, sodium carbonate, and analytical grade monobasic and dibasic sodium phosphates were purchased from BDH Inc., Toronto, ON. Peroxide solution for the analytical assays was prepared daily.

Naphthol Blue Black (dye content: ~80%), Acid Red 4 (~50%), Crocein Orange G (~ 70%) and Disperse Orange 3 (~90%) were purchased from Sigma Aldrich Chemicals (Milwaukee, WI) and used as delivered.

1,4-phenylenediamine (97%), 1,2-phenylenediamine (98%), 1,3-phenylenediamine (99%), and analytical grade trinitrobenzenesufonic acid (TNBS) (as picrylsulfonic acid

5% (w/v) aqueous solution, lot no. 107H5001) were obtained from Sigma Aldrich Chemicals, St. Louis, MO.

Iron metal was purchased from Fisher Chemicals (I57-500: lot no. 976195) as iron fillings (about 40 mesh size). Supplier data stated that the purity of the metal was 99.98% (on metal basis) with the major impurities being phosphorus (16 ppm), cobalt (14 ppm), nickel and manganese (both 10 ppm).

Cobalt chloride and ACS grade sodium sulfite was obtained from Fisher Scientific, Pittsburgh, PA.

Plastic syringes (6.0 mL) was purchased from Becton Dickinson & Co. Clifton, NJ. Syringe filters (bulk, non-sterile, size: 0.2 micrometer) were obtained from Nalge Nunc International Co. Rochester, NY.

3.2 Analytical equipment

A Hewlett Packard Diode Array Spectrophotometer, Model 8452A (wavelength 190 to 820nm with 2nm resolution), interfaced with a HP vectra ES/12 computer was used for all absorbance measurements. Quartz spectrophotometer cells (semi-micro having 1 cm optical path length) were supplied by Hellma (Canada) Ltd., Concord, ON. Disposable polystyrene semi-micro curvettes were used to measure the absorbance of the samples. They were purchased from Bio-Rad Laboratories, Hercules, CA.

High Performance Liquid Chromatography (HPLC) was used to analyze the product(s) of DO3 reduction by zero-valent iron. The equipment used was Waters System, including Waters 2487 dual λ Absorbance Detector, Waters 1525 Binary HPLC pump and Waters 717_{plus} Autosampler and was supplied by Waters Co., Milford, MA, USA. The column used was symmetry C18 (5μM, 4.6*150mm) operating with Breeze software.

3.3 Experiment procedure

In general:

- All reactions were carried out at room temperature, 18-22 °C, except where noted.
- Buffers, whenever used, were prepared following the recommendations of Gomori (1955).
- All solutions were prepared using deionized water.

3.3.1 Color reduction of azo dyes

The experiments in this study were conducted in batch reactors at room temperature, approximately 22°C. This study was designed to achieve the highest color reduction efficiency of the dyes dissolved in buffer solution by optimizing the following parameters: pH, enzyme concentration, hydrogen peroxide concentration, and reaction time.

The batch reactors were glass vials which contained 30 mL buffered mixture of dye, ARP, H_2O_2 and deionized water. The components were added in the following order: buffer, dye, enzyme and last the hydrogen peroxide to initiate the reaction. The solution was stirred using a magnetic stirrer and a teflon coated stir bar.

3.3.2 Color reduction of Disperse Orange 3 by zero-valent iron

Batch reactor runs were carried out typically in 50-mL vials sealed by screw-cap. The vials were shaken on a Burrel model 75 shaker (Burrell, Pittsburgh, PA) at maximum setting. This provided a wrist-like action to the vials for proper mixing. Solutions were made anaerobic by adding Na_2SO_3 along with $CoCl_2 \cdot 6H_2O$ as catalyst at 0.1 wt % of Na_2SO_3.

Prior to use, the Fe^0 was hand-sieved to obtain a size range of 30-40 mesh. This iron was always washed with HCl acid as recommended by Agrawal and Tratnyek (1996). The purpose of the pretreatment was to clean the surface of the iron to remove any (hydr)oxide. Subsequently, the excess HCl was removed by washing with carbonate buffer (pH 9.5); this was followed by washing with Na_2SO_3 solution to remove any remaining dissolved oxygen.

DO3 was first dissolved in 100% methanol to 1.0 mM and thereafter diluted to 50 μM in the reactor.

3.3.3 p-PD removal

Batch reactor runs were carried out typically in 30-mL vials. The vials were shaken on a Burrel model 75 shaker (Burrell, Pittsburgh, PA). This provided a wrist-like action to the vials for proper mixing. The samples were tested after filtration through a syringe filter.

3.3.4 Phenylenediamine and aniline TNBS tests

All solutions were prepared by using deionized water. 10 mM TNBS stock solution was prepared by diluting 586 uL of the 5% stock solution to 10 mL and it was stored in the refrigerator. Sodium sulfite solution was prepared fresh daily as a 20 mM stock solution.

3.4 Analytical methods

3.4.1 Color reduction of azo dyes

3.4.1.1 *Arthromyces ramosus* peroxidase assay

A colorimetric assay was used to measure the activity of the ARP enzyme. This assay used phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide (H_2O_2) as color generating substrates. The rate of reaction was proportional to the enzyme activity and was deduced from the rate of formation of a soluble product which absorbed light at a peak wavelength of 510 nm with an extinction coefficient relative to peroxide of 6000 $M^{-1}cm^{-1}$ (Artiss et al., 1979). One unit of activity (U) is defined as the number of micromoles of hydrogen peroxide converted in one minute at pH 7.4 and 25°C in an assay mixture consisting of 10 mM phenol, 2.4 mM AAP and 0.2 mM H_2O_2. The concentration of enzyme was determined by measuring the peak absorbance at 404 nm and a peroxidase extinction coefficient (molar absorbitivity) of 102,000 $M^{-1}cm^{-1}$ (Kjalke et al., 1992).

3.4.1.2 Dye remaining

Different volumes of reaction solution were taken with a pipette and were transferred into the 1.5 mL cuvettes. Deionized water was added if dilution was required and the absorbance was measured immediately with the spectrophotometer.

The degree of color reduction of dyes was determined by monitoring the decrease in absorbance at respective maximum wavelengths, and was expressed in terms of percentage color reduction as:

$$\text{Color reduction (\%)} = 100 * (A_{ini} - A_{obs})/A_{ini} \qquad \text{Eq. 3.1}$$

where:

A_{ini} = initial absorbance, and

A_{obs} = observed absorbance.

3.4.2 Color reduction of Disperse Orange 3 by zero-valent iron

The concentration of the p-PD was determined by TNBS test. In a final volume of 1.0 mL, 100 μL of 10 mM TNBS, 100 μL of 0.5M phosphate buffer of pH 7.4, 100 μL of 20 mM Na_2SO_3 and 800 μL of sample plus water were added. Samples were allowed to stand for 60 min. and then absorbance was measured using Hewlett-Packard diode array spectrophotometer Model 8451A, at 430 nm. Concentrations were calculated using an experimentally determined (see section 4.4) extinction coefficient (molar absorptivity) of 45,300 $M^{-1}cm^{-1}$.

The p-PD and the product of the zero-valent iron reduction of DO3 were also determined by High Pressure Liquid Chromatography. The column used was a symmetry C18 (5μM, 4.6*150mm). The elution was isocratic and the mobile phase consisted of 50% methanol and 50% deionized water. At a flow rate of 1 mL/min, the retention time for p-PD was 1.5 min, for DO3 was 25 min. The UV-Vis detector was set at 302 nm (Characteristic absorbance wavelength of p-PD) and 434 nm (Characteristic absorbance wavelength of dissolved DO3).

3.4.3 p-PD removal

Catalase was needed to stop the peroxidase reaction before testing. The concentration of the p-PD was determined by TNBS test as described above.

3.4.4 Phenylenediamines and aniline TNBS test

All experiments were carried out at room temperature, 18-22°C. In a final volume of 1.0 mL, 100 μL of 10 mM TNBS, 100 μL of 0.5 M phosphate buffer pH 7.4, 100 μL

of 20 mM sodium sulfite, 100 μL of different concentration of PDs or aniline, and 600 μL of H_2O were added. The blank was prepared in the same way as samples except that additional 100 μL of H_2O was added instead of the PD solutions. The solution absorbance was measured against the blank using the Hewlett-Packard Diode Array Spectrophotometer. The reaction time was set when stable compound was produced.

3.5 Sources of error

Systematic errors and random errors could occur in the experiments. Systematic errors are due to the measuring or analytical techniques and the instruments and random errors are due to the personal factors, such as careless. For instance, systematic errors can occur in the direct spectrophotometric method when very low concentrations are present in the cuvettes. For the direct UV method, the spectrum of the solution always changed after the start of the reaction, so different delays between taking the samples and starting the measurement could cause the personal error.

Although errors can not be prevented completely, they were reduced by taking the following precautions:

- Experiments were designed and run in such a way that all conditions were exactly the same except the parameter being investigated. For example, the test time and sampling time were controlled as precisely as possible.
- The protocol of the experimental design and analysis procedures were followed strictly.
- Same volume of sample in assay was used when relative values were desired.

4. RESULTS AND DISCUSSION

4.1 Color reduction of azo dyes by peroxidase plus peroxide

4.1.1 Effect of pH

The activity of an enzyme is dependent on pH, and each enzyme possesses an optimum pH. Enzyme often functions only when certain ionizable side chains are in a specific form. Since the characteristics of the ionizable side chains of amino-acids depend on pH, enzyme activity is usually pH dependent, i.e. the activity of the enzyme is at its maximum at a certain pH and any change in pH to higher or lower value causes significant lowering of activity (Palmer, 1995). Since ARP showed some catalytic ability below pH 6.0 and above 9.5 (Al-Kassim et al., 1993a), the pH range of 4.0 to 10.0 was chosen in this study. The initial substrate concentration was 50 μM for all the dyes. These experiments were designed so that the effect of pH could be investigated. Enzyme concentration was 0.1 U/mL for 50 μM dye concentration, hydrogen peroxide concentration used was 250 μM for Naphthol Blue Black (NBB), 150 μM for Crocein Orange G (COG), Acid Red 4 (AR4), and Disperse Orange 3 (DO3). A two hour reaction time was chosen.

The results for these experiments are shown in Figures 4.1.1 to 4.4.4. As seen, pH had different effects on the color reduction of these selected dyes. For Crocein Orange G, the reaction was strongly dependent on the pH and the high removal efficiency was achieved in a narrow range, from 7.0 to 8.2. Outside this range, the color reduction

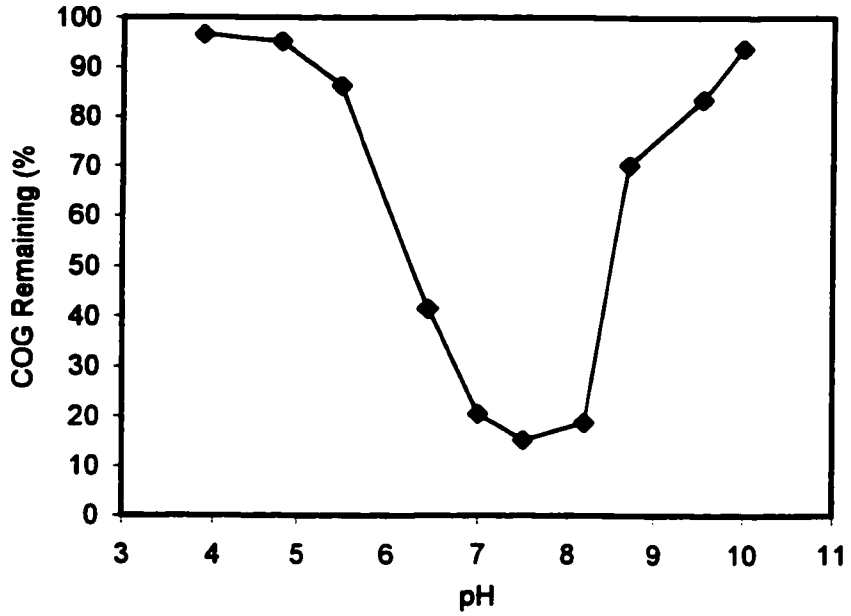


Figure 4.1.1 Effect of pH on the color reduction of Crocein Orange G ($[COG]_0$=0.05 mM, $[ARP]_0$=0.1 U/mL, $[H_2O_2]_0$=0.15 mM, Reaction time: 2 h)

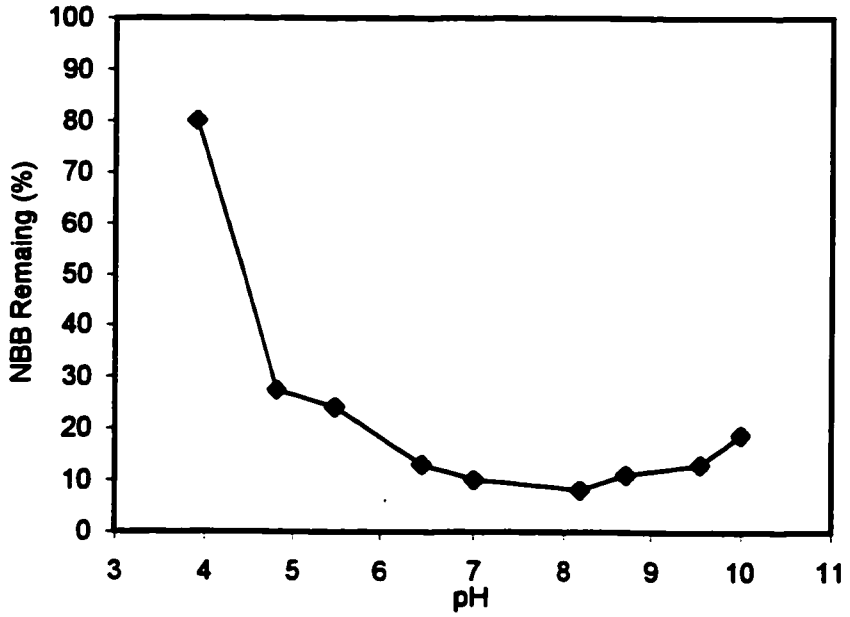


Figure 4.1.2 Effect of pH on the color reduction of Naphthol Blue Black ($[NBB]_0$=0.050 mM, $[ARP]_0$=0.1 U/mL, $[H_2O_2]_0$=0.25 mM, Reaction time: 2 h)

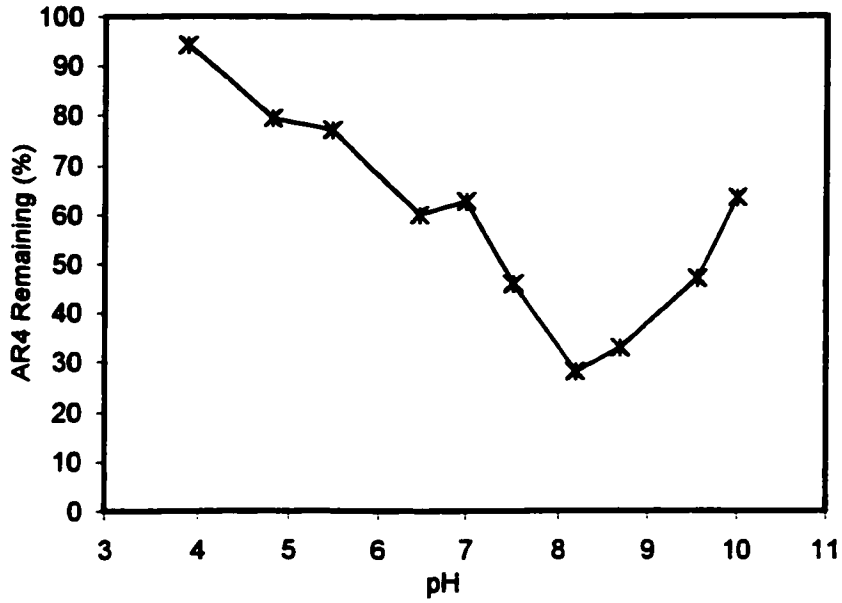


Figure 4.1.3 Effect of pH on the color reduction of Acid Red 4
($[AR4]_0$=0.05 mM, $[ARP]_0$=0.1 U/mL, $[H_2O_2]_0$=0.15 mM, Reaction Time=2 h)

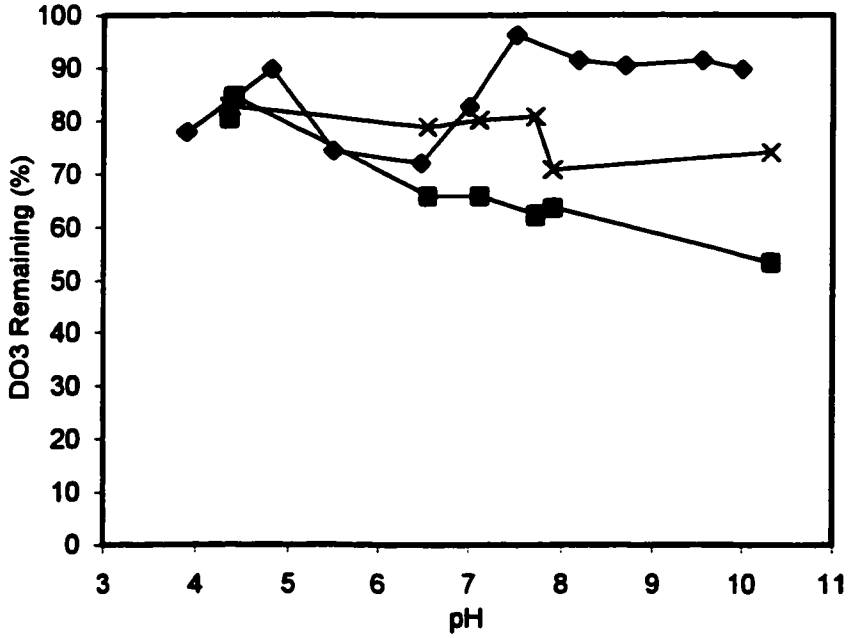


Figure 4.1.4 Effect of pH on the color reduction of Disperse Orange 3.
($[DO3]_0$=50 μM, $[ARP]_0$=0.1 U/mL, $[H_2O_2]_0$=150 μM, Reaction Time=2 h)
Experiments were conducted on ♦--June 17, 2001; ×--June23, 2001; ■--May 12, 2002.

efficiency dropped sharply. For example, at pH 8.2, 20% of COG was remaining, but at pH 8.7, more that 70% had not been reacted. By contrast, Naphthol Blue Black, had a broad optimum pH range, with a high color reduction efficiency at pH>4.7. However, the optimum pH range was located in the range of 6.4 to 8.7. For Acid Red 4, the highest efficiency was achieved when pH was 8.2.

Three sets of experiments were conducted to study the effect of pH on the color reduction of DO3. As seen from Figure 4.1.4., satisfactory results were not obtained. The removal efficiency was low and was not reproducible. The studies on the effect of ARP and hydrogen peroxide concentrations on the color reduction of DO3 were also conducted and similar results were obtained as for the pH effect. The main reason is that the DO3 is insoluble in water and has a nitro group in its structure which is outside the scope of peroxidase catalysis (Mantha et al., 2001). When Soares et al. (2002) studied the color reduction of disazo dyes using laccase, they got similar results; none of the water insoluble dyes that lacked the sulfonic group were decolorized by laccase. The optimum pH value for the color reduction of the four dyes is summarized in Table 4.1.1.

Analysis of NBB remaining with time for different pH's value is shown in Figure 4.1.5. With the same data, the results of NBB remaining vs time are shown in Figure 4.1.6. In order to obtain more information about the reaction time needed under various pH conditions and study the effect of pH on the time based on a wider pH range, the reaction time was set for 3 hours. The graph shows that pH mainly affected the reaction rate and not the final color reduction efficiency. At lower pH, such as 4.1 to 6.4, the color of NBB was reduced very quickly in the first 15 minutes, after that, little further reaction occurred, and the color reduction efficiency remained steady. On the other hand, at the higher pH, the lower reaction rate, but the higher color reduction efficiency was achieved.

In the range of pH from 4.1 to 6.4, most of the color removal had occurred in less than 20 minutes; whereas at pH value of 7.1, 7.6 and 8.4, the reactions reached their end points at 60, 90 and 210 minutes, respectively.

Table 4.1.1 Optimum pH values for different dyes

Dye	Optimum pH range	Recommended pH
Crocein Orange G	7.0-8.2	7.4
Naphthol Blue Black	6.4-8.7	7.6
Acid Red 4	7.4-8.7	8.2
Disperse Orange 3	No	No

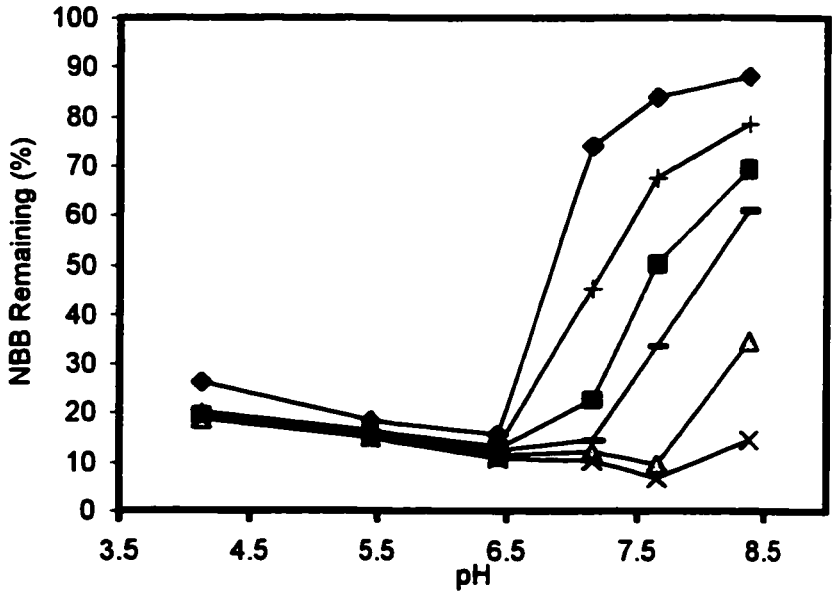


Figure 4.1.5 Effect of pH on the color reduction of Naphthol Blue Black.
$[NBB]_0$=0.05 mM, $[ARP]_0$=0.175 U/mL, $[H_2O_2]_0$=0.16 mM
◆--15 min; +--30 min; ■--45 min; – --60 min; ▲-- 120 min; ×--210 min

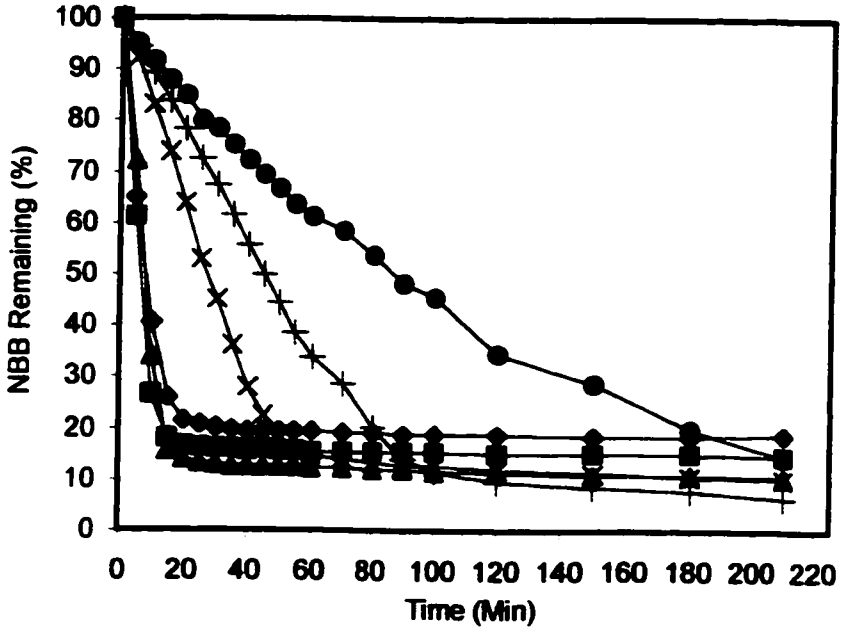


Figure 4.1.6 Effect of pH and time on the color reduction of Naphthol Blue Black. $[NBB]_0$=0.05 mM, $[ARP]_0$=0.175 U/mL, $[H_2O_2]_0$=0.16 mM
◆—pH=4.1; ■—pH=5.4; ▲—pH=6.4; ×—pH=7.2; +— pH=7.6; ●—pH=8.4

4.1.2 Effect of enzyme concentration

The ARP concentration experiments were conducted at the previously established optimum pH values shown in Table 4.1.1. The reaction time was set at 2 hours to carry the reaction to completion. The results are presented in Figure 4.1.7 to 4.1.9.

No color reduction was observed when either enzyme or hydrogen peroxide were added to the wastewater alone. Thus, the color reduction observed in this study is attributed to the combined action of ARP and hydrogen peroxide on dyes. These figures show the dependence of the color reduction efficiency on the enzyme concentration. The

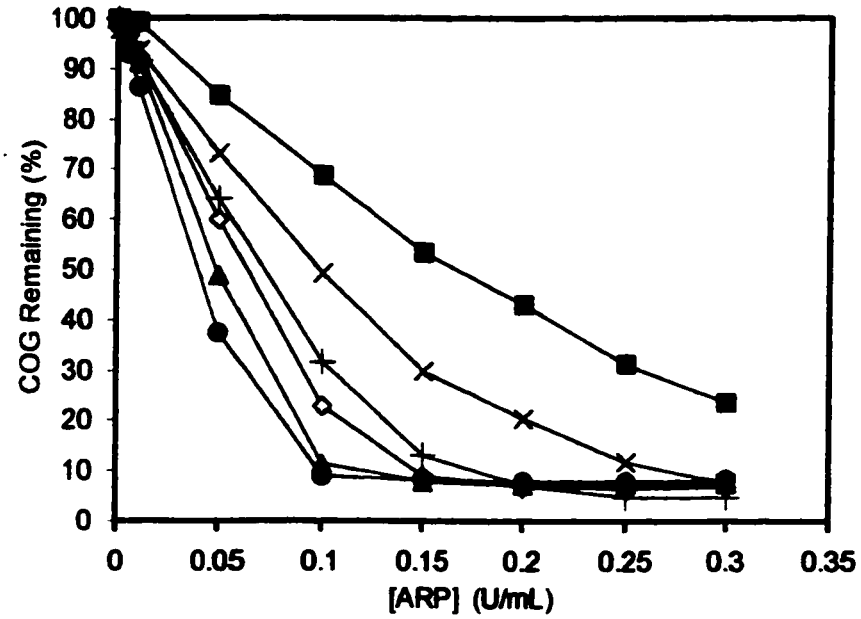


Figure 4.1.7 Effect of ARP concentration on the color reduction of Crocein Orange G
$[COG]_0$=0.05 mM, $[H_2O_2]_0$=100 μM, buffer pH=7.5
■— T=15 min, ×— T=30 min, +—T=45 min,
◇—T=60 min, ▲—T=90 min, ●—T=120 min.

color reduction efficiency increased with higher enzyme activity in the solution. For example, at 2 hours, 0.1 U/mL enzyme had enough color reduction efficiency for the 0.05mM COG, NBB and AR4. Additional enzyme present in the solution showed little improvement to the reaction which means that the extra enzyme at higher concentration was not used up completely in the catalytic process and hence the whole process will not be economical.

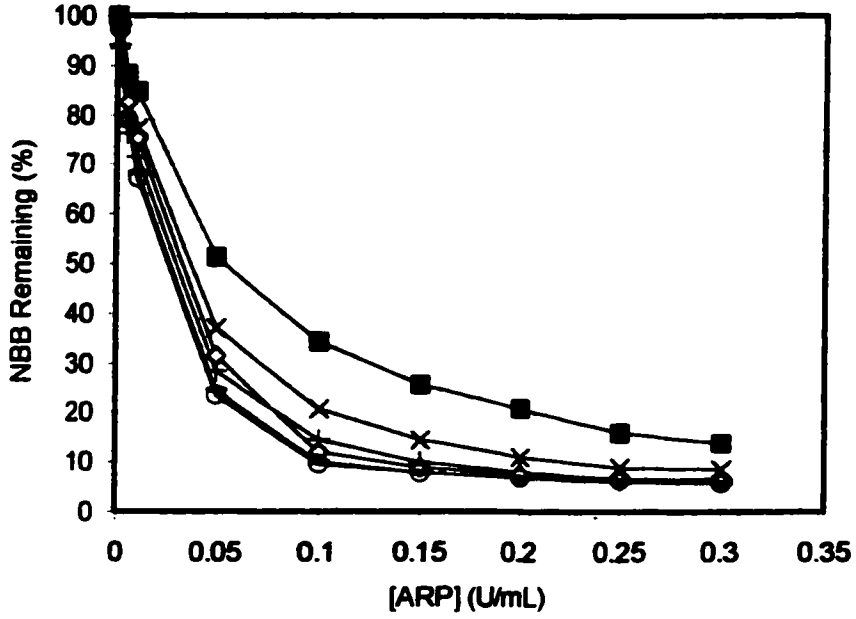


Figure 4.1.8 Effect of ARP concentration on the color reduction of Naphthol Blue Black. $[NBB]_0$=0.05 mM, $[H_2O_2]_0$=0.2 mM, Buffer pH=7.6. ■—T=15 min, ×—T=30 min, +—T=45 min, ◆—T=60 min, ○—T=120 min.

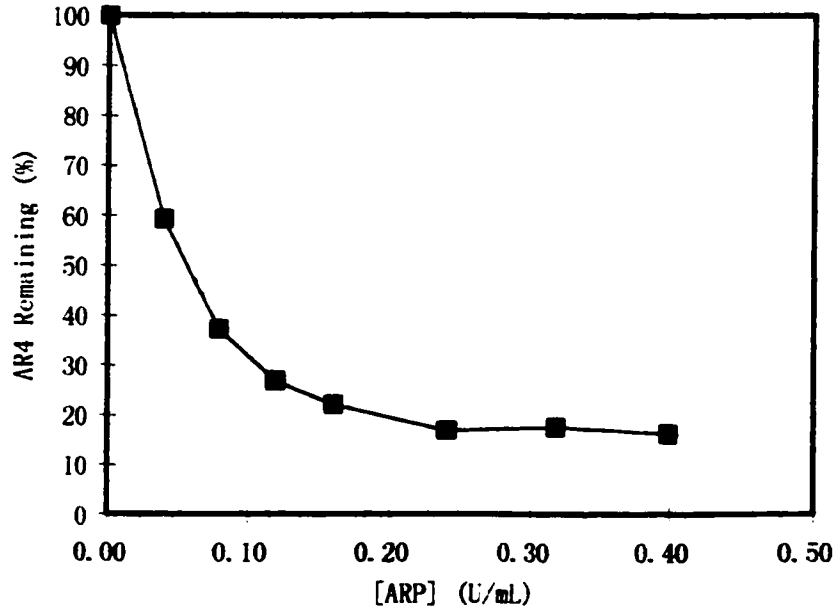


Figure 4.1.9 Effect of ARP concentration on the color reduction of Acid Red 4 $[AR4]_0$=0.05 mM, $[H_2O_2]_0$=0.1 mM, Buffer pH=8.2, Reaction time=2 h.

4.1.3 Effect of hydrogen peroxide concentration

Experiments were performed to determine the effect of the initial hydrogen peroxide concentration on the color reduction of dyes. The effect was studied with 0.05 mM dye concentration and at the optimum pH determined from the previous experiments. The results of these experiments are presented in Figures 4.1.10 to 4.1.12. These figures show that the remaining color decreased sharply with an increase in hydrogen peroxide concentration before reaching the optimum point (lowest remaining dye concentration). In this range, the low color reduction efficiency was attributed to insufficient H_2O_2.

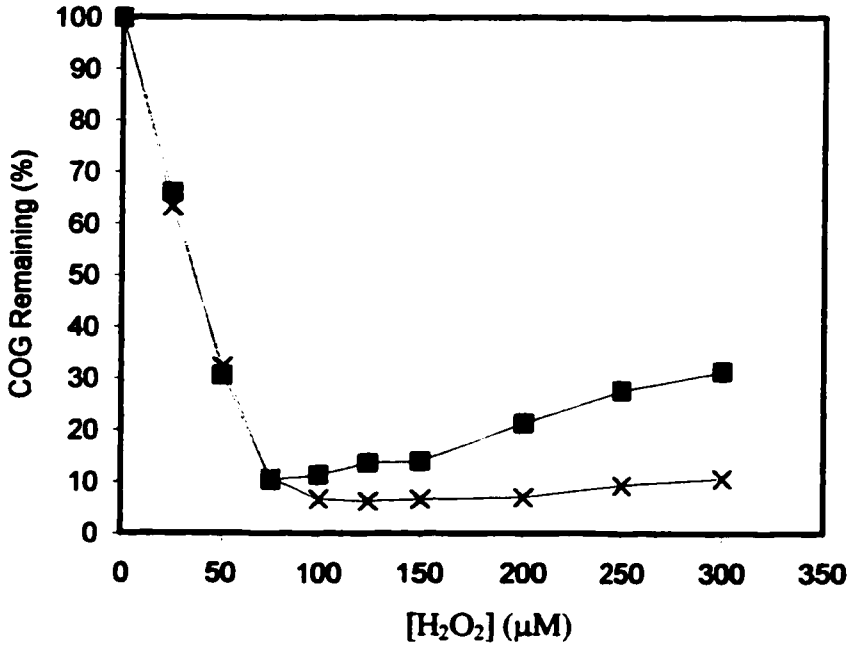


Figure 4.1.10 Effect of H_2O_2 concentration on the color reduction of COG.
$[COG]_0$=0.05 mM, $[ARP]_0$=0.1 U/mL, pH=7.5.
Reaction time: ■--- T=60 min, ×---T=120 min

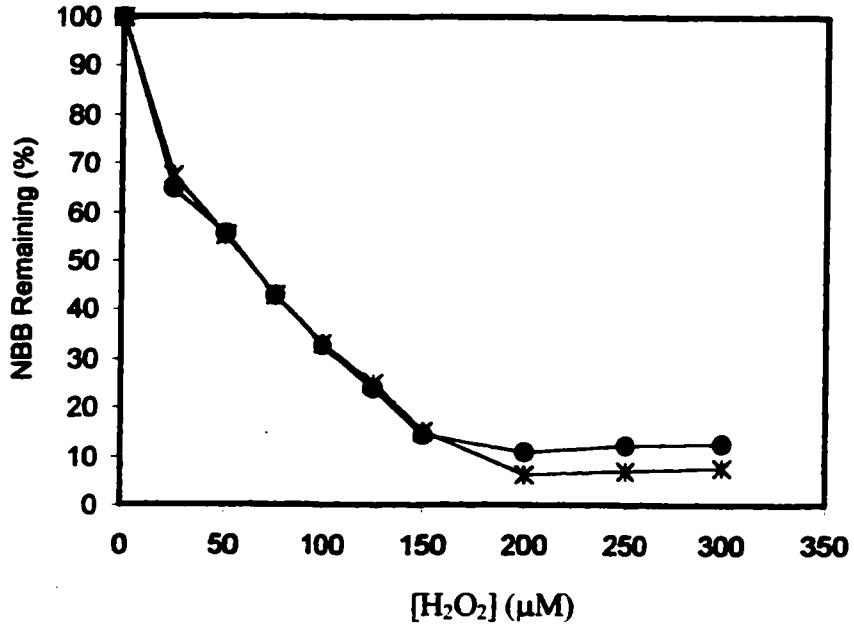


Figure 4.1.11 Effect of H_2O_2 concentration on the color reduction of NBB. $[NBB]_0$=0.05 mM, $[ARP]_0$=0.1 U/mL, pH=7.6. Reaction time: ●— T=60 min, ✱— T=120 min

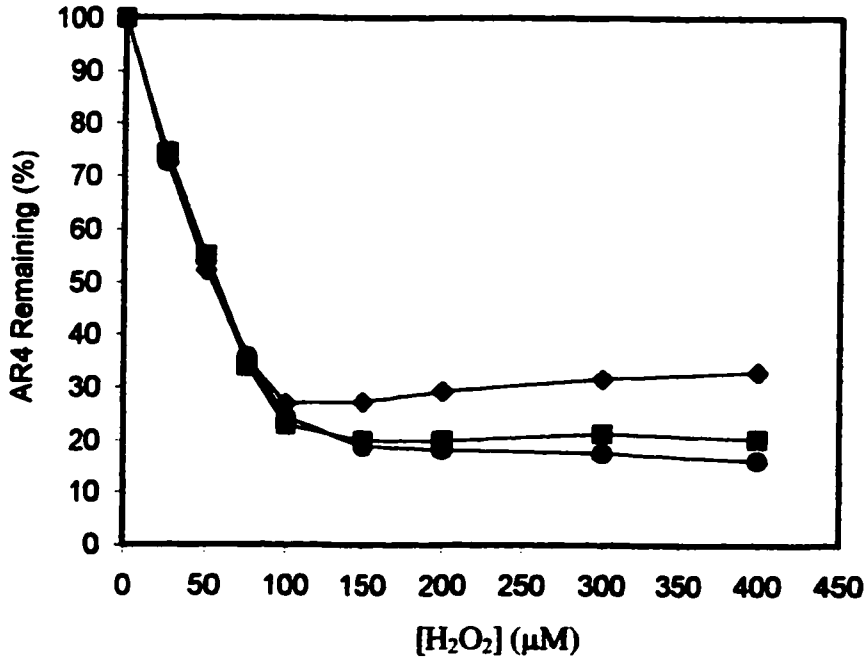


Figure 4.1.12 Effect of H_2O_2 concentration on the color reduction of Acid Red 4 $[AR4]_0$=0.05 mM, $[ARP]_0$=0.1 U/mL, Buffer pH=8.2. Reaction time: ◆— T=15 min, ■—30 min, ●—T=90 min

Beyond the optimum point, the remaining color increased with further increase in H_2O_2 concentration. It is concluded that any excess hydrogen peroxide was not effective in dye color reduction but increased the enzyme inactivation, especially in the beginning of reaction. With a longer reaction time, the difference in color remaining for different concentration of H_2O_2 was less pronounced. This is probably because the H_2O_2 concentrations dropped with the time and the inactivated enzyme had gone back to their activated state. For 0.05 mM AR4 and COG, it was observed that the optimum hydrogen peroxide concentration was nearly 0.1mM, which gives the optimum molar ratio of hydrogen peroxide to dye around 2. For 0.05 mM NBB, the results show that the optimum ratio was 4. It can be seen from the structure of the dyes that AR4 and COG have only one azo bond, whereas NBB has two in structure. Therefore, the molar ratio of color reduction of azo dyes between hydrogen peroxide and azo bond is nearly 2.

4.1.4 Effect of reaction time

Reaction time is an important engineering parameter along with the pH, enzyme concentration and hydrogen peroxide concentration. Therefore experiments were conducted to select the optimum reaction time. The results are shown in Figures 4.1.13 to 4.1.15.

Figure 4.1.13 shows the time effect on the color reduction of COG at different enzyme concentrations. The results indicate that the color was removed quickly in less than 15 minutes, and at high enzyme concentration, the reactions had almost reached their end points in this time. At lower enzyme concentration, the reaction continued at lower rate. Figure 4.1.14 shows similar behavior for color reduction of NBB. Figure 4.1.15 shows the color of AR4 was removed quickly in the first 15 minutes, while after that less improvement was achieved.

The optimum values for pH, enzyme concentration, H_2O_2 concentration and reaction time, as determined in these experiments for different dyes, are summarized in Table 4.1.2

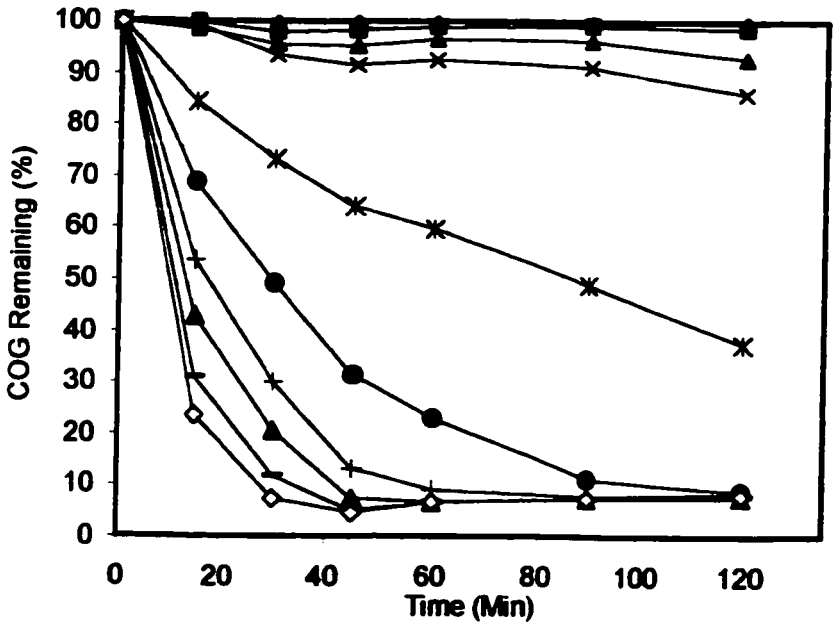


Figure 4.1.13 The effect of reaction time on the color reduction of Crocein Orange G.
$[COG]_0$=0.05 μM, $[H_2O_2]_0$=100 μM, pH=7.5
ARP concentration (U/mL):
◆—0 ■—0.001 ▲—0.005 ×—0.010 ✱—0.050
●—0.100 +—0.150 ▲—0.200 -—0.250 ◇—0.300

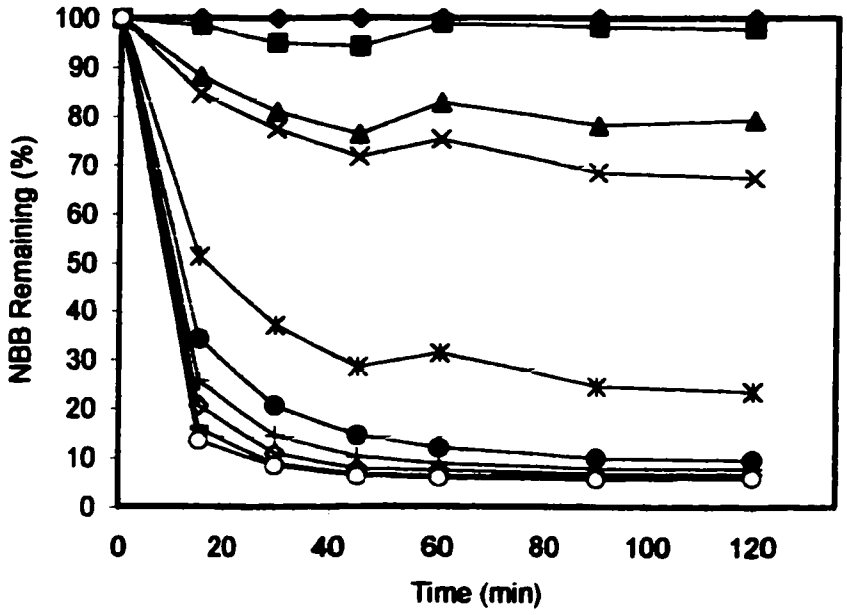


Figure 4.1.14 The effect of reaction time on the color reduction of Naphthol Blue Black.
$[NBB]_0$=0.05 mM, $[H_2O_2]_0$=0.2 mM, pH=8.7
ARP concentration (U/mL):
◆--- 0 ■---0.001 ▲--- 0.005 ×---0.010 ✳---0.050
●---0.100 +---0.150 ◇---0.200 ▬---0.250 ○---0.300

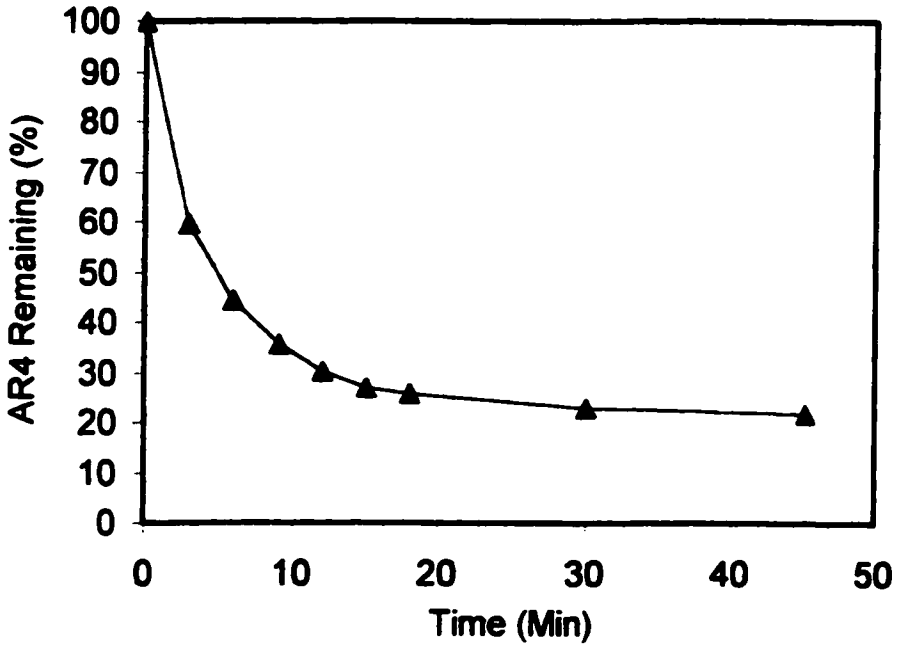


Figure 4.1.15 The effect of reaction time on the color reduction of Acid Red 4 $[AR4]_0$=0.05 mM, $[ARP]_0$=0.1 U/mL, $[H_2O_2]_0$=0.1 mM, Buffer pH=8.2

Table 4.1.2 Summary of optimum reactor parameters required for color reduction of different dyes

Dye	Naphthol Blue Black		Acid Red 4		Crocein Orange G	
Optimum pH	Range	Optimum value	Range	Optimum value	Range	Optimum value
	6.4~8.7	7.6	7.4~8.7	8.2	7.0~8.2	7.4
ARP concentration (U/mL)	0.1		0.2		0.1	
H_2O_2 concentration (μM) (for 50 μM dye)	200		100		100	
Highest color removal efficiency (%)	95		84		94	
Reaction time (min)	60		30		60	

4.2 Color reduction of Disperse Orange 3 by zero-valent iron

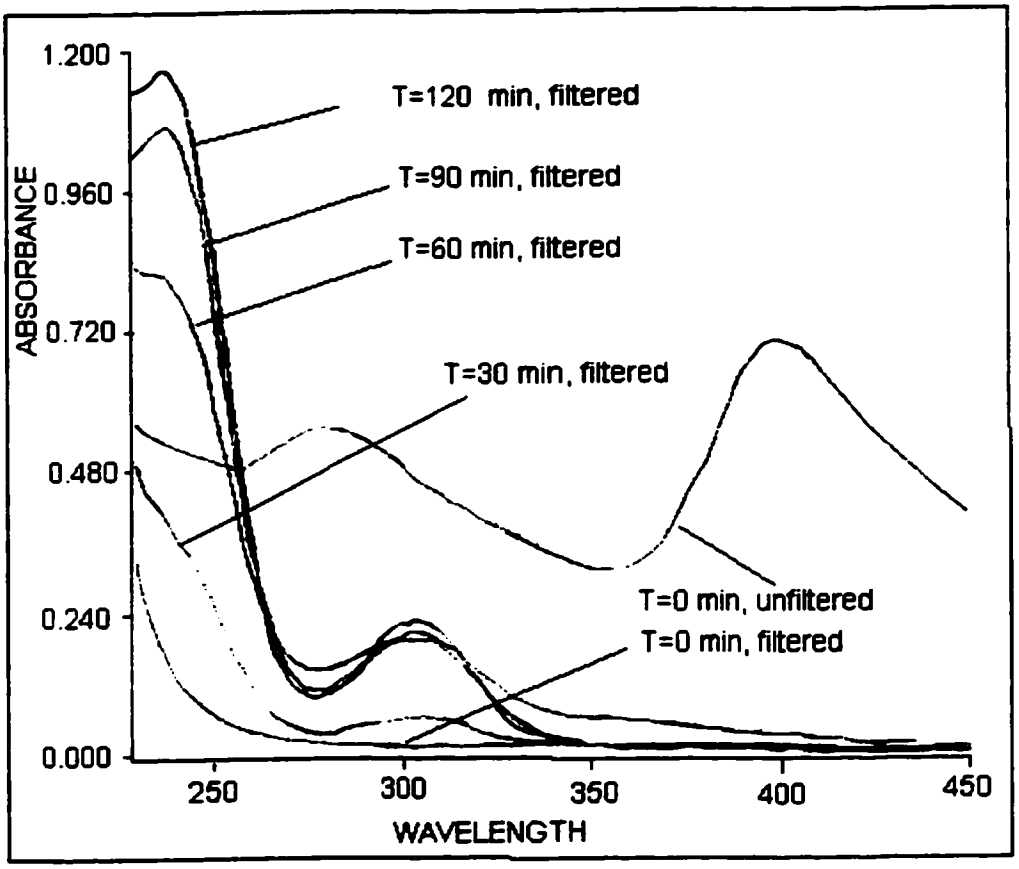
4.2.1 Color removal efficiency

The spectra during the reduction of DO3 by Fe^0 are shown in Figure 4.2.1. DO3 is not soluble in water. Before filtration, the mixture of DO3 and H_2O had a peak absorbance at 400 nm; however, after filtration, all the DO3 was filtered out and nothing remained in the solution. As seen from Figure 4.2.1, before reaction with Fe^0, the absorbance of DO3 at wavelength 400 nm was 0.7. This is an apparent absorbance since the mixture was not a true solution, but was a mixture of liquid and suspended solids. Since particles were formed during the reaction, the absorbance of the reaction liquid were measured after filtration. As seen in the figure, after reaction, there is no peak absorbance at 400 nm, but another peak absorbance formed at 302 nm. The absorbance turned to be 0.013 at the same wavelength. Thus the color reduction efficiency was more than 98% when calculated by using the peak wavelength absorbance. After 2 hours reaction, color of the mixture in the reactor turned from yellow to colorless and the spectrum of filtered solution showed two peaks formed at 240 and 302 nm.

4.2.2 Product(s) identification by UV-spectrum

Tests were done to determine the solubility of DO3 in methanol. It was found that DO3 will be completely dissolved to 1.0 mM when the methanol is equal or more than 50% and will have a same spectrum before or after filtration. The spectra of DO3 dissolved in 50% methanol, pure p-PD and the product of this reaction are shown in Figure 4.2.2. It is observed that the spectrum of the product of reduced DO3 had almost the same spectrum as that of the pure p-PD in the range of 220-600 nm. Both of them

have peak absorbance at 302 nm. Therefore, it was tentatively concluded that the DO3 had been reduced to p-PD.



4.2.1 Spectra of DO3 (filtered and unfiltered) and filtered product (s) of reduced DO3 at different reaction time

4.2.3 Product(s) identification by TNBS test

The product of reduced DO3 (assumed to be p-PD) produced at different times was tested at characteristic absorbance wavelength of 302 nm and also subjected to the TNBS test. The

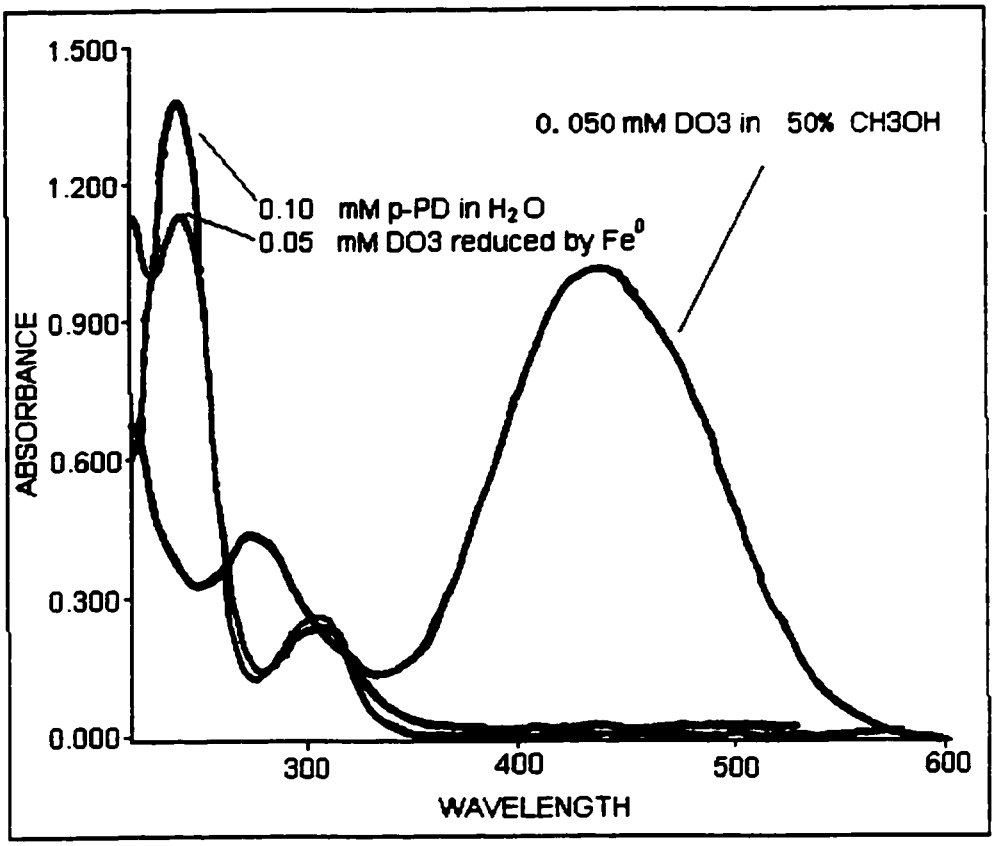


Figure 4.2.2 Spectra of 50% methanol dissolved DO3, pure p-PD and reduced DO3

results are shown in Figure 4.2.3. The results obtained by the two methods show clearly a similar pattern. The reason that p-PD product concentration calculated by absorbance was higher than that obtained by TNBS was possibly because of some other impurities in the dye (DO3 content: 90%).

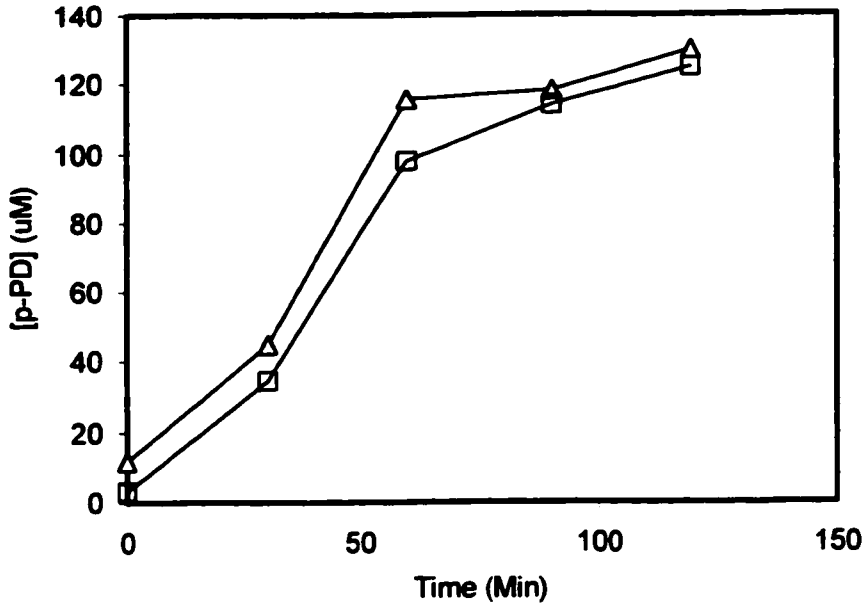


Figure 4.2.3 p-phenylenediamine produced by DO3 and Fe^0
$[DO3]_0$=0.0625 mM, $[Na_2SO_3]_0$=1.0 mM, 1.0 g Fe used.
□--- [p-PD] caculated by TNBS test
▲--- [p-PD] calculated by absorbance at 302 nm

4.2.4 Product(s) identification by HPLC

Another method used to identify the product(s) of DO3 reduction was High Performance Liquid Chromatography (HPLC). The HPLC analysis of DO3, p-PD and the product(s) of DO3 reduction by Fe^0 at 430 and 302 nm are summarized in Table 4.2.1 and Figures 4.2.4 to 4.2.9. It can be seen from these Figures that there is only one sharp peak absorbance detected at 1.64 min. Therefore, only one product was produced by this reaction. The results show that p-PD and DO3 reduction by Fe^0 had a very close retention time: 1.61 and 1.64 min. This gives evidence that the product of DO3 reduction by Fe^0 is p-PD. The retention time of DO3 is 25 min, but no peak was found near 25 min in the 60

min analysis of DO3 reduction by Fe^0 at 302 and 434 nm. Therefore, all the DO3 was converted to p-PD.

Table 4.2.1 HPLC analysis for DO3 reduced by Fe^0, pure DO3 and p-PD

	Wavelength(nm)	DO3	p-PD	DO3+Fe0
Initial conc. (μM)		50	100	54.5
RT (min)	302, 434	25.57 ± 0.63	1.61 ± 0.01	1.62 ± 0.01
Area (μV*Sec)	302	157127 ± 525	151707 ± 6562	149460 ± 7416
Area (μV*Sec)	434	635505 ± 19431	42980 ± 1053	40476 ± 3617
Height (μV)	302	2404 ± 81	12464 ± 615	12763 ± 899
Height (μV)	434	9508 ± 364	4534 ± 276	4691 ± 115

The calibration curve of p-pD HPLC analysis is shown in Figure 4.2.10.

Quautitatively, for 54.5 μM DO3, the HPLC analysis indicates:

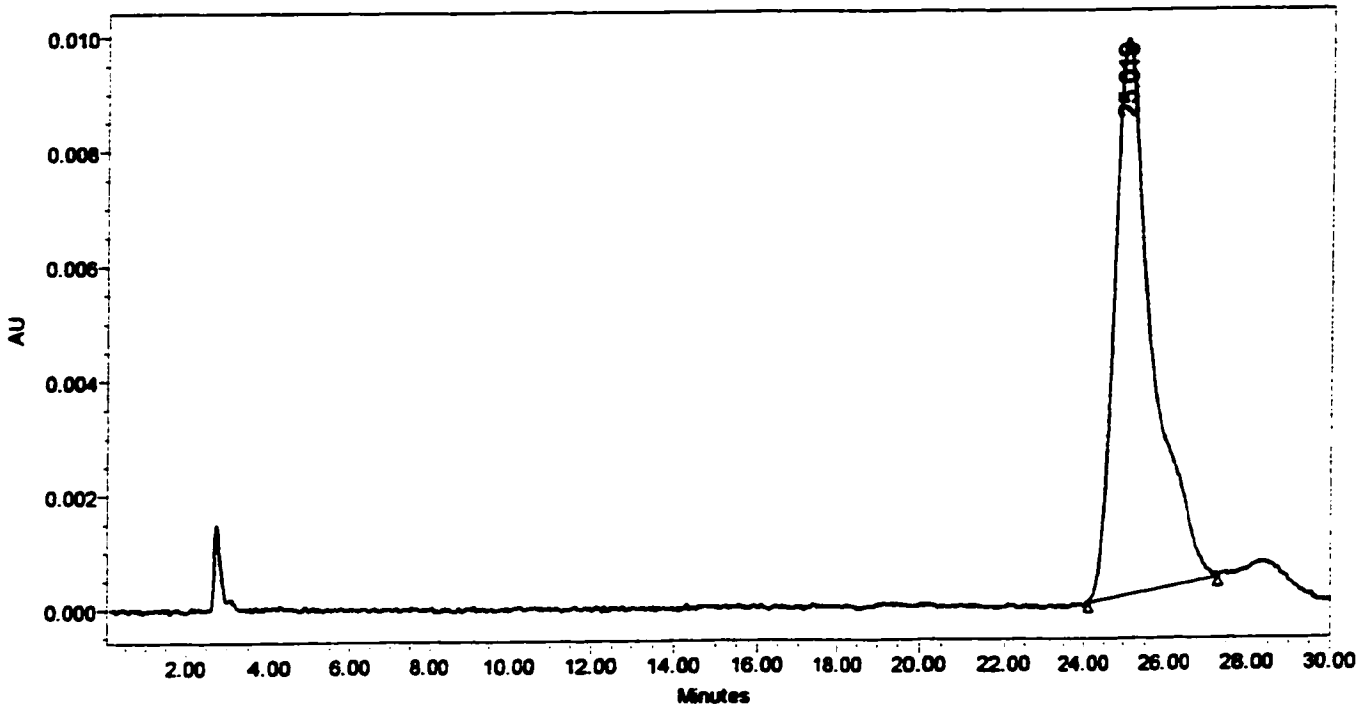
98.5% recovery* by area at 302 nm

94.17% recovery* by area at 434 nm

102.4% recovery* by height at 302 nm

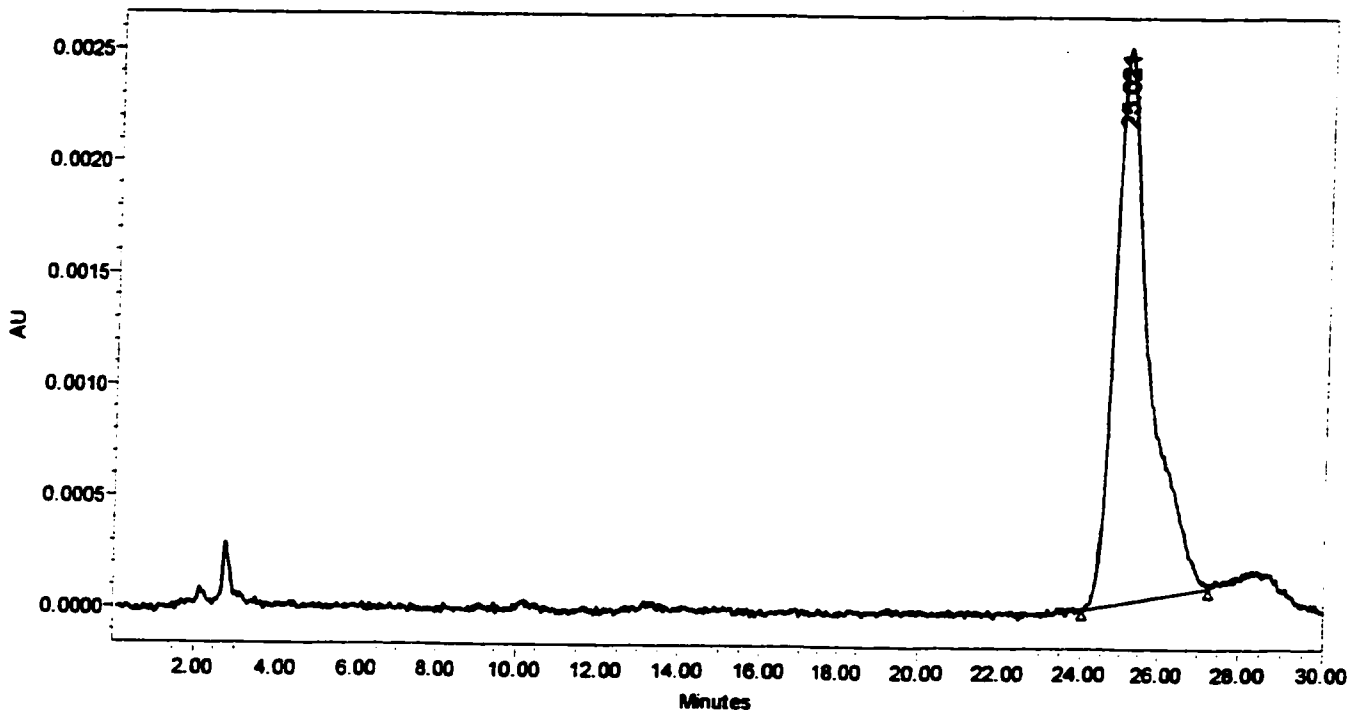
103.4% recovery* by height at 434 nm

*---- recovery of theoretical p-PD



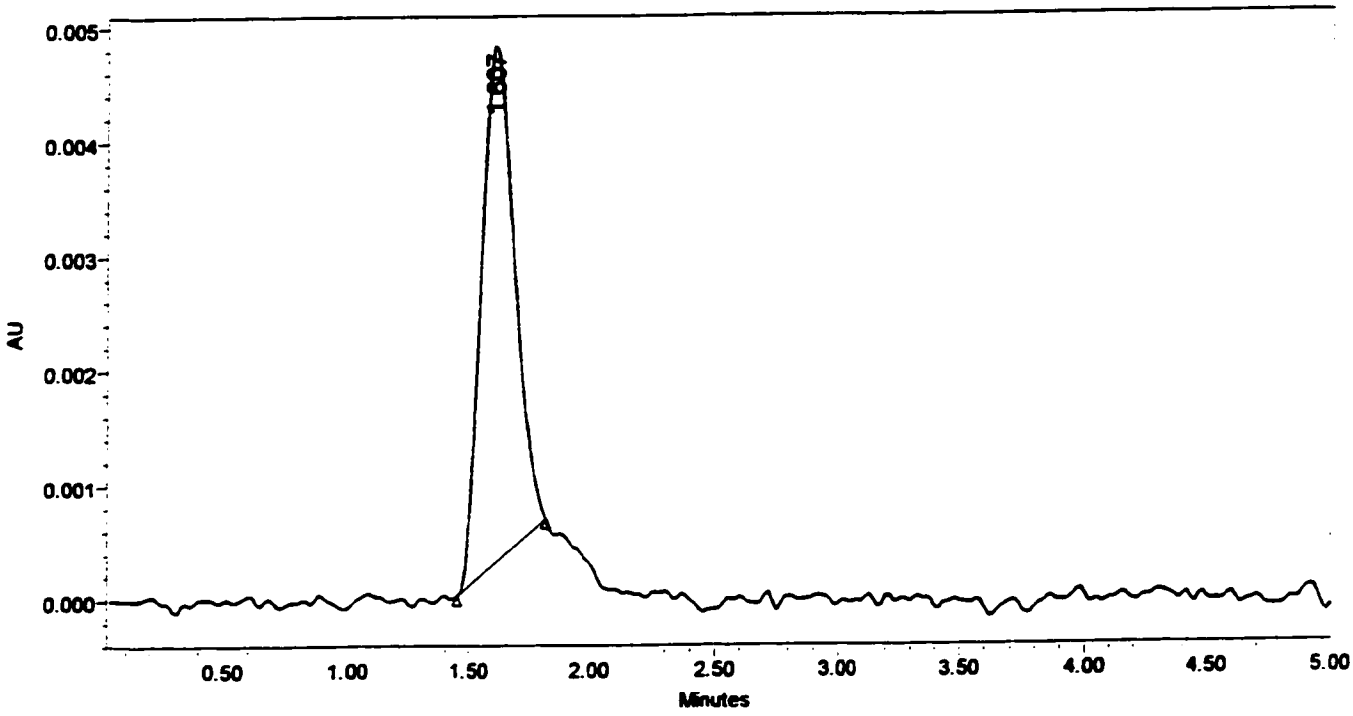
	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	25.019	622079	100.00	9736	100.00

Figure 4.2.4 HPLC analysis of DO3 at 434 nm
Injection volume: 10 μL, mobile phase: 50 % CH_3OH, 50% H_2O. [DO3]=50 μM



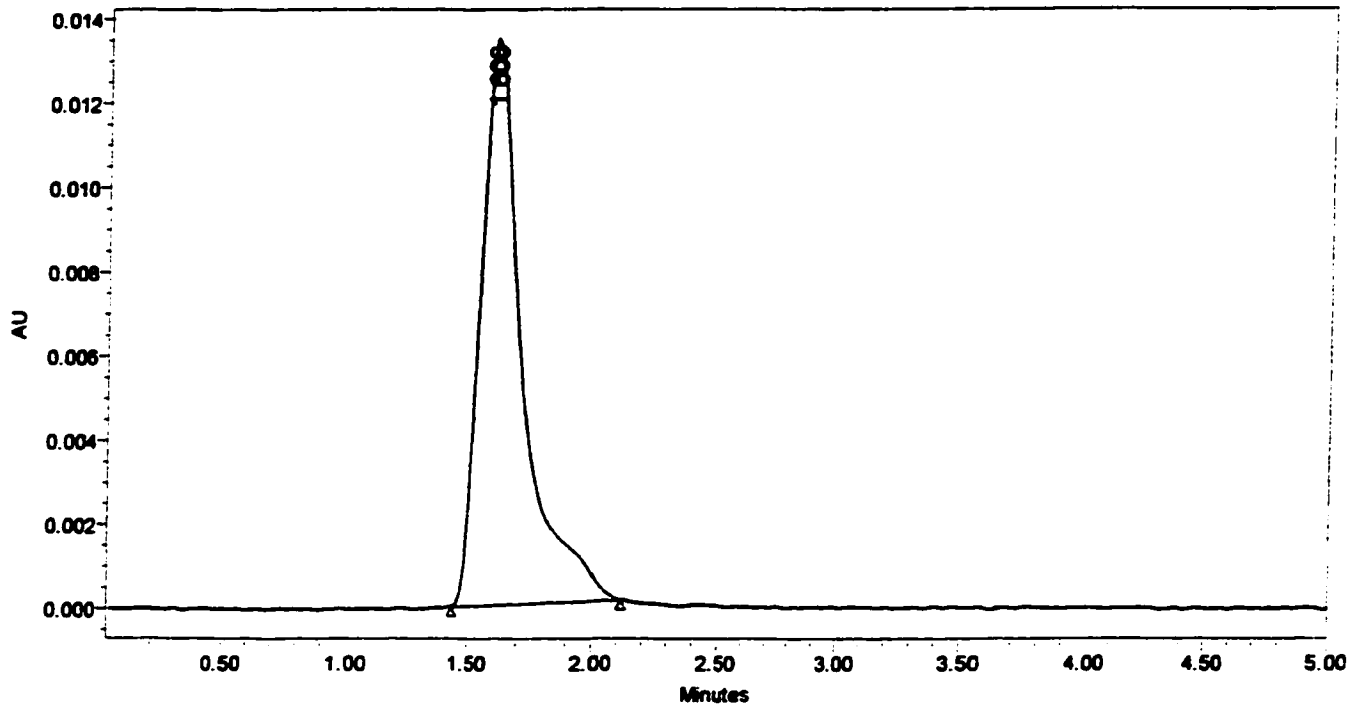
	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	25.021	158153	100.00	2487	100.00

Figure 4.2.5 HPLC analysis of DO3 at 302 nm
Injection volume:10 μL, mobile phase: 50 % CH_3OH, 50% H_2O. [DO3]=50 μM



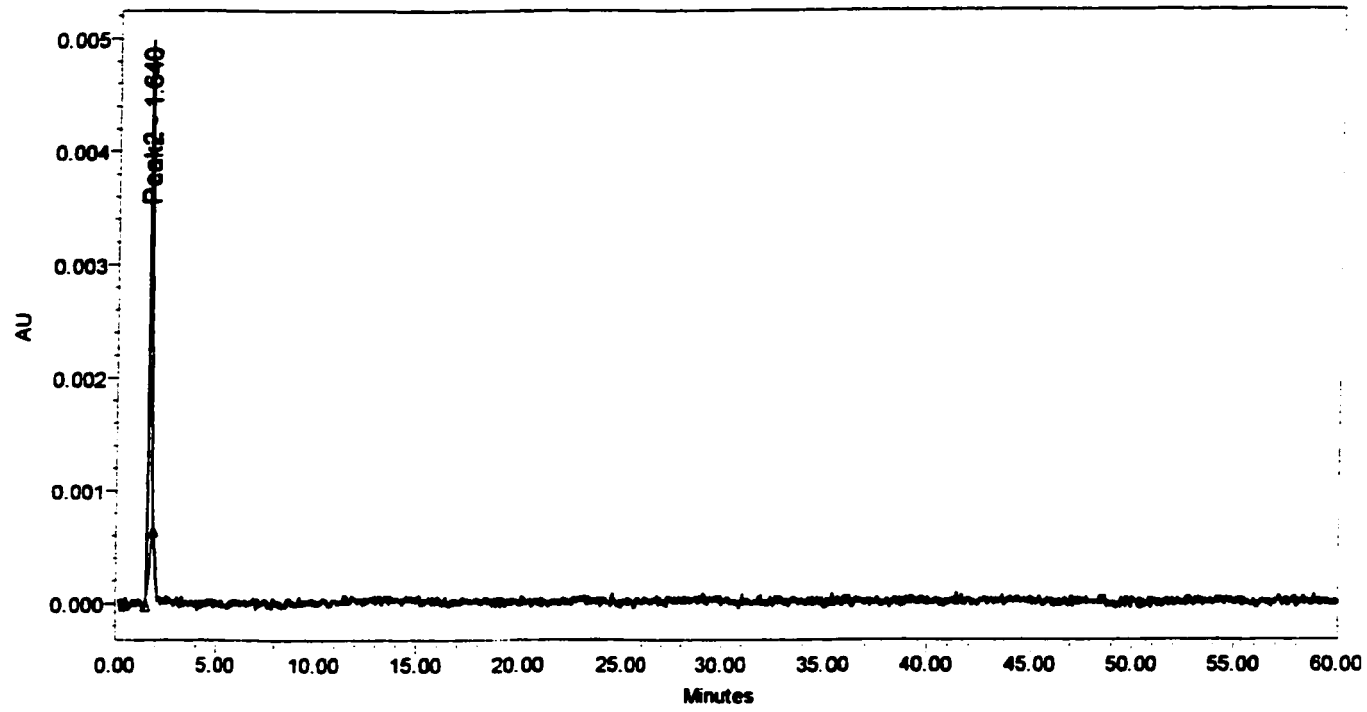
	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	1.607	42980	100.00	4534	100.00

Figure 4.2.6 HPLC analysis of pure p-phenylenediamine at 434 nm
Injection volume:10 µL, mobile phase: 50 % CH_3OH, 50% H_2O. [p-PD]=100 µM



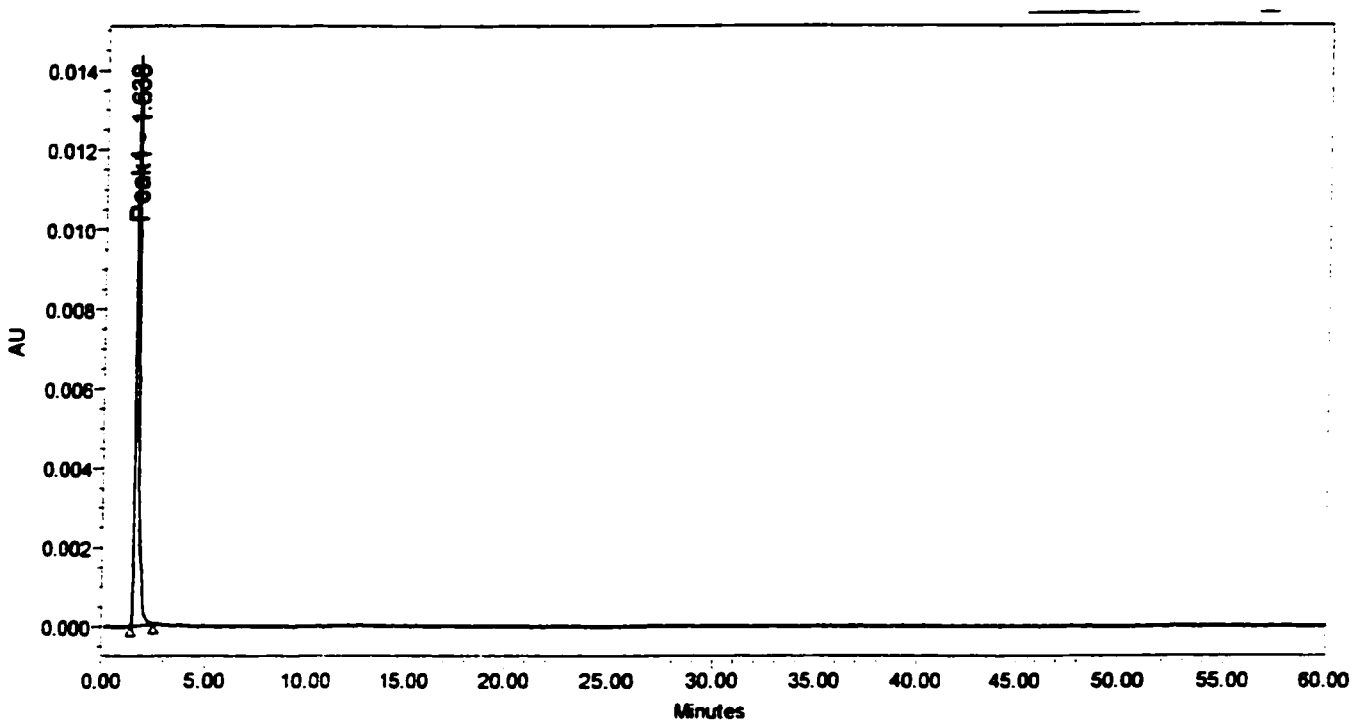
	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	1.606	158121	100.00	13499	100.00

Figure 4.2.7 HPLC analysis of pure p-phenylenediamine at 302 nm
Injection volume:10 μL, mobile phase: 50 % CH_3OH, 50% H_2O. [p-PD]=100 μM



	Peak Name	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	Peak2	1.640	40426	100.00	4714	100.00
2	Peak3	25.019				
3	Peak1	26.839				

Figure 4.2.8 HPLC analysis of reduced DO3 by Fe^0 at 434 nm
Injection volume:10 μL, mobile phase: 50 % CH_3OH, 50% H_2O. $[DO3]_0$=54.5 μM



	Peak Name	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	Peak1	1.638	158469	100.00	14365	100.00
2	Peak2	24.870				

Figure 4.2.9 HPLC analysis of reduced DO3 by Fe^0 at 302 nm
Injection volume: 10 μL, mobile phase: 50 % CH_3OH, 50% H_2O. $[DO3]_0$=54.5 μM

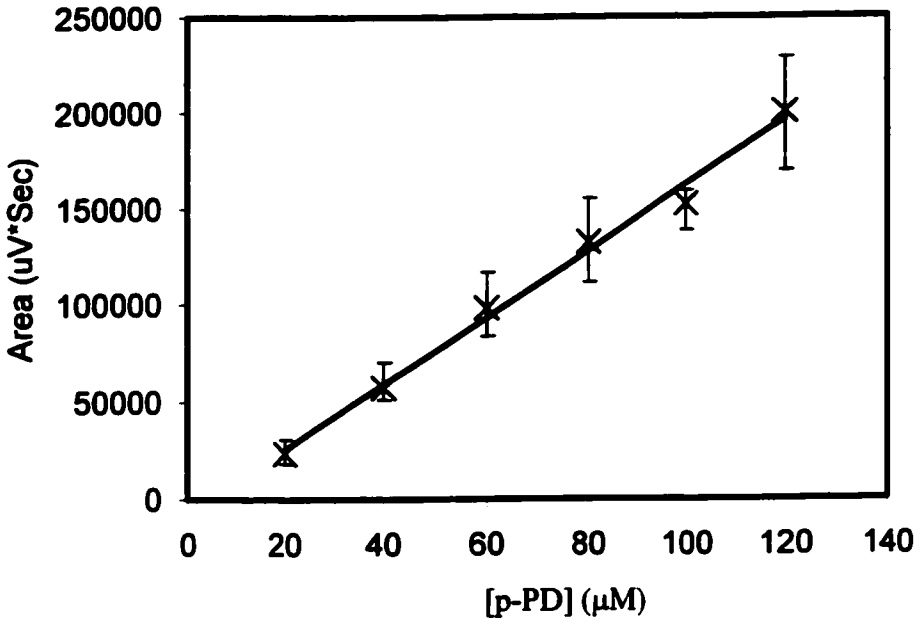


Figure 4.2.10 Results of p-PD Calibration of HPLC test by area at 302 nm
$y=17.085x-8889.3$, $R^2=0.9916$

4.3 Removal of p-PD by peroxidase plus peroxide

It was observed that when ARP and hydrogen peroxide were added to a p-PD solution, the mixture first turned to blue-violet color within a few minutes, which was dependent on both ARP and hydrogen peroxide being present. After about 1 hour, suspended particles were observed in the reactor and the color in the reactor gradually become lighter until it disappeared. When the mixture was filtered, black solids were seen on the filter and no color was observed in the filtered liquid. This behavior is the same as described by Cataldo (1996). The UV-vis spectrum shows clearly that in the beginning of the reaction, the mixture formed a product that had a peak wavelength near 440 nm, and the absorbance increased to a certain point; after that, the absorbance in the range of 300 to 600 nm dropped down gradually. It was observed that suspended particles appeared in the reactor after this point.

Cataldo (1996) reported that, during the oxidation of p-phenylenediamine, the first step during oxidation was the extraction of an electron from the p-PD molecule with the formation of a radical cation or semiquinone radical and this radical cation was intensely colored and had a tendency to polymerization.

4.3.1 Effect of pH on the removal of p-PD

Experiments were conducted to determine the optimum pH. Since ARP showed some catalytic ability between pH 6.0 and 9.5, (Al-Kassim et al., 1993), the pH range of 6.0 to 9.5 was chosen in this study. The results are shown in Figure 4.3.1. As seen, when the pH was in the range from 7.0 to 9.5, less than 15% of p-PD remained in the solution. However, when pH was less than 6.5, the p-PD removal efficiency dropped quickly. Thus, the suitable pH range for p-PD removal by ARP and hydrogen peroxide was in the

range from 7.0 to 9.5. The pH of 7.6 was selected as an optimum pH because the p-PD removal efficiency was high and this condition being near neutral is easy to obtain in the treatment of p-PD.

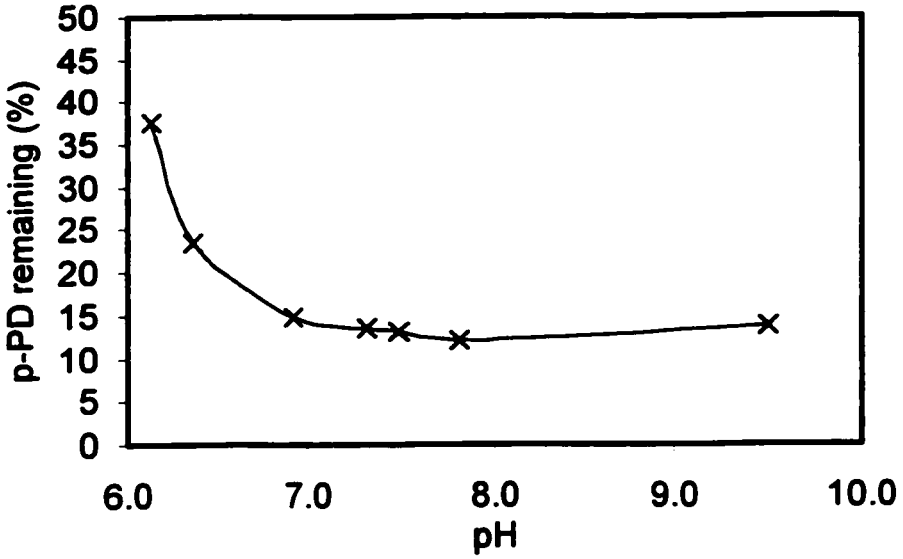


Figure 4.3.1 Effect of pH on the removal of p-phenylenediame by ARP and H_2O_2 (Analyses by TNBS test).
$[p\text{-}PD]_0$=0.3 mM, $[ARP]_0$=0.2 U/mL, $[H_2O_2]_0$=0.3 mM, Reation time=4 h.

4.3.2 Effect of enzyme concentration on the removal of p-PD

These experiments were conducted at the previously established optimum pH of 7.6. The reaction times were set at 2 and 4 hours. The results are presented in Figure 4.3.2 and 4.3.3 for 100 μM and 300 μM p-PD initial concentrations and 2 hours reaction time. These figures show the dependence of color reduction efficiency on the enzyme concentration. The p-PD removal efficiency increased with higher enzyme

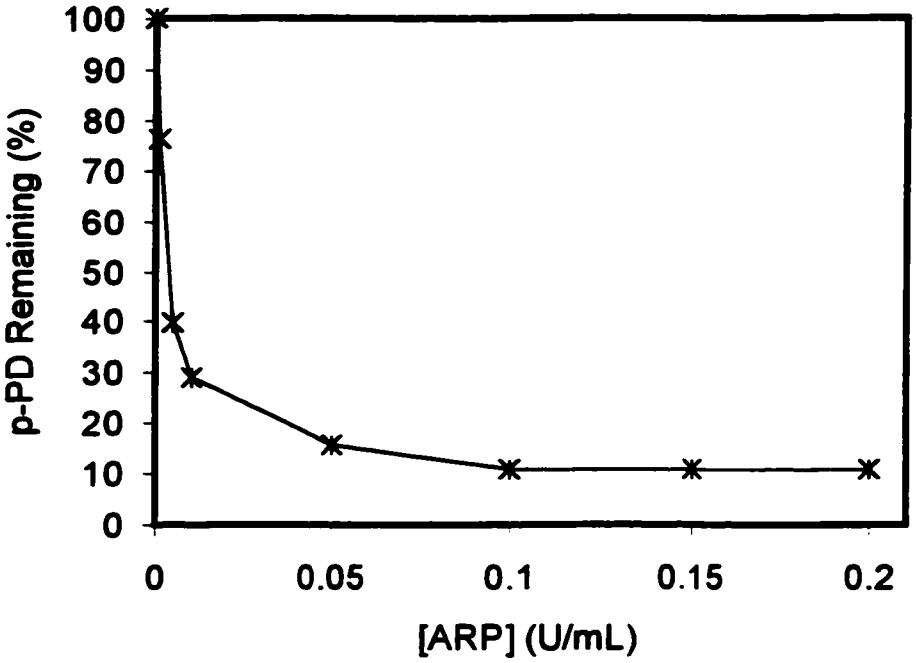


Figure 4.3.2 Effect of ARP concentration on the removal of 100μM p-PD (Analysis by TNBS test).
$[p\text{-}PD]_0$=0.1 mM, $[H_2O_2]_0$=0.1 mM, Reaction time=2 h, Buffer pH=7.6

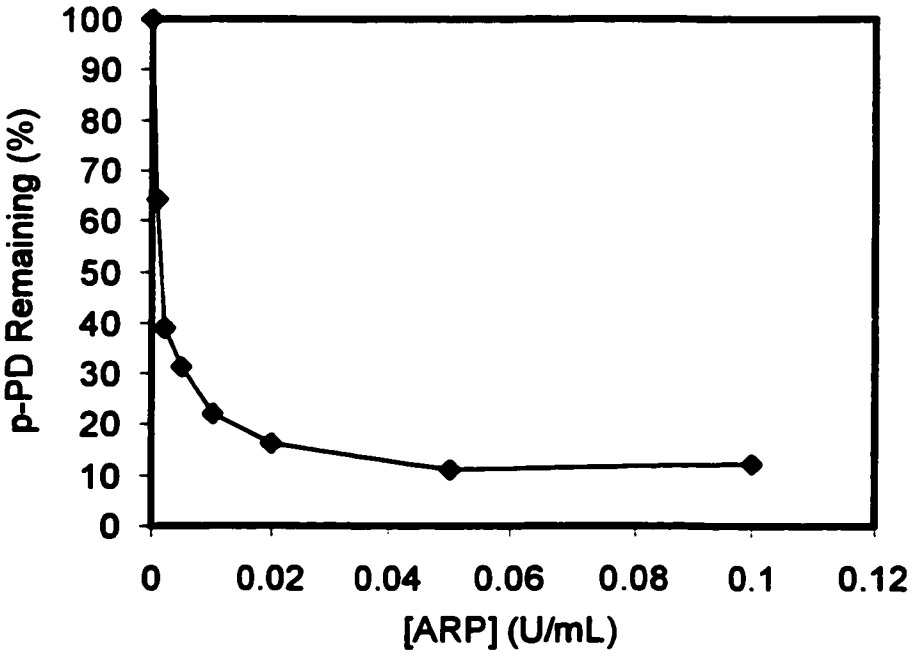


Figure 4.3.3 Effect of ARP concentration on the removal of 300μM p-PD (Analysis by TNBS test). $[p\text{-}PD]_0$=0.3 mM, $[H_2O_2]_0$=0.2 mM, Reaction time=2 h, Buffer pH=7.6

activity in the solution to a certain limit. As seen from these figures, 0.02 U/mL and 0.05 U/mL ARP resulted in more than 80% removal efficiency of p-PD in two hours. Additional enzyme present in the solution showed little improvement in the reaction. Figure 4.3.4 gives further evidence that for 300 μM p-PD with 4 h reaction time, the removal efficiency was mainly dependent on the enzyme concentration in the solution and different amounts of hydrogen peroxide over 0.2 mM had little effect on the removal of p-PD in 4 hours. It was noticed that there was a little removal even without enzyme being added. This may be because the p-PD was not stable under the experiment conditions and some uncertain reactions occurred.

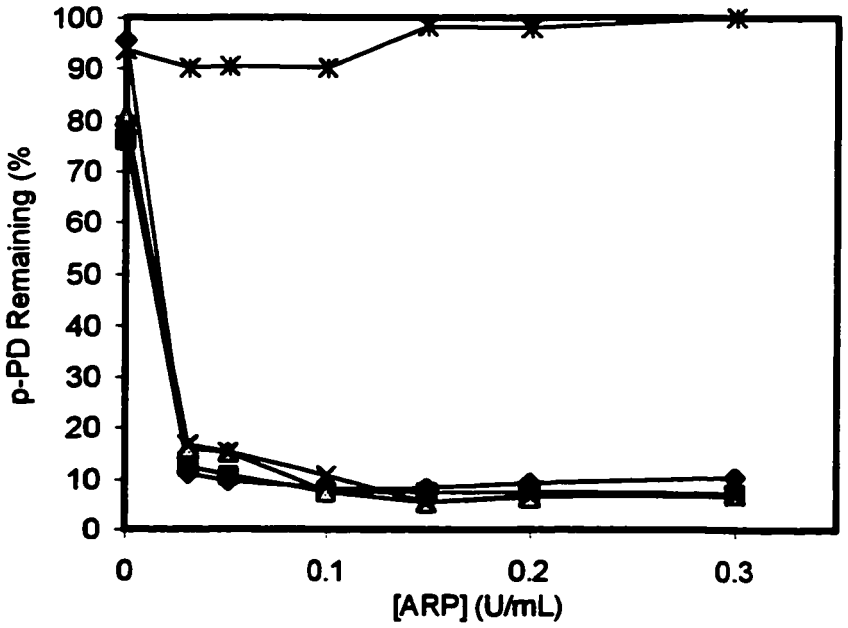


Figure 4.3.4 Effect of ARP concentration on the removal of p-PD at different H_2O_2 concentraions.
[p-PD]$_0$=0.3 mM, Reaction time=4 h (Analysis by TNBS test).

* --- [H_2O_2]$_0$=0 mM ♦---[H_2O_2]$_0$=0.2 mM ■ ---[H_2O_2]$_0$=0.3 mM
▲– [H_2O_2]$_0$=0.4 mM ×---[H_2O_2]$_0$=0.5 mM

4.3.3 Effect of hydrogen peroxide concentration

Experiments were performed to determine the effect of the initial hydrogen peroxide concentration on the removal of p-PD. The effect was studied at pH 7.6 with 100 μM and 300 μM p-PD concentrations. The results of these experiments are presented in Figure 4.3.5 to show the effect of varying reaction time and in Figure 4.3.6 show the effect of enzyme dose. These figures show that the p-PD remaining decreased sharply with an increase in hydrogen peroxide concentrations before reaching the optimum point (lowest remaining p-PD concentration). In this range, the low removal efficiency is attributed to insufficient H_2O_2 concentration. Beyond the optimum point, the remaining p-PD increased slowly with further increase in H_2O_2 concentration. It indicates that any excess hydrogen peroxide was not effective in p-PD removal but increased the enzyme inactivation, especially in the beginning of reaction. It was observed that the optimum hydrogen peroxide concentration was near 120 μM and 400 μM, for 100 μM and 300 μM p-PD, which gives the optimum molar ratio between hydrogen peroxide and p-PD of slightly more than 1.0 (i. e. 1.0 per amine group).

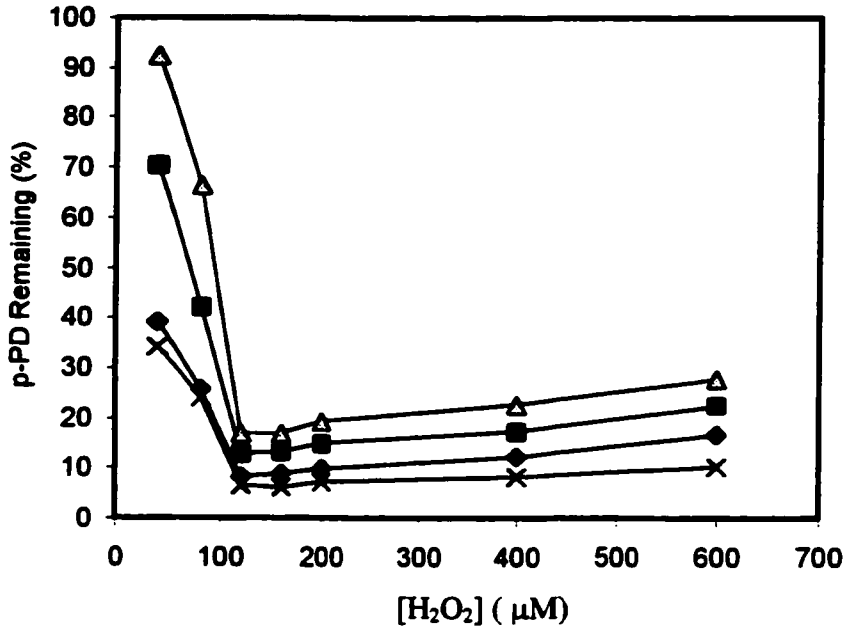


Figure 4.3.5 Effect of H_2O_2 concentration on the removal of 100 μM p-PD.
$[p\text{-}PD]_0$=0.1 mM, $[ARP]_0$=0.03 U/mL
▲— t= 30 min ■----t=60 min ♦----t=120 min ×----t=240 min

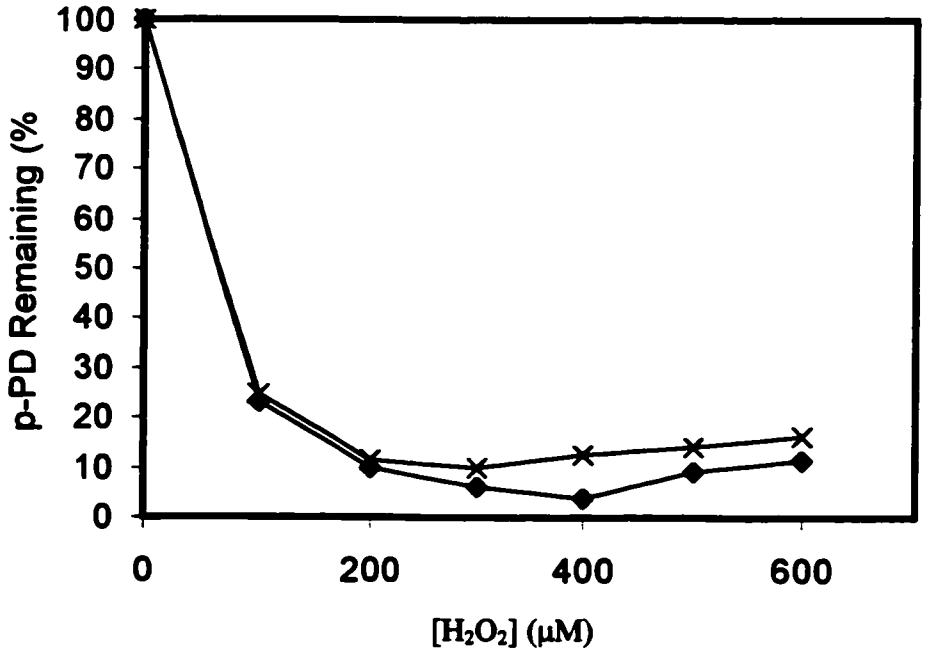


Figure 4.3.6 Effect of H_2O_2 concentration on the removal of 300 μM p-PD.
$[p\text{-}PD]_0$=0.3 mM, , Buffer pH=7.6, Reaction time: 4 h
♦----$[ARP]_0$=0.12 U/mL ×----$[ARP]_0$=0.09 U/mL

4.3.4 Effect of reaction time on the removal of p-PD

Experiments were conducted to determine the optimum reaction time to complete the reaction. Figures 4.3.7 and 4.3.8 show that the p-PD was removed quickly in the first 1~2 hours; after that, up to 6 hours, further removal of p-PD was achieved but at a lower rate. Considering both the high removal efficiency required in the industry and engineering economics, 4 hours reaction time is considered to be an optimum reaction time.

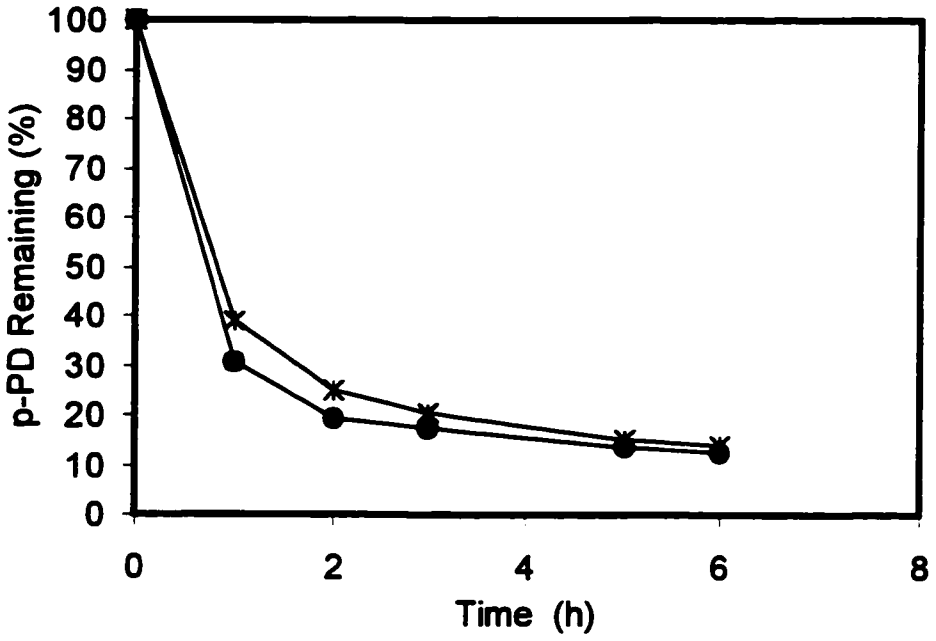


Figure 4.3.7 Effect of time on the removal of 100 μM p-PD.
$[p\text{-}PD]_0$=0.1 mM, $[ARP]_0$=0.01 U/mL
*—$[H_2O_2]_0$=0.1 mM ●—$[H_2O_2]_0$=0.0667 mM

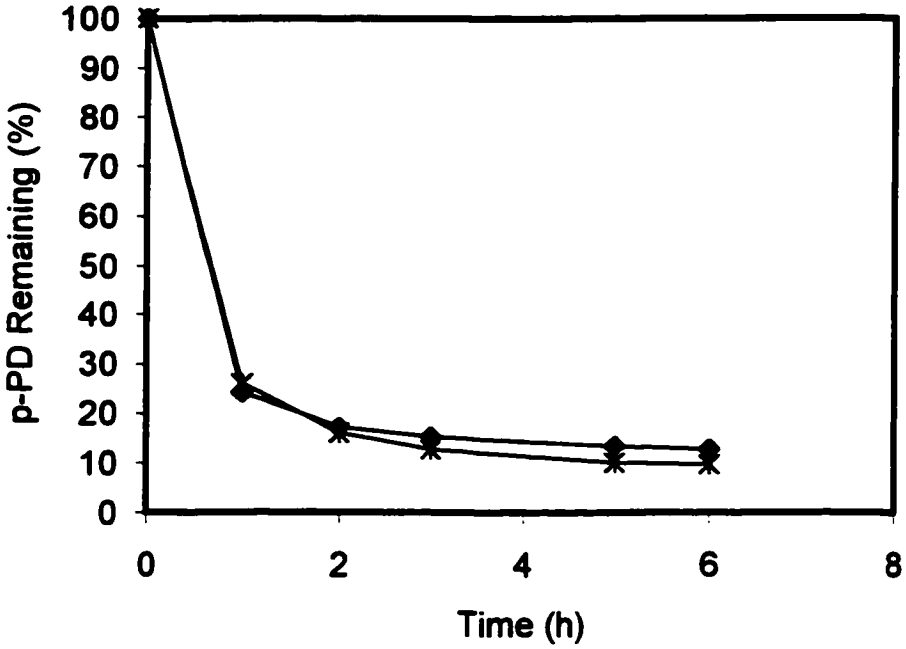


Figure 4.3.8 Effect of time on the removal of 300 μM p-PD.
$[p\text{-}PD]_0$=0.3 mM, $[ARP]_0$=0.03 U/mL
◆----$[H_2O_2]_0$=0.2 mM *----$[H_2O_2]_0$=0.3 mM

4.4 Phenylenediamine and aniline TNBS tests

4.4.1 TNBS test for P-PD under conditions similar to the aniline test

Monsef et al. (2000) developed a TNBS test method for aniline. In a final volume of 1.0 mL, 100 μL of 10 mM TNBS, 100 μL of 0.5 M phosphate buffer of pH 6.4 and 800 μL of sample plus water were added. Samples were allowed to stand for 30 min and then absorbance was measured at 384 nm against a reagent blank.

4.4.1.1 Without sodium sulfite

The absorbance was monitored with time by using 20 μM p-PD in the TNBS test at pH=6.4, with no sodium sulfite added. The products had an initial peak absorbance near 460 nm, which shifted to 430 nm and then, to the longer wavelength, finally, it stopped at 510 nm. The absorbance at 430 nm was not stable, rapidly reaching its maximum in 10 min and then gradually dropping down. The absorbance at 510 nm also increased rapidly in first 10 min and then gradually reached a maximum value at about 30 min, with a slow decline thereafter. Thus a 30 min reaction time was considered to be suitable for this test.

The absorbance for different concentrations of p-PD under the above conditions using 30 min test time showed good linear relationship at 510 nm. The extinction coefficient at 510 nm was 15,400$M^{-1} \cdot cm^{-1}$.

4.4.1.2 With sodium sulfite

It is known that TNBS readily forms an adduct with SO_3^{2-} that affects the analysis (Means et al., 1972). The spectra, Figure 4.4.1 were developed by using 20 μM p-PD and different concentrations of sodium sulfite with the TNBS in pH=6.4 phosphate buffer. The spectra rose with an increase in sodium sulfite concentration, to about 2.0 mM; the

spectra then became stable and showed little change with further increase in sodium sulfite concentration.

When the sodium sulfite concentration was less than 1.0 mM, the spectra showed no significant difference, and no peak absorbance was observed. However, when sodium sulfite concentration was 2 mM or greater, the extinction coefficient maximized, and, a peak absorbance appeared at 430 nm with a shoulder around 510 nm (Figure 4.4.1).

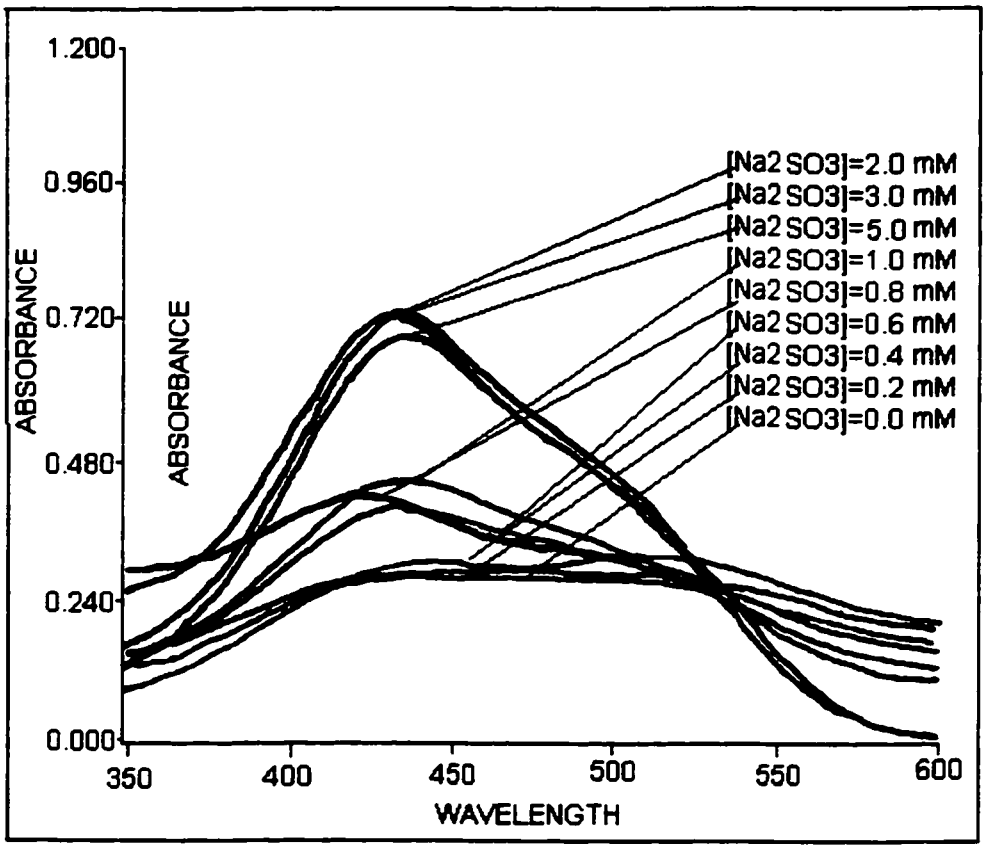


Figure 4.4.1 Effect of Na_2SO_3 to the p-PD TNBS test
$[p\text{-}PD]_0$=20 μM, reaction time =30 min

The change in peak values with time obtained with 20 μM p-PD in the TNBS test and the absorbances at 430, 510 nm are plotted in Figure 4.4.2. It can be seen that after a rapid reaction in the first 5 min, the absorbance increased slowly to a maximum at about

30 minutes, and decreased thereafter. Thus, 30 minutes reaction time was recommended also for the p-PD TNBS test in the presence of sodium sulfite at pH of 6.3.

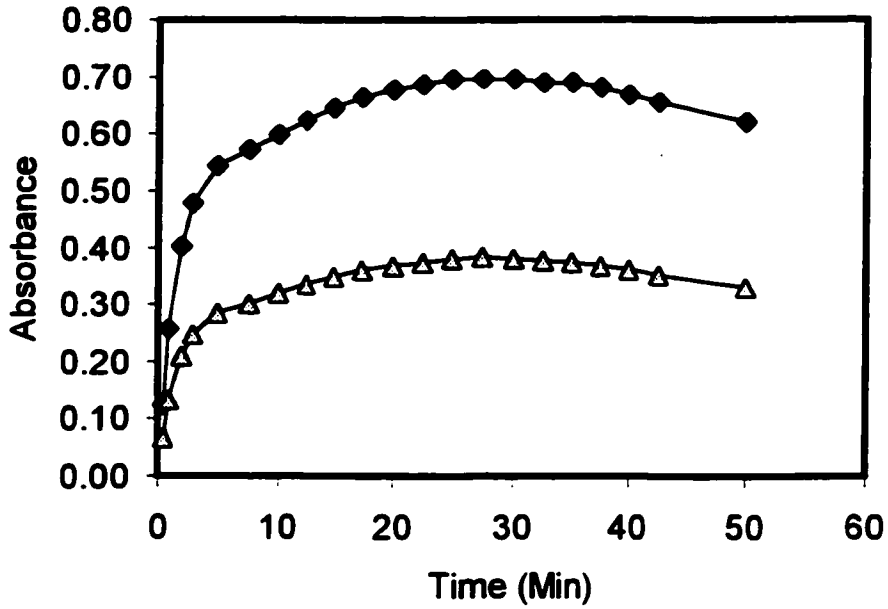


Figure 4.4.2 Absorbance of p-PD TNBS test and reaction vs time
$[p\text{-}PD]_0$= 20 μM, $[Na_2SO_3]_0$=2 mM. ▲----A510, ♦----A430

For the preliminary calibration curve, p-PD concentrations in the 2 μM to 30 μM range were used to obtain spectra. Plots of the absorbances at 430 and 510 nm vs p-PD concentration at 30 minutes are shown in Figure 4.4.3. The absorbance at 430 nm had the highest extinction coefficient (36,300 $M^{-1}\cdot cm^{-1}$) and an almost perfect linear relationship between the absorbance and the concentration of p-PD was obtained at both wavelengths. When the p-PD concentration was greater than 30 μM, the products formed turbidity and thus influenced the results.

4.4.2 Optimization of PD TNBS tests

4.4.2.1 Effect of pH

The pH has a significant effect on the absorbance of TNBS and the products of PDs' reaction with TNBS. Tests were conducted by using different pH buffers with 60

minutes reaction time for p-PD and m-PD, and 200 minutes for o-PD. Initial concentrations of 20 μM, 20 μM and 40 μM were chosen for p-PD, m-PD and o-PD, respectively. Test results, shown in Figures 4.4.4 to 4.4.7, indicate that when pH was in the range of 7.0-8.0, the PD TNBS tests had the highest absorbance at 430 nm. Therefore, pH 7.4 was chosen for the PD TNBS tests.

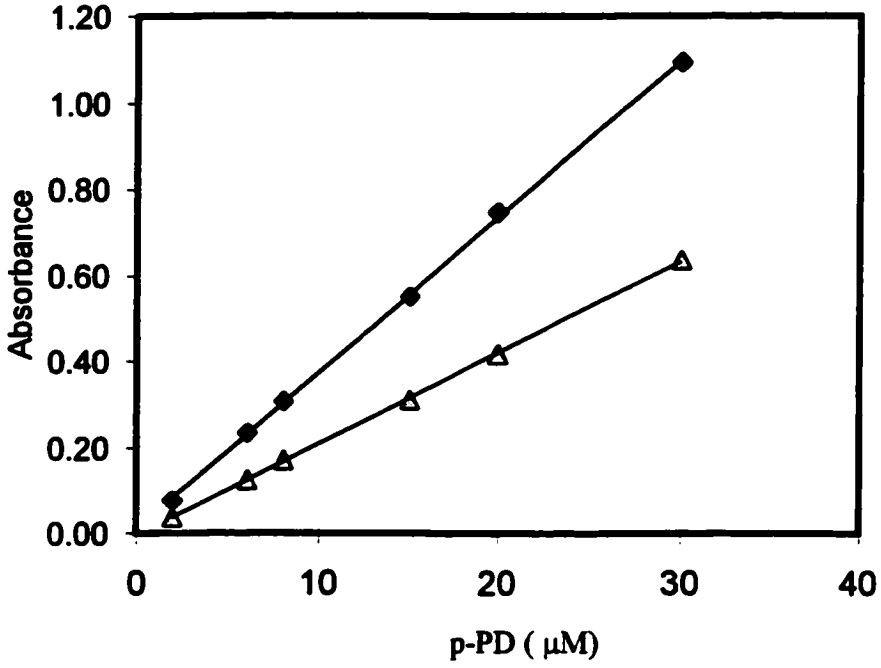


Figure 4.4.3 Calibration curve of p-PD
♦----A430, y=0.0363x+0.0093, R^2=0.9996
▲----A510, y=0.0212x+0.0042, R^2=0.9997

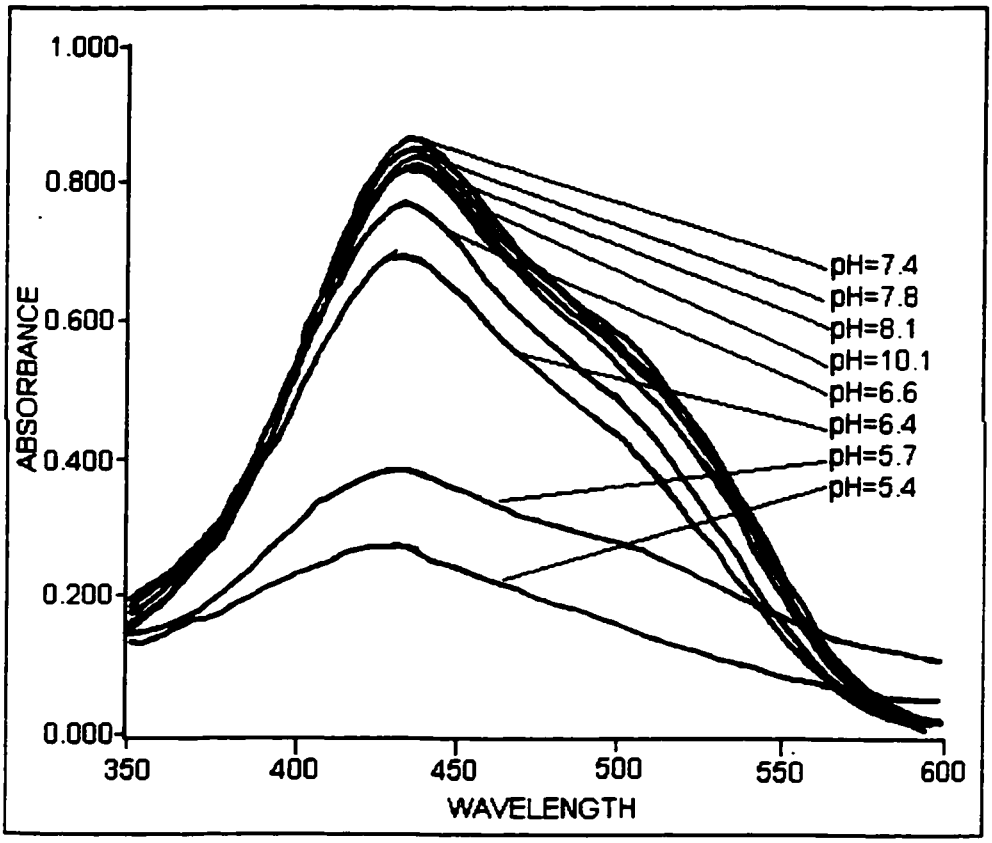


Figure 4.4.4 Effect of pH on the p-PD TNBS test
($[p\text{-}PD]_0$=20 μM, $[Na_2SO_3]_0$=2 mM, reaction time=60 min)

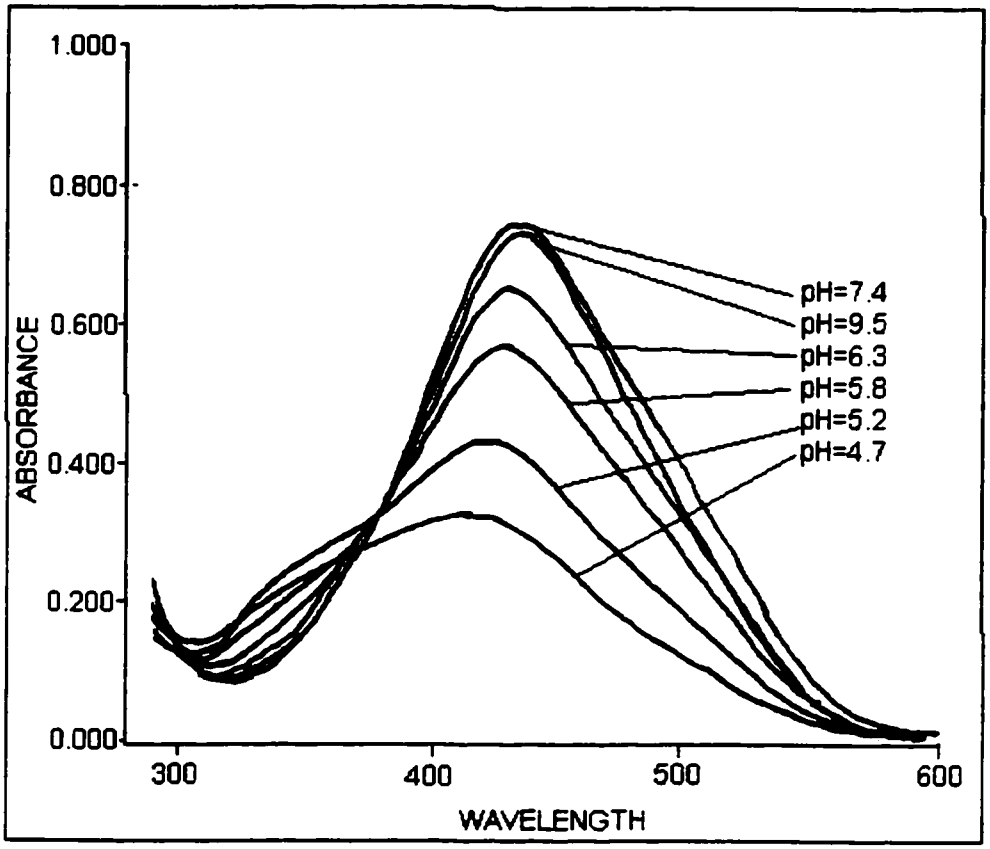


Figure 4.4.5 Effect of pH on the o-PD TNBS test
$[o\text{-}PD]_0$=40 μM, $[Na_2SO_3]_0$=2 mM, reaction time=60 min

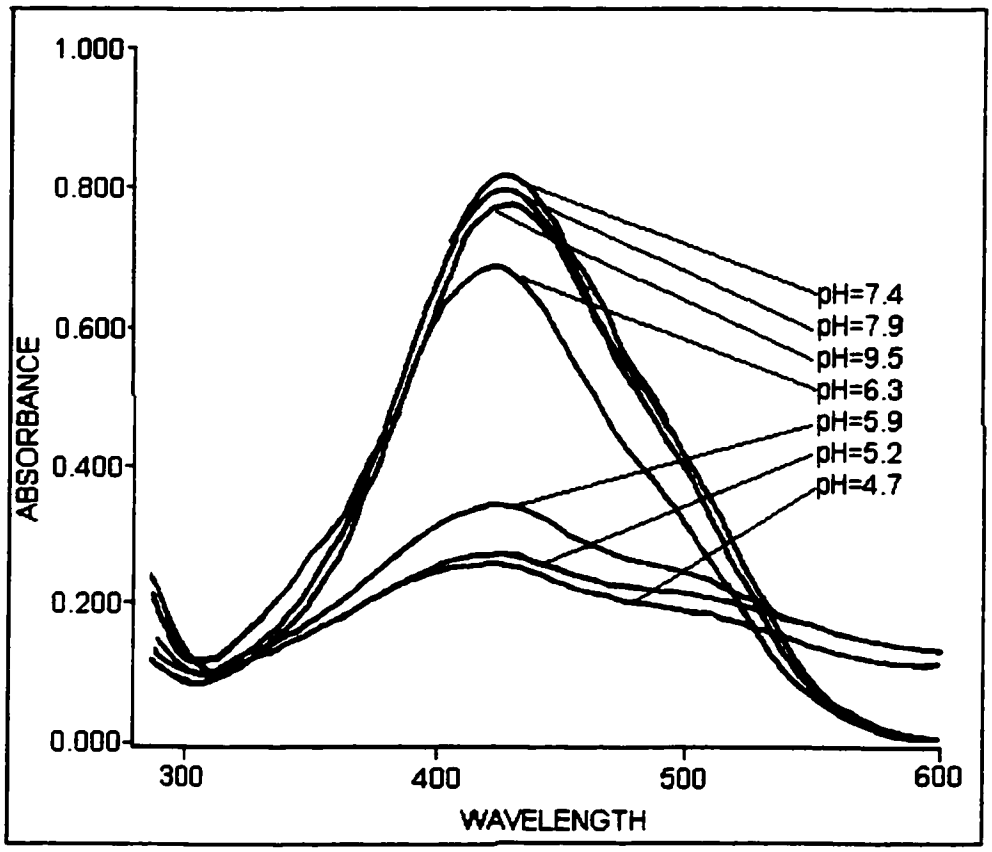


Figure 4.4.6 Effect of pH on the m-PD TNBS test
$[\text{m-PD}]_0$=20 μM, $[Na_2SO_3]_0$=2 mM, reaction time=60 min

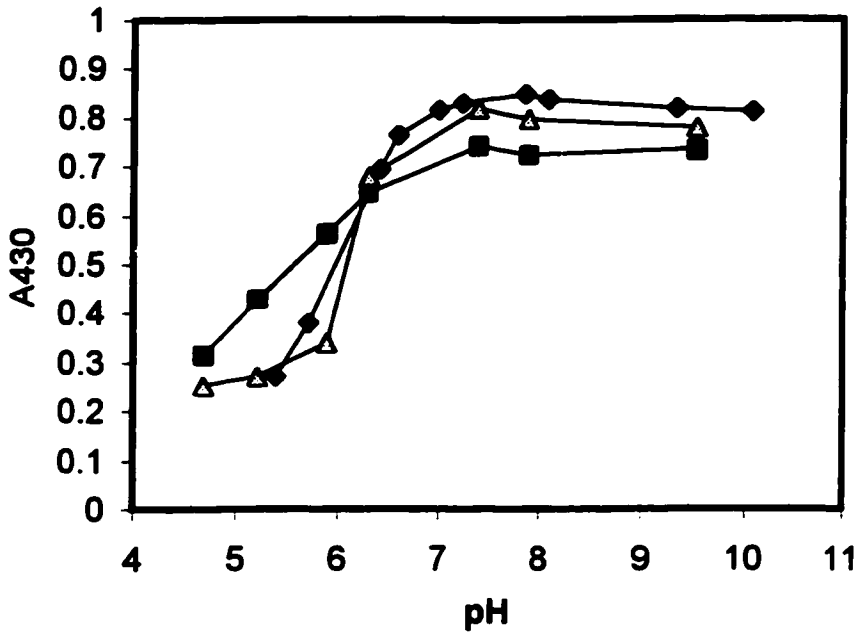


Figure 4.4.7 Effect of pH on the o-PD, m-PD and p-PD TNBS test
♦--p-PD (20 μM), 60 min ■--o-PD (40 μM), 200 min
▲—m-PD (20 μM), 60 min

4.4.2.2 Optimum sodium sulfite concentration

A series of experiments were conducted to choose the optimum sodium sulfite concentration and the response time at pH 7.4 and 60 min reaction time for p-PD, m-PD, 200 min reaction time for o-PD by using the same procedure with 20 μM p-PD and m-PD, 40 μM o-PD, and pH 7.4 buffer. The absorbance at 430 nm at various sodium sulfite concentrations was determined (Figure 4.4.8). From this figure, it is concluded that the 2 mM sodium sulfite is the appropriate concentration at pH of 7.4.

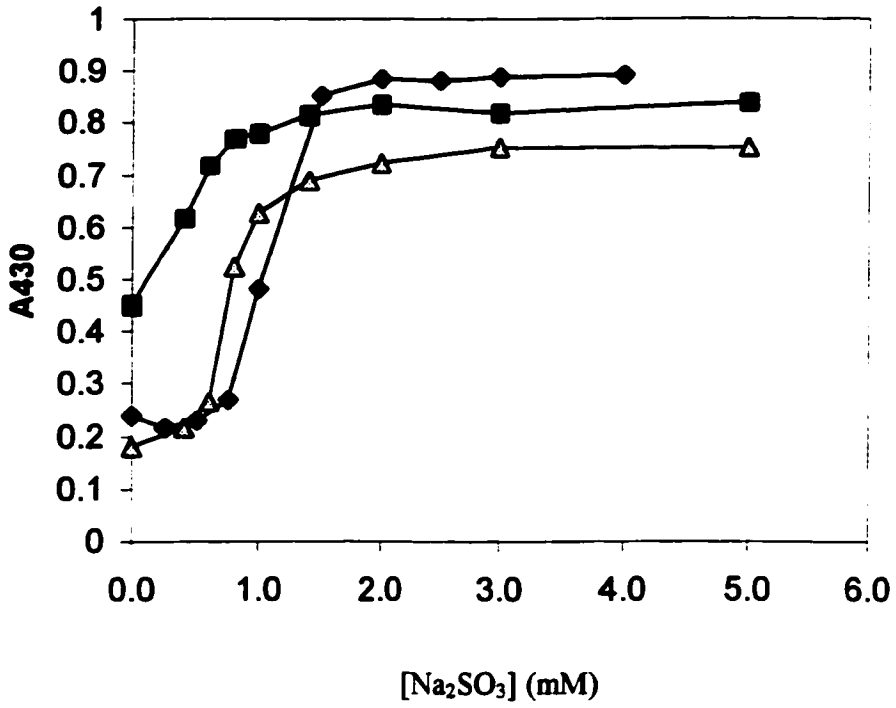


Figure 4.4.8 Effect of sodium sulfite concentration on the p-PD, o-PD and m-PD TNBS test . ♦----p-PD (20 μM), 60 min; ■----o-PD (40 μM) 200 min; ▲—m-PD (20 μM), 60 min

4.4.2.3 Optimum response time

TNBS tests were performed at pH=7.4, with 2 mM Na_2SO_3 for p-PD, m-PD, o-PD and aniline to determine the optimum reaction time. The results of absorbance at 430 nm with time (Figures 4.4.9 to 4.4.12) show that the absorbance increased quickly in the first 10 min, reached their maximum values and remained stable for a certain period of time, then decreased slowly thereafter. From these tests, 60, 60 and 200 minutes optimum response time were selected for p-PD, m-PD and o-PD respectively.

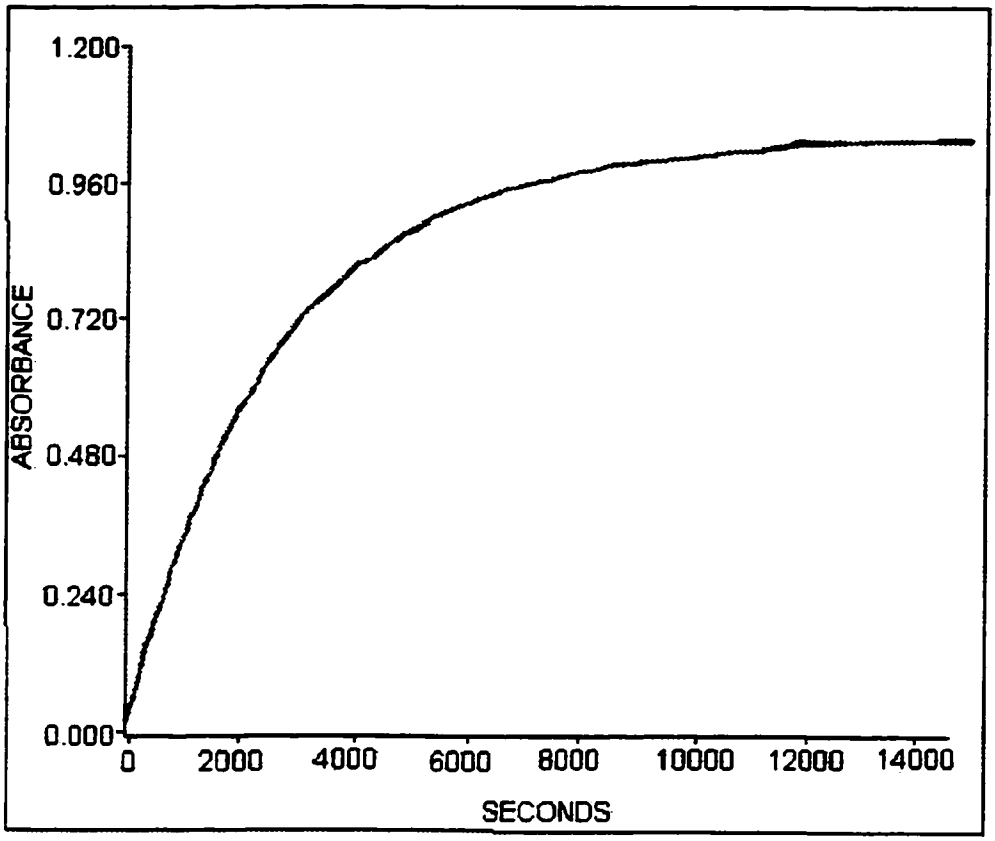


Figure 4.4.9 Effect of time on the 1,2-phenylenediamine TNBS test
[o-PD]=40 μM, Buffer pH=7.4, [Na_2SO_3]=2 mM

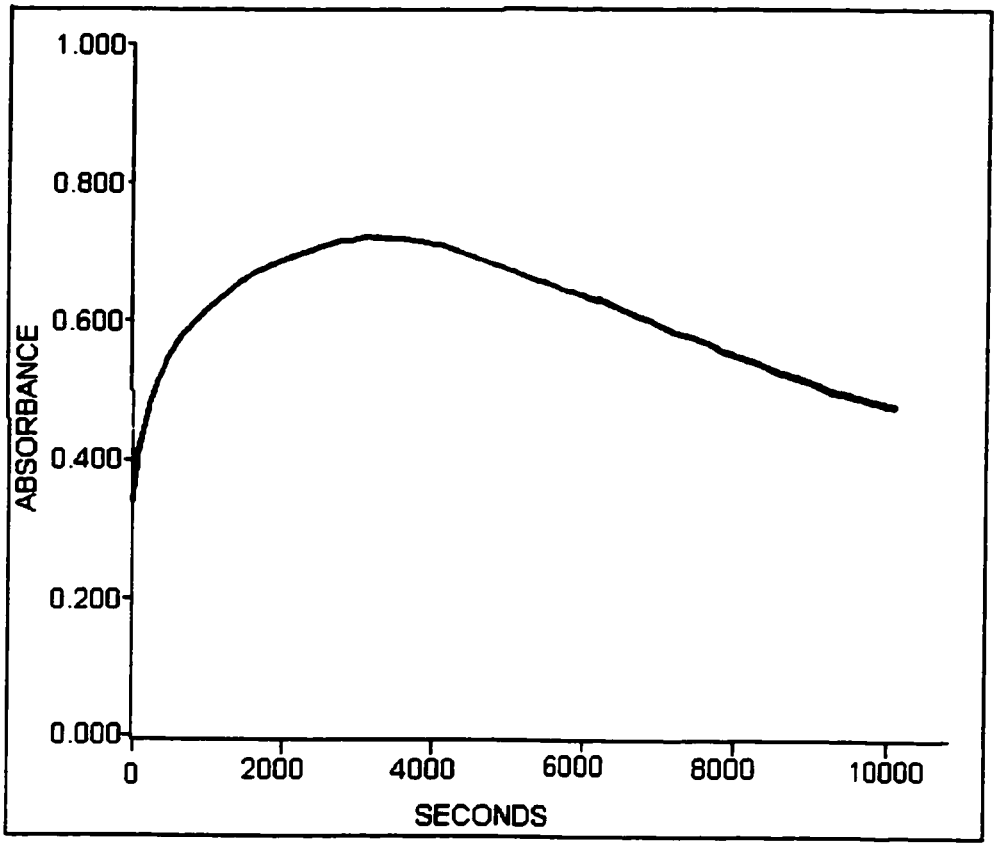


Figure 4.4.10 Effect of time on the 1,3-phenylenediamine TNBS test
[m-PD]=20 μM, Buffer pH=7.4, [Na_2SO_3]=2 mM

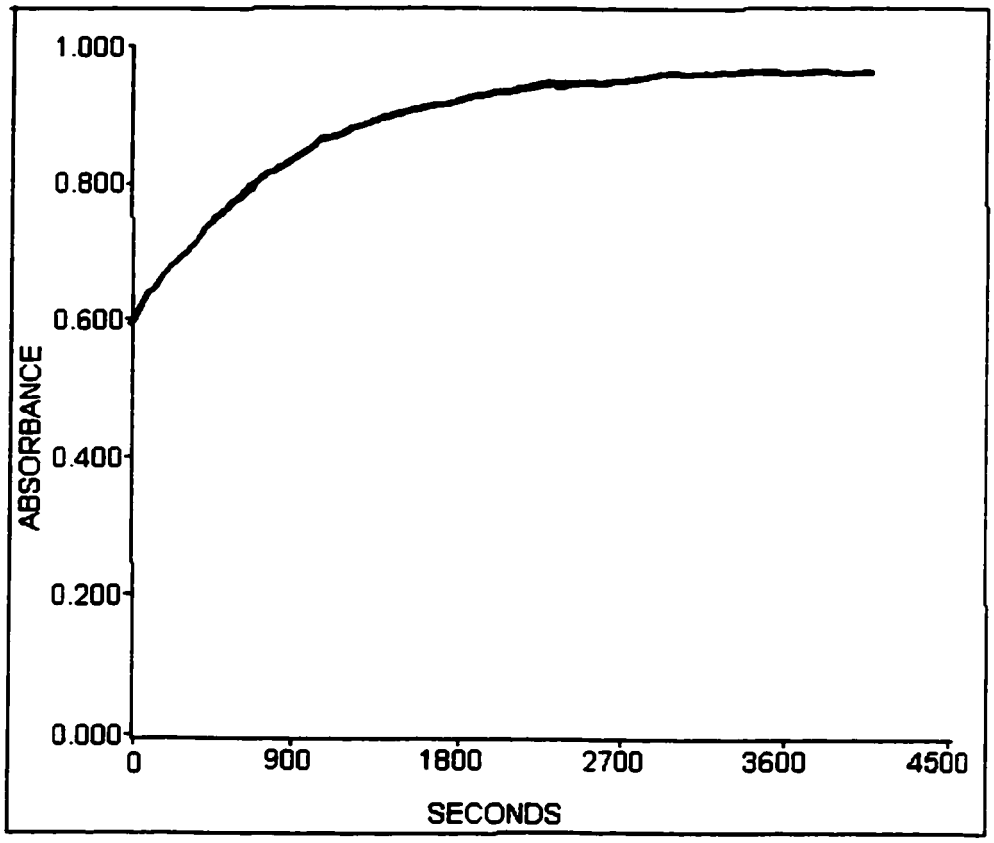


Figure 4.4.11 Effect of time on the 1,4-phenylenediamine TNBS test
[p-PD]=20 μM, Buffer pH=7.4, [Na_2SO_3]=2 mM

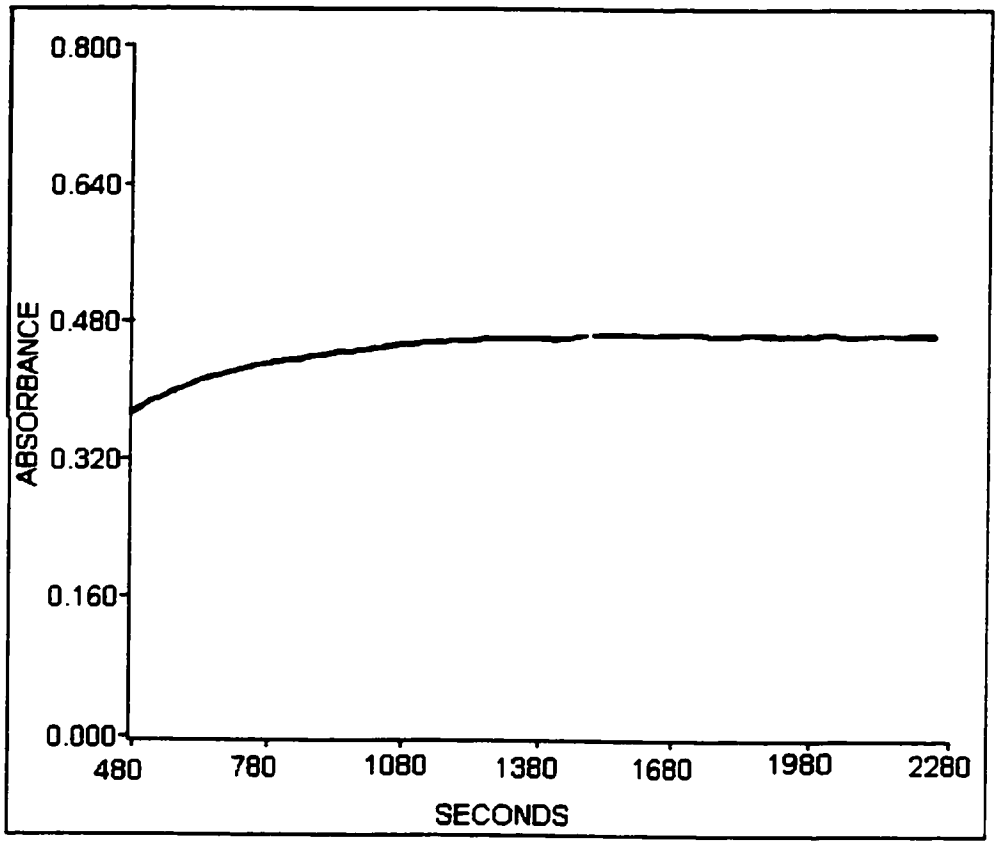


Figure 4.4.12 Effect of time on the aniline TNBS test
Aniline concentration was 20 μM, Buffer pH=7.4, [Na_2SO_3]=2 mM

4.4.2.4 Blank stability

The absorbance of the TNBS test blank was measured with time at 430 nm (Figure 4.4.13). The blank itself grew slowly with time; in 10 hours, the absorbance changed from 0.85 to 1.0. Therefore, it is recommended that the blank should have the same history as the sample.

4.4.2.5 Linear relationship between the absorbance and PD concentrations

Three parallel 2-20 μM p-PD TNBS calibration curves were obtained by using buffer of pH 7.4, 2mM sodium sulfite, and 60 minutes response time. The results are shown in Table 4.4.1 and Figure 4.4.14. They show a perfect linear relationship and both the slope and the intercept for the three tests are close. This provides confidence in the precision of this method. Linear relationships were also obtained for the m-PD, o-PD and aniline TNBS tests (Figure 4.4.15). Experiments showed that the extinction coefficient of the m-PD was close to that of p-PD, but for o-PD, the extinction coefficient was about half of that for the p-PD.

4.4.3 Optimization of aniline TNBS test

The TNBS test for aniline was performed by using the same procedure with pH of 7.4 buffer and 2mM sodium sulfite concentration. It was found that 30 minutes response time was enough to obtain a maximum steady absorbance (as shown in Figure 4.4.12).

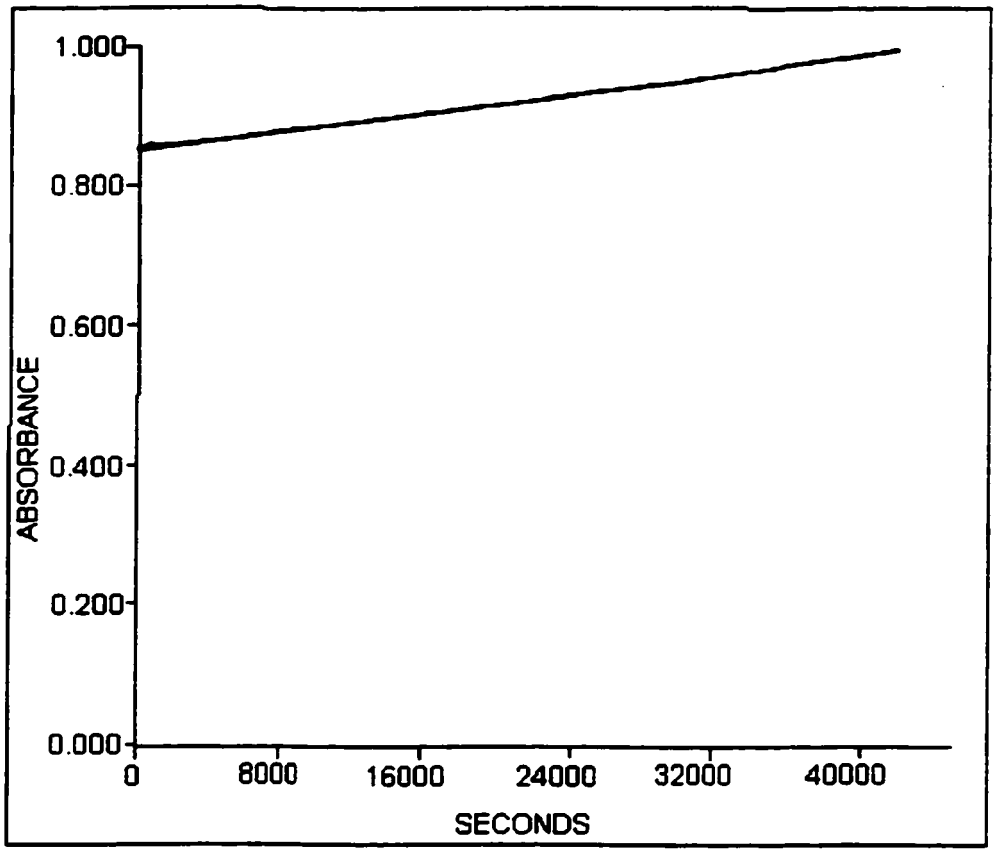


Figure 4.4.13 TNBS Blank stability

Table 4.4.1 Results of three parallel p-PD TNBS tests

p-PD (μM)	A430				Standard deviation (±)
	Test I	Test II	Test III	Average	
2	0.1014	0.0950	0.0986	0.0983	0.0032
4	0.1866	0.1938	0.1860	0.1888	0.0043
6	0.2917	0.2812	0.2791	0.2840	0.0068
8	0.3768	0.3755	0.3774	0.3766	0.0010
10	0.4705	0.4660	0.4682	0.4682	0.0023
12	0.5649	0.5492	0.5804	0.5648	0.0156
14	0.6542	0.6411	0.6446	0.6466	0.0068
16	0.7452	0.7308	0.7328	0.7363	0.0078
18	0.8313	0.8222	0.8229	0.8255	0.0051
20	0.9062	0.9097	0.9082	0.9080	0.0018

Table 4.4.2 Results of m-PD and p-PD TNBS calibration

Concentration (μM)	A430 for m-PD	A430 for p-PD
2	0.0672	0.0986
4	0.1535	0.1860
6	0.2433	0.2791
8	0.3221	0.3774
10	0.4029	0.4682
12	0.4933	0.5804
14	0.5825	0.6446
16	0.6727	0.7328
18	0.7551	0.8229
20	0.8466	0.9032

Table 4.4.3 Results of o-PD and aniline TNBS calibration

Concentration (μM)	A430 for o-PD	A430 for aniline
5	0.1211	0.1177
10	0.2368	0.2343
15	0.3587	0.3505
20	0.4748	0.4688
25	0.5957	0.5774
30	0.6994	0.6994
35	0.8245	0.8256
40	0.9314	

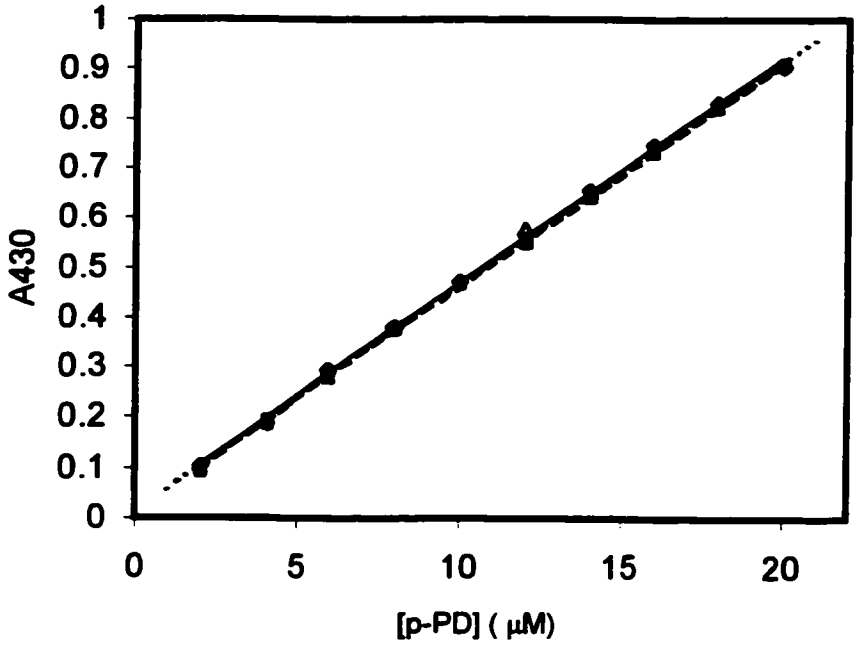


Figure 4.4.14 Three parallel p-PD TNBS calibration tests
(◆---- test I, y=0.0453x+0.0145, R^2=0.9993
■----test II, y=0.0452x+0.0093, R^2=0.9998
▲----test III, y=0.0452x+0.0123, R^2=0.9987
[p-PD]=20 μM, [Na_2SO_3]=2.0 mM, reaction time=60 min)

Table 4.4.4 Calibration curve parameters for three parallel p-PD TNBS tests

	Test I	Test II	Test III	Average	Standard deviation (±)
A	0.0453	0.0452	0.0452	0.0452	5.77E-05
B	0.0145	0.0093	0.123	0.0489	0.0642
R^2	0.9993	0.9998	0.9987	0.9993	0.00055

Note: A—the slope of the calibration curve; B—the intercept of the calibration cure Y=Ax+B. R^2--- square of the Pearson product moment correlation coefficient.

An excellent linear correlation was obtained as shown in Figure 4.4.15. The extinction coefficient for aniline was nearly one-half of that for the p-PD TNBS test.

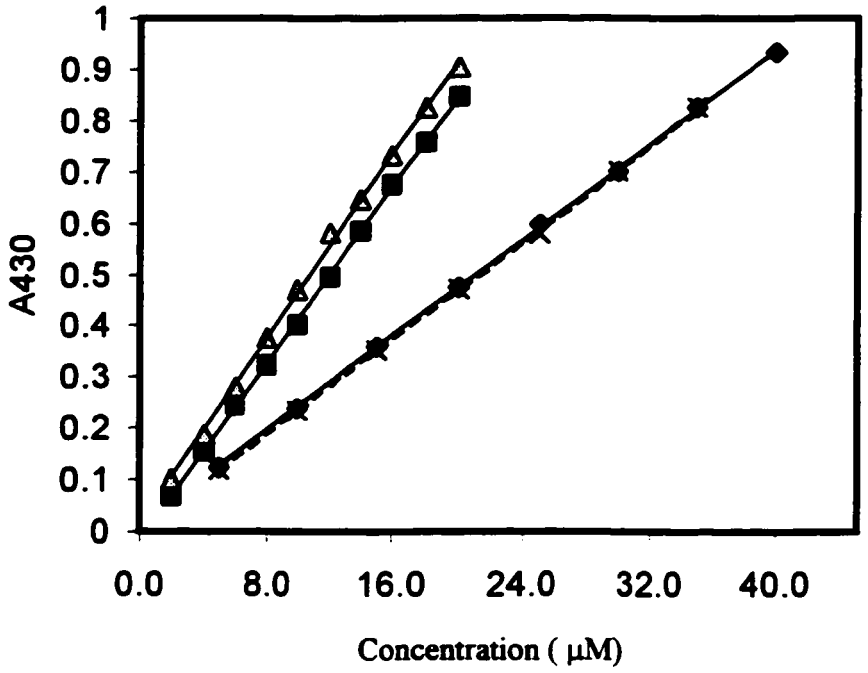


Figure 4.4.15 Calibration curve of p-PD, m-PD, o-PD and aniline TNBS test
(◆----o-PD, y=0.0232x+0.0078, R^2=0.9997;
▲----p-PD, y=0.0452x+0.0093, R^2=0.9998;
■----m-PD, y=0.0432x+0.0209, R^2=0.9998;
*----Aniline, y=0.0234x-0.001, R^2=0.9997.)

4.4.4 p-PD TNBS test compared to p-PD HPLC analysis

The p-PD was also analyzed by High Performance Liquid Chromatography (HPLC). The results of three parallel p-PD calibration curves calculated by area and height are shown in Figure 4.4.16 and 4.4.17, respectively. By comparing these plots to those obtained by TNBS test (Figure 4.4.14 and 4.4.15), it can be seen that the TNBS test provided better linear relationship than the HPLC analysis. Also, because the TNBS test

has more than 4 times the extinction coefficient at 430 nm than that of the p-PD at 240 nm and 20 times more than that of the p-PD at 302 nm, the TNBS test can detect very lower concentrations of p-PD and be more accurate than the HPLC analysis.

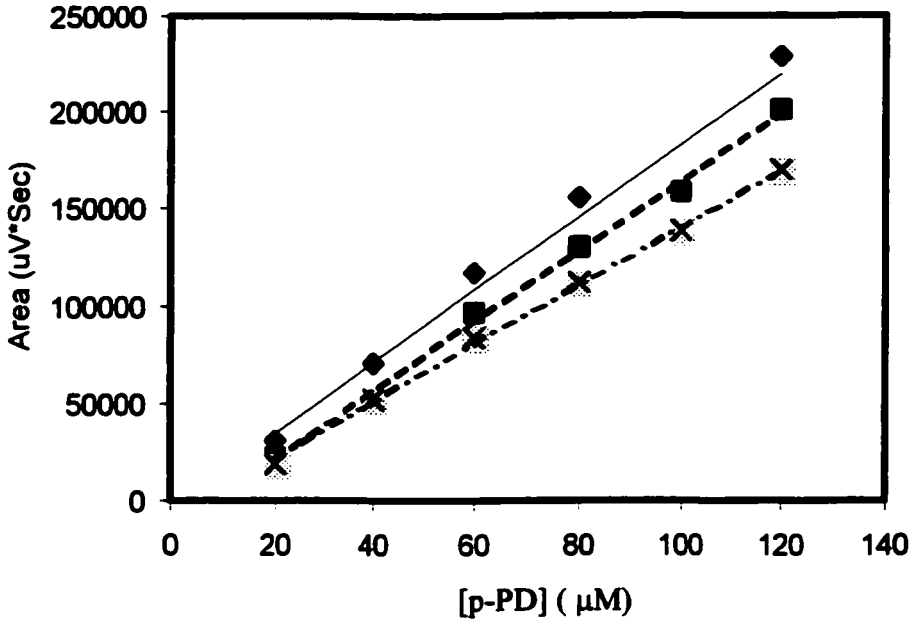


Figure 4.4.16 p-PD Calibration curve of HPLC analysis by area
◆---test I y=1850.4x-2822.5, R^2=0.9659
■---test II y=1783.8-14982, R^2=0.9961
×---test III y=1491.8x-8863.1, R^2=0.9986

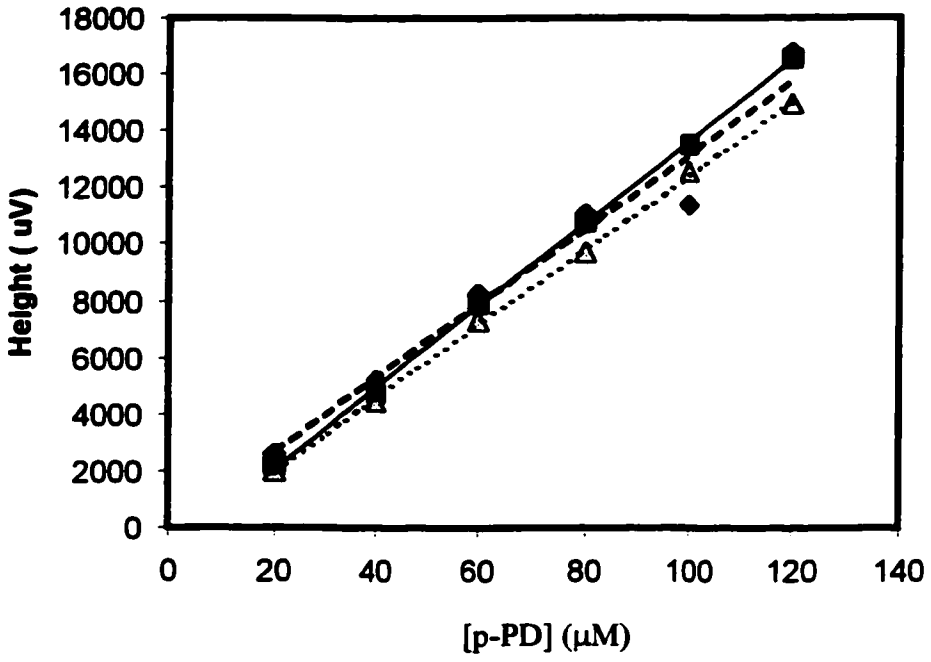


Figure 4.4.17 p-PD Calibration curve of HPLC analysis by height
◆---test I y=131.33x+5.8667, R^2=0.9643
■---test II y=144.4x-834.67, R^2=0.9993
▲---test III y=130.48x-659.33, R^2=0.9957

4.4.5 Summary of TNBS test conditions

The optimum conditions for testing different phenylenediamines and aniline are summarized in Table 4.4.5

Table 4.4.5 Summary of optimum test conditions

Chemical	Buffer pH	[Na_2SO_3] (mM)	Time (min)	Extin. Coeff. At A430($M^{-1} \cdot cm^{-1}$)	Intercept in calibration Equations	Suitable Conc. (μM)
p-PD	7.4	2	60	45300±1200	0.0093±0.0003	2-20
o-PD	7.4	2	200	23200±500	0.0078±0.0003	2-40
m-PD	7.4	2	60	43200±900	0.0209±0.0010	2-20
Aniline	7.4	2	30	23440±400	-0.001±0.0004	2-40

4.5 Error analysis

Three sets of experiments on the effect of hydrogen peroxide concentration on the color reduction of AR4, conducted on different dates, were chosen to determine the reliability of the results in this study. Concentrations of AR4 and ARP were 0.05 mM and 0.1 U/mL respectively, buffer pH was 8.2, and reaction time was 2 h. The results are shown in Table 4.5.1.

Table 4.5.1 Error analysis for color reduction

$[H_2O_2]$	Color remaining (%)			Average	Percent deviation(%)			Standard deviation
(μM)	Test I	Test II	Test III		Test I	Test II	Test III	(± %)
0	100	100	100	100.0	0.00	0.00	0.00	0.00
25	67.6	72.1	68.4	69.4	-2.55	3.94	-1.39	3.45
50	55.3	59.1	57.3	57.2	-3.38	3.26	0.12	3.32
75	43.0	46.0	44.8	44.6	-3.59	3.14	0.45	3.36
100	33.1	32.8	34.3	33.4	-0.90	-1.80	2.69	2.40
150	15.3	16.1	15.9	15.8	-2.96	2.11	0.85	2.53
200	6.4	6.7	6.6	6.6	-2.54	2.03	0.51	3.03
250	7.0	7.2	7.2	7.1	-1.87	0.93	0.93	1.41
300	7.9	8.1	7.9	8.0	-0.84	1.67	-0.84	1.25

Note: Test I, II, III were conducted on separate days

The results indicate that the deviation was less than ± 4%, which is within an acceptable range.

Similarly, error analysis was carried on TNBS test, by using the data presented in Figure 4.4.14 and is shown in Table 4.5.2. It again indicates that the overall error range was less than ±4%, and when the p-PD concentration was larger than 8.0 μM, the error

range was less than 2%. The standard deviation values of these tests were also very small. Therefore, the results obtained in this study are considered be accurate and reliable.

Table 4.5.2 Error analysis for TNBS test

p-PD (uM)	Absorbance at 430 nm			Average	Percent deviation(%)			Standard deviation (± %)
	Test I	Test II	Test III		Test I	Test II	Test III	
2	0.1014	0.095	0.0986	0.0983	3.12	-3.39	0.27	3.26
4	0.1866	0.1938	0.1860	0.1888	-1.17	2.65	-1.48	2.28
6	0.2917	0.2812	0.2791	0.2840	2.71	-0.99	-1.73	2.40
8	0.3768	0.3755	0.3774	0.3766	0.06	-0.28	0.22	0.26
10	0.4705	0.466	0.4682	0.4682	0.48	-0.48	-0.01	0.49
12	0.5649	0.5492	0.5804	0.5648	0.01	-2.77	2.76	2.76
14	0.6542	0.6411	0.6446	0.6466	1.17	-0.86	-0.31	1.05
16	0.7452	0.7308	0.7328	0.7363	1.21	-0.74	-0.47	1.06
18	0.8313	0.8222	0.8229	0.8255	0.71	-0.40	-0.31	0.62
20	0.9062	0.9097	0.9082	0.9080	-0.20	0.18	0.02	0.20

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 Color reduction of azo dyes by ARP and H_2O_2

On the basis of the experiments conducted with *Arthromyces ramosus* peroxidase (ARP) on color reduction of different dyes, the following conclusions are drawn:

- This study demonstrates the applicability of *Arthromyces ramosus* peroxidase for decolorizing the selected dyes, Acid Red 4, Crocein Orange G and Naphthol Blue Black. The highest color removal efficiencies obtained were 84%, 94% and 95% respectively.
- Due to the poor solubility and the nitro group in the structure of Disperse Orange 3, ARP did not reduce the color.
- The reaction parameters optimized were pH, ARP concentration, hydrogen peroxide concentration, and reaction time.
- The optimum pH for the enzyme-based color reduction was generally in the basic range; however, NBB had a relatively broad optimum pH range from 4.0 to10.0. For Crocein Orange G, the results showed the decolorizing reaction was strongly dependent on the pH and the optimum pH range was narrow as compared to NBB and AR4.
- High enzyme concentration had no significant effect on the color reduction of dyes; however, limiting the amount of ARP resulted in lower color removal efficiency.
- As the molar ratio of peroxide to dye increased, the color removal efficiency increased until the optimum ratio was reached. The optimum H_2O_2 stoichiometry with

respect to dyes depended on the number of azo bonds in the dyes. For AR4 and COG, which have only one azo bond, the optimum H_2O_2 to dye molar ratios was 2. For NBB, which has two azo bonds in its structure, the optimum H_2O_2 to dye molar ratio was 4. Therefore, the optimum H_2O_2 stoichiometry with respect to azo group was 2. Beyond the optimum ratio, there was a decrease in color removal efficiency.

- The reaction time was dependent on the pH and enzyme concentrations. With higher enzyme concentration, the reactions reached their end point faster.

5.1.2 Color reduction of Disperse Orange 3 by zero-valent iron

Through the experiments of color reduction of Disperse Orange 3 by zero-valent iron, the following conclusions can be drawn.

- The results of experiments showed the color of DO3 was successfully reduced by zero-valent iron. The efficiency of conversion was more than 98 %.
- Three different methods, the characteristic absorbance, the TNBS test and HPLC analysis were used to identify the product of reduced DO3. These methods gave evidence that the DO3 had been reduced to p-PD.
- A comparison between the HPLC method and the TNBS method for p-PD shows that the TNBS test is more accurate and reliable because it had a higher extinction coefficient and better linear correlation.

5.1.3 Removal of 1,4-phenylenediamine by ARP and H_2O_2

On the basis of the experiments conducted with *Arthromyces ramosus* peroxidase (ARP), the following conclusions are drawn:

- The results of this study demonstrate the applicability of using *Arthromyces ramosus* peroxidase for removal p-PD. The highest removal efficiency 95% can be achieved.
- The reaction parameters optimized were pH, ARP and hydrogen peroxide concentrations, and reaction time.
- When pH was in the range from 7.0 to 9.5, the enzyme efficiently catalyzed the polymerization of p-PD and removed it from the solution. The optimum pH of 7.6 was determined for highest removal efficiency and operation convenience.
- High enzyme concentration had no significant effect on the removal of p-PD. Experiments showed that even at low concentrations of enzyme, for example 0.02 U/mL for 100 μM p-PD and 0.05 U/mL for 300 μM p-PD, the p-PD removal efficiencies were more than 95 %.
- As the molar ratio between H_2O_2 and p-PD increased, the removal efficiency increased until the optimum ratio was reached. Under similar reaction conditions, 120 μM and 400 μM hydrogen peroxide reached the highest removal efficiencies for 100 μM and 300 μM p-PD, respectively. Thus the optimum H_2O_2 stoichiometry with respect to p-PD was a little higher than 1.0 even when there are two NH_2- groups in one p-PD structure. Beyond the optimum ratio, there was a decrease in color removal efficiency.
- Most of the p-PD could be removed in the first 1 ~ 2 hours. After that, higher removal efficiency was achieved with increase in reaction time, but the reaction rate was slower.

5.1.4 Colorimetric method for phenylenediamines and aniline

It is shown that the p-PD, m-PD, o-PD and aniline can be measured accurately by using the TNBS test. The optimum conditions for testing each of the chemicals are tabulated earlier in Table 4.4.5. With pH 7.4 buffer and in the presence of 2 mM sodium sulfite, the PDs and aniline react with TNBS to form products which have a peak absorbance at 430 nm. The absorbance shows linear relationships between the concentration of PDs and aniline. With 2 mM sodium fulfite, the extinction coefficients are twice higher than that of the methods developed by Monsef et al. (2000). Therefore, this method shows higher sensitivity. Since this method is simple and convenient, it is also better suited for site or field test. This method has more than 4 times the extinction coefficient at 430 nm than that of the p-PD at 240 nm and 20 times more than that of the p-PD at 302 nm, the TNBS test can detect very lower concentrations of p-PD and be more accurate than the HPLC analysis.

5.2 Recommendations

5.2.1 Color reduction of azo dyes by ARP and H_2O_2

The results of this study have shown that *Arthromyces ramosus* peroxidase can efficiently reduce the color the azo dyes Acid Red 4, Naphthol Blue Black and Crocein Orange G. In order to implement the enzymatic method of treatment to full-scale industrial application, following other aspects must be considered.

- The mechanisms and kinetics of color reduction of azo dyes by using *Arthromyces ramosus* peroxidase and hydrogen peroxide need to be studied.

- The potential toxicity and other properties of the final products and by-products should be investigated. Once the nature of these products is determined, a suitable disposal method can be chosen.
- Further studies should be conducted with the real industrial wastewater using a continuous flow system.
- Comprehensive cost analysis should be carried out once the parameters have been optimized.

5.2.2 Color reduction of Disperse Orange 3 by zero-valent iron

This study showed that the color of Disperse Orange 3 can be efficiently reduced by zero-valent iron. For future industrialization, other disperse dyes or other non-water soluble dyes should be studied using this method.

5.2.3 Removal of 1,4-phenylenediamine by ARP and H_2O_2

- Further study of the mechanism and kinetics of the enzyme catalyzed polymerization of p-PD is necessary before it can be used by industry.
- The properties of the polymerized product are still unclear and further studies are recommended.
- The p-PD used in this study was pure p-PD. Further studies using product(s) from the reduced DO3 or other dyes should be conducted.
- Further studies about the combination of zero-valent iron color reduction and enzymatic waste removal by ARP and H_2O_2 should be conducted.
- Comprehensive cost analysis should be carried out once all the parameters have been optimized.

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APPENDIX A

ARP Activity Assay

General

The purpose of the assay is to determine the amount of active enzyme present in a sample. The assay uses saturating concentrations of phenol, 4-aminoantipyine (AAP), and appropriate concentration of hydrogen peroxide (H_2O_2) such that the initial reaction rate is proportional to enzyme activity. The initial rate is measured by observing the rate of color formation in a solution in which a reaction between phenol and hydrogen peroxide is catalyzed by ARP such that the products of the reaction react with AAP to form a red-colored solution which absorbs light at a peak of 510 nm with an extinction coefficient of 6000 M^{-1} cm^{-1}.

1. Preparation of reagents

a. Phosphate buffer (0.5 M NaPP, pH 7.4)

In a 1000 mL flask , add the following

13.796 g of monobasic sodium phosphate ($NaH_2PO_4.H_2O$)

56.78 g of dibasic sodium phosphate(Na_2HPO_4)

distilled water to make up to 1 L.

b. Phenol (0.1 M phenol) in 0.5 M phosphate buffer pH 7.4

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

c. Hydrogen peroxide (100 mM H_2O_2)

Dilute 556.5 mL of 30% (w/v/) hydrogen peroxide to 50 mL with distilled water

d. Assay mixture

In a beaker , add the following:

50 mg of AAP

10 mL of 0.1 M phenol in 0.5 M phosphate buffer

0.2 mL of 100 mM H_2O_2

dilute with distilled water to a final volume of 25 mL

2. Procedure

The total assay volume is 1 mL, and the assay should be conducted before the substrate depletion become significant. Therefore, in a semi-micro cuvette, place solution in the following order:

250 μL of the assay mixture

50 to 750 μL sample containing ARP

distilled water, to make up to a total volume of 1.0 mL.

The activity of ARP in the cuvette is proportional to the rate of change in absorbance at 510 nm. As soon as the ARP has been added, shake the cuvette and monitor change in absorbance with time at 510 nm.

3. Calculations

One unit of activity is defined as the number of micromoles of hydrogen peroxide utilized in one minute at pH 7.4 and 25 °C in an assay mixture consisting of 10 mM phenol, 2.4 mM AAP, and 0.2 mM H_2O_2. The activity of ARP in the cuvette is obtained from the average slope (absorbance unit per minute) within the linear range. Therefore,

the activity within the cuvette, in units of μmol min^{-1} mL^{-1} (i.e U/mL) is calculated according to:

$$\text{Activity (U/mL)} = \frac{\text{slope (au/min)}}{\text{6000 au L/mol}} \times \frac{10^6\ \mu\text{mol}}{\text{mol}} \times \frac{1\text{L}}{\text{1000 mL}} \qquad \text{(A.1)}$$

In which au represents absorbance units and 6000 au L/mol relates color development to peroxide consumption.

Calculate the activity of the sample according to:

$$\text{Sample activity (U/mL)} = \text{activity U/mL} \times \frac{1000}{\text{Sample volume, mL}} \qquad \text{(A.2)}$$

APPENDIX B

Hydrogen peroxide assay

General

This end-point colorimetric assay was used to measure the concentration of hydrogen peroxide in a sample .The assay uses *Arthromyces ramosus* peroxidase as a catalyst and 4-aminoantipyrine as a color generating cosubstrate in combination with an aromatic substrate, phenol, in the assay mixture. In this assay, the amount of hydrogen peroxide introduced into the assay sample is the only limiting reactant; therefore, the degree of the color developed in the reaction is proportional to the amount of peroxide in the sample. Once the maximum amount of color has developed, the absorbance at 510 nm is converted to the hydrogen peroxide concentration in the cuvette by means of a calibration curve. The hydrogen peroxide concentration in the sample is then calculated according to the dilution the sample has undergone in the cuvette.

1. Preparation of reagents

a. phosphate buffer (0.5 M Na PP, pH 7.4)

In a 1000 mL flask, add the following

13.796 g of monobasic sodium phosphate ($NaH_2PO_4.H_2O$)

56.78 g of dibasic sodium phosphate (Na_2HPO_4)

add distilled water make up to 1 L.

b. phenol (0.1 M phenol) in 0.5 M phosphate buffer pH 7.4

Dissolve 9.411gm of phenol in 1000 mL of 0.5 M phosphate buffer solution

c. Assay mixture

In a beaker , add the following:

41 mg of 4-aminoantipyrine (AAP)

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

200 mL of ARP stock solution (~2000 U/mL)

9.8 mL distilled water

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

2. Calibration Procedure

Make up a stock solution of hydrogen peroxide with a concentration of 1.0 mM. From this stock solution prepare standards ranging from0 to 1.0 mM. In a test tube place the following solutions:

200 mL of the assay reagent

750 mL distilled water

50 mL of standard sample

The total volume of the assay mixture must be 1 mL and hydrogen peroxide concentration in the assay mixture should be below 50 mM. Immediately after the addition of hydrogen peroxide standard, shake the tube and then wait until the color is fully developed (mostly after 10 minutes). Put the assay mixture in a semi-micro cuvette and read the standards, using triplicate measurements. Make a plot of absorbance versus hydrogen peroxide concentration in the cuvette and determine the slope of the trace using linear regression.

3. Measurement of hydrogen peroxide

200 mL of the assay reagent

50 mL to 800 mL of sample

distilled water to make up to 1.0 mL total volume

Immediately after addition of sample, shake the cuvette and then wait for the full development of color. Read the maximum amount of absorbance at the peak wavelength of 510 nm against a reagent blank. Determine the cuvette H_2O_2 concentration from the calibration curve.

4. Calculations

Calculate the sample hydrogen peroxide concentration from:

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \text{ Ç } (1000 \text{ mL} / \text{ Sample volume mL}) \quad \text{(B.1)}$$

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