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Removal of various aromatic compounds from synthetic and refinery wastewater using soybean peroxidase

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Removal of Various Aromatic Compounds from Synthetic and Refinery Wastewater using Soybean Peroxidase

By

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ABSTRACT

Crude soybean peroxidase (SBP), isolated from soybean seed hulls, catalyzed the oxidative polymerization of hazardous aqueous pollutant aryldiamines, aryldiols, 2-mercaptobenzothiazole and phenol in the presence of hydrogen peroxide. Experiments were conducted to investigate the optimum operating conditions including pH, hydrogen peroxide-to-substrate concentration ratio and the minimum SBP concentration required to achieve at least 95% conversion of these pollutants in synthetic and refinery wastewaters. In addition, the effect of PEG_{3350} on enhancing the conversion efficiency was studied. The substrate conversion and hydrogen peroxide consumption were monitored over the period of the reactions. The enzymatically-generated polymeric products from aryldiamines could be removed with surfactant, sodium dodecyl sulphate (SDS), whereas polyvalent metal cation salt, aluminium sulphate (Alum), was able to remove the products from aryldiols. Enzyme-catalyzed polymerization using SBP and the methods for removal of the generated polymeric products can provide an alternative means to the conventional treatment methods for treating aromatic wastewater pollutants.

DEDICATION

This thesis is dedicated to

My Parents

Dr. ***Abdul Elah Al-Ansari*** - Dr. ***Haifa. Al-Assam***

&

My Sisters & brother

Hadeel - Nazik - Laith

For all the love, endless support and encouragement they have always given to me

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CHAPTER 1

INTRODUCTION

1.1 Pollutants

1.1.1 Industrial applications

Phenylenediamines are commonly used as a raw material intermediate in the synthesis of dyes, polyamides and maleimides [48]. O-phenylenediamine is employed in paints, dyestuffs, rubbers, coating production and in the synthesis of fungicides, pigments and corrosion inhibitors [47]. It has also been utilized in mining ores and the chemical processing industry to remove elemental sulfur and aldehyde impurities [49]. M-phenylenediamine and p-phenylenediamine are used in preparing synthetic fibers (Aramid fibers) for aerospace and military applications. They are also being used in enamel wire coating, as an antioxidant in rubber compounds, aldehyde impurities removal, photographic products, printer/facsimile inks and in hair, leather and textiles dyes [49, 50, 51]. Polymers based on phenylenediamine have unique properties such as high temperature stability, high strength, and high electron-conducting ability. Polymers of phenylenediamine have been used as a catalyst, functional glucose biosensor, creation of electrochromic films and microfibriles [53, 52].

Phenols and aryldiols are used in many industries such as petroleum refining, coal conversion, wood preservation, metal casting, pulp, paper, dyes, resin, plastics and textiles manufacturing. Phenol is one of the early compounds that have been used in the industry since the 1860s. It was used as antiseptic and then later in the synthesis of dyes, aspirin and picric acid (explosives) [54]. Nowadays, phenol is utilized in the production of phenolic resins that are used in the plastic (such as melamine and bakelite), plywood (as a bonding agent), metal casting, construction, automotive, and appliance industries [54-56].

Phenol is also being employed in the synthesis of other phenolic compounds such as bisphenol A and caprolactam that are used in the production of epoxy resins and nylon, respectively. Phenolic compounds are also being used in pharmaceuticals products (such as ear/nose drops and throat lozenges) [54], perfumes, cigarettes smoke and smoked products [57]. Aryldiols (catechol, resorcinol and hydroquinone) are used as developing agents in photography, lithography and x-ray films. They are also being used as intermediates in the production of fur dyes, antioxidants in rubbers and lubricating oil. They are also being utilized as a topical antiseptic reagents and antifungal preservatives [58] [55]. Polymers of phenolic compounds have also been widely used in resin industry as a result of its unique properties [59].

2-Mercaptobenzothiazole is used mostly in the rubber industry. It is used as a corrosion inhibitor, fungicide, additive in greases and as a vulcanization accelerator for rubber tire and carcasses. It is present in goggles, latex gloves, wet suits and diving masks [60].

1.1.2 Health hazards

Phenylenediamines have been shown to have an extreme effect on human health upon direct absorption by the skin, ingestion and inhalation exposure. Phenylenediamines can cause severe dermatitis, urticaria, hyperreflexia, hyporeflexia, chemosis, lacrimation, exophthalmos, ophthalmia and even permanent blindness to the eye [60]. They can also cause bronchial asthma, gastritis, hypertension, kidney failure, liver damage, urinary bladder infection, anemia and transudation into serious cavities, dizziness, tremors, coma and death. The p-phenylenediamine is more toxic and irritating than o- and m- isomers.

The o- and m- isomers are less toxic than p-phenylenediamine but found to cause DNA damage in human lymphocytes. Phenol, catechol, resorcinol, hydroquinone and 2-mercaptobenzothiazole have been shown to have similar effects on human health to that of phenylenediamines. Phenylenediamines, phenol, aryldiols and 2-mercaptobenzothiazole has not classified as human carcinogens (inadequate evidence). These compounds have not only effect on human health but also on animals and aquatic life. They can cause respiratory disturbance, tremors, convulsions, increased excitability and irritation in skin, nose, stomach and liver. No evidence of carcinogenicity exists for phenol, resorcinol, hydroquinone, 2-mercaptobenzothiazole, m- and p-phenylenediamines, but catechol and o-phenylenediamine have been classified as animal carcinogens [60].

1.1.3 Environmental contamination

The aromatic compounds studied are considered to be toxic and have been classified as hazardous pollutants. There is clear evidence that high concentrations of these compounds are corrosive, absorbed rapidly and produce severe toxicity that may result in death during dermal or oral exposure in humans. Those aromatic compounds are discharged into wastewater streams from various industries (Table 1.1). In water, aromatic compounds can be adsorbed strongly into suspended solids and sediments. Phenol and 2-mercaptobenzothiazole were found to be adsorbed, while aryldiols and phenylenediamines were not suspected to adsorb [60-62]. All of these compounds have high affinity binding to organic material in water [60, 63]. Volatilization of those compounds or their ionized forms is not expected to occur as a result of their low Henry's law constants, vapor pressure and solubility (Tables 1.2-1.5) [44, 45, 60, 64, 65]. Phenylenediamines react

rapidly with sunlit water via photooxidation-produced hydroxyl and peroxy radicals [60, 66]. Hydrolysis is not expected to occur in water environment [60].

Table 1.1: Chemical discharges (lb)			
Chemical (TRI Database, 2004)	Total On-site Disposal or Other Releases	Total Off-site Disposal or Other Releases	Total On- and Off-site Disposal or Other Releases
phenol	6,294,310 (2,855,051 Kg)	1,551,747 (703,861 Kg)	7,846,057 (3,558,912 Kg)
catechol	33,201 (15,060 Kg)	2,711 (1,230 Kg)	35,911 (16,289 Kg)
hydroquinone	565,236 (256,387 Kg)	9,697 4,398	574,933 260785
1,2-phenylenediamine	2,859 (1,297 Kg)	7,234 (3,281 Kg)	10,093 (4,578 Kg)
1,3-phenylenediamine	5,562 (2,523 Kg)	10,457 (4,743 Kg)	16,019 (7,266 Kg)
1,4-phenylenediamine	17,321 (7,857 Kg)	346 (157 Kg)	17,667 (8,014 Kg)
2-mercaptobenzothiazole	215,877 (97,920 Kg)	428,729 (194,468 Kg)	644,605 (292,388 Kg)

In soil, all of the studied aromatic compounds have high mobility except 2-mercaptobenzothiazole. The mobility of these compounds is expected to be reduced as a result of the high affinity of the aromatic compounds to humus or organic matter in the soil [60, 63]. The phenylenediamines and 2-mercaptobenzothiazole are found to be very slow or not subject to degradation in soil. Phenol and aryldiols are expected to biodegrade in soil [60, 67, 68]. In the atmosphere, phenylenediamines have the potential of direct photolysis as a result of their high absorption. The vapor-phase compounds can be degraded as a result of photooxidation-produced hydroxyl and peroxy radicals [60, 69, 70].

1.1.4 Current wastewater treatments methods

Over the past years, different waste treatment methods have been used to remove aromatic compounds but these methods suffer from serious drawbacks such as high cost,

incomplete removal, formation hazardous byproducts, low efficiency, intensive energy requirements or applicability to a low level concentration range [71]. The current treatment methods for phenol, aryldiols, aryldiamines and 2-mercaptobenzothiazole compounds are physical/chemical or biological treatment systems.

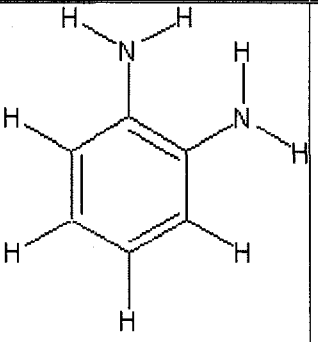
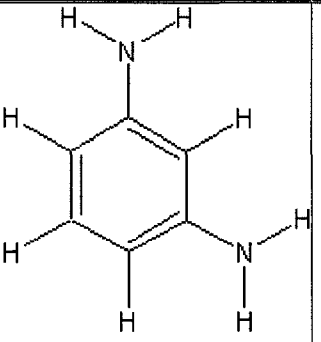
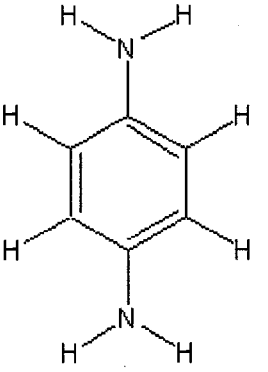
The physical/ chemical treatment methods used are activated carbon adsorption, ozone oxidation, Fenton reaction, landfilling, coagulation, chlorine/chlorine dioxide oxidation, photocatalytic oxidation, potassium permanganate oxidation and incineration. The drawbacks in physical and chemical treatment systems are the insufficient removal of the organic compounds from wastewater, unselectivity in terms of the range of pollutant removed during the treatment, high cost especially for concentrated waste, high energy input and the production of toxic byproduct compounds [71, 72].

The biological treatment methods used are activated sludge, bio-oxidation, aerated lagoon, stabilization, trickling filter, oxidation ditch and rotating biological contactors. The drawback in biological treatment is the difficulty in handling and storing of the microorganism. Microorganisms require sufficient food, oxygen and stable environmental conditions, including pH, salt content and temperature in order to maintain its optimum efficiency. Also, high strength waste will kill the microorganisms due to shock loading effect. Therefore, biological methods are costly, require high energy and time [71, 72].

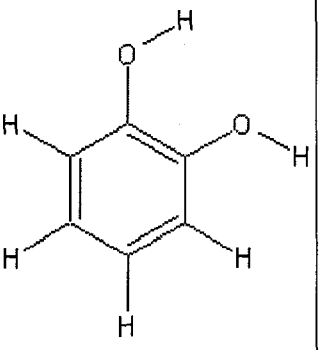
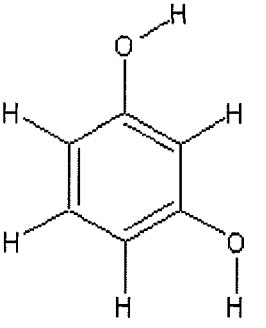
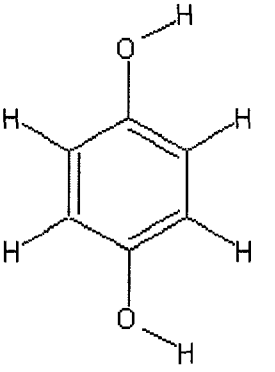
None of the traditional physical-chemical and biological treatment methods has been found to be very suitable for the removal of the aromatics and their color. In contrast, enzymatic treatment overcomes these drawbacks and is more suitable for their removal.

1.1.5 Chemical and physical properties

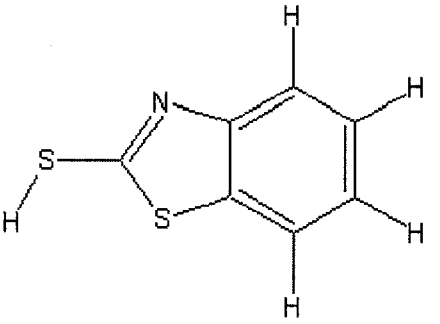
Aryl amine:

Table 1.2: Chemical and physical properties of aryldiamines			
	o-phenylenediamine	**m-phenylenediamine**	**p-phenylenediamine**
Molecular Structure			
Synonyms	1,2-diaminobenzene	1,3-diaminobenzene	1,4-diaminobenzene
Molecular Formula	$C_6H_4(NH_2)_2$	$C_6H_4(NH_2)_2$	$C_6H_4(NH_2)_2$
Molecular Mass	108.1 g/mol [43]	108.1 g/mol [43]	108.1 g/mol [43]
Appear-ance	Red-brown solid [44]	Brown-gray solid [44]	Brown-purple solid [44]
Chemical stability	Stable under normal temperature and pressure [44]	Light and air sensitive; Darkens on exposure [44]	Light and air sensitive; Darkens on exposure [44]
Melting Point	100-102 °C [43]	64-66 °C [43]	138-143 °C [43]
Boiling point	256-258 °C [43]	282-284 °C [43]	267 °C [43]
Solubility in water	36g/L (25°C) [45]	240 g/L (25°C) [45]	37g/L (25°C) [45]
Henry's law constant	7.2×10^{-9} atm-cu m/mole [64, 60]	1.3×10^{-9} atm-cu m/mole [64, 60]	6.7×10^{-10} atm-cu m/mole [64, 60]
vapor pressure	2.1×10^{-3} mm Hg [64, 60]	2.1×10^{-3} mm Hg [64, 60]	5.0×10^{-3} mm Hg [64, 60]

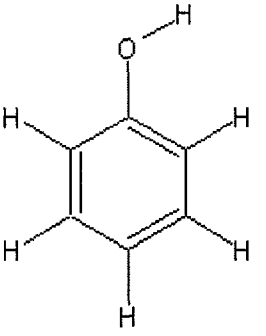
Aryldiols:

Table 1.3: Chemical and physical properties of aryldiols			
	Catechol	**Resorcinol**	**Hydroquinone**
Molecular Structure			
Synonyms	Pyrocatechol 1,2-dihydroxybenzene benzene-1,2-diol 2-hydroxyphenol o-benzenediol	Resorcin 1,3-dihydroxybenzene benzene-1,3-diol 3-hydroxyphenol m-benzenediol	Quinol 1,4-dihydroxybenzene benzene-1,4-diol 4-hydroxyphenol p-benzenediol
Molecular Formula	$C_6H_4(OH)_2$	$C_6H_4(OH)_2$	$C_6H_4(OH)_2$
Molecular Mass	110.1 g/mol	110.1 g/mol	110.1 g/mol
Appear-ance	White to brown[44]	White to light peige[44]	White to off-white
Chemical stability	Light and air sensitive[44]	Hygroscopic (absorbs moisture); Light and air sensitive; discolor on exposure[44]	Stable under normal temperature and pressure; Light and air sensitive [44]
Melting Point	104 °C[44]	109-111 °C[44]	170-174 °C[44]
Boiling point	245 °C[44]	281 °C[44]	285-287 °C[44]
Solubility in water	430g/L	1400g/L[44]	70g/L[44]
Henry's law constant	1.2×10^{-9} atm-cu m/mole [60, 65]	9.9×10^{-11} atm-cu m/mole [60, 65]	1.32×10^{-9} atm-cu m/mol [60, 65]
vapor pressure	3.66×10^{-3} mm Hg [60, 65]	4.9×10^{-4} mm Hg [60, 65]	6.7×10^{-4} mm Hg [60, 65]

Mercaptobenzothiazole:

Table 1.4: Chemical and physical properties of 2-mercaptobenzothiazole	
Molecular Structure	
Synonyms	2-benzothiazolethiol, benzothiazole-2-thiol
Molecular Formula	$C_7H_5NS_2$
Molecular Mass	167.2 g/mole
Appearance	Light yellow solid
Chemical stability	Stable
Melting Point	172-180 °C [46]
Boiling point	243 °C [46]
Solubility in water	0.118 g/L [45]

Phenol:

Table 1.5: Chemical and physical properties of phenol	
Molecular Structure	
Synonyms	carbolic Acid, benzenol, hydroxybenzene, phenic acid
Molecular Formula	C_6H_5OH
Molecular Mass	94.11 g/mole
Appearance	White solid [44]
Chemical stability	Hygroscopic (absorbs moisture) [44]
Melting Point	39-41 °C [44]
Boiling point	182 °C [44]
Solubility in water	80 g/L [44]
Henry's law constant	3.33 x^{10-7} atm cu m/mol [60]
vapor pressure	0.35 mm Hg [60]

1.2 Peroxidases:

An enzyme-based treatment method to remove organic compounds from aqueous wastewater was first proposed by Klibanov and co-workers in 1980. The proposed treatment method employed the enzyme-peroxidase from horseradish (HRP) in the presence of hydrogen peroxide catalyzing the oxidation of over 30 different phenols and aromatic amines [71]. During the same period, Bollag and co-workers started investigating the enzyme laccase catalyzing the oxidation of phenols by oxygen [74, 73]. Both enzymes will catalyze the oxidization of phenols and aromatic amines into aromatic radicals in presence of hydrogen peroxide (for peroxidases) or oxygen (for laccases). Those radicals diffuse from the active site of the enzyme into solution where they couple non-enzymatically to form dimers. If the dimers are soluble and still phenolic or arylamine, then they become substrates of the enzyme for another enzymatic cycle, forming higher oligomers. The cycle continues until the polymer generated reaches its solubility limit and precipitates out of solution, which can later be removed by filtration or sedimentation. The potential of other enzymes such *Coprinus cinereus* peroxidase (CCP), *Coprinus macrorhizus* peroxidase (CMP), *Arthromyces ramosus* peroxidase (ARP) and soybean peroxidase (SBP) were investigated later [1-7, 12, 75].

The enzyme-based treatment method has several advantages over other treatment methods as a result of the enzyme active-site's high specificity to the target pollutant, high efficiency in pollutant removal, lower cost and ease of handling and storage of the enzyme. The advantages of enzymatic treatment over biological treatment are the ability of enzyme to remove high concentrations of toxic compounds with no shock loads effect) that usually kill the microbes in biological treatment. The enzyme treatment operates

over wide temperature, pH and salinity ranges. The applicability of the enzyme to remove a broad range of compounds in the presence of other substances those are usually toxic to microbes. It's also a faster process since no delay occurs for acclimatization of biomass and there is no biomass generation resulting in reduction sludge volume. The enzyme-based treatments provide better defined system with simpler process control. The advantages of enzyme based treatments method over physical/chemical treatments are its operation under less corrosive conditions, trace level organic compounds and on organics not removed by existing chemical/physical processes. It reduces consumption of oxidants and the amounts of adsorbent material for disposal [72, 76].

1.2.1 Peroxidases as an enzyme

Enzymes are biological catalysts, which increases chemical reactions rates that take place in living cells. Enzymes are proteins that are composed of polymers of amino acids arranged in a specific sequence and linked by peptide bonds. Enzymes are tightly regulated, highly specific and act under mild conditions of pressure, temperature and pH. The active site of an enzyme dictates specificity to only one type of chemical group and type of reaction. The substrate that binds to the enzyme interacts in a stereo-specific manner (fit) with high affinity. Furthermore, the substrate has to react either by forming or breaking bonds. The active site of the enzyme consists of the catalytic site and the binding site. The catalytic site is where the reaction occurs and the binding site is the area that holds the substrate in a proper conformation. Normally, enzyme uses weak non-covalent interactions to hold the substrate in a place [77].

The peroxidases were among the first enzymes to be discovered in the 19th century. Peroxidases are distributed throughout the plant and animal kingdom and also found in bacteria and fungi. Peroxidases have been classified into three superfamilies: plant, animal and catalase peroxidases. The classification was based on the amino acid sequence, three-dimensional structure and biological function [8].

The plant peroxidase superfamily was subdivided further into three families based on their sequence homologies. Class I peroxidases are of prokaryotic origin such as yeast cytochrome *c*, chloroplast and ascorbate peroxidase. They have no calcium ions, cystine bridges, carbohydrate and no signal peptide for secretion. Classes II peroxidases are secreted fungal peroxidases such as lignin, manganese and *Arthromyces ramosus* peroxidase. They have two calcium ions, cystine bridges (four conserved) and a signal peptide for secretion. Class III peroxidases are classical secretory plant enzymes such as horseradish, peanut and soybean peroxidases. They have two calcium ions, cystine bridges (different location than class II), extra helices and a signal peptide for secretion [8, 78].

The animal peroxidase superfamily is larger than plant peroxidase one with larger differences in primary, tertiary structure. The animal peroxidases are similar to plant peroxidases in the helices that makeup the heme binding pocket. Examples of animal peroxidases are lactoperoxidase, myeloperoxidase, and eosinophil peroxidase [8].

The catalase peroxidase superfamily has been classified as one of the peroxidase subfamilies as a result of catalases ability to act as a peroxidase and the ability of Class II and III plant peroxidases to act as catalases. Catalases scavenge hydrogen peroxide, converting it to water and oxygen via a two-step enzymatic cycle. Catalases have similar

heme groups to that of peroxidase but the catalases have different structure and primary sequence than those of animal and plant peroxidases [8].

1.2.2 Peroxidase active site

Peroxidases have a common active site structure that contains very similar prosthetic groups and similar amino acid residues. The prosthetic group for all plant peroxidases is ferriprotoporphyrin IX, which is also called heme when coordinated with iron ion (Figure 1.1).

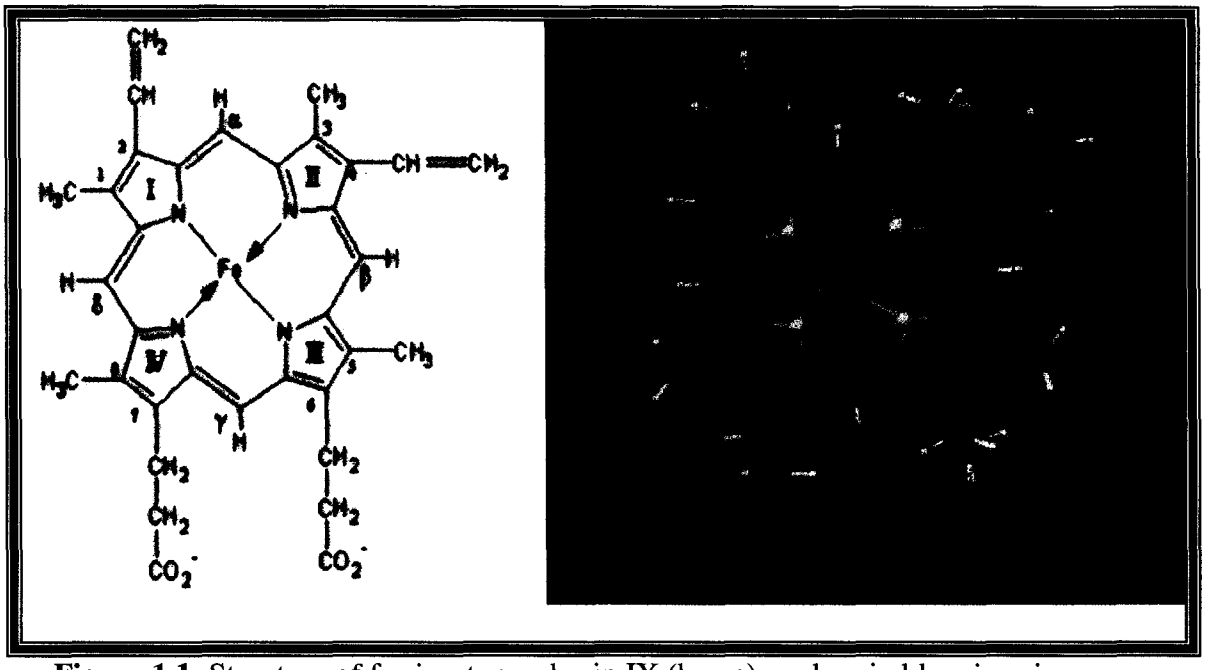


Figure 1.1: Structure of ferriprotoporphyrin IX (heme); carbon in blue, iron in gray, nitrogen in yellow, oxygen in red and hydrogen in white; ACDLABS-software [8]

Ferriprotoporphyrin IX consists of four pyrrole rings joined by methane-bridge (carbon atom labeled α, β, γ and δ) with iron (III) in the center of the molecule. The ferriprotoporphyrin IX also carries eight side chains, four methyl groups (positions 1, 3, 5

and 8; Figure 1.2), two vinyl groups (positions 2 and 4; Figure 1.2), and two propionate groups (positions 6 and 7; Figure 1.2) [8].

Figure 1.2 illustrates six possible positions for iron coordination in the active site of peroxidases. Four pyrrole nitrogen atoms occupy positions 1-4, that is also shown in Figure 1.1. The imidazole side chain of a histidine residue occupies position 5 which is located on the proximal side of the heme. Position 5 plays an important role in the linkage of the heme to the apoprotien. The linkage of the histidine residue to the iron (III) is best described as a covalent bond (linkage can be broken by using an acid). Position 6 is located on the distal side of the heme where all peroxidase reactions usually occur. In the native resting enzyme, the iron (III) has five coordination where position 6 is vacant. The enzyme can be inhibited by ligand that can coordinate with the iron (III) in position 6 such as cyanide or azide [8].

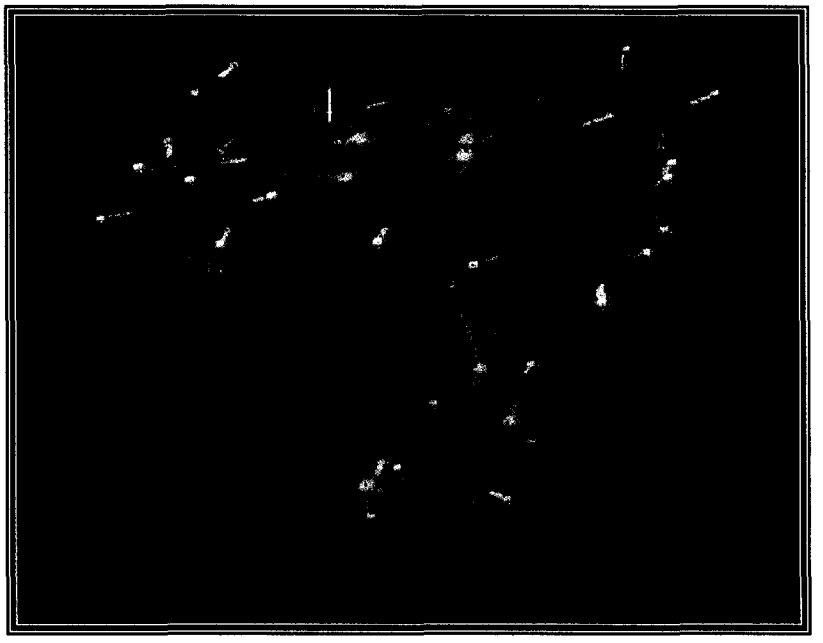


Figure 1.2: Imidazole side chain of a histidine residue bonding to the iron atom of the heme; carbon in blue, iron in gray, nitrogen in yellow, oxygen in red and hydrogen in white; ACDLABS-software [8]

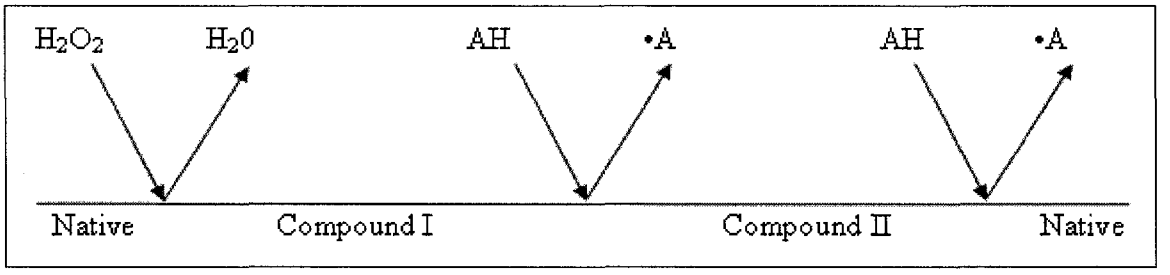
1.2.3 Peroxidase mechanism

The peroxidase reaction cycle is a modified bi-bi ping-pong mechanism (Scheme 1.1). The native form of the enzyme (E_N) reduces hydrogen peroxide to water and is converted to Compound I (E_1), active form of the enzyme (Equation 1.1). The Compound I is capable of oxidizing a reducing substrate (AH) such as phenols and arylamines, generating a free radical ($A^{\bullet}$) and is converted to Compound II (E_2) of the enzyme (Equation 1.2). The Compound II is capable of oxidizing another reducing substrate, generating another free radical and returning the enzyme to its native form (Equation 1.3) [8].

Native peroxidase (E_N) + H_2O_2 ⟶ Compound I (E_1) + H_2O (1.1)

Compounds I (E_1) + AH ⟶ Compound II (E_2) + •A (1.2)

Compounds II (E_2) + AH ⟶ Native peroxidase (E_N) + •A + H_2O (1.3)



Scheme 1.1: Proposed ping-pong mechanism for peroxidases

The major different between the peroxidase ping-pong mechanism and the conventional ping-pong mechanism is the reaction reversibility. The conventional ping-pong mechanism is reversible while the peroxidase ping-pong mechanism is irreversible. A reversible reaction indicates that there is a finite upper limit in rate. Therefore, when the substrates are in low concentrations, the rate-limiting step is the substrate binding.

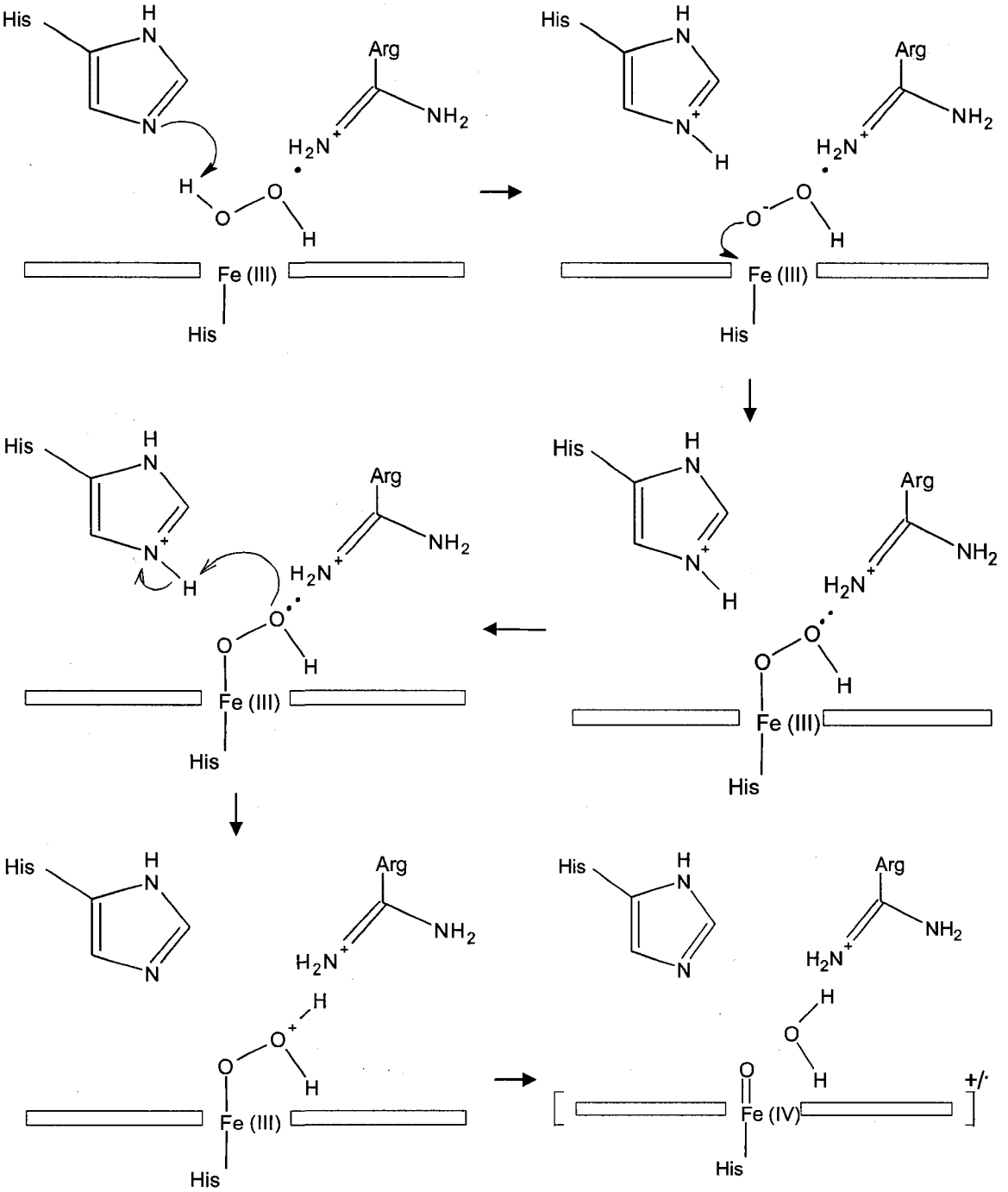
While, when the substrates are in a saturated level to the enzyme, the rate-limiting step is the product release. An irreversible reaction, in the peroxidase mechanism, appears to have no upper limit in rate. The generated enzyme-peroxide complex is short-lived, i.e. as fast as the peroxide comes in contact with the enzyme, the product departs directly. Hence, the higher the peroxide concentration, the higher is the rate of reaction. This does not mean that the reverse reaction does not occur but it's just not measurable. Saturation or enzyme deactivation (by hydrogen peroxide) could result as the peroxide concentration increases [8, 78].

Mechanism of Compound I formation:

In the active site of the enzyme, two amino acid residues play a significant role in the transfer of oxygen atom from hydrogen peroxide to iron (III) in the mechanism of Compound I formation. The two amino acids are histidine and arginine located on the distal side of the heme (position 6). Distal histidine accepts a proton from the hydrogen peroxide (involved in acid-base catalysis) where distal arginine acts as a charge stabilizer. The distal histidine and arginine feature is conserved through the plant peroxidase superfamily such as in HRP, ARP and SBP [8].

The mechanism for the heterolytic cleavage of the O-O bond of hydrogen peroxide is shown in Scheme 1.2. The distal histidine accepts a proton from the oxygen atom of unionized hydrogen peroxide. Formation of the Fe-OOH intermediate result from the negative charge on the peroxide and the proton on the proximal histidine that facilities the electron flow. The heterolytic cleavage of the O-O bond leading to the formation of the ferryl group (Fe =O) is facilitated by the proton transfer from distal

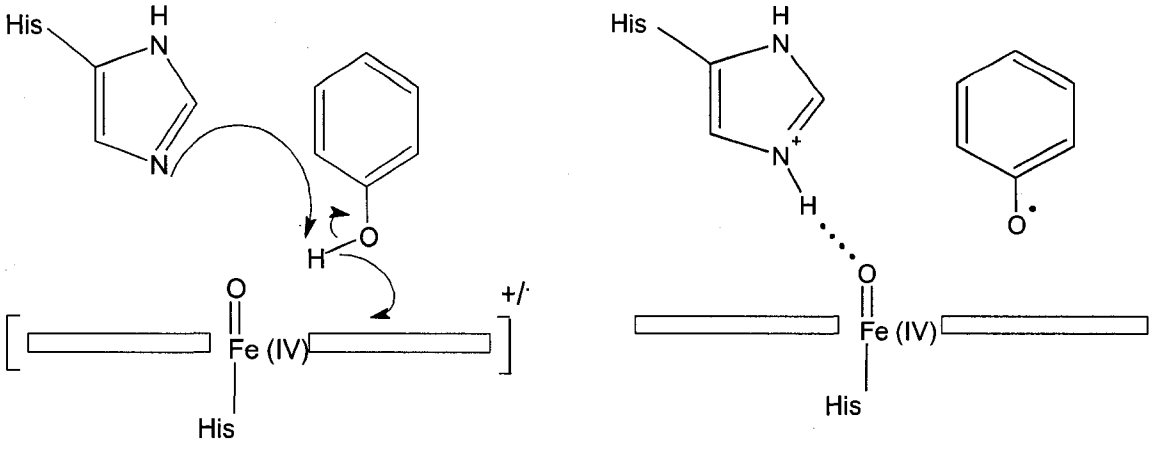
histidine to peroxides. This mechanism was proposed by Poulos and Dawson and was described as an electron push-pull mechanism [8, 78].



Scheme 1.2: Proposed mechanism for the formation of Compound I

Reaction of Compound –I:

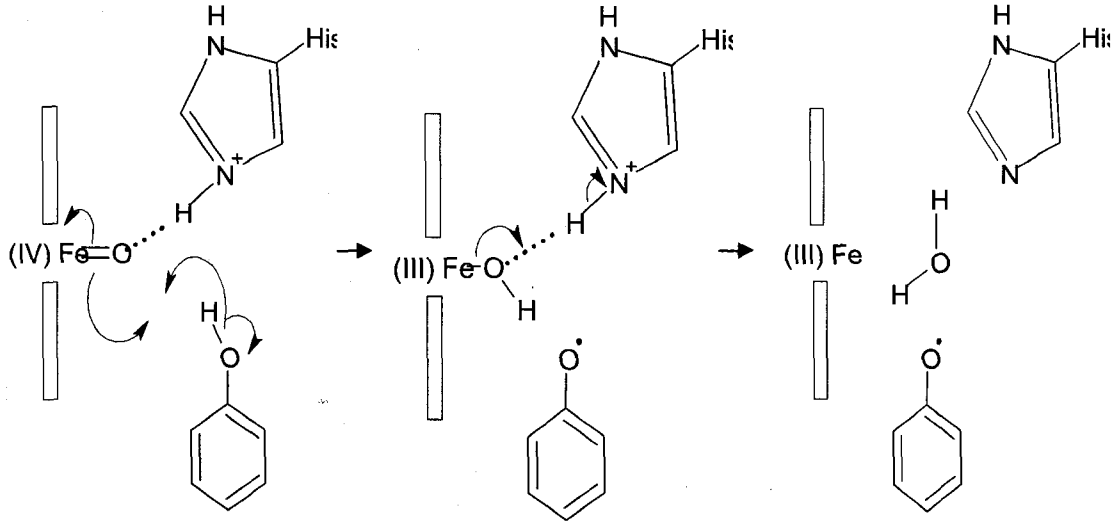
Reducing substrates such as phenols and arylamines act as hydrogen donors, in which a proton is accepted by a distal histidine as shown in Scheme 1.3. The electron is transferred simultaneously from the hydroxyl group to the neutralized porphyrin ring. As a result, Compound II is formed as arylradical. It is believed that a hydrogen bond is formed between the acidified distal group and the ferryl oxygen atom of the newly formed Compound-II [8, 78].



Scheme 1.3: Proposed mechanism for the formation of Compound II

Reaction of Compound –II:

The final step in the peroxidase mechanism is based on the fact that one proton is supplied by the distal histidine and the other by the reducing substrate. Electron transfer from the ferryl bond reduces iron (IV) to iron (III). A new bond is formed between the ferryl oxygen and proton from the reducing substrate causing the formation of a radical that leaves the active site, followed by the formation of a water molecule as a result of proton abstraction from distal histidine as shown in Scheme 1.4 [8, 78]. The Compound II is returned to its native state.



Scheme 1.4: Proposed mechanism for the formation of the native enzyme back

1.2.4 Soybean peroxidase

Soybean peroxidase is an oxidoreductase class enzyme, extracted from the soybean seed coat. It belongs to class III of the plant peroxidase superfamily. Soybean peroxidase has 3 polymer chains (A, B and C; Figure 1.3), 912 residues and 7182 atoms [9].

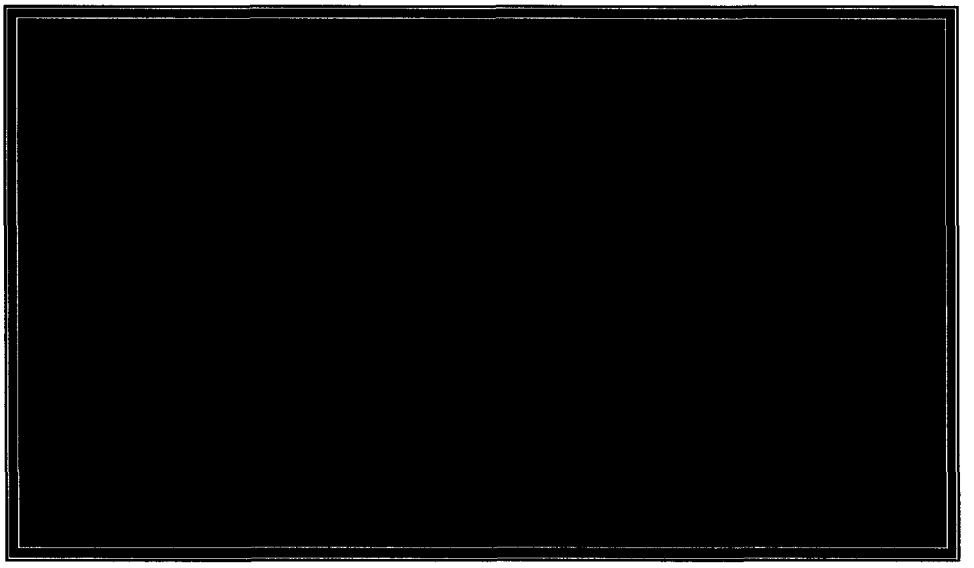


Figure 1.3: The three polymer chains of SBP; the heme group in the middle and two calcium atoms, the diagram is generated using ZMM-software using the information from protein data bank[9]

Each of the three polymers has a heme group (Fe (III) protoporphyrin IX), catalytic mechanism, conserved catalytic residue, 2 calcium ions, 4 disulfide bonds, 8 glycans, 1 tryptophan (Trp 117), 304 amino acids, a molecular mass of 33155.3 g /mole and a theoretical pI of 6.77 [9]. Figure 1.4 shows the protein structure of soybean peroxidase with the active site and the conserved catalytic residues. The top left picture shows the protoporphyrin IX prosthetic group and the two calcium ions (in green). The

middle top picture shows the heme group with the iron (III) in the middle (in green-bright ball). The right top picture shows the histidine amino acid present in the active site, where they interact with the heme group (in yellow). The right bottom picture shows the arginine amino acid (red), and the middle picture shows the cysteine amino acid (orange), which form a disulfide-bonds. The right bottom picture shows the alpha and beta helix folding.

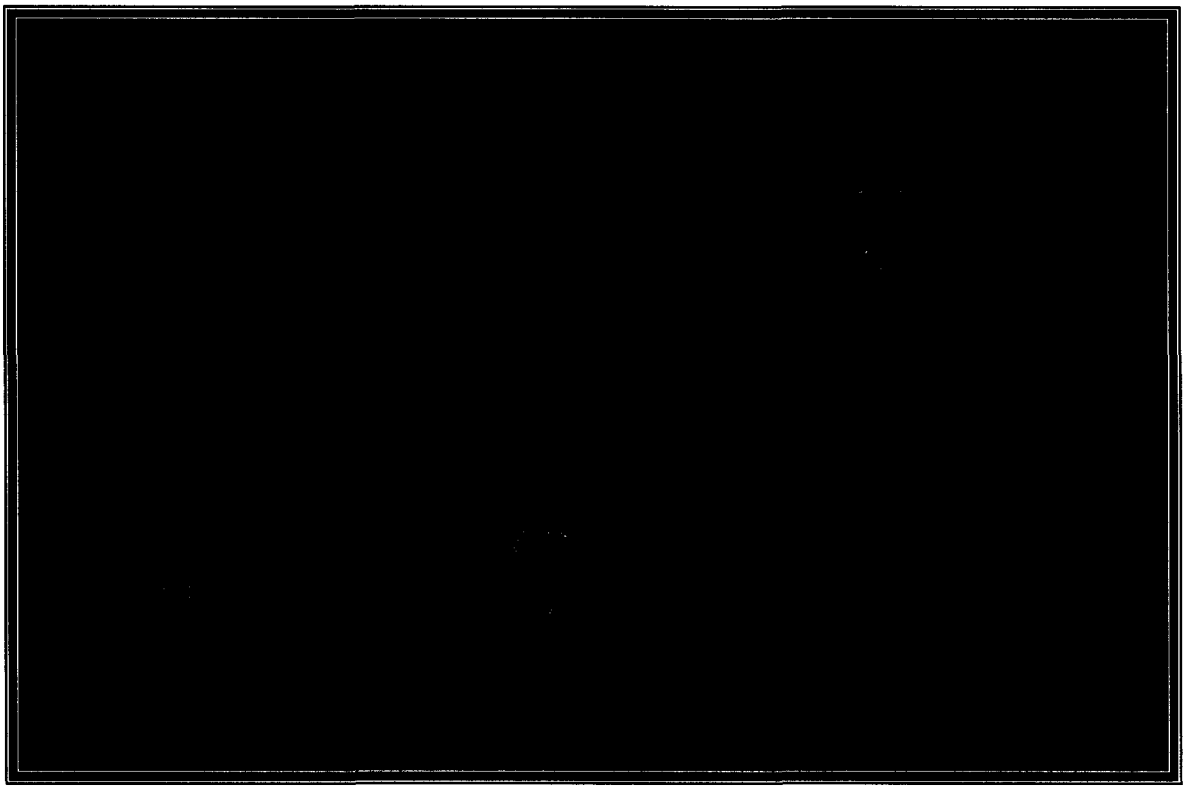


Figure 1.4: SBP structure view; heme group with iron are shown in green, histidine in yellow, arginine in red, cysteine in orange and the remaining amino acid in blue color, the diagram is made using Razmol-software using the information from protein data bank [9]

Figure 1.5 shows a close look on the important amino acid interacting with the active side of the enzyme. The imidazole side chain of **His 169** (in green) occupies

position 5 that is located on the proximal side of the heme. **His 42** (in red) is located on the distal side of the heme that acts as a proton acceptor from hydrogen peroxide. **Arg 38** (in yellow) is located on the distal side of the heme that acts as a charge stabilizers. **Arg 175** (in blue), **Arg 173** (in violet) and **Arg 175** (in white gray) were also found to be highly interacting with the active site. The two calcium ions and the disulfide-bond present increase the protein stability [9, 8, 78].

Figure 1.5: Close look at the heme group and the nearby amino acid residues; the white with the light blue structure are the heme molecules, His 169 in green, Arg 38 in yellow, His 42 in red, Arg 175 in blue, Arg 173 in violet, Arg 175 white gray and the green line represent the rest of the amino acid. The diagram also shows the two calcium ion in red circles. The diagram is made using ZMM-software using the information from Protein Data Bank [9]

1.3 Enzymes in Wastewater Treatment

The most research time and effort have been devoted to horseradish peroxidase (HRP) among all the peroxidases [8]. HRP has been found to be effective in removal of phenols and aromatic amines with 95% or higher efficiency in aqueous wastewater [71]. The main drawback in using HRP was the short catalytic lifetime which was attributed to the high inactivation of the enzyme. This drawback made the enzymatic treatment with HRP to be not economically feasible method. As a result, a number of other peroxidases are being investigated such as *Coprinus cinereus* peroxidase (CCP), *Coprinus macrorhizus* peroxidase (CMP), *Arthromyces ramosus* peroxidase (ARP) and soybean peroxidase (SBP). [1-7, 12, 75].

SBP was found to be a better alternative for enzymatic treatments than HRP for several reasons. First, SBP is cheaper in price, since it's extracted from the soybean seed coats that are usually a waste product of food industry [93]. SBP is also found in the root and leaf tissue of soybean but it has lower specific activity than the seed coat [82]. In addition, SBP is easy to isolate and does not require purification since crude SBP simple seed-hulls extract has been found to be more efficient than the purified one [83, 86]. Secondly, the catalytic efficiency (k_{cat}/ k_M) was found to be higher than that of HRP (~20 fold) [10]. Thirdly, the conformational stability is greater over broad range of pH (3.5-8.0) than that of HRP and other peroxidases, being in its maximal conformational flexibility (solvent-exposed heme active site) at pH 5.5 [1, 5, 10, 26]. Fourthly, SBP has a higher thermal stability (being active at 70°C) not only to that of HRP but to all plant peroxidases [84, 85]. Fifthly, SBP is less susceptible to inactivation when compared to HRP and other peroxidases such as ARP [1].

Peroxidase inactivation represents one of the drawbacks in enzyme application. There are three possible pathways by which the enzyme is deactivated. First, permanent inactivation can be due to the free radical generation during the catalytic process. The free radicals can return to the active site of the enzyme and form a covalent bond that prevents further substrates molecules from accessing the active site due to proximity [83, 92]. Second, inactivation can be due to end-product polymers formed during the catalytic process [20, 88, 5]. It has been proposed that the polymeric product can adsorb the enzyme on them and co-precipitate it when they exceed their solubility limit, resulting in a lower soluble free enzyme concentration. Third, inactivation can be due to excess hydrogen peroxide causing what is known as a suicide inhibition [89, 91]. It has been proposed that in the presence of excess of hydrogen peroxide or when substrate concentrations are low, the Compound II (E_2) is oxidized to the Compound III (E_3) which is catalytically inactive but its formation does not represent a terminal inactivated since it can decompose to the native form of the enzyme (Equations 1.4 and 1.5).

$$\text{Compound II } (E_2) + H_2O_2 \longrightarrow \text{Compound III } (E_3) + H_2O \quad (1.4)$$

$$\text{Compound III } (E_3) \longrightarrow \text{Native peroxidase } (E_N) + O_2^- + H^+ \quad (1.5)$$

$$\text{Compound III } (E_3) + AH \longrightarrow \text{Compound I } (E_1) + \bullet A \quad (1.6)$$

$$\text{Compound I } (E_1) + H_2O_2 \longrightarrow \text{Native peroxidase } (E_N) + O_2 + H_2O \quad (1.7)$$

$$\text{Compound I } (E_1) + H_2O_2 \longrightarrow \text{P-670} \quad (1.8)$$

The Compound III (E_3) can also be reduced though a one-electron reduction to the Compound I (E_1) generating a radical from which the Compound I (E_1) can be oxidized

back to the native enzyme (E_N) or though a series of reactions from the terminally inactive verdohemoprotein referred to as P-670 due to the shift in absorbance from 405 nm to 670 nm (Equations 1.6-1.8) [89]. Both the E_3 form and the P-670 contribute to suicide inhibition.

Attempts have been made to prevent peroxidase inactivation and increase the catalytic life. Different additives have been investigated for this purpose such as polyethylene glycol (PEG), gelatin and certain polyelectrolytes [79]. Most of the studied additives were found to be effective in reducing the peroxide inactivation, with PEG being found to be better than other additive in terms of the practical additive dose range (PADR), no negative effect on the efficiency of the reaction removal, easily separated from solution as precipitate with the enzymatic products formed, non toxic and more competitive than others in term of cost [19, 20, 79-81, 88]. PEG effectiveness in the protection of enzyme has been observed on various peroxidases such as HRP, ARP SBP and other enzymes such as laccase. PEG protection efficiency varies depending on the concentration and average molecular mass of PEG. Previous study has proven that PEG of an average molecular mass of 400 g/mole or less is inefficient in protecting the enzyme from inactivation. As the molecular mass increases (above 600 g/mole) and concentration increases, the efficiency also increases. PEG protection efficiency also depends on the enzymes used in the treatment and the aromatic substrates being treated [20]. The exact mechanism of PEG in protecting the enzyme is not fully understood but it is believed to follow the "sacrificial polymer" theory. The PEG can provide a barrier between the active side of the enzyme and the product generated. Hence, it prevents the free radical generated during the catalytic process from covalently binding to the active

side or it prevents the adsorption of the enzyme into the polymeric products [20]. Different attempts have also been made to prevent peroxidase inactivation by hydrogen peroxide. It has been suggested that inactivation of peroxidases by excess hydrogen peroxide can be reduced by a step feeding of hydrogen peroxide rather than single feed [7, 41]. Overcoming the peroxidases inactivation factors can solve one of the main problems in the enzymatic treatment. The solution for peroxide inactivation might be a heterogeneous reactor system of high concentration of aromatic pollutant flowing past peroxidase immobilized on different reactor matrices such as cellulose filter paper, nylon tubing and nylon balls [90].

Other oxidoreductase class enzymes, such as laccase and tyrosinase, have also been investigated as a result of their ability to oxidize phenols and aromatic amines, analogous to peroxidases. Laccases are glycosylated multicopper oxidases found in most plants and fungi in which fungal laccases are more reactive than plant laccases. Laccase require molecular oxygen for their catalytic cycle in which four one-electron oxidations of aromatic substrates occurs along with the irreversible four-electron reduction of molecular oxygen to water. The exact mechanism is not fully understood but it is believed to follows the modified ping-pong mechanism. Laccase was found to be as efficient as peroxidase in removal of phenols and aromatic amines. In addition, it is less resistant to enzyme inactivation [21, 73, 74]. The drawback in laccase is that it is slower than peroxidases and of higher cost. Tyrosinase is also a glycosylated multicopper oxidase found in most plants, bacteria and fungi. It follows different mechanism in oxidizing the aromatic substrates, instead of generating free radicals, they catalyze the hydroxylation of various monophenols to the o-diphenols by the oxygenated form of the enzyme which

then can be oxidized to o-quinones. The quinones are highly reactive and non-enzymatically couple to intermediate products that slowly polymerize to form insoluble polyphenolic compounds [94-96].

The efficiency of the enzymatic treatments in removal the aqueous aromatic pollutants can depend on several factors such as pH, temperature, minimum enzyme concentration, hydrogen peroxide concentration (for peroxidase), oxygen concentration (for laccases, tyrosinases), initial substrate concentrations and its type, PEG concentration, reaction time and the reactor configuration [6, 7, 87].

1.4 Polymeric Products

As described earlier, the aromatic radicals formed from the enzymatic reaction couple non-enzymaticly to form dimers. Those dimers can be substrates of the enzyme for another enzymatic cycle to form larger polymers that have reduced solubility and can be separated from solution by filtration or sedimentation. This is not always the case because some of these polymers are soluble and stay in solution causing a color change. Several coagulating/flocculating agents can be employed to induce removal of their colored products.

1.4.1 Hydrolysing coagulants

Aluminum sulphate (alum), poly-aluminum chloride (PAC), ferric sulphate and ferric chloride are used extensively as coagulants in wastewater treatment. They play a fundamental role in removing a wide range of impurities from contaminated water such as colloidal particles, natural organic matter (NOM) and dissolved organic substances

(DOC) [102-106]. Two mechanisms are proposed for the role of hydrolyzing coagulants in removing impurities. The first mechanism is based on charge neutralization of negatively charged colloids by cationic hydrolysis of products causing the destabilization of the colloidal particles and greatly reducing their solubility. This mechanism is called "charge neutralization". The second mechanism is based on the adsorption of organic matter on the amorphous hydroxide precipitate (usually referred to as aluminum hydroxide gel). This mechanism is called "sweep-floc coagulation" [102, 105]. In aqueous solution, alum dissolves in water resulting in a series of hydrolysis species. The metallic ion Al^{3+} is hydrated and hydrolysed to form soluble $Al(OH)^{2+}$ and $Al(OH)_2^+$ below pH 6.7, that participate in a "charge neutralization" mechanism (Equation 2.1 and 2.2). As the pH increases, insoluble aluminum hydroxide gel is formed ($Al(OH)_3$), that participates in a "sweep-floc coagulation" mechanism (Equation 2.3). Further increasing the pH will cause the formation of soluble $Al(OH)_4^-$ (Equation 2.4) [102, 105].

$$Al^{3+} + H_2O \rightleftharpoons Al(OH)^{2+} + H^+ \qquad pK_1 = 4.95 \qquad (2.1)$$

$$Al(OH)^{2+} + H_2O \rightleftharpoons Al(OH)_2^+ + H^+ \qquad pK_2 = 5.60 \qquad (2.2)$$

$$Al(OH)_2^+ + H_2O \rightleftharpoons Al(OH)_3 + H^+ \qquad pK_3 = 6.7 \qquad (2.3)$$

$$Al(OH)_3 + H_2O \rightleftharpoons Al(OH)_4^- + H^+ \qquad pK_4 = 7.6 \qquad (2.4)$$

Therefore, the effectiveness of the two mechanisms is highly pH-dependent and also depends on the kind and concentration of coagulant agent and the pollutant(s). The drawback in using alum is its long time effect on human health. Residual aluminum

present in the solution after the treatments was suspected to be linked to Alzheimer's disease [102, 105, 106].

1.4.2 Surfactants

Surfactant-mediated separation methods have been used in water treatment as means of removal of organic species in solution, such as hemimicellar/admicellar systems, micelle enhanced ultra filtration (MEUF), cloud point separation (phase change) and the most recent, adsorption micellar flocculation (AMF). AMF has advantages over other methods in term of cost, lower energy and lower pressure requirements. AMF is a two-step process, by which Al^{3+} or Fe^{3+} adsorbs into anionic surfactant such as sodium dodecyl sulphate (SDS) (Figure 1.6) or ά-olefin sulphonate (AOS). Upon binding, Al^{3+} or Fe^{3+} neutralizes the electrostatic repulsion between the negatively charged micelles causing the formation of an Al-SDS amorphous aggregate which flocculates and can be easily filtered [98]. Anionic solutes such as 2,4-dichlorophenoxyacetic acid and benzoic acid can adsorb into the Al-SDS aggregate and then are removed with the floc. In addition, non-ionic species such as phenol and polyaniline may partition into the micelles in a process known as solubilisation and then be removed with the floc [97, 100, 101]. The process is highly dependent on the pH, critical micellar concentration (CMC), SDS to Al^{3+} ratio and the kind of surfactant used. The limitation in using AMF results from the high concentrations of heavy metal used and the presence of hydrocarbon in the process that can be toxic [99]. Many studies are being conducted to overcome these problems such as the use of marble dust [100].

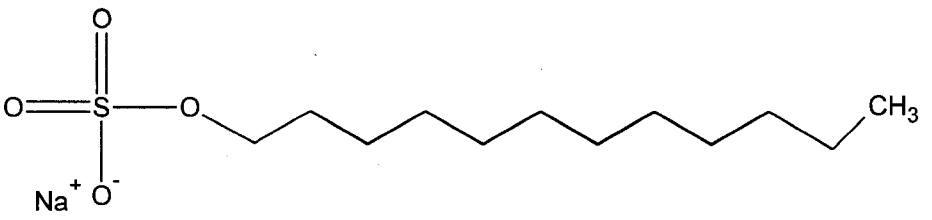


Figure 1.6: Structure of sodium dodecyl sulphate

Previous studies have shown the ability of AMF by using SDS as a surfactant and Al^{3+} polyvalent cations in removing colored polymeric product after the enzymatic treatment of diphenylamine and diaminotoluenes. SDS alone (without Al^{3+}) was not effective in removing the polymeric products, similarly to the results obtained using alum alone (no SDS) [30, 32, 33].

Other studies have monitored the effect of SDS during the enzymatic reaction and have found that SBP activity increased to three-fold from that of un-reacted SBP. The apparent increase in activity resulted from different proposed factors such as higher exposure of the active site of the enzyme to the substrates, diminished SBP aggregation with an increases SBP solubility and the partition of the products into the micelles preventing them from adsorption on to the enzymes and deactivating it. It is also mentioned that no SBP deactivation was seen even at high detergent concentrations up to 20% (w/v) [83]. Another study has reported the ability of SDS to stabilize the intermediate formed in the peroxidase oxidation of o-dianisidine, 3,3',5,5'-tetramethylbenzidine and o-phenylenediamine. The cause of the stabilization was the electrostatic interaction of the positively-charged intermediate and the negatively-charged micelles. In both studies, no information was given on the colored polymeric product precipitation [36].

CHAPTER 2

OBJECTIVES

Objectives

The objectives of this study are divided into three parts:

Part I:

1. Investigate the feasibility of using crude SBP to catalyze the removal of aryldiamines (o-PD, m-PD and p-PD) and aryldiols (catechol, resorcinol and hydroquinone) in synthetic wastewater.
2. Investigate the optimal operating conditions to achieve at least 95% conversion of aryldiamines and aryldiols in a 3-hour reaction period. The conditions include the effect of pH, hydrogen peroxide-to-substrate concentration ratio and enzyme concentration.
3. Investigate the effect of additive, PEG, in improving the conversion efficiency.
4. Monitor the substrate conversion over time and monitoring the hydrogen peroxide consumption by the substrate in a 3-hour period.
5. Investigate the removal of the polymeric colored products resulting from SBP-catalyzed polymerization by using SDS and alum as a flocculating-coagulating agents.
6. Investigate the effect of pH on the removal of the polymeric colored products.

Part II:

1. Investigate the feasibility of using crude SBP to catalyze the removal of 2-mercaptobenzothiazole in synthetic wastewater.
2. Investigate the optimal operating conditions to achieve at least 95% conversion in a 3-hour reaction period. The conditions include the effect of pH, hydrogen peroxide-to-substrate concentration ratio, enzyme concentration and substrate conversion over time.
3. Investigate the effect of additive, PEG, in improving the conversion efficiency.

Part III:

1. Investigate the feasibility of using crude SBP to catalyze the removal of phenol in synthetic and refinery wastewaters.
2. Investigate the optimal operating conditions to achieve at least 95% conversion in a 3-hour reaction period. The conditions include the effect of pH, hydrogen peroxide-to-substrate concentration ratio and enzyme concentration.
3. Investigate the effect of PEG in improving the conversion efficiency.
4. Monitor the substrate conversion over time and monitor the hydrogen peroxide consumption by the substrate in a 3-hour period.
5. Provide a pre-treatment method to improve the SBP-catalyzed polymerization in refinery wastewater.
6. Investigate the influence of reducing anions, halides and cyanide on the SBP-catalyzed polymerization in synthetic wastewater.

CHAPTER 3

METHODOLOGY AND EXPERIMENTAL SETUP

3.1 Materials:

Enzymes and PEG: Crude dry solid SBP (E.C. 1.11.7, Industrial Grade lot #18541NX) was obtained from Organic Technologies (Coshocton, OH). Liquid ARP (SP-502, activity 2000 U/mL) was a developmental preparation donated by Novzymes Inc. (Franklinton, NC). Dry solid bovine liver catalase (E.C. 1.11.1.6, lot #120H7060, 19,900 U/mg) was purchased from Sigma Chemical Company Inc. (St. Louis, MO). The enzymes were stored at a temperature of -15°C, while the aqueous stock solutions were stored at 4°C. Polyethylene glycol (PEG), with an average molecular mass of 3350 g/mole, was also obtained from Sigma Chemical Company Inc. (St. Louis, MO) and stored at room temperature of 22.0°C.

Aromatic Compounds (Substrates): Phenol, o-phenylenediamine, m-phenylenediamine, p- phenylenediamine, catechol, resorcinol, hydroquinone, p-benzoquinone and 2-MBT (all having a purity $\geq$ 98.0%) were obtained from Aldrich Chemical Corporation (Milwaukee, WI). The compounds were stored at room temperature of 22.0°C.

Reagents: 2,4,6-Trinitrobenzensulfonic acid solutions (TNBS) (~1.0 M in H_2O) were purchased from Sigma Chemical Company Inc. (St. Louis, MO) and were stored at -15.0°C. 4-aminoantipyrine (4-AAP) and potassium ferricyanides were supplied by BDH Inc. (Toronto, ON) and were stored at room temperature. Hydrogen peroxide (30.0% w/v) was also purchased from BDH Inc. and was stored at 4.0°C.

Reducing Anions: Sodium salts of sulphite, sulphate, thiosulphate, chloride, fluoride, iodide, bromide, nitrate, acetate, phosphate and cyanide were all obtained from Aldrich Chemical Corporation (Milwaukee, WI) and BDH Inc. All sodium anions were stored at room temperature.

Metals: Cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) stock solutions were made from EnviroConcentrate™ (10,000μg/mL) in dilute HNO_3 supplied by ULTRA Scientific (North Kingstown, RI). Iron (Fe) stock solution was made from concentrate (1025 μg/mL) in 1.0 wt % HCl solution supplied by Sigma-Aldrich Chemical Corporation (Milwaukee, WI). Magnesium (Mg) stock solution was made from $MgCl_2$ in 1.0 wt% HCl. All metal solutions were all stored at room temperature.

Coagulating /flocculating Agents: Aluminum sulphate (Alum) was purchased from BDH Inc. (Toronto, ON). Sodium dodecyl sulphate (SDS) was obtained from Sigma Chemical Company Inc. (St. Louis, MO). These materials were stored at room temperature.

Refinery Wastewaters: Five samples of refinery wastewater were donated by a confidential source.

All other chemicals obtained and used in theses studies were of analytical grade and purchased either from Aldrich Chemical Corporation (Milwaukee, WI) or BDH Inc. (Toronto, ON).

3.2 Analytical Equipment:

3.2.1 High performance liquid chromatography (HPLC)

Aromatic compound concentrations were analyzed with an HPLC System obtained from Waters Corporation (Milford, MA). The system consisted of binary HPLC pump (model 1525), autosampler (model 717), dual λ absorbance detector (model 2487) and C_{18} reverse phase column (5 μM, 4.6 x 150 mm) operated by Breeze software. Elutions were isocratic with a mobile phase consisting of various solvents depending on the aromatic compound used. Table 3.1 summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector wavelength for each aromatic compound. The injection volume was 10 μL, flow-rate was 1.0 mL/min and the column temperature was 40°C, additional information is provided in Appendix H.

Table 3.1: HPLC conditions

Aromatic Substrates	Mobile Phase Solvents		Mobile Phase Solvents Ratio (%)		Wave-Length (nm)
	Pump A	Pump B	Pump A	Pump B	
o-PD	Methanol	Buffer (50mM) pH 6.7	60	40	290
m-PD			40	60	290
p-PD			40	60	304
Catechol	Acetonitrile	Acetic Acid (0.1%)	40	60	276
Resorcinol			30	70	274
Hydroquinone			20	80	290
p-benzoquinoe			20	80	248
2-MBT			60	40	310
Phenol			40	60	276

3.2.2 Ultraviolet-visible spectroscopy (UV-Vis)

A Hewlett Packard Diode Array Spectrometer (Model 8452A) with wavelength range between 190 to 820 nm and 2 nm resolution and controlled by Hewlett Packard

Vectra ES/12 computer was used to measure sample absorbance. Quartz spectrometer cells with 1 cm optical path length were supplied by Hellma Canada Limited (Concord, ON), additional information on the measurement of substrates by direct absorbance is provided in Appendix G.

3.2.3 Total organic carbon (TOC)

A Shimadzu TOC-V CSH Total Carbon Analyzer, supplied by Shimadzu Scientific Instruments (Columbia, MD), was used to measure the total organic carbon in the samples. The total organic carbon has been defined based on the subtraction of inorganic carbon (IC) from total carbon (TC). The samples were purged by using nitrogen gas; acidified with phosphoric acid, oxidized with oxygen, and detected by using a non-dispersive infrared spectrophotometer (NDIR). The combustion chamber had a temperature between 680-700°C.

3.2.4 Atomic absorption spectrometer (AA)

A Shimadzu atomic absorption spectrometer (6800 series) was used to analyze metal concentrations. The spectrometer is equipped with a single autosampler (ASC-6100). The lamp current was 5 mA, the oxidant was air and the fuel was acetylene. Table 3.2 summarizes the wavelengths, slit width and the optimum working range for each metal used in this study.

Table 3.2: AA conditions

Metal	Wavelength (nm)	Optimum working range (µg/mL)	Slit Width (nm)
Fe	248.3	0.06-15.0	0.2
Mg	285.2	0.05-5.0	0.5
Cu	324.7	0.03-10.0	0.5
Pb	217.0	0.1-30.0	1
Zn	213.9	0.01-2.0	1
Co	240.7	0.05-15.0	0.2
Cd	324.7	0.03-10	0.5

3.2.5 Additional equipment:

pH Meter: An expandable Ion Analyzer EA940, manufactured by Orion Research (U.S.A), was used to measure the pH of the samples and the buffers. Calibration buffer solutions (pH 4.00, 7.00 and 10.00) were supplied by BDH Inc. (Toronto, ON).

Centrifuge: IEC Centra-8 Centrifuge was supplied by International Equipment Company (USA). The centrifuge speed was maintained at 3000 rpm for duration of 30 min.

Magnetic stir-plates: Micro V magnetic stirrers (0-1100 rpm, Model 4805-00) and VWR MAGSTIRRER (100-1500 rpm, Model 82026-764) were supplied by VWR International Inc.(Mississauga, ON). The magnetic stir bars were supplied by Cole-Parmer (Chicago, IL).

Microfilters and Plastic syringe: 0.2 μm HT Tuffryn membrane filters, purchased form Gelman Labs (Mississauga, ON), were used for microfiltering the samples. The plastic syringes were purchased form Becton Dickinson Corporation (Clifton, NJ).

3.3 Analytical Methods:

3.3.1 Colorimetric assay for soybean peroxidase activity

This assay determines the amount of active enzyme (SBP) present in solution. Enzyme catalytic activity (U) is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 23°C. The enzyme activity is reported in U/mL. The enzyme activity can be determined by monitoring the initial rate of color formation resulted from the oxidative coupling of phenol and 4-AAP in the presence of hydrogen peroxide using SBP as catalyst. The product of such reaction is a pink

chromophore with an absorption maximum at 510 nm and an extinction coefficient of 6000 $M^{-1}cm^{-1}$realtive to hydrogen peroxide [7]. In this assay all reagents (hydrogen peroxide, 4-AAP and phenol) were present in excess except SBP in order to ensure that the initial rate of reaction was directly proportional to the concentration of the enzyme present. Additional information is provided in Appendices A, B and C.

3.3.2 Colorimetric assay for aromatic amines

The concentrations of phenylenediamines are expressed as molar quantities, where one millimolar is equal to 108 mg/L. The phenylenediamine concentrations can be determined by measuring the color intensity formation resulting from the nucleophilic substitution on a color-generating substance trinitrobenzenesulfonic acid (TNBS) by the arylamine in the presence of sodium sulphite at pH 7.4 (phosphate buffer) and 23°C [29]. The relatively stable compound of such reaction has a yellow color with maximum adsorption maximum at ~430 nm where the color generated is directly proportional to the concentration of the phenylenediamine compounds. Additional information is provided in Appendix D.

3.3.3 Colorimetric assay for aromatic phenols

The concentrations of phenolic compounds in solution can be determined by measuring the color intensity formation resulting from the electrophilic substation of 4-AAP on the para-position of phenolic compounds followed by the oxidation of the intermediate by potassium ferricyanide at a pH between 8.0 and 10.0. The relatively stable compound of such reaction has a pink quinoneimine chromophore with an adsorption maximum at 510 nm where the color generated is proportional to the phenolic compound concentration [21]. Additional information is provided in Appendix E.

3.3.4 Colorimetric assay for hydrogen peroxide

This assay determines the concentration of hydrogen peroxide present in the solution by measuring the color intensity resulting from oxidative coupling of phenol and 4-AAP in the presence of hydrogen peroxide using ARP as the enzymatic catalyst at pH 7.4. The product of such reaction produces a pink quinoneimine chromophore with an absorption maximum at 510 nm and is proportional to the aqueous hydrogen peroxide concentration [41]. This assay is similar to the colorimetric assay for SBP activity. Additional information is provided in Appendix F.

3.3.5 Buffer preparation

The buffers used in the pH optimization study were prepared according to a previous study [40]. Two main buffers were used in the study depending on the choice of pH range. Acetic acid-sodium acetate buffer was used in the pH range of 4.0-5.8 and monobasic-dibasic sodium phosphate buffer was used in the pH range of 5.6-9.0.

3.3.6 Cyanide and sulphide test strips

Cyanide levels in the wastewater samples were detected with EM Quant Test Strips. The test strip contained barbituric acids that reacted with glutaconic dialdehyde and produced a red polymethine dye. Glutaconic dialdehyde formation resulting from the reaction of cyanide ion with chlorine to form cyanogen chloride that caused the opening of pyridine ring giving glutaconic dialdehyde. The pH of the sample was adjusted to pH 6-7 with sulfuric acid. The strips were stored at 20-25°C. The detection range of the test strip was 0-30 mg/L.

Dissolved sulphide in the wastewater samples was detected by using lead acetate test strips. The dissolved sulphide reacted with lead acetate and formed lead sulphide that

produced a gray-black color. The test strip could detect as low as 5.0 mg/L. The strips were stored at 20-25°C.

3.4 Experimental Protocols:

The first part of this study was to optimize the reaction conditions for the removal of aromatic substrate in the presence of SBP and peroxide. The experiments in this study were carried out in 20 or 30 mL aerated batch reactors (open glass vials) at room temperature of about 23°C. The components of the sample mixture were added in the following order to a total volume of 20.0 mL: water (distilled, tap or refinery wastewater), buffer with 40 mM acetate or phosphate buffer, 1.0 mM aromatic substrate, appropriate concentration of SBP enzyme and at the end, hydrogen peroxide with appropriate concentration to initiate the reaction. The batch reactors were stirred gently for three hours by using a magnetic stirrer plate and stir bars (Figure 3.1). After reaction, the samples were quenched with catalase of excess concentration to quickly consume all the hydrogen peroxide, microfiltered and then tested for the residual concentration by using a colorimetric assay while taking into account the color correction, if necessary, or HPLC or TOC. All reactions were carried out in triplicate. The study was designed to achieve at least 95% conversion of aromatic substrate by optimizing the following parameters: pH, SBP concentration, hydrogen peroxide-to-substrate concentration ratio and PEG concentration. Experiments were also conducted to monitor the substrate and hydrogen peroxide consumption over time.

The second part of this study was the removal of colored polymeric products that were produced from the SBP enzymatic reaction. The experiments were conducted under

the optimum enzymatic conditions to achieve at least 95% conversion of aromatic substrates. Alum or SDS was added in the appropriate concentrations and allowed to stir for 30 min and then the precipitates were allowed to settle for 1 to 2 hours. Then the supernatants were analyzed with a UV-Vis-spectrophotometer. The study was designed to achieve a high removal of polymeric colored products by optimizing the following parameters: pH, SDS concentration and alum concentration.

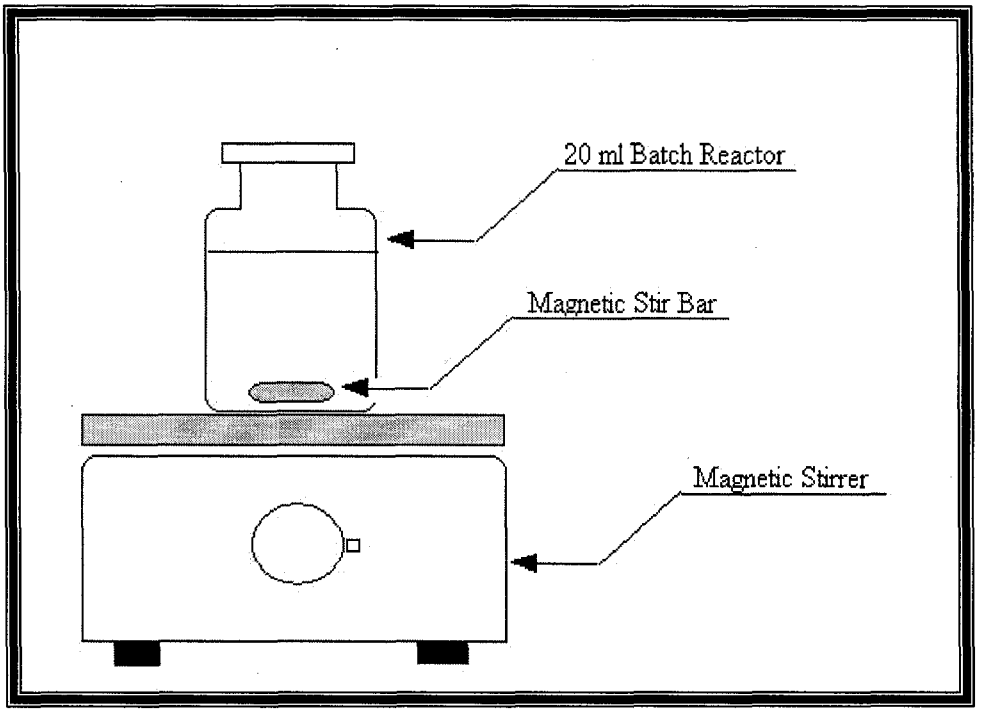


Figure 3.1: The reaction system

3.4.1 SBP polymerization reactions

Optimum pH: Experiments were conducted to determine the optimum pH condition for the enzymatic conversion of the aromatic substrates in both synthetic and refinery wastewaters. The pH examined ranged from 3.0 to 10.0: Acetate buffer in the pH

range of 3.0-5.8, phosphate buffer in pH range of 5.6-9.0 and sodium hydroxide with phosphate buffer for the pH range higher than 9.0 were used. The aromatic substrate concentrations in the synthetic studies were 1.0 mM while for the aromatic substrate (phenol) in the refinery wastewater samples were between 0.8 and 0.9 mM. The hydrogen peroxide amounts added in these studies were in the optimum range or slightly higher for each substrate [2]. The pH studies were run under stress SBP conditions in order to better observe any trends. Control experiments were conducted to monitor the effect of pH alone, without SBP and hydrogen peroxide, on substrate conversion and also the effect of hydrogen peroxide and pH, without SBP, on the substrate conversion.

Optimum hydrogen peroxide to substrate concentration ratio: Experiments were conducted to determine the optimum hydrogen peroxide-to-substrate concentration ratio for the enzymatic conversion of the aromatic substrates in both synthetic and refinery wastewaters. The hydrogen peroxide to substrate ratio range examined was between 0.25-8.0 mM at the optimum pH for each substrate. The aromatic substrate concentrations were about 1.0 mM for both synthetic and refinery wastewaters. The experiments were run at various concentrations of SBP. Control experiments were conducted to monitor the effect of different hydrogen peroxide concentrations alone, without SBP, on substrate conversion.

Minimum SBP concentration: Experiments were conducted to determine the minimum SBP concentration required for the enzymatic conversion of the aromatic substrates in both synthetic and refinery wastewaters. The SBP concentration range examined was 0.001-3.0 U/mL, depending on the substrate studied, at the optimum pH condition for each substrate. The aromatic substrate concentrations were about 1.0 mM

for both synthetic and refinery wastewaters. The experiments were run at several hydrogen peroxide-to-substrate concentration ratios, including the optimum condition.

PEG additive effect: Experiments were conducted to investigate the effect of PEG (molecular mass of 3350 g/mole) on reducing the enzyme concentration required for 95% conversion of the aromatic substrates in both synthetic and refinery wastewaters. The PEG concentration range examined was 50-400 mg/L at the optimum conditions of pH and hydrogen peroxide concentration, depending on the substrates studied. The studies were run under stress and optimum SBP concentrations. The aromatic substrate concentrations were about 1.0 mM for both synthetic and refinery wastewaters.

Consumption of the aromatic substrates over time: Experiments were conducted to monitor the consumption of aromatic substrates during the first 3 hours of the enzymatic reaction for both synthetic and refinery wastewaters. The reactions were run under the optimum conditions of pH, hydrogen peroxide and SBP concentrations. Similar experiments were also run under stress SBP concentrations. The samples were tested at 2 min intervals for the first 10 min; at 5 min intervals between 10 and 30 min; and at 30 min intervals between 30 and 180 min.

Consumption of the hydrogen peroxide over time: Experiments were conducted to monitor the hydrogen peroxide consumption by the aromatic substrates during the first 3 hours of the enzymatic reaction. The reactions were run under the optimum conditions of pH, hydrogen peroxide and SBP concentrations. Similar experiments were also run under stress SBP concentrations. The samples were tested at 2.5 min intervals for the first 10 min; at 5 min intervals between 10 and 30 min; and at 30 min intervals between 30 and 180 min. Control experiments were conducted to monitor

the hydrogen peroxide consumption by the aromatic substrates alone without SBP. In this study, the samples were not quenched with catalase.

The effect of reducing anions, halides and cyanide: Experiments were conducted to investigate the effect of reducing anions, halides and cyanide, on the enzymatic conversion of phenol in synthetic wastewater. The reactions were run under the optimum conditions of pH 7.0 and 1.2 mM hydrogen peroxide, and under a stress SBP concentration of 0.5 U/mL. Reducing anions, halides and cyanide were made from their respective salts and were present at 200 mg/L. Control experiments were conducted to monitor the hydrogen peroxide consumption by the reducing anions, halides and cyanide in the presence of phenol alone without SBP.

The pretreatment of refinery wastewater samples: Hydrogen peroxide pretreatment experiments were conducted on refinery wastewater Samples 2-4. The wastewater samples were allowed to react with hydrogen peroxide for 24 hours to complete hydrogen peroxide demand. The hydrogen peroxide concentrations demands were in the range 10.0-30.0 mM. The phenol concentrations were approximately 1.0 mM. The pretreatments were followed by enzymatic optimization for SBP and hydrogen peroxide as described earlier. Control experiments were conducted to monitor the hydrogen peroxide consumption by the wastewater samples alone, with no SBP, during the 24 hour reactions.

3.4.2 Polymeric colored products removal

Effect of pH: Experiments were conducted to investigate the effect of pH on the removal of the polymeric colored products that were produced in the enzymatic reaction. The pH range examined was 2.0-11.5. Sodium hydroxide and sulfuric acid were used to adjust the pH. The pH effects were examined also in the presence of excess alum at 2.7 mM (for aryldiamine and aryldiol compounds) and also in presence of excess SDS at 0.5 mM (for aryl amine compounds).

Minimum alum concentration: Experiments were conducted to determine the minimum alum concentration required for the removal of the polymeric colored products after the enzymatic conversion of the aryldiol compounds. The alum concentration range examined was 0.1-2.0 mM. The samples were adjusted to pH ~ 7.0 after the alum addition.

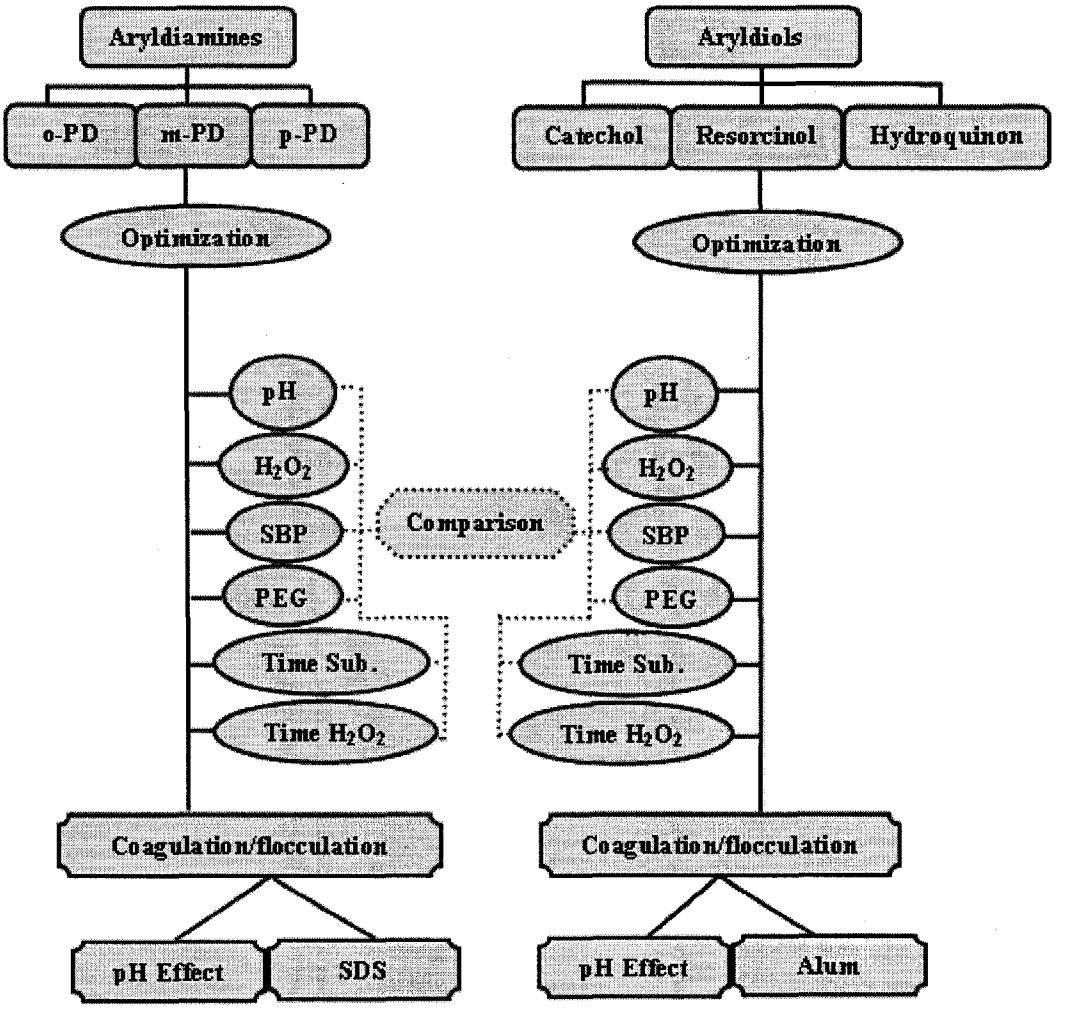
Minimum SDS concentration: Experiments were conducted to determine the minimum SDS concentration required for the removal of the polymeric colored products after the enzymatic conversion of the arylamine compounds. The SDS concentration range examined was 0.01-0.6 mM. The samples were run under the optimum pH condition.

CHAPTER 4

ANALYSIS OF RESULTS

4.1 Aryldiamines and Aryldiols Results

In the first section, the optimal conditions for the enzymatic treatment of aryldiamines and aryldiols in synthetic water and the methods for removing enzymatically-generated polymeric products are discussed. Scheme 4.1, summarizes the sequential approach for the results and discussion.



Scheme 4.1: Analysis approach for aryldiamines and aryldiols

4.1.1 Optimum pH

Optimum pH is defined as the pH at which the enzyme possesses maximum catalytic activity. The activity of an enzyme is dependent on pH, which dictates that the enzyme possesses an optimum pH. pH dependence suggests that acid-base catalysis plays a significant role in the enzymatic reaction. There are key amino acid residues involved in the catalysis at the active site of SBP. The protonation of the residues under improper conditions affects the catalytic efficiency. The distal histidine (His 42) must be in a deprotonated form in order to act as a catalytic base, while an arginine (Arg 38) must be in a protonated form in order to function as a charge stabilizer [9]. Thus, the optimum pH equals a value that satisfies both amino acids' ionization state conditions. The pH can also affect the three-dimensional structure of SBP by disturbing the pH-dependent forces that stabilize the protein's tertiary structure such as salt bridges and hydrogen bonds [10]. An optimum pH ensures that the enzyme exists in its best conformational catalytic state. The pH can also affect the substrates in certain cases.

Phenylenediamines:

The pH range studied for phenylenediamines was 3.2-8.8. The hydrogen peroxide concentration was maintained in a sufficient amount for the enzymatic reaction. The phenylenediamines concentration was 1.0 mM and the experiments were conducted under both stress and sufficient SBP concentrations during 3.0 h reaction. The implication of stress SBP concentration provides clearer and easier discerning of the optimum pH. Among the three phenylenediamine isomers under stress SBP concentration, o-PD had the broader optimum pH whereas the m-PD and p-PD showed narrow pH optima (Figures 4.1, 4.2 and 4.3). However, when the SBP concentrations

were increased, the SBP became more insensitive to the pH changes. The optimum pH values of 4.5-5.2, 5.4 and 5.6 for o-PD, m-PD and p-PD, respectively, provided the proper ionization of catalytic residues of the SBP as it ensured the protonation of arginine residue (pK_a ~12.0, as a free amino acid) and the deprotonation of the distal histidine (pK_a 3.2 in SBP and pK_a 6.0 as a free amino acid) [11]. The pK_a of arginine and histidine, as free amino acids, compared to being residues in the enzyme may be altered due to the unique microenvironment of the active site. The results have demonstrated a sharp reduction in the percent conversion of the substrates in the basic region when compared to the acidic region. This may have resulted from an improper ionization of catalytic residues of the enzyme at high pH.

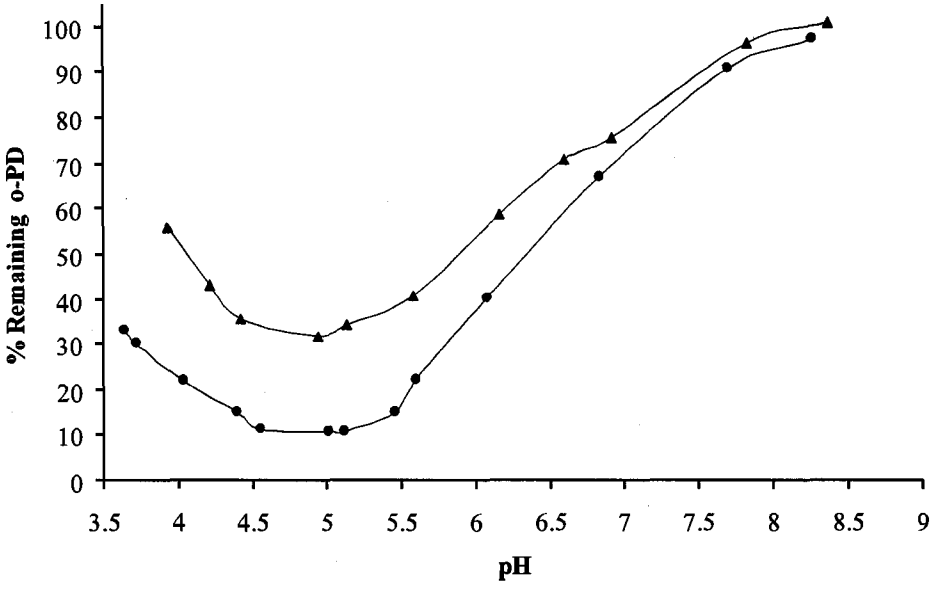


Figure 4.1: pH optimization for enzymatic conversion of o-PD; **Conditions:** 1.0 mM o-PD, 2.0 mM H_2O_2 and 40.0 mM buffer; **Test method:** TNBS
0.0015 U/mL SBP 0.0005 U/mL SBP

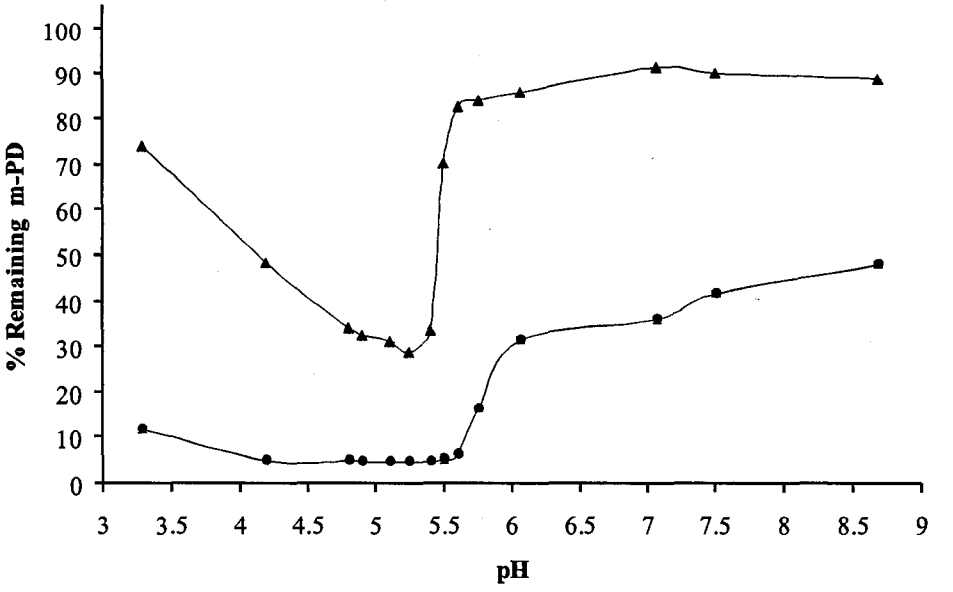


Figure 4.2: pH optimization for enzymatic conversion of m-PD; **Conditions:** 1.0 mM m-PD, 3.0 mM H_2O_2 and 40.0 mM buffer; **Test method:** TNBS
0.05 U/mL SBP 0.003 U/mL SBP

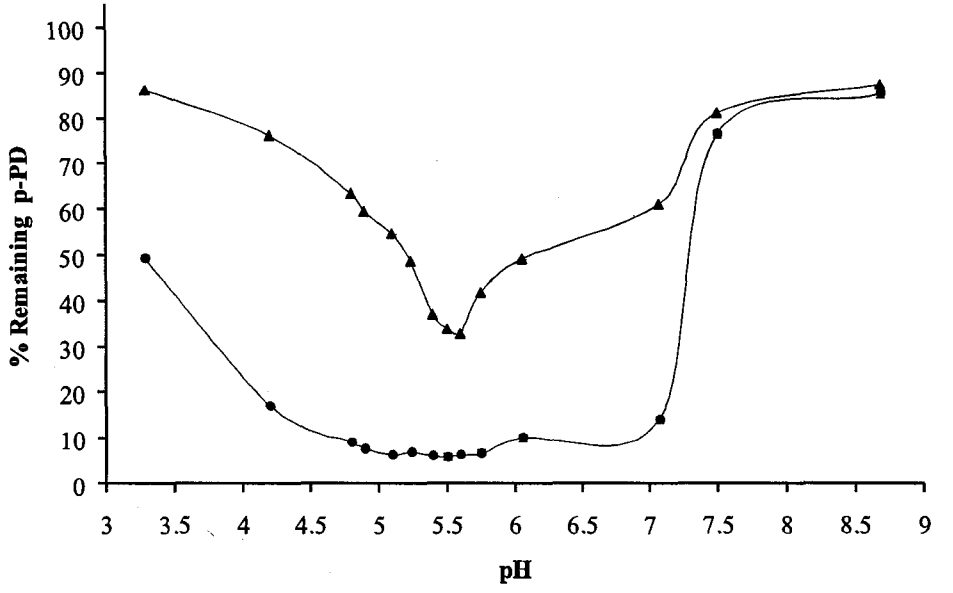


Figure 4.3: pH optimization for enzymatic conversion of p-PD; **Conditions:** 1.0 mM p-PD, 3.0 mM H_2O_2 and 40.0 mM Buffer; **Test method:** TNBS
0.005 U/mL SBP 0.0003 U/mL SBP

Aryldiols:

The pH range studied for aryldiols was 3.5-11.0. The hydrogen peroxide concentration was maintained in a sufficient amount for the enzymatic reaction. The aryldiol concentration was 1.0 mM and the experiments were conducted under both stressed and sufficient SBP concentrations during 3.0 h reaction. Experiments were also conducted to investigate the effect of pH, without SBP and hydrogen peroxide, on the aryldiol conversion (Control 1) and the effect in the presence of hydrogen peroxide without SBP (Control 2).

The effect of pH alone on the catechol, resorcinol and hydroquinone conversion (Control 1) was insignificant (5.0-10.0% conversion) in the pH range 3.75-7.75, 3.75-8 and 3.5-6.5, respectively (Figures 4.4 and 4.6). The conversion of aryldiols significantly increased as the pH was increased with 80-90.0% conversion at pH 10.0-11.0. Similar results were obtained in the presence of hydrogen peroxide without SBP (Control 2). This high percent conversion can result from the chemical transformation of catechol and hydroquinone to quinone or semi-quinone structure with dissociation constants for catechol pK_{a1} 9.48 and pK_{a2} 12.08 [14]; hydroquinone pK_{a1} 9.8 and pK_{a2} 11.4 [15]. Similarly, deprotonation of resorcinol can occur with dissociation constant for resorcinol pK_{a1} 9.32 and pK_{a2} 11.27 [13]. As the pH increased above pH 8.0 for catechol, 9.5 for resorcinol and 6.5 for hydroquinone, the enzymatic oxidations became limited due to the chemical transformation of aryldiols to a new form that could not be oxidized by the SBP.

Among the three aryldiol isomers under stress SBP concentration, catechol and hydroquinone had the broader optimum pH whereas the resorcinol showed a narrow

optimum pH (Figures 4.4, 4.5 and 4.6). As the SBP concentration increased, the SBP became more resistant to pH change for catechol and hydroquinone. The optimum pH values of 6.5-7.5, 7.75-8.25 and 4.0-6.5 for catechol, resorcinol and hydroquinone, respectively, presumably provided the proper ionization of catalytic residues of the SBP. The results also demonstrate an increase in the percent conversion of the substrates in the basic region in the presence of enzyme similar to that in the controls. This demonstration did not result from the enzymatic conversion of these substrates but from the chemical transformation of the substrates at high pH to a quinone structure form, as previously discussed for Controls 1 and 2. It is to be noted that the position of the hydroxyl groups play an important role in the reactivity of these compounds. Catechol and hydroquinone can be easily transformed to o-benzoquinone or p-benzoquinone at high pH. However, resorcinol can not be converted to a benzoquinone unless it undergoes hydroxylation or hydroxyl transformation.

HPLC analysis confirmed the formation of p-benzoquinone at Retention Time (RT) of 1.34 min basic pH of 10.0. The formation of p-benzoquinone did not result from enzymatic reaction of hydroquinone (RT 2.53 min) but rather from the chemical transformation due to pH change. While analyzing the retention time of the products of the enzymatic reaction of hydroquinone, a similar peak was observed to that of p-benzoquinone stock solution at low pH (pH 5.0; RT 3.92 min). This peak can be speculated to result from the formation of p-benzosemiquinone (RT 3.92 min) rather than p-benzoquinone (RT 1.34 min). Quinones may be as toxic as the staring substrates; therefore conversion of such substrates to a quinone form is not solving the problem unless it is being removed after treatments.

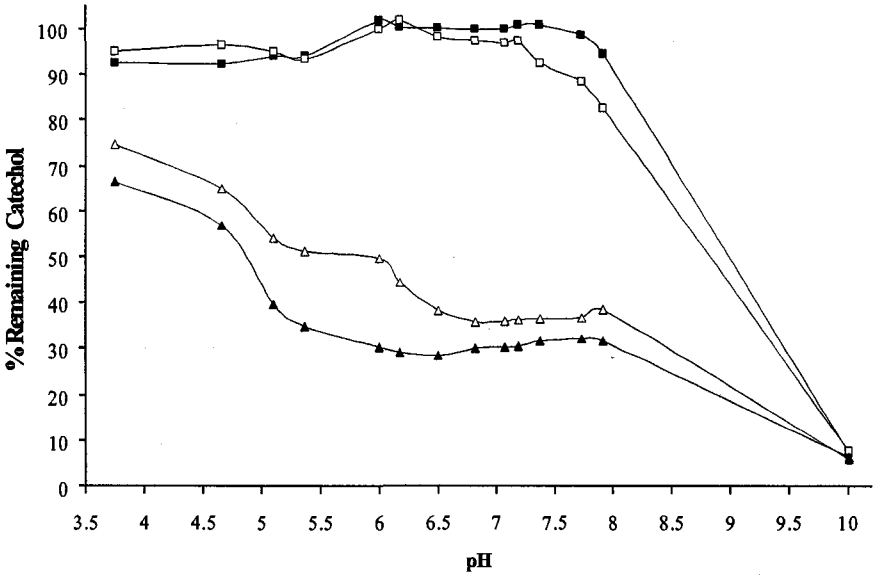


Figure 4.4: pH optimization for enzymatic conversion of catechol; **Conditions:** 1.0 mM catechol, 2.5 mM H_2O_2 and 40.0 mM Buffer; **Test method:** HPLC

0.0075 U/mL SBP — 0.005 U/mL SBP — No SBP — No SBP and H_2O_2

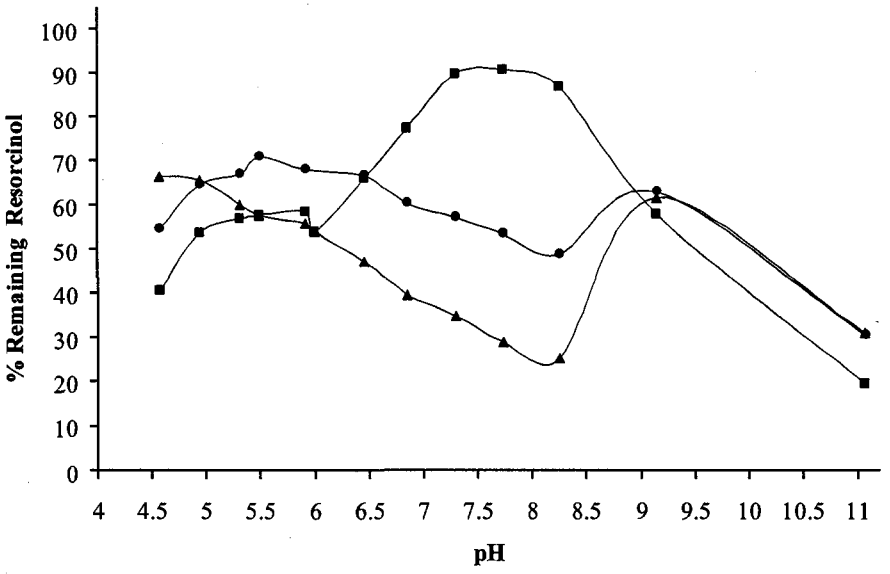


Figure 4.5: pH optimization for enzymatic conversion of resorcinol; **Conditions:** 1.0 mM resorcinol, 2.0 mM H_2O_2 and 40.0 mM Buffer; **Test method:** HPLC

0.1 U/mL SBP — 0.05 U/mL SBP — No SBP

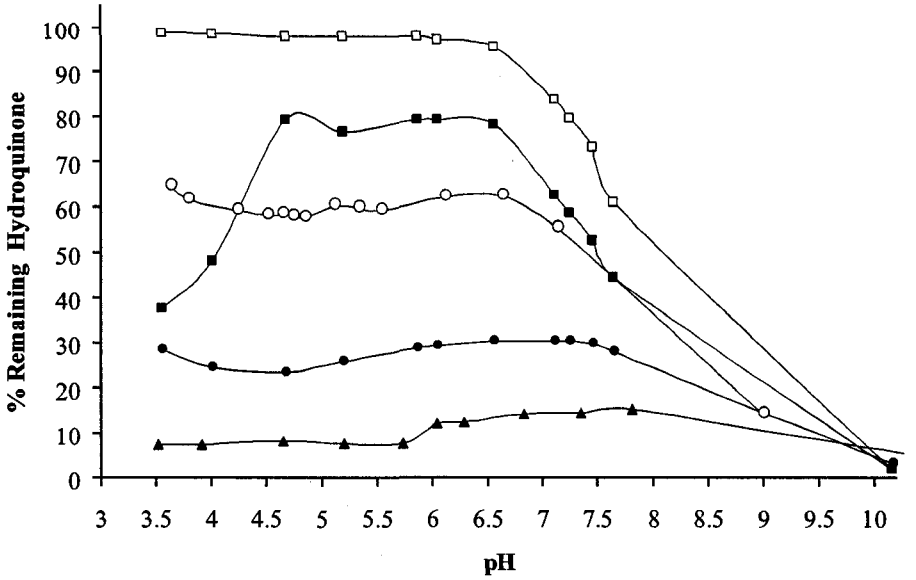


Figure 4.6: pH optimization for enzymatic conversion of hydroquinone; **Conditions:** 1.0 mM hydroquinone, 2.0 mM H_2O_2 and 40.0 mM Buffer; **Test method:** HPLC
0.5 U/mL SBP 0.0025 U/mL SBP 0.001 U/mL SBP
No SBP No SBP and H_2O_2

Comparing phenylenediamines and aryldiols compounds: The choice of the optimum pH was based on the highest percent conversion of the substrates by the SBP, taking into consideration that at this pH, the substrate has the lowest chemical reactivity with hydrogen peroxide alone or chemical transformation by pH effect alone. The optimum pH for the enzymatic conversion of phenylenediamine compounds was found to be in the acidic region, 4.5-5.6, while the optimum pH for aryldiol compounds was in the alkaline region, 6.5-8.0, except for hydroquinone which showed a broad optimum (Table 4.1). Previous work with SBP and anilino compounds had shown an optimum pH similar to that of phenylenediamine. 2, 4- and 2, 6-diaminotoluenes had an optimum pH range 5.0-5.5 [1]. Aniline and toluidines (o-, m- and p-) had an optimum pH range 5.0-8.0 [12], whereas SBP and phenolic compounds had shown an alkaline optimum pH range similar

to that for aryldiols. Phenol, chlorophenols (o-, m- and p-), cresol (o-, m- and p-), dichlorophenol (2,4-) and bisphenol A had optima in the pH range of 6.0-8.0 [5]. It is concluded that the optimum pH does not only depend on the proper ionization of the catalytic residues of SBP in the enzymatic reaction but also dependent on the kind of the aromatic substrate. These experiments were important to find an environment for the enzyme to show its maximum catalytic activity to be used for the following optimization experiments.

Table 4.1: Optimum pH

Aromatic Compounds	Optimum pH under stress SBP concentrations	Optimum pH at high SBP concentrations
o-PD	4.5-5.2	4.25-5.5
m-PD	5.4	4.25-5.6
p-PD	5.6	4.5-7.0
catechol	6.5-7.5	5.0-7.5
resorcinol	7.5-8.25	7.5-8.25
hydroquinone	4.0-6.5	3.5-6.5

4.1.2 Optimum hydrogen peroxide-to-substrate concentration ratio

According to the peroxidase reaction mechanism, for every one mole of hydrogen peroxide being consumed, two moles of the aromatic functional groups are converted to aromatic radicals that couple non-enzymatically to form dimers and through subsequent enzyme cycles form larger polymers [8]. Phenylenediamines and aryldiols possess two potential sites for reaction with the activated forms of the enzyme (Compound I and Compound II). Therefore, it is possible for the soluble dimers formed to be substrate(s) of the enzyme in a second enzymatic cycle for higher polymer formation, thereby causing an increase in the stoichiometric ratio of hydrogen peroxide to substrates [16, 19]. Other

factors can also alter the stoichiometric ratio, such as the catalase activity of peroxidase to reduce hydrogen peroxide to water [8], and the presence of other peroxide consuming substances in a waste stream.

Phenylenediamines:

The molar concentrations of hydrogen peroxide studied were between 0.1 and 8.0 mM and the phenylenediamine concentration was 1.0 mM. The experiments were conducted at the previously determined optimum pH, whereas the initial SBP concentrations varied from stress conditions to sufficient for the 95% conversion. The experiments were run for 3.0 h duration. In comparing the three phenylenediamine isomers, o-PD and p-PD required the same optimum hydrogen peroxide concentration of 1.5 mM while m-PD required higher hydrogen peroxide concentration of 2.0 mM for 95% conversion (Figures 4.7, 4.8 and 4.9). The same trends were observed when HPLC was used for the analysis. The o-PD and p-PD deviated from the TNBS color test by 5-10% in the substrate conversion, while m-PD showed similar percent conversion. A control experiment was conducted to investigate the effect of various hydrogen peroxide concentrations, without SBP, on the o-PD conversion. The control experiment showed an insignificant effect of hydrogen peroxide alone on the o-PD conversion (Figure 4.7).

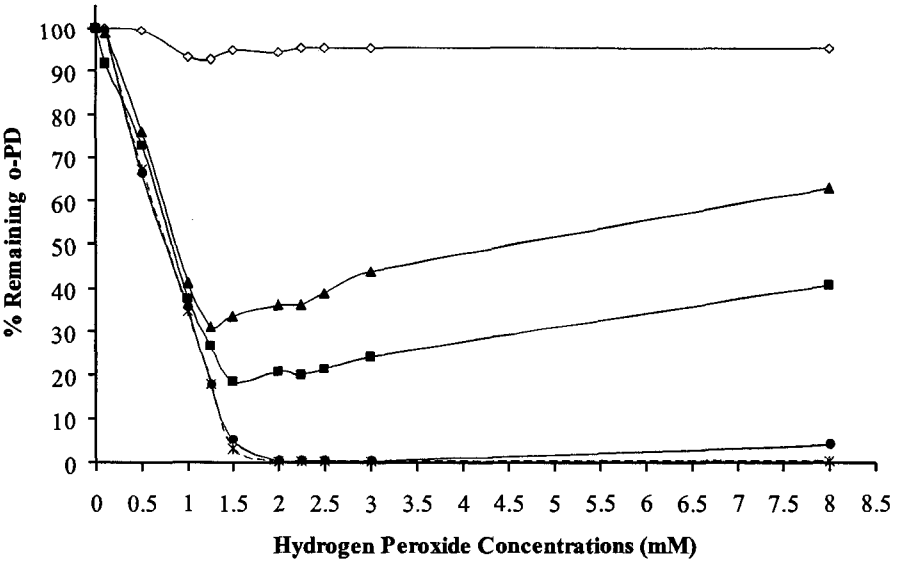


Figure 4.7: Effect of hydrogen peroxide concentration on the enzymatic conversion of o-PD; **Conditions:** 1.0 mM o-PD and 40.0 mM acetate buffer pH 5.1; **Test method:** TNBS

0.2 U/mL SBP 0.003 U/mL SBP 0.001 U/mL SBP 0.0005 U/mL SBP No SBP

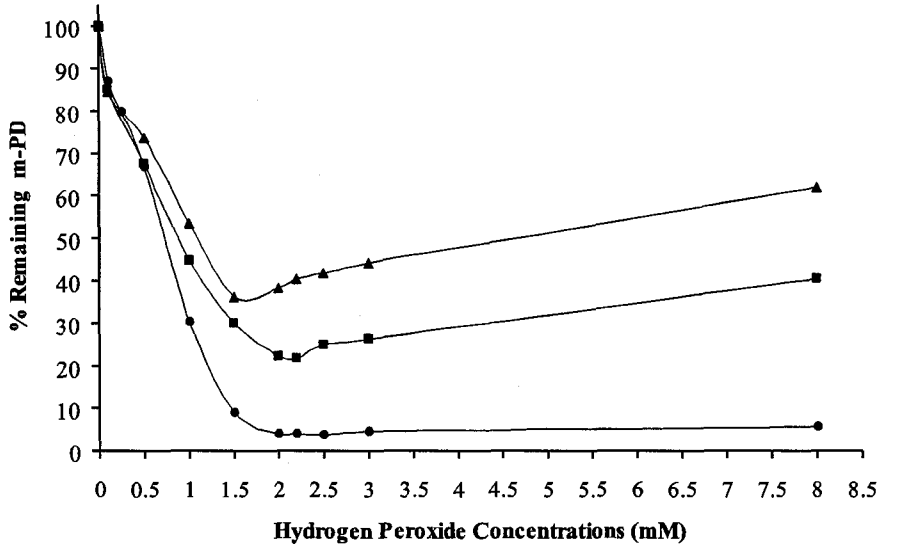


Figure 4.8: Effect of hydrogen peroxide concentration on the enzymatic conversion of m-PD; **Conditions:** 1.0 mM m-PD and 40.0 mM acetate buffer pH 5.4; **Test method:** TNBS

0.03 U/mL SBP 0.005 U/mL SBP 0.003 U/mL SBP

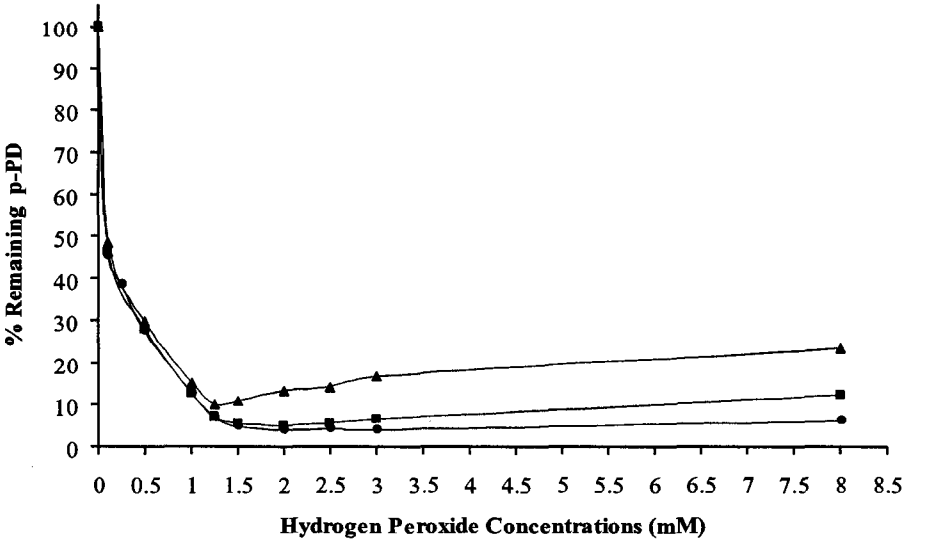


Figure 4.9: Effect of hydrogen peroxide concentration on the enzymatic conversion of p-PD; **Conditions:** 1.0 mM p-PD and 40 mM acetate buffer pH 5.6; **Test method:** TNBS

0.01 U/mL SBP 0.005 U/mL SBP 0.001 U/mL SBP

Aryldiols:

The molar concentrations of hydrogen peroxide studied were between 0.1 and 6.0 mM and the aryldiol concentration was 1.0 mM. The experiments were conducted at the previously determined optimum pH, whereas the initial SBP concentrations varied from stress conditions to sufficient for the 95% conversion. A control experiment was conducted to investigate the effect of various hydrogen peroxide concentrations, without SBP, on the substrate conversion. The experiments were run for 3.0 h time duration. In comparing the three hydroxyl isomers, catechol required the highest optimum hydrogen peroxide concentration of 2.5 mM (Figure 4.10), whereas resorcinol required 2.0 mM (Figure 4.11) and hydroquinone required lowest optimum of 1.5 mM for 95% conversion of 1.0 mM substrate (Figure 4.12). The hydrogen peroxide to substrate ratio was also

investigated in tap water, with no buffer and pH was adjusted by using NaOH and H_2SO_4. Similar results were observed; catechol, resorcinol and hydroquinone had optimum requirements of 2.5, 1.75 and 1.5 mM, respectively. The control experiment shows an insignificant effect of hydrogen peroxide alone on the resorcinol conversion, while 10 to 30% conversion was noticed for catechol and hydroquinone.

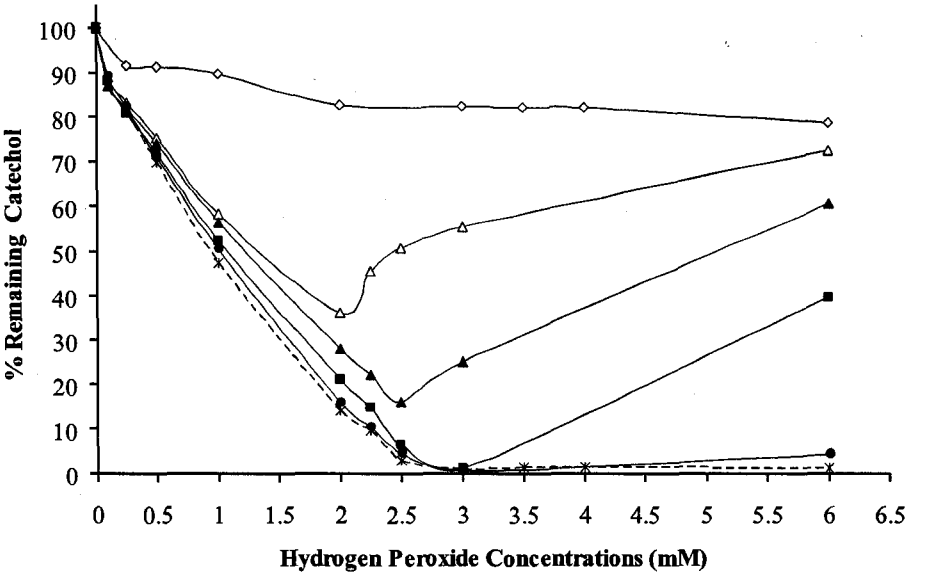


Figure 4.10: Effect of hydrogen peroxide concentration on the enzymatic conversion of catechol; **Conditions:** 1.0 mM catechol and 40.0 mM phosphate buffer pH 7.28; **Test method:** HPLC

0.1 U/mL SBP 0.05 U/mL SBP 0.02 U/mL SBP

0.01 U/mL SBP 0.005 U/mL SBP No SBP

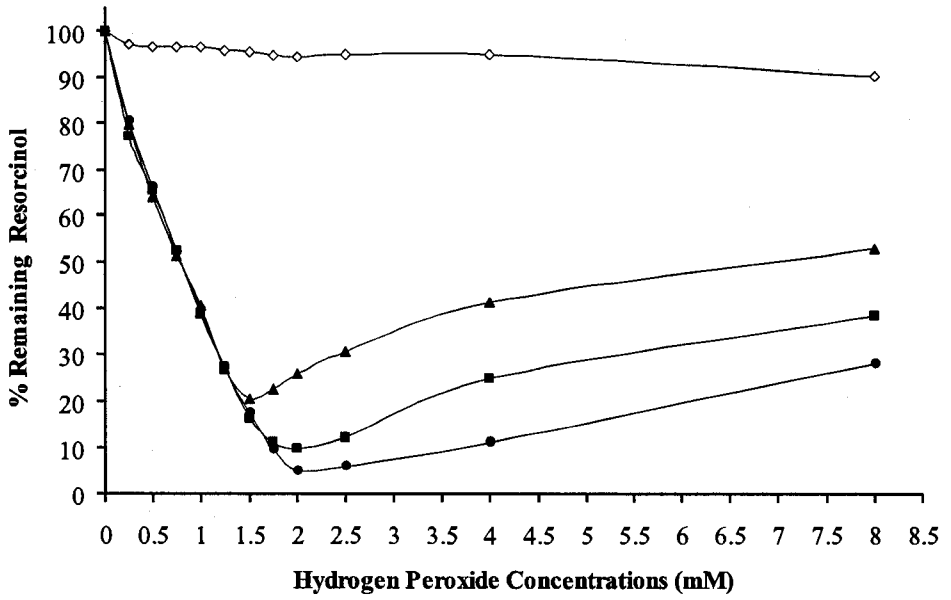


Figure 4.11: Effect of hydrogen peroxide concentration on the enzymatic conversion of resorcinol; **Conditions:** 1.0 mM resorcinol and 40.0 mM phosphate buffer pH 7.73; **Test method:** HPLC

0.2 U/mL SBP 0.15 U/mL SBP 0.1 U/mL SBP No SBP

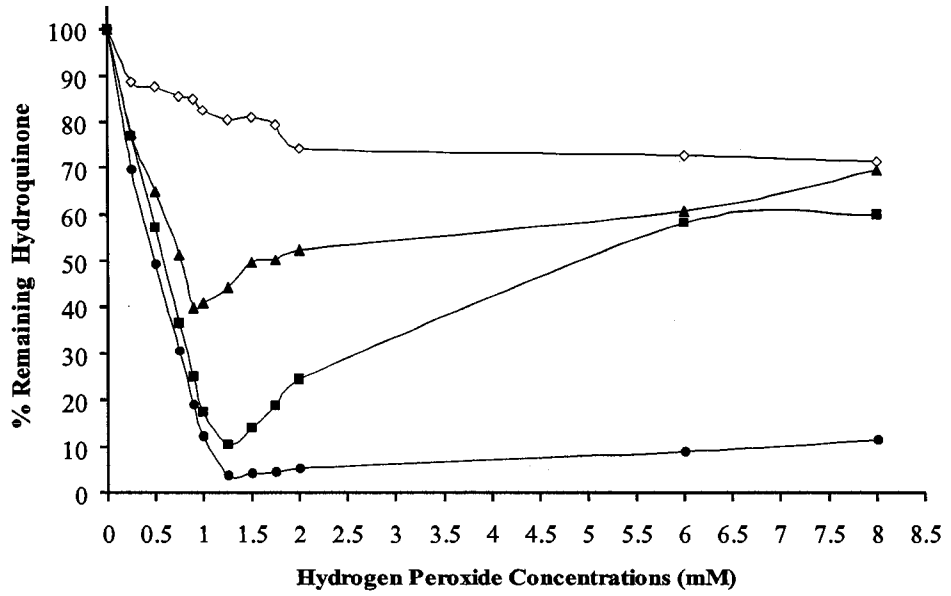


Figure 4.12: Effect of hydrogen peroxide concentration on the enzymatic conversion of hydroquinone; **Conditions:** 1.0 mM hydroquinone and 40.0 mM acetate buffer pH 5.2; **Test method:** HPLC

0.05 U/mL SBP 0.0025 U/mL SBP 0.001 U/mL SBP

No SBP

Comparing phenylenediamines to hydroxyl diols compounds:

The three isomers each of phenylenediamines and aryldiols consumed hydrogen peroxide within the same concentration range of 1.5- 2.5 mM for 1.0 mM substrate (Table 4.2). The para-occupied functional group for phenylenediamine and aryldiols exhibited the minimum hydrogen peroxide demand for the 95% conversion. The hydrogen peroxide demand for all the substrates was more than what is expected theoretically. This could be explained from the fact that a portion of the hydrogen peroxide available for reaction was converted to water molecules by catalase ability of peroxidase, resulting in an increase in the observed hydrogen peroxide optimum concentration [8]. In addition, soluble dimers produced may have become substrates for the enzyme [16]. The higher demands of hydrogen peroxide were also observed with other phenolic and anilino compounds [1] [5]. As the hydrogen peroxide to substrate ratio increased beyond the optimum, aryldiol compounds demonstrated a higher reduction in conversion when compared to phenylenediamines. This finding was not observed at the optimum SBP condition for phenylenediamines but only at stress SBP conditions, as shown in Figures 4.7, 4.8 and 4.9. Aryldiols showed deactivation under stress SBP conditions as well as at the optimum SBP condition, as shown in Figures 4.10, 4.11 and 4.12. Generally, peroxidases get deactivated as hydrogen peroxide concentration increases beyond the optimum. This can be due to the transformation of the active form of the enzyme (Compound II) to catalytically slow form (Compound III) [17] or the formation of an inactive form of the enzyme due to the free radicals released from Compound I and Compound II of the peroxidase that have the potential to react back with the active site of Compound II [18]. Upon reaction, a covalent bond is formed between the

active site of the enzyme and an aromatic bulky group which prevents further substrate molecules from accessing the active site due to steric hindrance [8, 19]. Hence, it is speculated that the aryldiol radicals had a higher ability than phenylenediamine radicals to diffuse to the active site of the enzyme and deactivate the enzyme. The deactivation of SBP in the presence of aryldiols can be overcome by the addition of extra SBP above the optimum (0.05 to 0.1 U/mL SBP), as shown in Figure 4.10 for catechol.

In comparing phenols and anilines in terms of the SBP deactivation by hydrogen peroxide, previous studies with aniline compounds, such as diaminotoluenes, showed no enzyme deactivation as the hydrogen peroxide increased [1], while previous studies with phenolic compounds such as phenol, chlorophenols (2-, 3- and 4-), cresols (o-, m- and p) and 2,4-dichlorophenol showed a deactivation with higher hydrogen peroxide-to-substrate concentration ratios [5].

Table 4.2: Optimum hydrogen peroxide to substrate ration

Aromatic Compounds	Optimum $[H_2O_2]$ for 1.0 mM Substrate	Deactivation due to high hydrogen peroxide concentration	
		@ optimum SBP concentration	@ Stress SBP concentration
o-PD	1.5 mM	No	yes
m-PD	2.0 mM	No	yes
p-PD	1.5 mM	No	yes
catechol	2.5 mM	yes	yes
resorcinol	2.0 mM	yes	yes
hydroquinone	1.5 mM	yes	yes

4.1.3 Minimum SBP concentrations and PEG effect

The minimum SBP concentrations required for the 95% conversion of the aromatic substrates are investigated in the following set of experiments. The enzyme cost in the enzymatic treatments can be a limiting factor in the real wastewater applications [7] [2]. Therefore, determining the minimum SBP concentration at its optimum conditions is important. Experiments were also conducted to investigate the effect of PEG_{3350} in the treatment, given that it may reduce the enzyme concentration required for the same substrate conversion efficiency [21]. The theory of PEG in protecting the enzyme is not well known but it has been proposed to follow a "sacrificial polymer" theory. PEG_{3350} can provide a shielding effect to the enzyme by attracting some of the insoluble polymers and radicals to it and preventing them from adsorbing the free enzyme or covalently bonding to it [19] [20].

Phenylenediamine:

The SBP concentration ranges studied for o-PD, m-PD and p-PD were 0.0001-0.01 U/mL, 0.001-0.1 U/mL and 0.001-0.1 U/mL, respectively. The phenylenediamine concentration was 1.0 mM and the pH was at the optimum value. The experiments were conducted both under stress and optimum hydrogen peroxide concentrations during 3.0 h reaction time. Experiments were also conducted to investigate the effect of PEG_{3350} in the conversion of phenylenediamines. Among different phenylenediamines, m-PD required the highest SBP concentration of 0.01 U/mL while o-PD and p-PD required 0.002 and 0.005 U/mL, respectively (Figures 4.13, 4.14 and 4.15). The same trend was observed while using the HPLC for analysis. The o-PD and p-PD deviated from the TNBS color test by 5-10% in the substrate conversion, while m-PD showed similar

percent conversion. PEG showed an insignificant effect on the conversion of these three isomers. Previous work with anilino compounds, such as 2,4- and 2,6-diaminotoluenes, also showed no PEG effect [1].

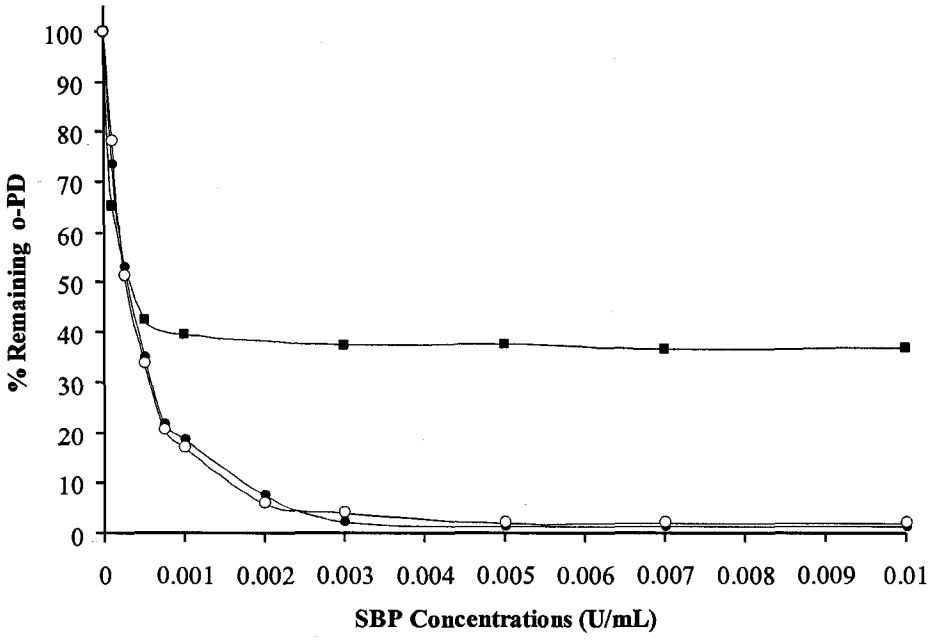


Figure 4.13: Minimum SBP concentration required for 95% conversion of o-PD; **Conditions:** 1.0 mM o-PD, 40.0 mM acetate buffer pH 5.1; **Test method:** TNBS
1 mM H_2O_2 2 mM H_2O_2 2 mM H_2O_2/ PEG (150mg/L)

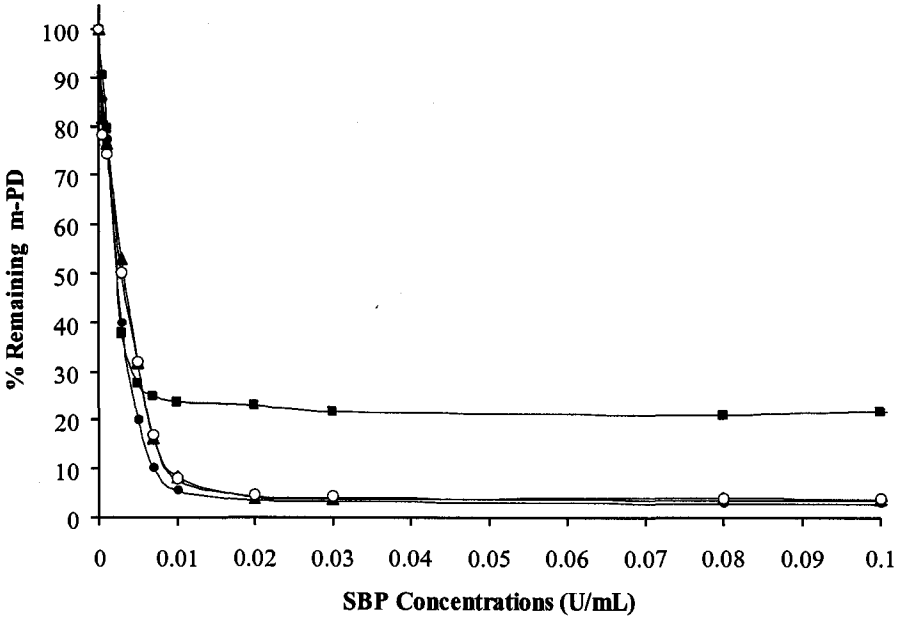


Figure 4.14: Minimum SBP concentration required for 95% conversion of m-PD; **Conditions:** 1.0 mM m-PD, 40.0 mM acetate buffer pH 5.4; **Test method:** TNBS

1 mM H_2O_2 2 mM H_2O_2 2 mM H_2O_2/ PEG (150mg/L)

2 mM H_2O_2/ PEG (50mg/L)

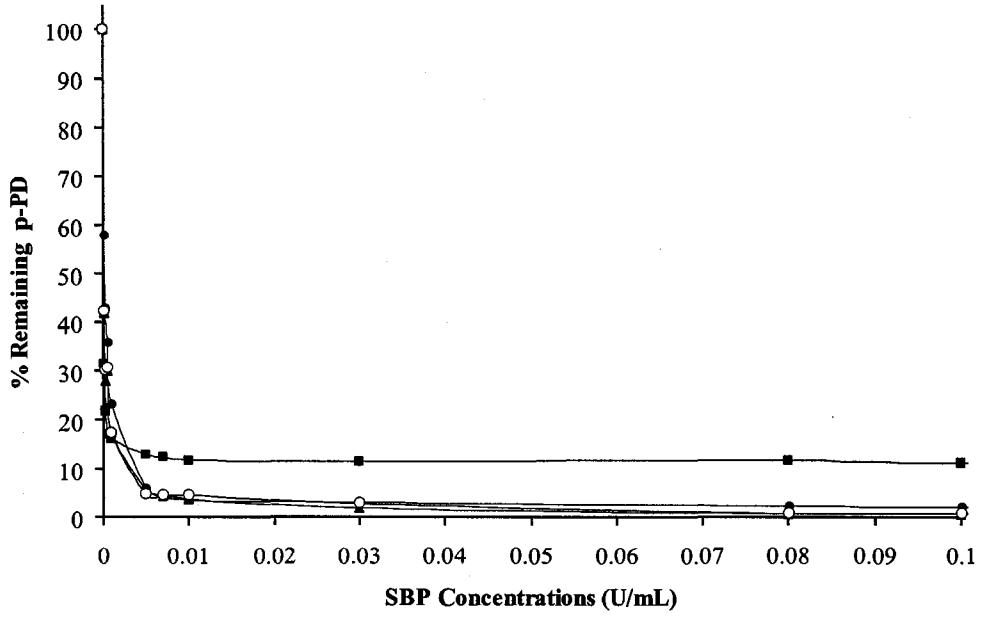


Figure 4.15: Minimum SBP concentration required for 95% conversion of p-PD; **Conditions:** 1.0 mM p-PD, 40.0 mM acetate buffer pH 5.6; **Test method:** TNBS

1 mM H_2O_2 2 mM H_2O_2 2 mM H_2O_2/ PEG (150mg/L)

2 mM H_2O_2/ PEG (50mg/L)

Aryldiols:

The SBP concentration ranges studied for catechol, resorcinol and hydroquinone were 0.01-0.5 U/mL, 0.01-1.0 U/mL and 0.01-1.0 U/mL, respectively. The aryldiol concentration was 1.0 mM and the pH was maintained at the optimum value. The experiments were conducted both under stress and optimum hydrogen peroxide concentrations during 3.0 h reaction time. Experiments were also conducted to investigate the effect of PEG_{3350} in the conversion of phenylenediamines. Among the aryldiols, resorcinol required the highest SBP concentration of 0.2 U/mL while hydroquinone required the lowest of 0.005 U/mL (Figures 4.17 and 4.18). Catechol required 0.025 U/mL SBP (Figure 4.16). PEG showed an insignificant effect on the conversion of these three isomers. The minimum SBP requirement was also investigated in tap water no buffer; pH adjusted using NaOH and H_2SO_4. Similar results were observed; catechol, resorcinol and hydroquinone had an optimum of 0.025, 0.25 and 0.01 U/mL, respectively. The conversion with hydroquinone was limited to 90%, excess SBP did not improve the conversion efficiency.

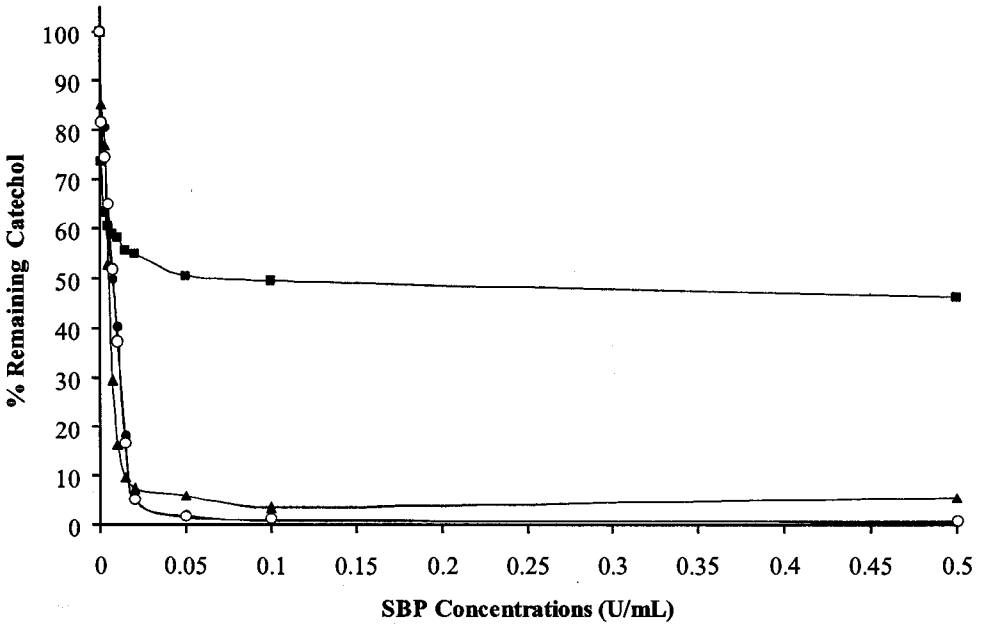


Figure 4.16: Minimum SBP concentration required for 95% conversion of catechol; **Conditions:** 1.0 mM catechol, 40.0 mM phosphate buffer pH 7.28; **Test method:** HPLC

—■— 1.0 mM H_2O_2 —▲— 2.5 mM H_2O_2 —●— 3.5 mM H_2O_2 —○— 3.5 mM H_2O_2/ PEG (150mg/L)

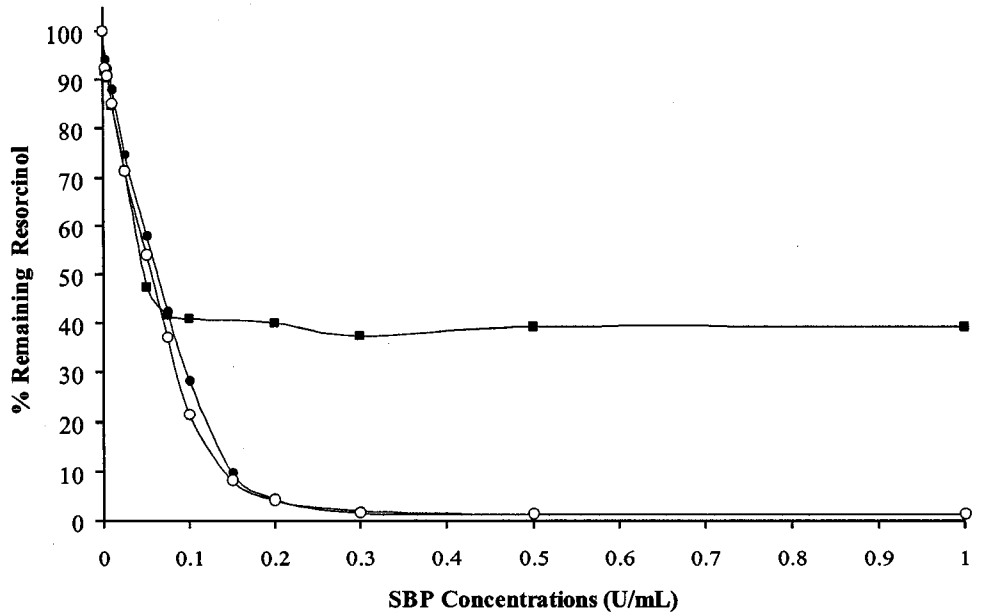


Figure 4.17: Minimum SBP concentration required for 95% conversion of resorcinol; **Conditions:** 1.0 mM resorcinol, 40.0 mM phosphate buffer pH 7.73; **Test method:** HPLC

—■— 1.0 mM H_2O_2 —●— 2.0 mM H_2O_2 —○— 2.0 mM H_2O_2/ PEG (150mg/L)

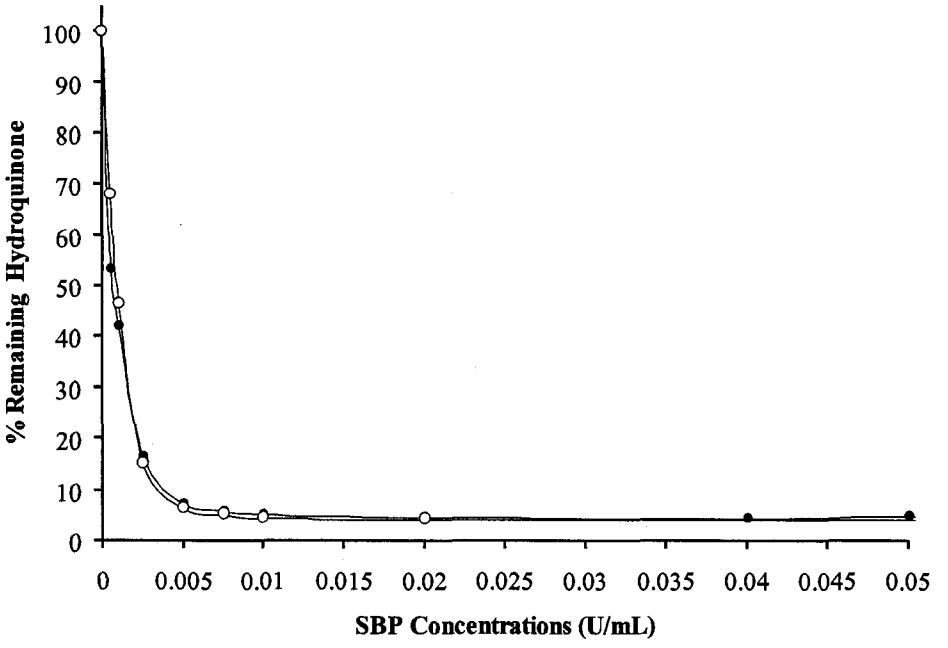


Figure 4.18: Minimum SBP concentration required for 95% conversion of hydroquinone; **Conditions:** 1.0 mM hydroquinone, 40.0 mM acetate buffer pH 5.2; **Test method:** HPLC

—●— 1.5 mM H_2O_2 —○— 1.5 mM H_2O_2/ PEG (150mg/L)

Comparing phenylenediamines to aryldiols compounds: The three isomers of phenylenediamine required less SBP than those of aryldiols for 95% conversions of the substrates (Table 4.3). The meta-substituted functional groups for phenylenediamine and aryldiols exhibit the highest SBP demand. Hence, it can be speculated that the interaction of the active site of the SBP with the meta-substituted functional groups is lower than that with the ortho- or para-substituted functional groups. The experiments conducted under stress hydrogen peroxide conditions exhibited a lower percent conversion of all the substrates as a result of limited formation of Compound I of the enzyme. As SBP concentration increased above the optimum, no significant effect was observed on the percent conversions of phenylenediamines and aryldiols; however, phenylenediamines

exhibited better precipitate formation. PEG_{3350} showed an insignificant effect on the conversion of phenylenediamines and aryldiols.

4.3: Minimum SBP concentrations and PEG effect		
Aromatic Compound	**Minimum SBP concentrations for 95% conversion of 1.0 mM substrate**	**PEG effect**
o-PD	0.002 U/mL	No
m-PD	0.01 U/mL	No
p-PD	0.005 U/mL	No
catechol	0.025 U/mL	No
resorcinol	0.2 U/mL	No
hydroquinone	0.005 U/mL	No

4.1.4 Consumption of aromatic substrates over time

The substrate conversion achieved in a reactor is dependent on the reaction time. It is generally observed that the peroxidases are faster in oxidizing substrates than laccase. Also, it has been observed that peroxidases were inactivated more rapidly [(2)]. Reaction time is one of the main parameters which determine the economics of an enzyme reactor. Therefore, the times required for the 95% conversion of the substrates were investigated under optimum conditions that were determined previously.

Phenylenediamines:

The conversion of phenylenediamines was monitored during the first three hours of the reaction. The experiment was conducted under the previously determined optimum pH and hydrogen peroxide concentration. The experiments were performed

both with the minimum SBP concentration required for the 95% conversion of 1.0 mM phenylenediamines and also at stress SBP concentration. The time required for 95% conversion of 1.0 mM o-PD, m-PD and p-PD at the optimum conditions were 60.0, 45.0 and 10.0 minutes, respectively (Figures 4.19, 4.20 and 4.21). It is observed that 65% conversion of o-PD was obtained during the first 15minutes, 85% conversion of m-PD was obtained during the first 15minutes and 70% conversion of o-PD was obtained during the first 2.5 minutes.

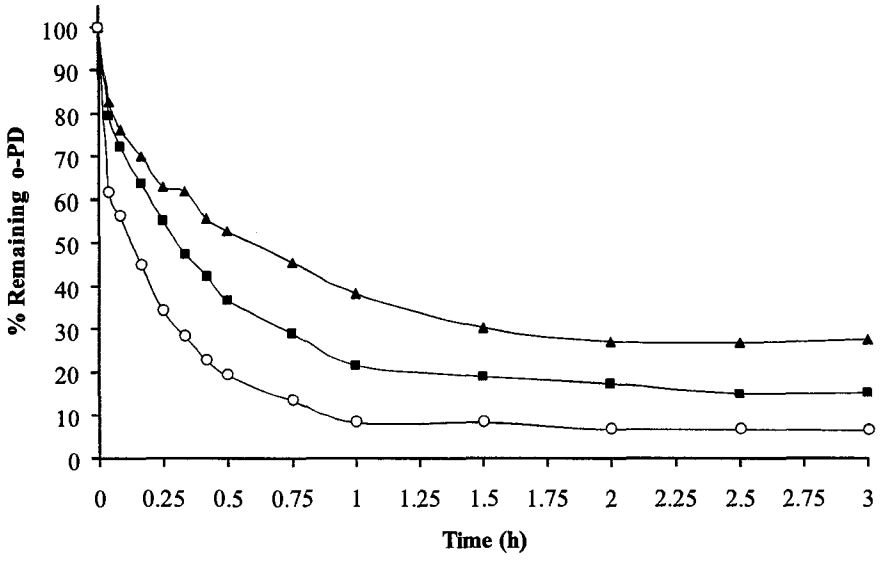


Figure 4.19: Conversion of o-PD over time; Conditions: 1.0 mM o-PD, 1.5 mM H2O2 and 40.0 mM acetate buffer pH 5.1; Test **method:** TNBS
—▲— 0.0005 U/mL SBP —■— 0.001 U/mL SBP —○— 0.002 U/mL SBP

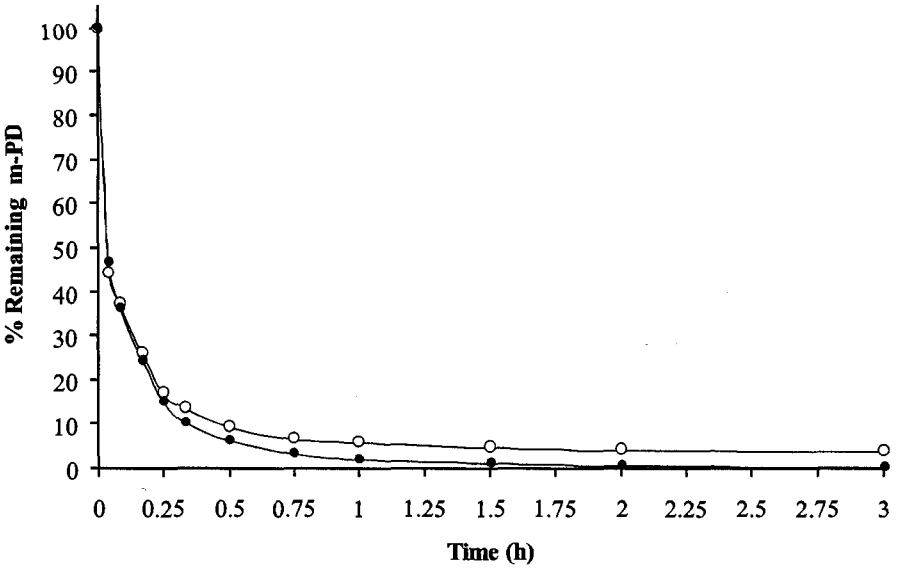


Figure 4.20: Conversion of m-PD over time; **Conditions:** 1.0 mM m-PD, 0.01 U/mL SBP, 2.5 mM H_2O_2 and 40.0 mM acetate buffer pH 5.4; **Test method:** HPLC and TNBS

HPLC Color Test (TNBS)

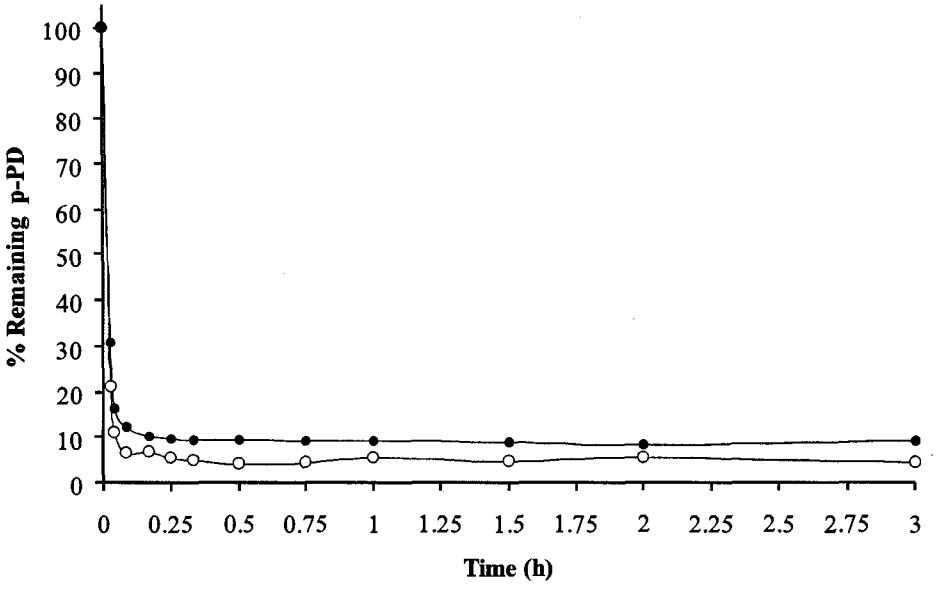


Figure 4.21: Conversion of p-PD over time; **Conditions:** 1.0 mM p-PD, 0.005 U/mL SBP, 1.5 mM H_2O_2 and 40.0 mM acetate buffer pH 5.6; **Test method:** HPLC and TNBS

HPLC Color Test (TNBS)

Aryldiols:

The conversion of aryldiols was monitored during the first three hours of the reaction. The experiments were conducted under the previously determined optimum pH and hydrogen peroxide concentrations. The experiments were performed both with the minimum SBP concentration required for 95% conversion of 1.0 mM aryldiols and also at stress SBP concentration. The time required for 95% conversion of the 1.0 mM catechol, resorcinol and hydroquinone at the optimum conditions were 45, 90 and 60 minutes, respectively (Figures 4.22, 4.23 and 4.24). It is observed that 82% conversion of the catechol was obtained during the first 15 minutes, 60% conversion of the resorcinol was obtained during the first 15 minutes and 80% conversion of the hydroquinone was obtained during the first 5 minutes.

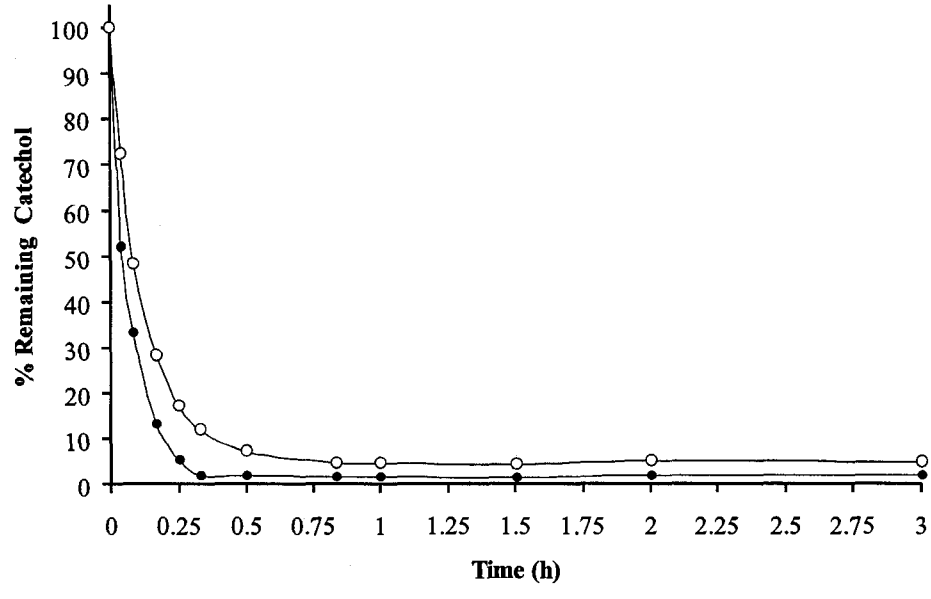


Figure 4.22: Conversion of catechol over time; **Conditions:** 1.0 mM Catechol, 3.5 mM H_2O_2 and 40.0 mM phosphate buffer pH 7.28; **Test method:** HPLC
—●— 0.1 U/mL SBP —○— 0.05 U/mL SBP

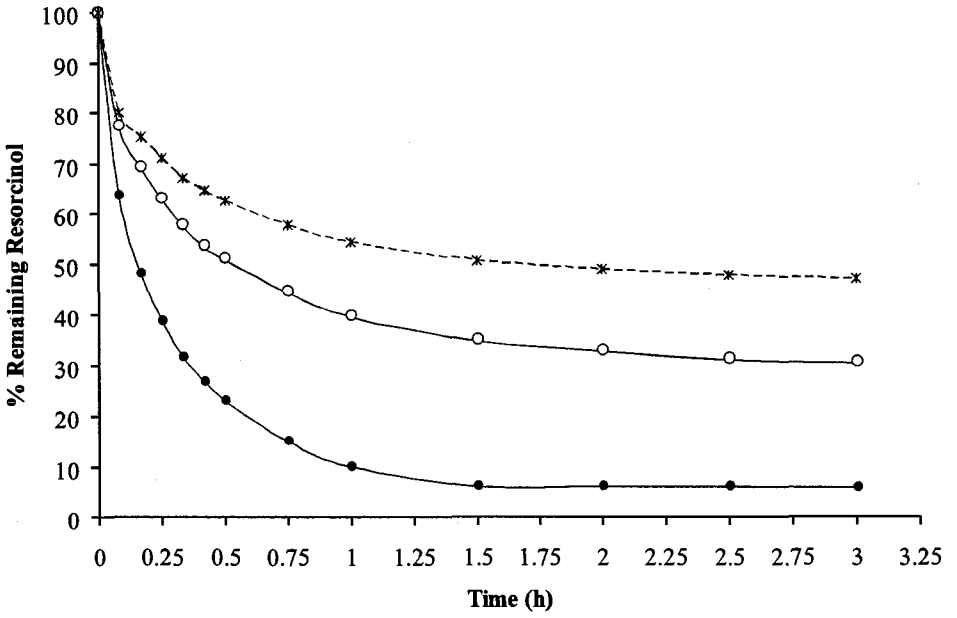


Figure 4.23: Conversion of resorcinol over time; **Conditions:** 1.0 mM resorcinol, 2.0 mM H_2O_2 and 40.0 mM phosphate buffer pH 7.73; **Test method:** HPLC
0.2 U/mL SBP 0.1 U/mL SBP 0.05 U/mL SBP

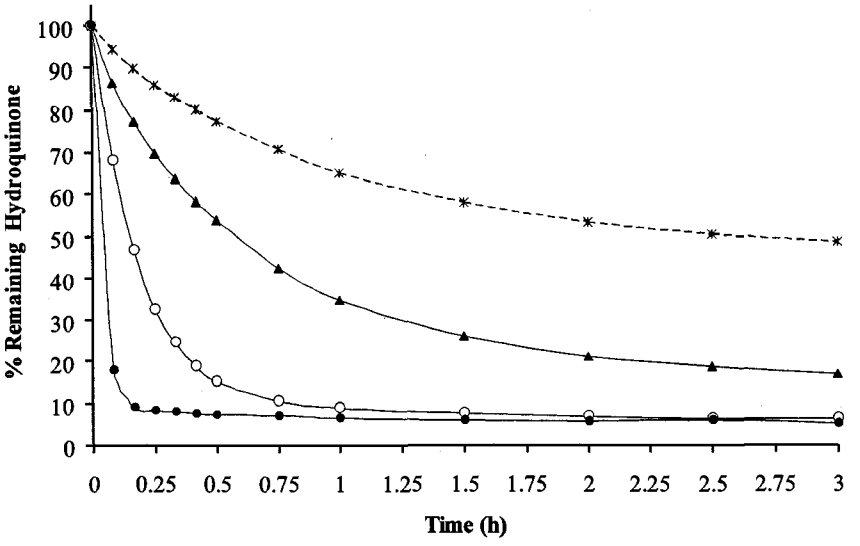


Figure 4.24: Conversion of hydroquinone over time; **Conditions:** 1.0 mM hydroquinone, 1.5 mM H_2O_2 and 40.0 mM acetate buffer pH 5.2; **Test method:** HPLC
0.05 U/mL SBP 0.01 U/mL SBP 0.0025 U/mL SBP
0.001 U/mL SBP

Comparing phenylenediamine to aryldiol compounds: Under the optimum conditions, 65- 95% of the three isomers of phenylenediamine got converted during the initial 15 minutes of the reaction. While, 60-85% of the three isomers of aryldiols got converted within the same time. During the initial 15 minutes of the reactions, the rates of conversion of both types of substrates were nearly the same with phenylenediamines showing little bit higher rates. The conversion of the substrates continued at a slower rate after the first 15 minutes. The slower conversion can be due to competitive conversion of dimers and higher polymers. It can also be due to the enzyme inactivation caused by the highly reactive radicals produced that reacted with the active site of the enzyme as discussed in Section 4.1.2. It is noticed that the rate of reaction is directly proportional to the enzyme concentration, as the enzyme concentration decreased the rate of reaction also decreased [8]. Experiments were conducted both in the presence of excess and stress SBP concentrations. It was observed that as the SBP concentration increased, the rate of conversion also increased. This is clearly shown in Figures 4.19, 4.23 and 4.24. Thus, an increase in SBP concentration can significantly reduce the time for the completion of the reaction.

Previous research has demonstrated that as the enzyme concentration increased, the rate of reaction also increased. Nevertheless, as the enzyme concentration increased, the enzyme inactivation increased proportionally. Thus, the enzyme turnover was low when enzyme concentration was high [22, 23].

4.1.5 Consumption of hydrogen peroxide over time

Hydrogen peroxide plays an important role in enzymatic treatment. Inadequate hydrogen peroxide results in insufficient formation of the active form (Compound I) of the enzyme and hinders the substrate conversion, while excess hydrogen peroxide can cause the deactivation of the enzyme [8]. Therefore, monitoring the consumption of hydrogen peroxide over time at the optimum conditions is important.

Phenylenediamines:

The hydrogen peroxide consumption by the enzymatic reaction of o-PD and m-PD was monitored during the first three hours of the reaction. The experiment was conducted under the previously determined optimum pH and hydrogen peroxide concentration. The experiments were performed both with the minimum SBP concentration required for 95% conversion of 1.0 mM substrates and also at stress SBP concentrations. The reaction time required for 95% consumption of hydrogen peroxide was found to be approximately 45 minutes for o-PD and p-PD. At the optimum SBP condition, o-PD consumed 75% of the hydrogen peroxide during the first 15 minutes while p-PD consumed 90% during the same time (Figures 4.25 and 4.26). The hydrogen peroxide consumption for m-PD could not be determined due to the interference with the color test by-products formed

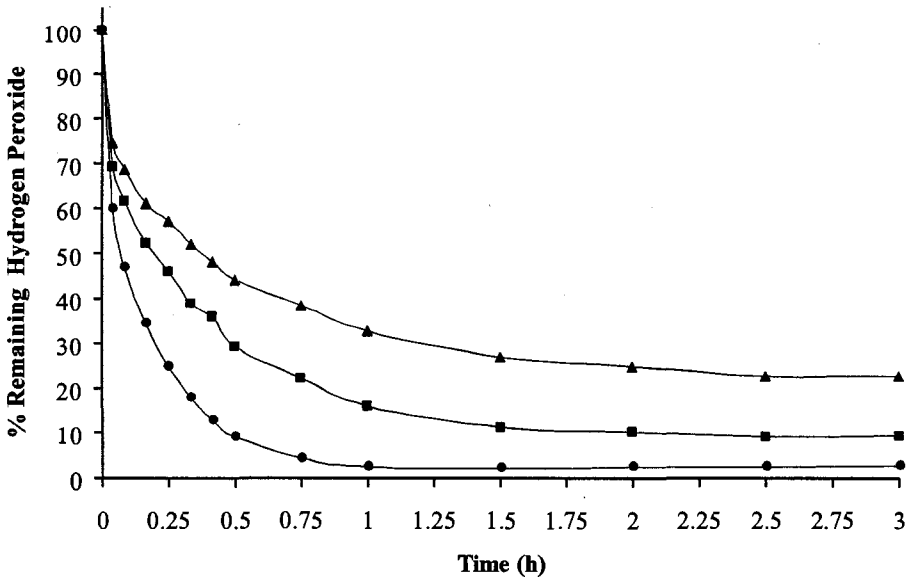


Figure 4.25: Hydrogen peroxide consumption over time for o-PD; **Conditions:** 1.0 mM o-PD, 1.5 mM H_2O_2 and 40.0 mM acetate buffer pH 5.1; **Test method:** hydrogen peroxide color test

0.0005 U/mL SBP 0.001 U/mL SBP 0.002 U/mL SBP

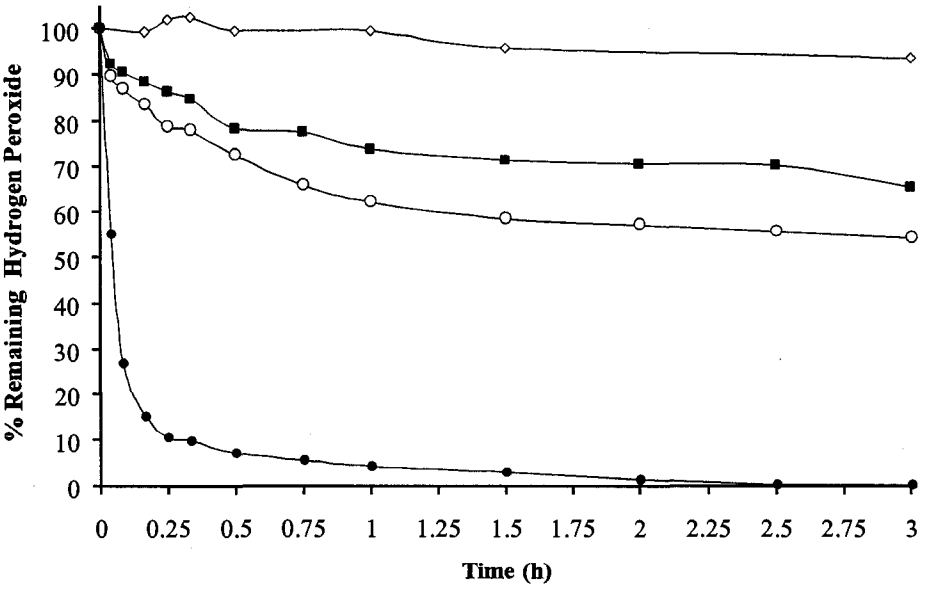


Figure 4.26: Hydrogen peroxide consumption over time for p-PD; **Conditions:** 1.0 mM p-PD, 1.5 mM H_2O_2 and 40.0 mM acetate buffer pH 5.6; **Test method:** hydrogen peroxide color test)

0.007 U/mL SBP 0.0005 U/mL SBP 0.0001 U/mL SBP

No SBP

Aryldiols:

Hydrogen peroxide consumption by the enzymatic reaction of catechol and resorcinol was monitored during the first three hours of the reaction. The experiment was conducted under the previously determined optimum pH and hydrogen peroxide concentration. The experiments were performed both with the minimum SBP concentrations required for 95% conversion of 1.0 mM substrates and also at stressed SBP concentration. The reaction time required for 95% consumption of hydrogen peroxide was found to be approximately 60.0 minutes for catechol and 90.0 minutes for resorcinol. Catechol consumed 75% of the hydrogen peroxide during the first 15 minutes while resorcinol consumed 45% during the same time (Figures 4.27 and 4.28). The hydrogen peroxide consumption for hydroquinone could not be determined due to the product interference with the color test by-products formed

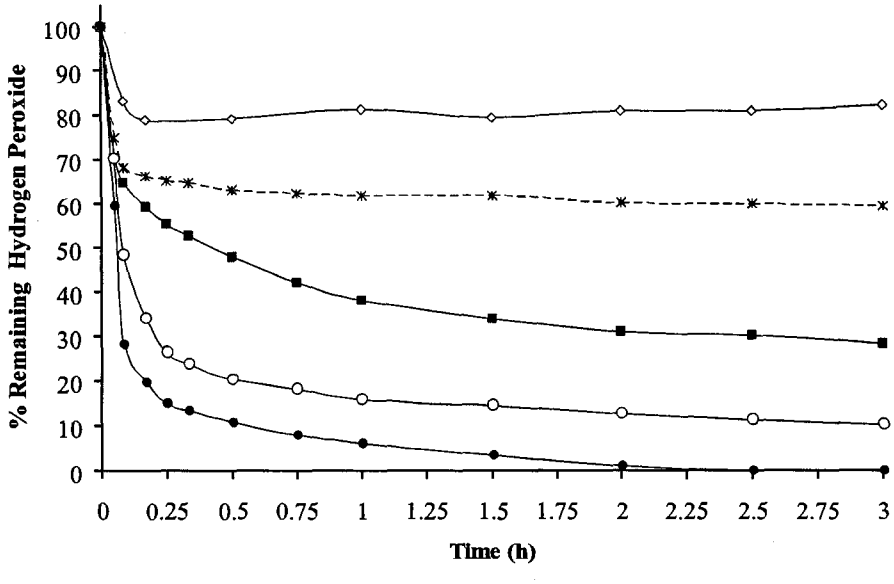


Figure 4.27: Hydrogen peroxide consumption over time for catechol; **Conditions:** 1.0 mM catechol, 3.5 mM H_2O_2 and 40.0 mM phosphate buffer pH 7.28; **Test method:** hydrogen peroxide color test

—●— 0.1 U/mL SBP —○— 0.05 U/mL SBP —■— 0.01 U/mL SBP

—✳— 0.001 U/mL SBP —◇— No SBP

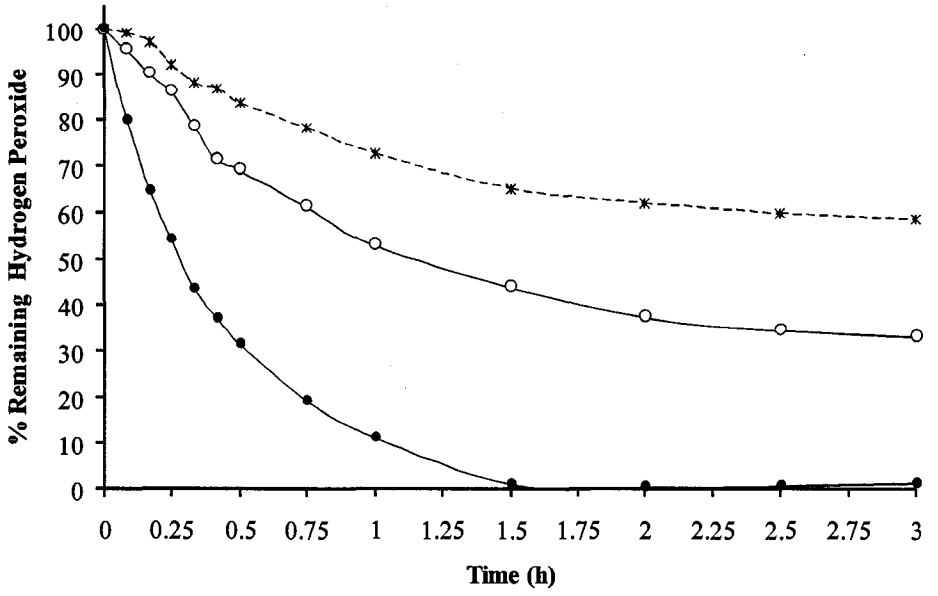


Figure 4.28: Hydrogen peroxide consumption over time for resorcinol; **Conditions:** 1.0 mM resorcinol, 2.0 mM H_2O_2 and 40.0 mM phosphate buffer pH 7.73; **Test method:** hydrogen peroxide color test

—●— 0.2 U/mL SBP —○— 0.1 U/mL SBP —✳— 0.05 U/mL SBP

Comparing phenylenediamine to aryldiols compounds: Under the optimum conditions, for the two isomers of phenylenediamine, 75-90% of the hydrogen peroxide got converted during the initial 15 minutes of the reaction, while 45-75% of the hydrogen peroxide got converted within the same time for the two isomers of aryldiols. During the initial 15.0 minutes of the reactions, the rates of conversion of phenylenediamines were higher than that with the aryldiols. While comparing the consumption of the substrates with the consumption of hydrogen peroxide during the first 15 minutes, hydrogen peroxide was consumed at a faster rate than the substrate. The higher rate of consumption of the hydrogen peroxide may have resulted from the competitive conversion of dimers and higher polymers as discussed in Section 4.1.4. The consumption of the hydrogen peroxide continued at a slower rate after the first 15

minutes, similar to the substrate conversion. The slower rate of consumption may be due to the enzyme inactivation from the highly reactive radicals produced that reacted with the active site (discussed in Section 4.1.2).

4.1.6 Removal of the Polymer Products

The enzymatic treatment uses peroxidases in the presence of hydrogen peroxide to form phenloxyl or arylamine radicals. These radicals are coupled to form dimers and then polymers. The dimers and polymers formed can be substrates for the enzyme for another enzymatic cycle as long as they do not exceed their solubility limit. In that case, they precipitate out and can be separated by filtration or sedimentation [8]. It is suspected that color arises from quinone-liked products, whether of monomers or polymers remaining in solution [30]. Therefore, removing the colored soluble products is important, especially when applied to real samples. Different flocculation and coagulation agents have been used for such purpose.

4.1.6.1 Removal of the Polymer Products of Phenylenediamines by SDS alone

Previous research has reported that polymeric colored products result from the ARP- and SBP-catalyzed polymerization of diaminotoluenes and laccase-catalyzed polymerization of diphenylamine can be removed by using SDS with alum via adsorption micellar flocculation (AMF). The previous research has illustrated that the polymer products were able to partition into the micelles causing a bridge-like structure formation between the SDS micelles. The large bridged SDS micelle complex could be

microfiltered but it did not settle until alum was added [30, 32, 33]. In this study, SDS alone was found to be able to settle the polymer products without using alum. Experiments were conducted to investigate the effect of SDS on the polymeric colored product removal from phenylenediamine reaction supernatants after 3.0 hours of enzymatic treatment under the optimal conditions for 95% conversion of the monomers.

In the enzymatic reaction of SBP with phenylenediamines, after the optimal conditions, the solution color changed to yellow-orange, dark reddish/black and dark brownish/black for o-PD, m-PD and p-PD, respectively. The colored solutions had absorbance maxima at 434 nm, 304 nm, and 444 nm for o-PD, m-PD and p-PD, respectively. No precipitates were observed after the enzymatic reaction and SDS was added to remove color. The SDS concentration range studied was 0.005-0.6 mM (1.44-173.0 mg/L). The pH of the solution was kept the same as after the enzymatic reaction. The samples were stirred and flocs were allowed to settle. The supernatants, microfiltered and non-microfiltered, were analyzed at their absorbance maxima. The minimum SDS required to remove of >90% of the SDS-polymer complex after settling (non-microfiltered samples) was found to be 0.5 mM (144.2 mg/L) , 0.25 mM (72.1 mg/L) and 0.2 mM (57.7 mg/L) for o-,m- and p- PD, respectively (Figures 4.29, 4.30 and 4.31). It was observed that the SDS-polymer complexes were formed at lower SDS concentrations also but did not settle until enough SDS was added. These samples were microfiltered sample for m- and p-PD (Figures 4.30 and 4.31). It is speculated that SDS-polymer complexes had to reach a certain molecular mass in order to settle. It was previously demonstrated that the SDS concentration had to be higher than the critical micellar

concentration (CMC) in order for micelles to form [34]. The CMC of SDS in water is 8.3 mM and it is reduced to 1.5 mM in the presence of 50.0 mM phosphate buffer [35, 36]. The CMC is further reduced in the presence of non-electrolyte species such as phenylenediamine polymers [34]. Also, it was found that species that partitioned into the micelles further reduced the CMC [34]. The CMC was not determined in this study.

Two previously proposed hypotheses on how the polymer product might bridge the SDS micelles together were either by the partition of the polymer into the different micelles or by the electrostatic interaction of the amino groups of the protonated polymer onto the surface of the micelles [30]. In order to investigate which hypothesis is more reasonable, the effect of pH of the SDS-polymer complex was studied.

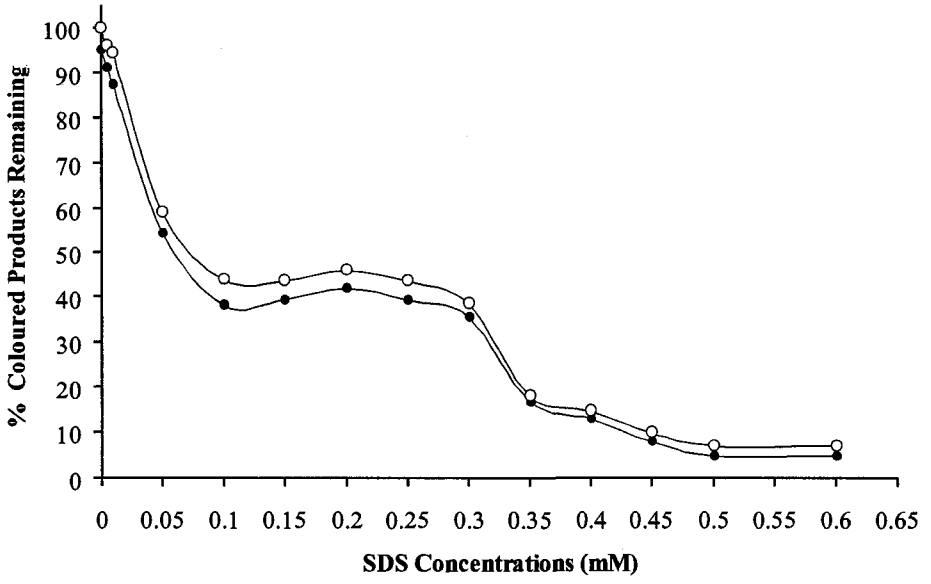


Figure 4.29: Effect of SDS concentration on the removal of o-PD polymeric color products; **Conditions:** Total volume 22.0mL and pH ~ 5.1; **Test method:** Direct Absorbance @ 434 nm

—●— Micro-filtered —○— Settled Floc

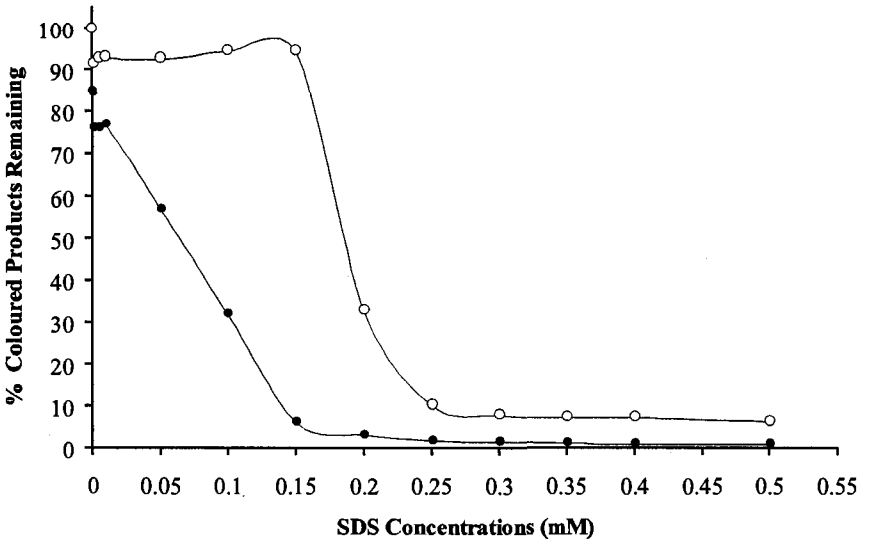


Figure 4.30: Effect of SDS Concentration on the removal of m-PD polymeric color products; **Conditions:** Total volume 22mL and pH ~ 5.4; **Test method:** Direct Absorbance @ 304 nm

—●— Micro-filtered —○— Settled Floc

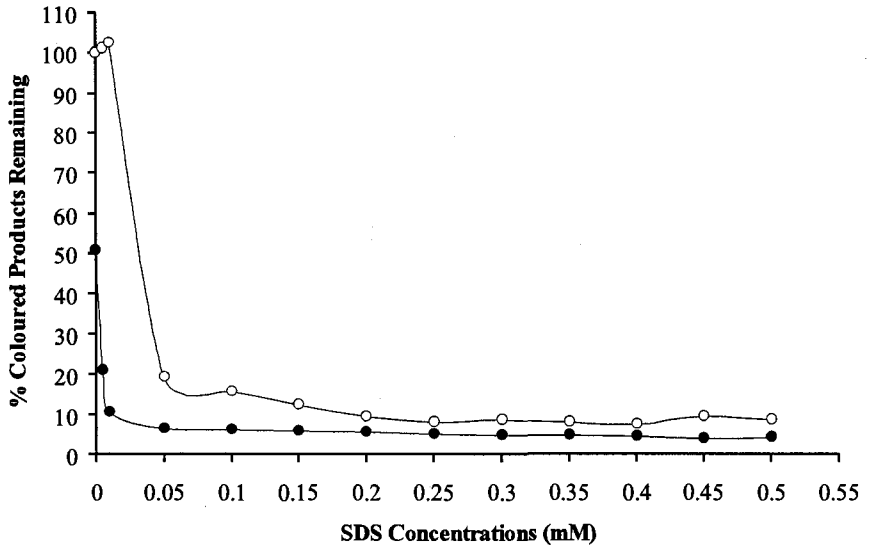


Figure 4.31: Effect of SDS Concentration on the removal of p-PD polymeric color products; **Conditions:** Total volume 22mL and pH ~ 5.6; **Test method:** Direct Absorbance @ 444 nm

—●— Micro-filtered —○— Settled Floc

4.1.6.2 Effect of pH on the removal of phenylenediamine-SDS complexes

Several sequential experiments were conducted to determine the effect of pH on the removal of polymeric colored products in the presence and absence of SDS. The pH was adjusted with sulphuric acid or sodium hydroxide. All the experiments were conducted after 3.0 hours of enzymatic treatment under optimal conditions for 95% conversion of the monomers. This study helped in clarification of the hypothesis of bridge formation of the SDS-polymer complexes. When the pH of the solution exceeded the pK_a of the polymers, and if the positively charged amino groups of the polymer interact with the negatively-changed SDS (electrostatic interaction), then it would have been neutralized thereby causing desorbing of the polymers from the SDS; whereas if the pH had no effect on the SDS-polymer complex, the interaction can be speculated to result from polymer partition into the micelles.

o-PD: In the absence of SDS, the polymeric colored products from the enzymatic reaction seem to be affected by the pH change (Figure 4.32). The color of polymeric product changed from orange to yellow as the pH increased above 5.1. When the pH decreased below 5.1, the polymeric products color changed from orange to dark orange. No precipitate formation was observed at any pH in the absence of SDS. It can be speculated that the color change from orange to yellow may have resulted from the formation of deprotonated polymers as the pH increased above the pK_a, while as the pH decreased below the pK_a, the color change from orange to dark orange resulted from more protonated polymers in the samples.

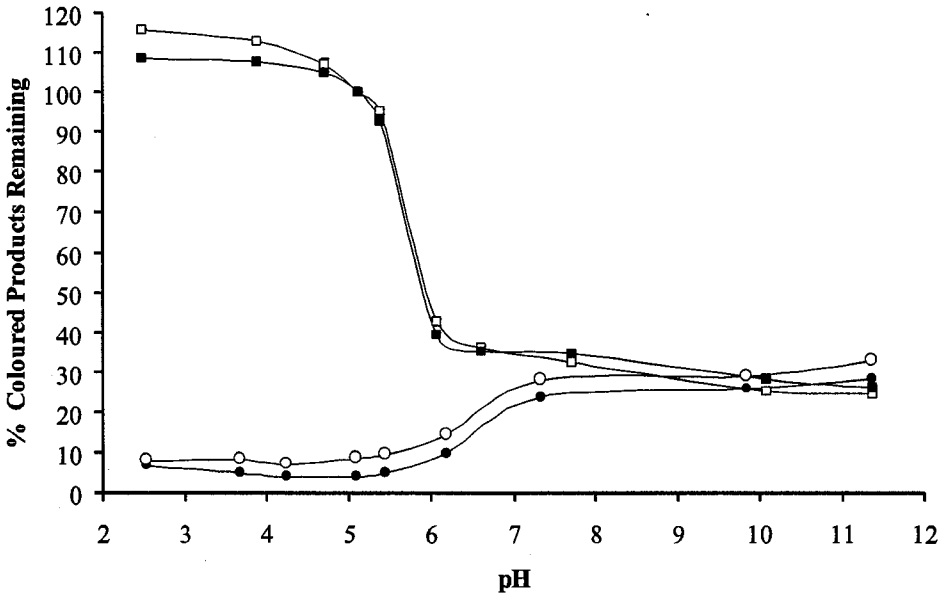


Figure 4.32: Effect of pH on the removal of o-PD polymeric color products; **Conditions:** 2.7 mM Alum, 0.5 mM SDS, total volume 22.0 mL and pH adjusted using NaOH and H_2SO_4; **Test method:** Direct Absorbance @ 434 nm

—●— Only SDS (Micro-filtered) —○— Only SDS (Settled Floc)

—■— No SDS (Micro-filtered) —□— No SDS (Settled Floc)

In the presence of SDS (0.5 mM), more than 90% of the polymeric colored products were removed in the pH range 2.5-5.1. As the pH increased from 5.4 to 7.0, the apparent removal decreased as a result of new polymeric product formation (yellow color) at higher pH that could not be removed with SDS. As the pH increased above 7.0, no SDS effect was observed (no precipitates) which is similar to the pH effect in the absence of SDS. Hence, it is speculated that the SDS complex can not be formed with the deprotonated polymers and the SDS-polymer complexes resulted from electrostatic interaction of the SDS and the protonated amino or imines groups of the polymers of o-PD.

m-PD: In the absence of SDS, the polymeric colored products from the enzymatic reaction seem to be affected by the pH changes (Figure 4.33). Precipitate started forming but did not settle when the pH was above 7.0 or below 5.5. As the pH decreased below 3.5, precipitate starts settling. This phenomenon may have resulted from polymer hydrolysis but it is hard to explain without polymer product characterization.

In the presence of SDS (0.5 mM), more than 90% of the polymeric colored products were settled in the pH range 1.8-10.8. No pH effect on the SDS-polymer complexes was observed. Hence, it can be speculated that the bridge formation between the SDS micelles may have resulted from the partition of the polymers into the different micelles and not from electrostatic interaction.

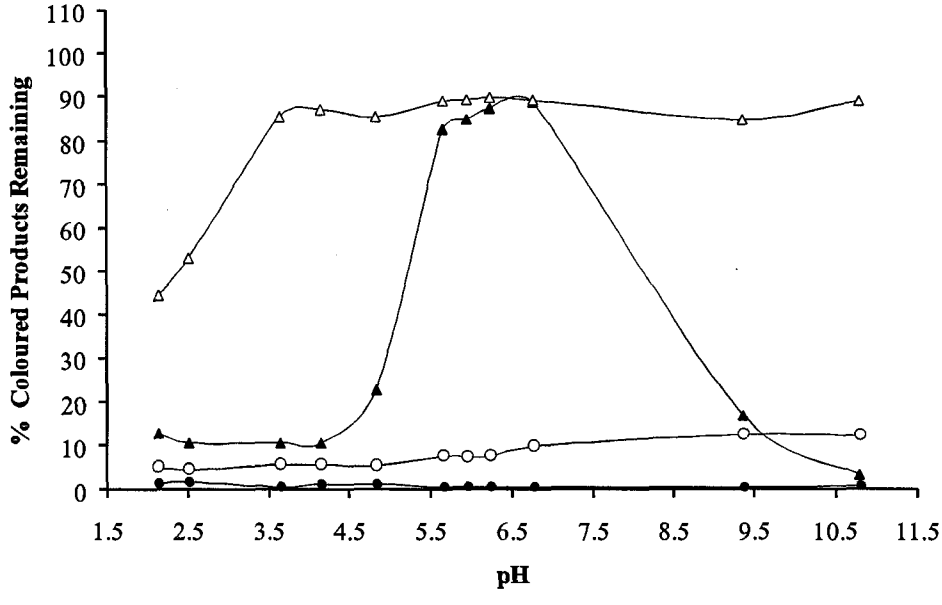


Figure 4.33: Effect of pH on the removal of m-PD polymeric color products; **Conditions:** 2.7 mM Alum, 0.5 mM SDS, total volume 22.0 mL and pH adjusted using NaOH and H_2SO_4; **Test method:** Direct Absorbance @ 304 nm

—●— Only SDS (Micro-filtered) —○— Only SDS (Settled Floc)

—▲— No SDS (Micro-filtered) —△— No SDS (Settled Floc)

p-PD: In the absence of SDS, the polymeric colored products from the enzymatic reaction seems to be affected by the pH changes (Figure 4.34). In the pH range between 5.5 and 8.0, precipitates formed but did not completely settle when the microfiltered and non-microfiltered samples are compared. Precipitate increased and settled when the pH was above 8.0 or below 5.5, similar to what was found with m-PD.

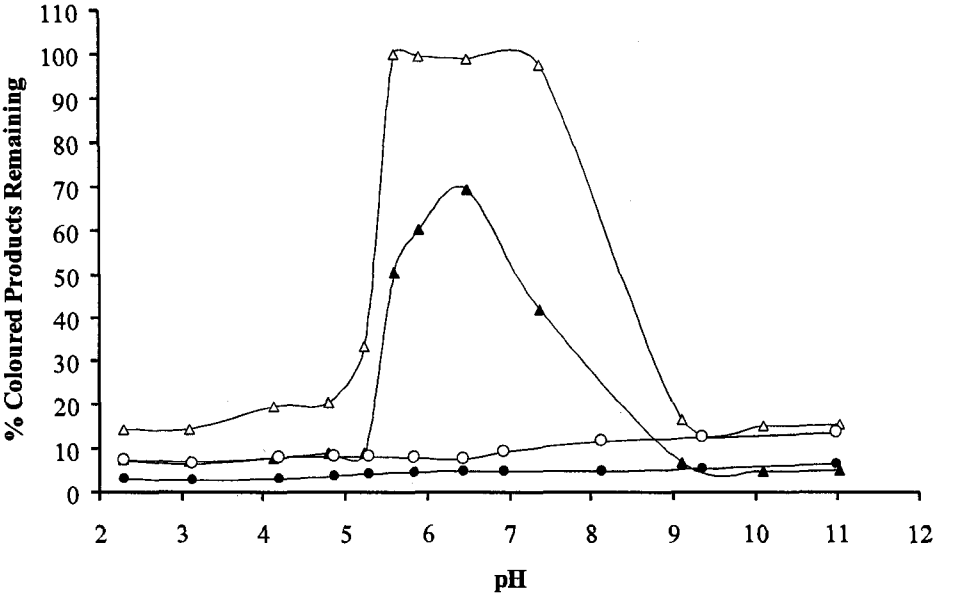


Figure 4.34: Effect of pH on the removal of p-PD polymeric color products; **Conditions:** 2.7 mM Alum, 0.5 mM SDS, total volume 22.0 mL and pH adjusted using NaOH and H_2SO_4; **Test Method:** Direct Absorbance @ 444 nm

—●— Only SDS (Micro-filtered) —○— Only SDS (Settled Floc)

—▲— No SDS (Micro-filtered) —△— No SDS (Settled Floc)

In the presence of SDS (0.5 mM), more than 90% of the polymeric colored products were settled in the pH range 2.2-11.0. No pH effect on the SDS-polymeric product was observed in this range. Hence it can be speculated that the bridge formation between the SDS micelles have may resulted from the partition of the polymers into the

different micelles and not from electrostatic interaction similar to what was found with m-PD.

4.1.6.3 Removal of the Polymer Products of aryldiols by Alum

Previous research has shown that polymeric colored products resulting from the ARP-catalyzed polymerization of phenol and laccase-catalyzed polymerization of bisphenol A could be removed by using alum [7, 37]. In aqueous solution, when the aluminum salt is dissolved, the metal ions (Al^{3+}) are hydrolysed to form aluminum hydroxide gel-like amorphous structure at alkaline pH. The resulting gel-like structure has large surface area and positives charges [38]. The partially negative polymers from the enzymatic reaction can be adsorbed into the gel and be removed with the gel by filtration or sedimentation. In the present study, experiments were conducted to investigate the effect of alum on the removal of polymeric colored products from aryldiol reactions. In the first attempt, experiments were conducted after the 3.0 hours of enzymatic treatment for catechol and resorcinol in phosphate buffers. It was observed that no gel formation occurred in the alkaline pH, but rather in an acidic pH of 5.5. The gel formation was inefficient in removing the polymeric products for resorcinol but was able to remove about 70% of the polymeric products from catechol at pH 5.5. It is believed that in the presence of phosphate buffer, aluminum hydroxide gel was not formed but rather aluminum phosphate gel was formed. Control experiments were conducted to confirm this phenomenon. In the presence of alum and only distilled water, a gel-like structure was observed in the pH range between 4.82 and 9.23, while in the presence of phosphate buffer, gel formation was observed between 3.67 and 6.11. As a result of this

phenomenon, the enzymatic reactions were run in tap water instead of phosphate buffer for 3.0 hours under optimal conditions, as discussed in Sections 4.1.2 and 4.1.3, and then the alum was added.

After the enzymatic reaction of SBP with aryldiols, the solution color changed to dark brownish/black, red and light red for catechol, resorcinol and hydroquinone, respectively. The color solutions had an absorbance at 440 nm and no precipitate was observed after the enzymatic reactions. The aluminium ion concentration range studied was 0.27-2.2 mM (7.34-59.4 mg/L). The pH of the solution was adjusted to 7.0. The samples were stirred and flocs were allowed to settle. The supernatants, both microfiltered and non-microfiltered were analyzed for absorbance at 440 nm. Approximately 90 to 95% of the microfiltered colored product for catechol and resorcinol were removed when using 0.7 mM alum (18.9 mg/L) and 0.6 mM alum (16.2 mg/L), respectively, while 80 to 90% of settled floc colored product for catechol and resorcinol are removed using 1.35 mM alum (36.4 mg/L) and 0.8 mM alum (21.6 mg/L), respectively (Figures 4.35 and 4.36). The results illustrate that the floc formed at lower aluminium ion concentrations did not settle until enough alum was added. Only 60% of the colored products for hydroquinone were removed with 0.7 mM alum (18.9 mg/L; Figure 4.37).

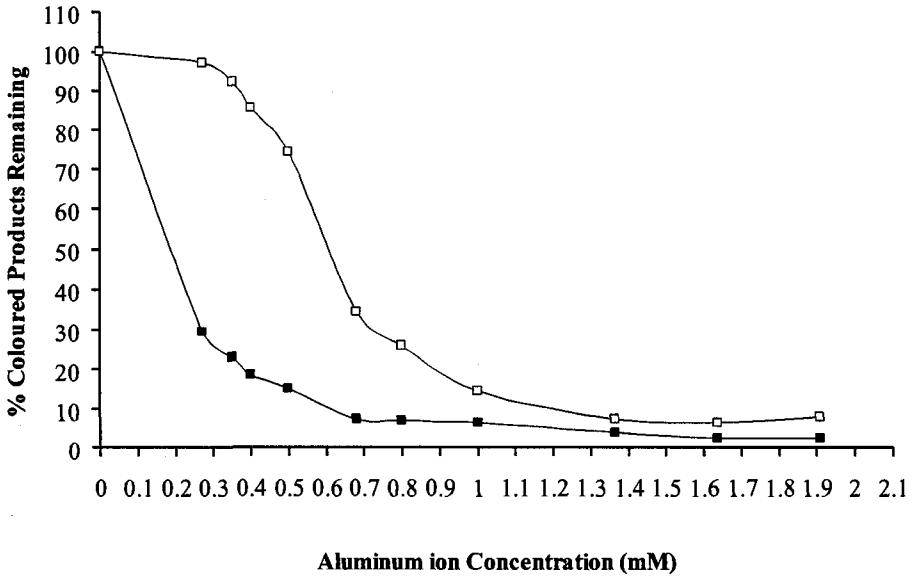


Figure 4.35: Effect of alum concentration on the removal of catechol polymeric colored products; **Conditions:** Total volume 22.0 mL and pH ~ 7.0; **Test method:** Direct Absorbance @ 440 nm

—●— Micro-filtered —○— Settled Floc

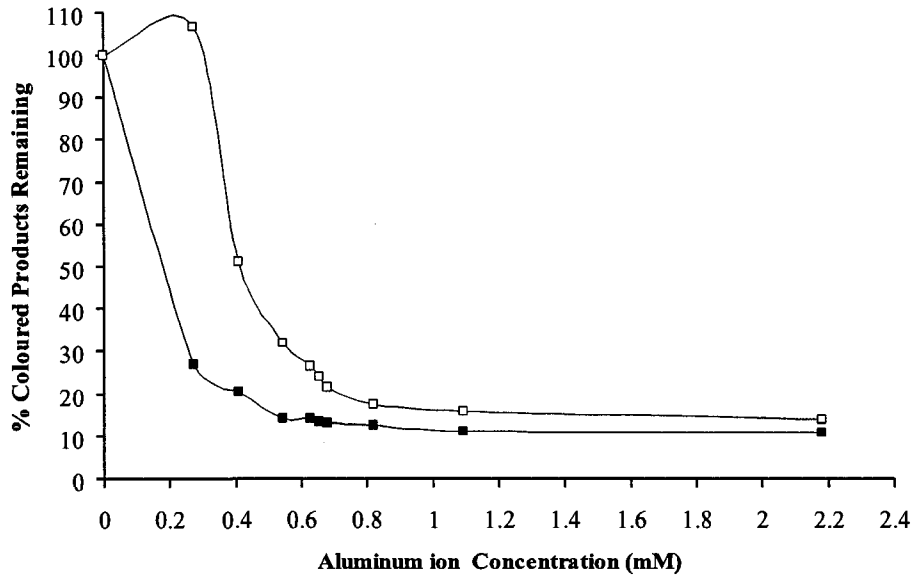


Figure 4.36: Effect of alum concentration on the removal of resorcinol polymeric colored products; **Conditions:** Total volume 22.0 mL and pH ~ 7.0; **Test method:** Direct Absorbance @ 440 nm

—●— Micro-filtered —○— Settled Floc

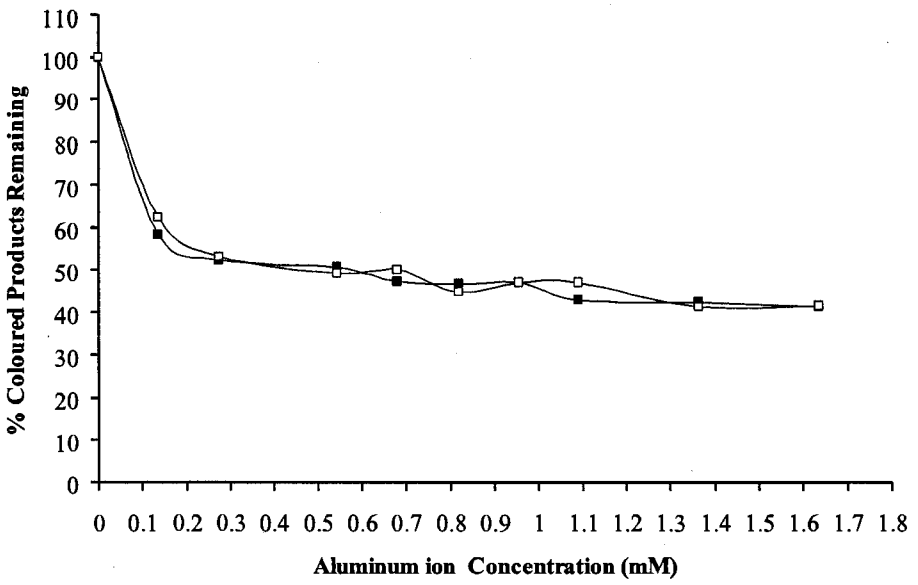


Figure 4.37: Effect of alum concentration on the removal of hydroquinone polymeric colored products; **Conditions:** Total volume 22 mL and pH ~ 7.0; **Test method:** Direct Absorbance @ 440 nm

—●— Micro-filtered —○— Settled Floc

4.1.6.4 Effect of pH on the removal of aryldiols in the presence of alum

Several sequential experiments were conducted to determine the effect of pH on the removal of polymeric colored products in the presence and absence of aluminum ions. All the experiments were conducted after the 3.0 hours of enzymatic treatment under optimal conditions for 95% conversion of the monomers. The pH was adjusted by using sulphuric acid or sodium hydroxide.

Catechol: In the absence of aluminum ions, the polymeric colored products from the enzymatic reaction seem to be affected by the pH changes (Figure 4.38). When the pH was above 7.0, the color becames darker and no precipitate was observed. As the pH

decreased below 7.0, precipitate started forming. In the presence of aluminum ions, 2.7 mM, more than 95% of the polymeric colored products settled in the pH range of 4.5-8.5. As the pH increased above 8.0, a decrease in the aluminum ion effect was observed.

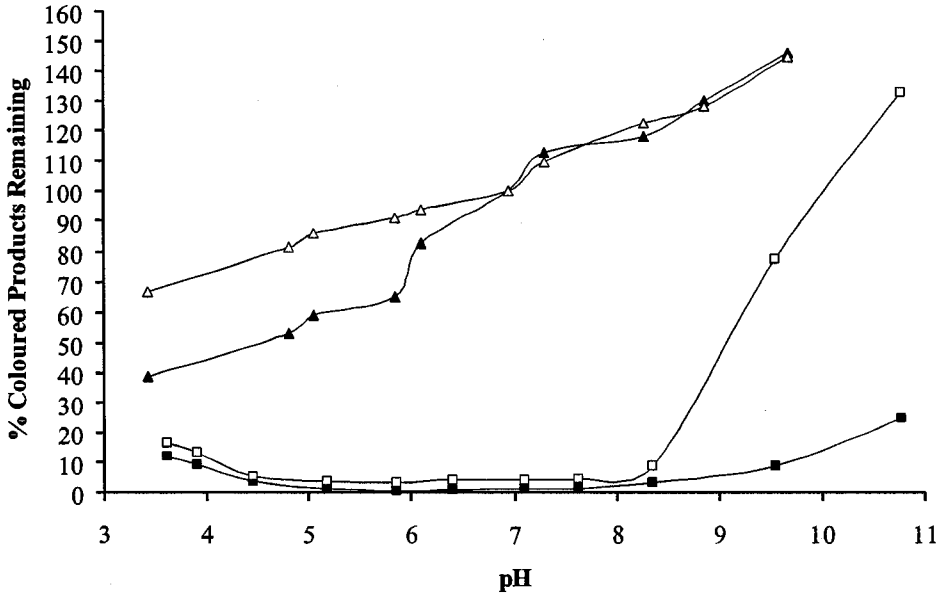


Figure 4.38: Effect of pH on the removal of catechol polymeric colored products; **Conditions:** 2.7 mM alum, total volume 22 mL and pH adjusted using NaOH and H_2SO_4; **Test method:** Direct Absorbance @ 440 nm

Only Alum (Micro-filtered) Only Alum (Settled Floc)

No Alum (Micro-filtered) No Alum (Settled Floc)

Resorcinol: In the absence of aluminum ions, the polymeric colored products from the enzymatic reaction seem to be affected by the pH changes (Figure 4.39). When the pH was changed to above 8.0, the color becames darker and no precipitate was observed. As the pH decreased below 5.5, precipitate started forming. In the presence of aluminum ions, 2.7 mM, more than 90% of the polymeric colored products settled in the

pH range of 2.2-8.5. At the pH above 9.0, a decrease in the aluminum ions effect was observed.

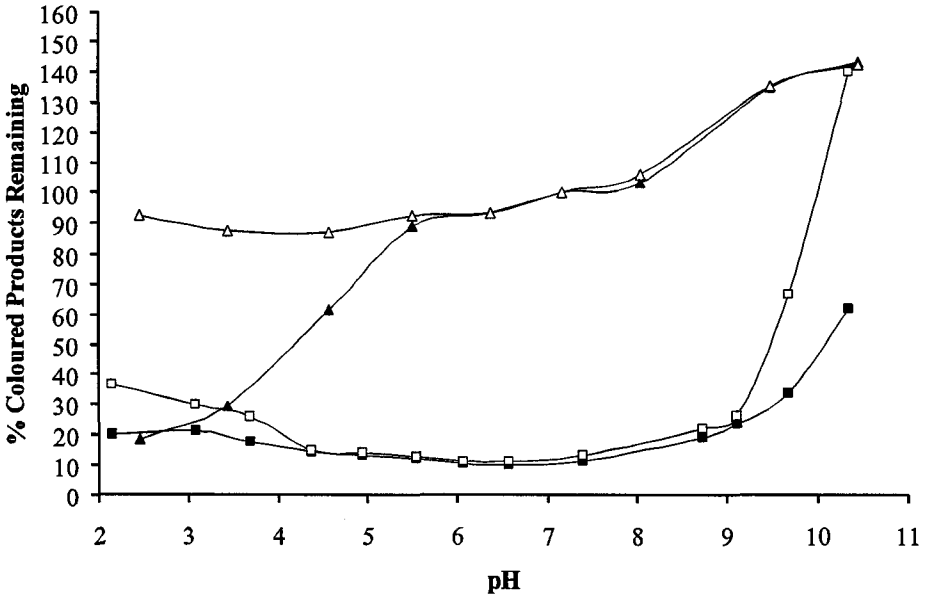


Figure 4.39: Effect of pH on the removal of resorcinol polymeric colored products; **Conditions:** 2.7 mM alum, total volume 22.0 mL and pH adjusted using NaOH and H_2SO_4; **Test method:** Direct Absorbance @ 440 nm

Only Alum (Micro-filtered) Only Alum (Settled Floc)

No Alum (Micro-filtered) No Alum (Settled Floc)

Hydroquinone: In the absence of aluminum ions, the polymeric colored products from the enzymatic reaction seem to be affected by the pH changes (Figure 4.40). When the pH was above 8.0, the color became darker and no precipitate was observed. As the pH decreased below 3.0, precipitate started forming. In the presence of aluminum ions, 2.7 mM, more than 70% of the polymeric colored products settled in the pH range of 5.0-8.0. At pH above 9.0, a decrease in the aluminum ion effect was observed.

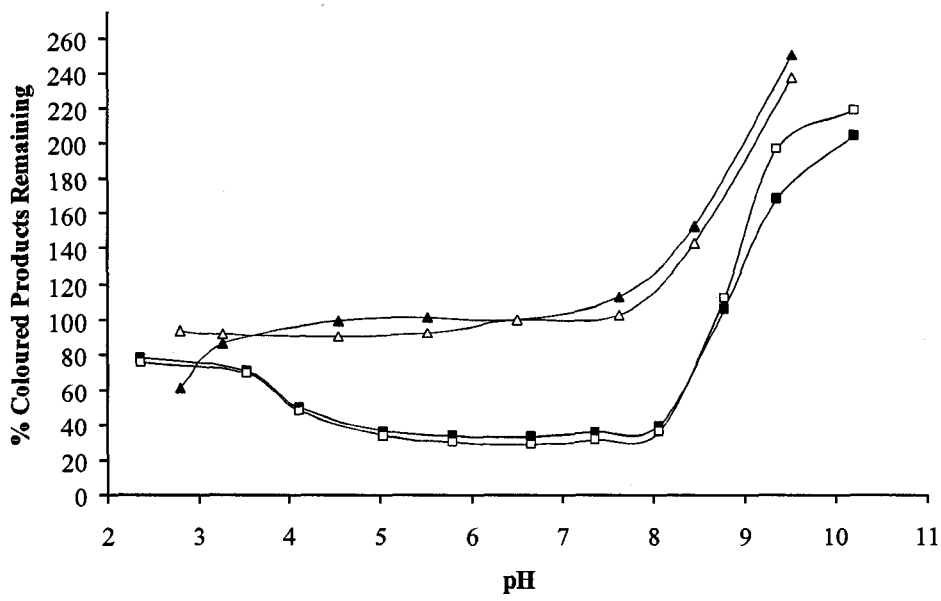


Figure 4.40: Effect of pH on the removal of hydroquinone polymeric color products; **Conditions:** 2.7 mM Alum, total volume 22.0 mL and pH adjusted using NaOH and H_2SO_4; **Test method:** Direct Absorbance @ 440 nm

—■— Only Alum (Micro-filtered) —□— Only Alum (Settled Floc)

—▲— No Alum (Micro-filtered) —△— No Alum (Settled Floc)

4.1.6.5 Effect of SDS and alum on the removal of aromatic monomers

Previous research has shown that ferric chloride can be used as a coagulant-flocculant agent in removing aromatic compounds from water [31]. Ferric chloride has similar functionality as aluminum sulphate in a coagulant-flocculant process.

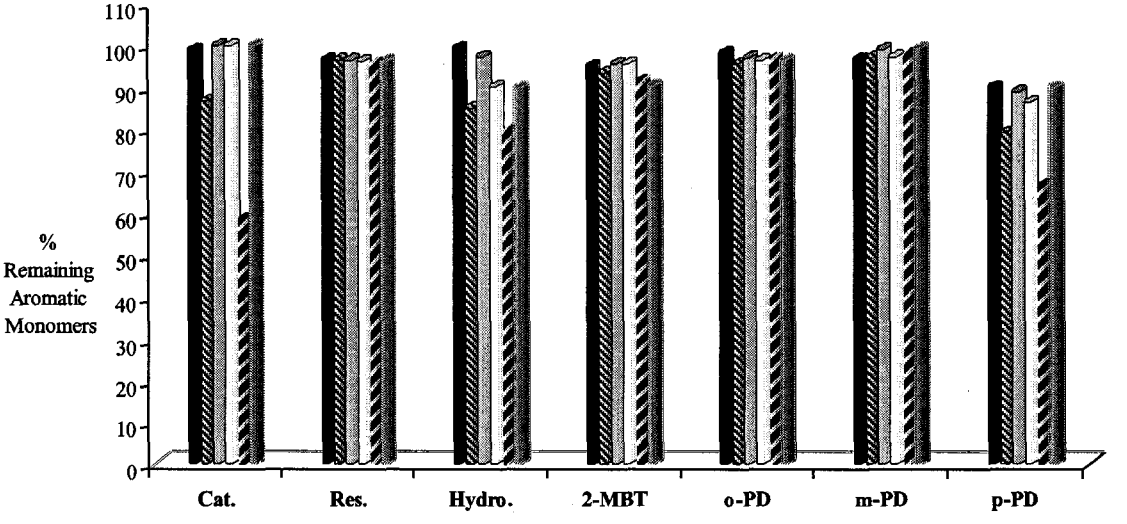


Figure 4.41: Effect of Alum and SDS on the removal of aromatic monomers; **Conditions:** 1.0 mM aromatic monomers, 2.7 mM alum, 0.5 mM SDS and pH adjusted using NaOH and H_2SO_4; **Test method:** HPLC: catechol, resorcinol and hydroquinone ; TNBS: o-PD, m-PD and p-PD

■ pH 5.0 (control) ▨ pH 5.0-Alum ▩ pH 5.0-SDS
□ pH 7.0 (control) ▨ pH 7.0-Alum ▩ pH 7.0-SDS

Hence, control experiments were conducted to investigate the effect of alum, 2.7 mM, and SDS, 0.5 mM, in removing 1.0 mM aromatic monomers at pH 5.0 and 7.0 (Figure 4.41). The results demonstrate no significant effect of SDS on the removal of any of the aromatic compounds at both pH values. Similarly, alum showed no significant

effect in the removal of resorcinol, 2-MBT (to be considered in section 4.2, below), o-PD and m-PD at both pH values, while 5 to 15% removal was observed for hydroquinone and p-PD. The most significant effect was observed for catechol in which 40% removal was observed at pH 7.0 and about 10% at pH 5.0. The results confirm the previous work in which resorcinol showed no removal, while catechol showed the greatest removal [31].

4.1.6.6 Monitoring the total carbon after the treatments

Phenylenediamines: experiments were conducted to monitor the total carbon in the batch reactors after the enzymatic treatment carried out under optimal condition for >95.0% conversion and after the polymeric colored product removal by SDS under optimum conditions for best removal. All the samples were microfiltered before testing. As shown in Figure 4.42, 5 to 30% removal of the carbon was observed after the enzymatic reaction. Hence it is speculated that most of the reaction polymer products produced were still in soluble form (low molecular mass; dimers or trimers) that could not be removed by microfiltration. After the flocculation process by using SDS, about 65 to 80% of the carbon was removed. The remaining carbon may have also resulted from the insoluble SDS that did not settle after the treatment.

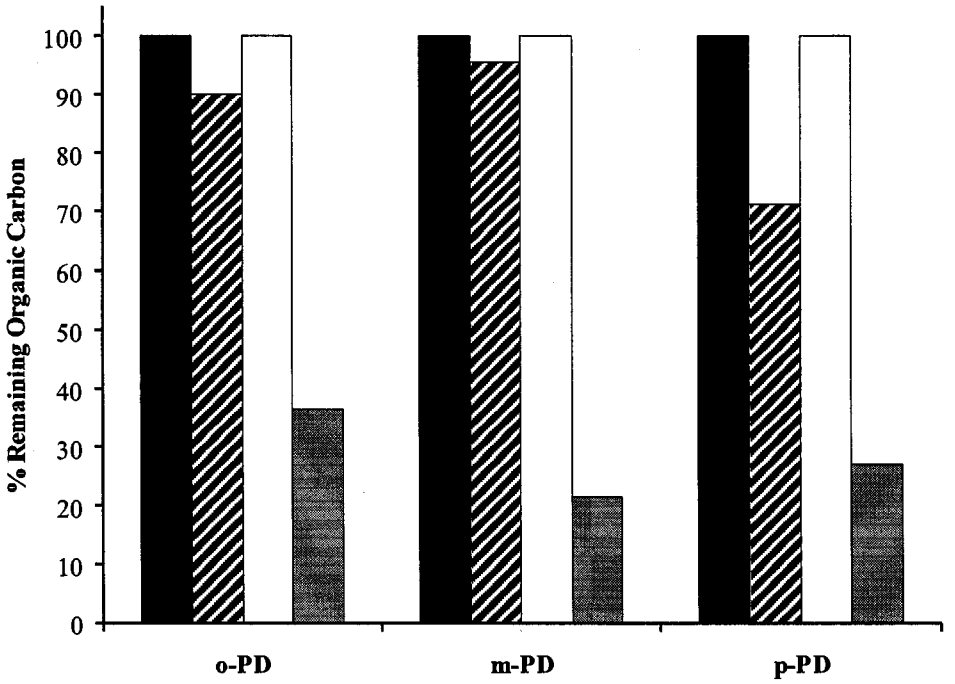


Figure 4.42: Monitoring the total organic carbon for aryldiamines; **Conditions:** 1.0 mM Aromatic monomers, Optimum Enzymatic conditions and Optimum SDS; **Test method:** TOC and TIC

■ Initial Aromatic Monomers ▨ After Enzymatic Reaction

□ After Enzymatic Reaction Plus SDS ▩ After Flocculation by SDS

Aryldiols: experiments were conducted to monitor the total carbon in the batch reactors after the enzymatic treatment carried out under optimal condition for >95% conversion and after the polymeric colored products removal by alum under optimum condition for best removal. All the samples were microfiltered before testing. As shown in Figure 4.43, no significant removal of the carbon was observed after the enzymatic reaction. Hence it is speculated that most of the reaction polymer products produced were still in soluble form (low molecular mass; dimers or trimers) that could not be removed by microfiltration (discussed in section 4.1.6). After the flocculation process by using

alum, about 80% of the carbon was removed in the catechol and resorcinol batch reactors, while only 20% of the carbon was removed in hydroquinone sample. Hence it can be speculated that alum can not remove the products of hydroquinone oxidation.

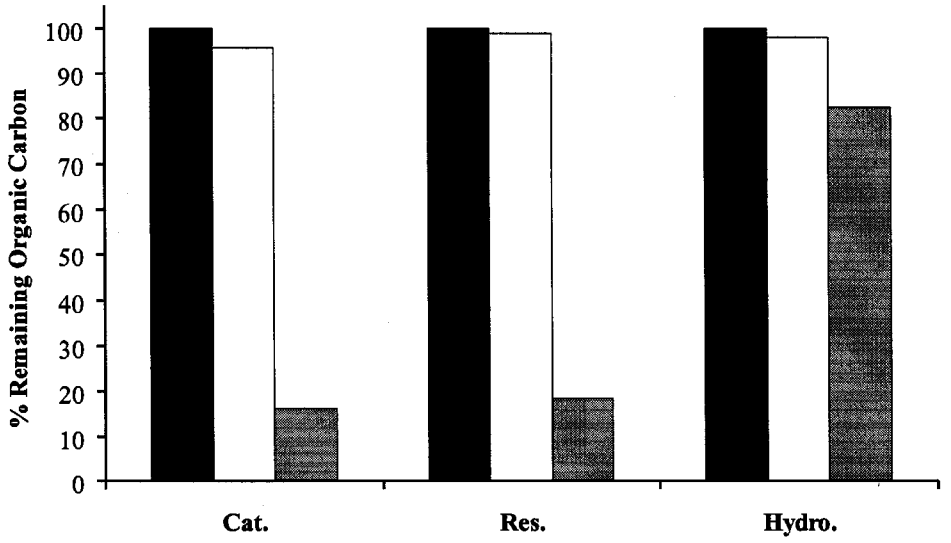


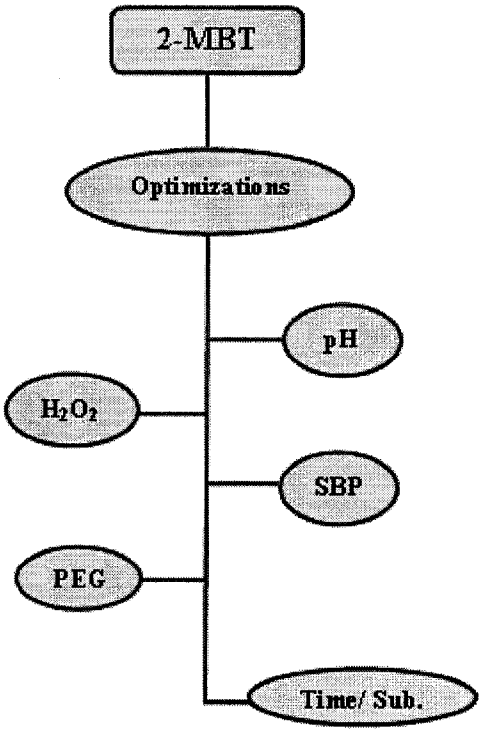
Figure 4.43: Monitoring the total organic carbon for aryldiols; **Conditions:** 1.0 mM aromatic monomers, optimum enzymatic conditions and optimum alum; **Test method:** TOC

■ Initial Aromatic Monomers □ After Enzymatic Reaction

▨ After Flocculation by Alum

4.2 2-Mercaptobenzothiazole Results

In this section, the optimal operation conditions for enzymatic treatment of 2-mercaptobenzothiazole (2-MBT) in synthetic water was determined (Scheme 4.2).



Scheme 4.2: Analysis approach for 2-mercaptobenzothiazole

4.2.1 Optimum pH

The optimum pH for the enzymatic conversion of 2-MBT was investigated in this study. The pH range was 3.8-9.8, the hydrogen peroxide concentration was maintained at 1.0 mM and the 2-MBT concentration was 1.0 mM. The experiments were conducted with SBP concentrations of 0.5 and 0.1 U/mL during 3.0 h reaction. The results of this study are shown in Figure 4.44.

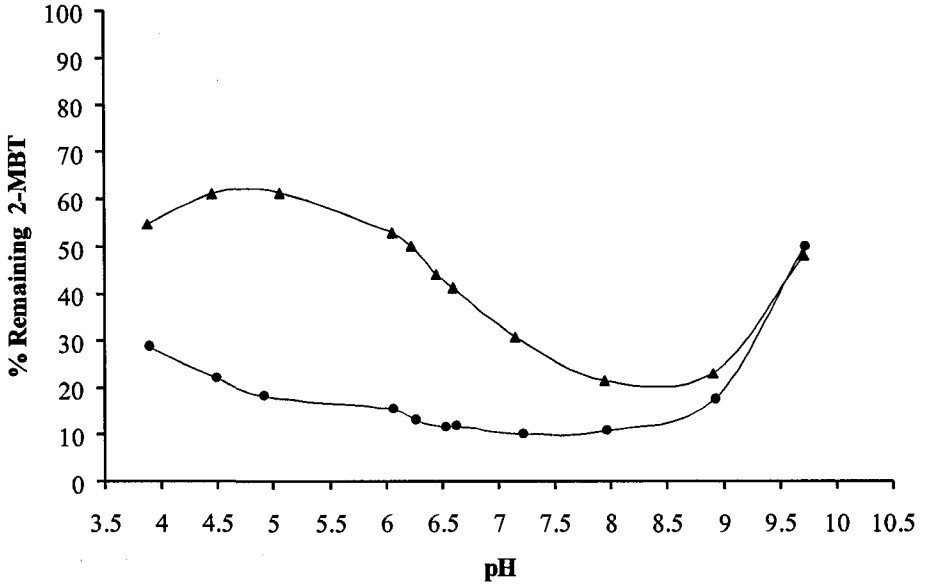


Figure 4.44: pH optimization for enzymatic conversion of 2-MBT; **Conditions:** 1.0 mM 2-MBT, 1.0 mM H_2O_2 and 40.0 mM phosphate buffer; **Test method:** HPLC
—●— 0.5 U/mL SBP —▲— 0.1 U/mL SBP

The optimum pH for the enzymatic conversion of 2-MBT compounds was found to be in the alkaline-basic region 7.5-9.0 while using 0.1 U/mL SBP. As the SBP concentration was increased to 0.5 U/mL SBP, the SBP became more resistant to pH change. As shown in Figure 4.44, the optimum pH plot showed a broader optimum pH between 6.0 and 9.0. The implication of stress SBP concentration provides easier

discerning of the optimum pH. Previous work with SBP and 2,4-dimethylphenol had shown similar high basic optimum pH [25].

4.2.2 Optimum hydrogen peroxide to substrates ratio

Previous study has shown that 2-MBT was oxidized by hydrogen peroxide alone [24]. Hence, a control experiment was conducted first to investigate the effect of various hydrogen peroxide concentrations, without SBP, on the substrate conversion. Figure 4.45 shows a plot of the percent remaining versus the hydrogen peroxide concentration when 2-MBT concentration was 1.0 mM. The concentrations of hydrogen peroxide studied were between 0.25 and 20.0 mM. The results showed that 95% of 2-MBT could be chemically converted by using 3.0 mM hydrogen peroxide in a period of 3.0 hours reaction.

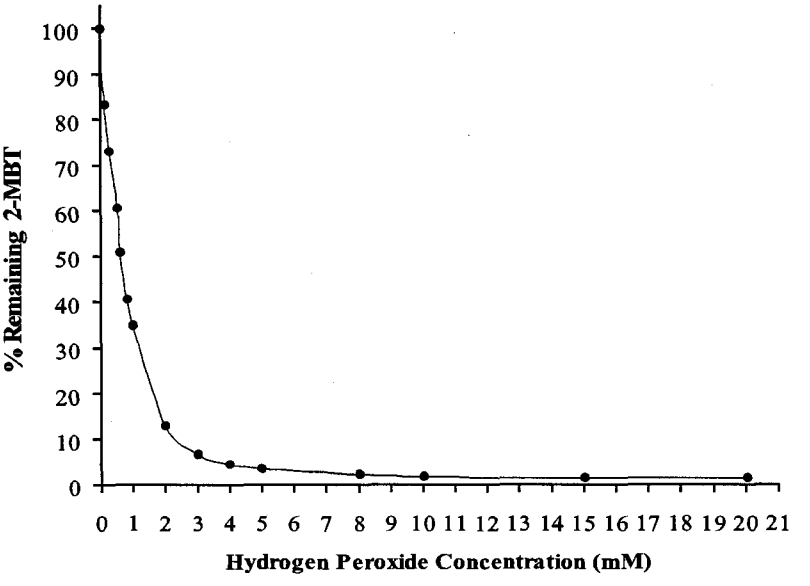


Figure 4.45: Effect of hydrogen peroxide concentration on the conversion of 2-MBT without SBP; **Conditions:** 1.0 mM 2-MBT and 40.0 mM phosphate buffer pH 7.1; **Test method:** HPLC

Next the enzymatic treatment, in presence of SBP, was conducted. The molar concentrations of hydrogen peroxide studied were between 0.1 to 2.0 mM and the 2-MBT concentration was 1.0 mM. The experiments were conducted at the previously determined optimum pH of 7.1, whereas the initial SBP concentrations were 0.05, 0.1, 0.5 and 1.0 U/mL. The experiments were run for 3.0 h. The plot of 2-MBT percent remaining versus hydrogen peroxide concentration for 1.0 mM 2-MBT is shown in Figure 4.46.

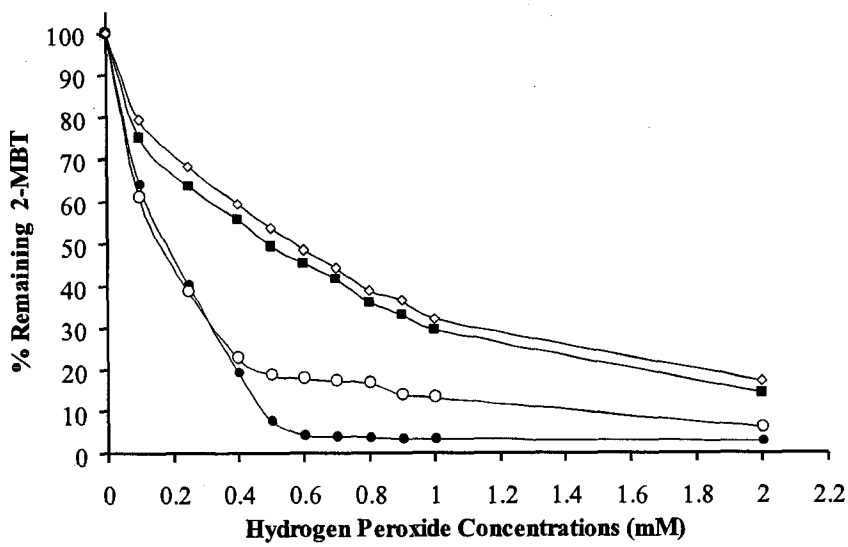


Figure 4.46: Effect of hydrogen peroxide concentration on the enzymatic conversion of 2-MBT; **Conditions:** 1.0 mM 2-MBT and 40.0 mM phosphate buffer pH 7.1; **Test method:** HPLC

—●— 1 U/mL SBP —○— 0.5 U/mL SBP —■— 0.1 U/mL SBP

—◇— 0.05 U/mL SBP

The optimum hydrogen peroxide concentration was found to be 0.6 mM while using 1.0 U/mL SBP. Increasing the hydrogen peroxide concentration beyond the optimum showed no reduction in percent conversion that usually results from the formation of a deactivated form of the enzyme [17]. Under the stressed SBP concentrations

of 0.05 and 0.01 U/mL, the percent conversion increased as the hydrogen peroxide concentration increased beyond the optimum. This may be speculated due to the chemical reaction of hydrogen peroxide with the remaining 2-MBT [24].

According to the peroxidase reaction mechanism, for every one mole of hydrogen peroxide consumed, two moles of the aromatic functional groups should be converted to aromatic radicals that couple non-enzymatically to form dimers then polymers [8]. In this study, the optimum ratio of hydrogen peroxide to 2-MBT was found to be close to the theoretical peroxidase mechanism. In which for every 1.2 moles of hydrogen peroxide, two moles of 2-MBT were converted. The slight increase in hydrogen peroxide demand could be due to the catalase ability of peroxidases, to convert a portion of the hydrogen peroxide available for reaction to water molecules as discussed in Section 4.1.2.

4.2.3 Minimum SBP concentrations and PEG effect

The SBP concentrations studied were in the range 0.01-1.5 U/mL, the 2-MBT concentration was 1.0 mM and the pH was at the optimum of 7.1. The experiments were conducted both under excess and optimum hydrogen peroxide concentrations of 1.0 and 0.6 mM, respectively. Experiments were also conducted to investigate the effect of PEG, 50.0 and 150.0 mg/L, on the removal of 2-MBT. The experiments were run for 3.0 h duration. The plot of 2-MBT percent remaining versus the SBP concentration is shown in Figure 4.47. The minimum SBP concentration required for 95% conversion was found to be 0.75 U/mL. 50 mg/L of PEG_{3350} reduced the enzyme concentration for the same substrate conversion up to 60%. The optimum in the presence of PEG was found to be 0.3 U/mL SBP. As discussed in Section 4.1.3 PEG_{3350} provided a shielding effect to the enzyme by attracting some of the insoluble polymers or radicals to it and preventing them

from adsorbing to the free enzyme or covalently bond to it [19, 20]. Increasing the PEG concentrations to 150 mg/L showed no significant improvement in enzyme reduction. Table 4.4 summarizes the optimum conditions for enzymatic conversion of 1.0 mM 2-MBT.

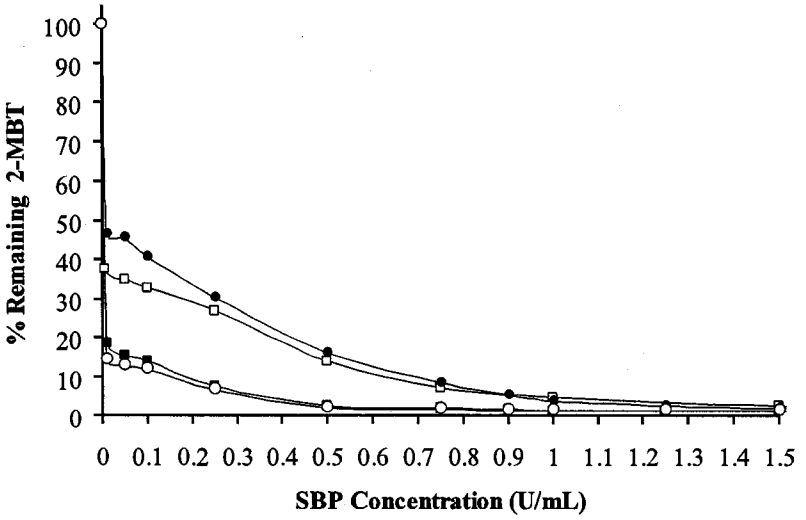


Figure 4.47: Minimum SBP concentration required for 95% conversion of 2-MBT; **Conditions:** 1.0 mM 2-MBT, 40.0 mM phosphate buffer pH 7.1; **Test method:** HPLC

—□— 1.0 mM H_2O_2 —●— 0.6 mM H_2O_2 —○— 0.6 mM H_2O_2/ PEG (150mg/L)

—■— 0.6 mM H_2O_2/ PEG (50mg/L)

After the enzymatic reaction and chemical reaction, white precipitate products were formed. The samples after the enzymatic reaction were more turbid in comparing to the chemical reaction. The products can be removed by filtration or sedimentation.

Table 4.4: Optimum conditions of 1.0 mM 2-MBT

Optimum pH	Optimum H_2O_2]/[Substrate]	Minimum SBP concentration	Minimum SBP in presence of 50mg/L PEG
7.5-9.0	0.6	0.75 U/mL	0.3 U/mL

4.2.4 Consumption of the aromatic substrates over time

The conversion of 2-MBT during the first three hours of the reaction was monitored. Control experiments were conducted to monitor the chemical conversion of 2-MBT in the absence of SBP by using 3.0 mM hydrogen peroxide. In control experiments, 95% of the 2-MBT was converted in 3.0 h reaction (Figure 4.48) with gradual increase in the percent conversion during the 3.0 h duration. Whereas in the presence of 1.0 U/mL SBP and 1.0 mM hydrogen peroxide, 70% of the 2-MBT was converted in 2.5 minutes (Figure 4.48). The conversion of the substrate continued at a slower rate after the first 2.5 minutes. The slower rate of conversion may be due to the decreases in the rate of conversion as the SBP concentration had decreased. It is speculated, that after 2.5 minutes, a combination of enzymatic reaction and chemical conversion by hydrogen peroxide caused the conversion of 2-MBT.

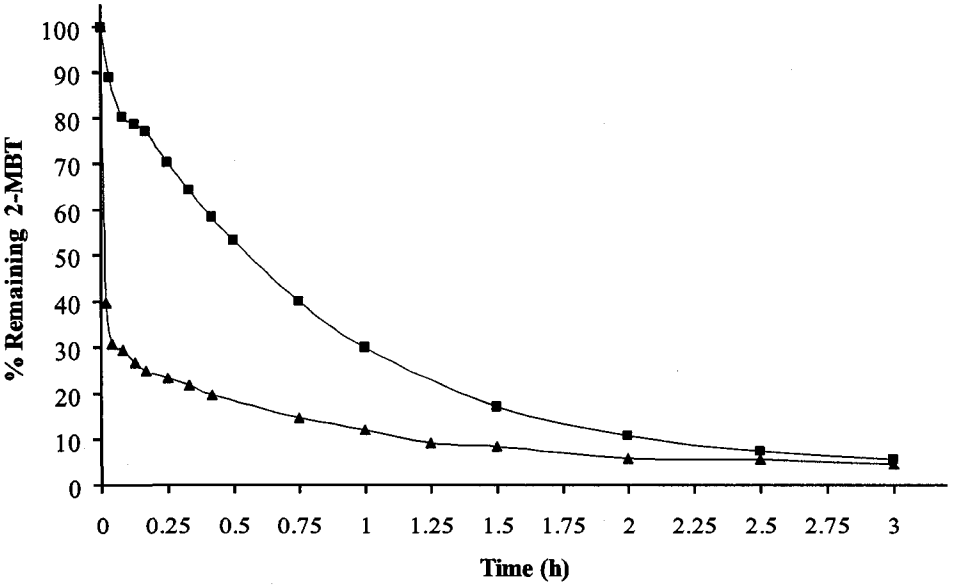
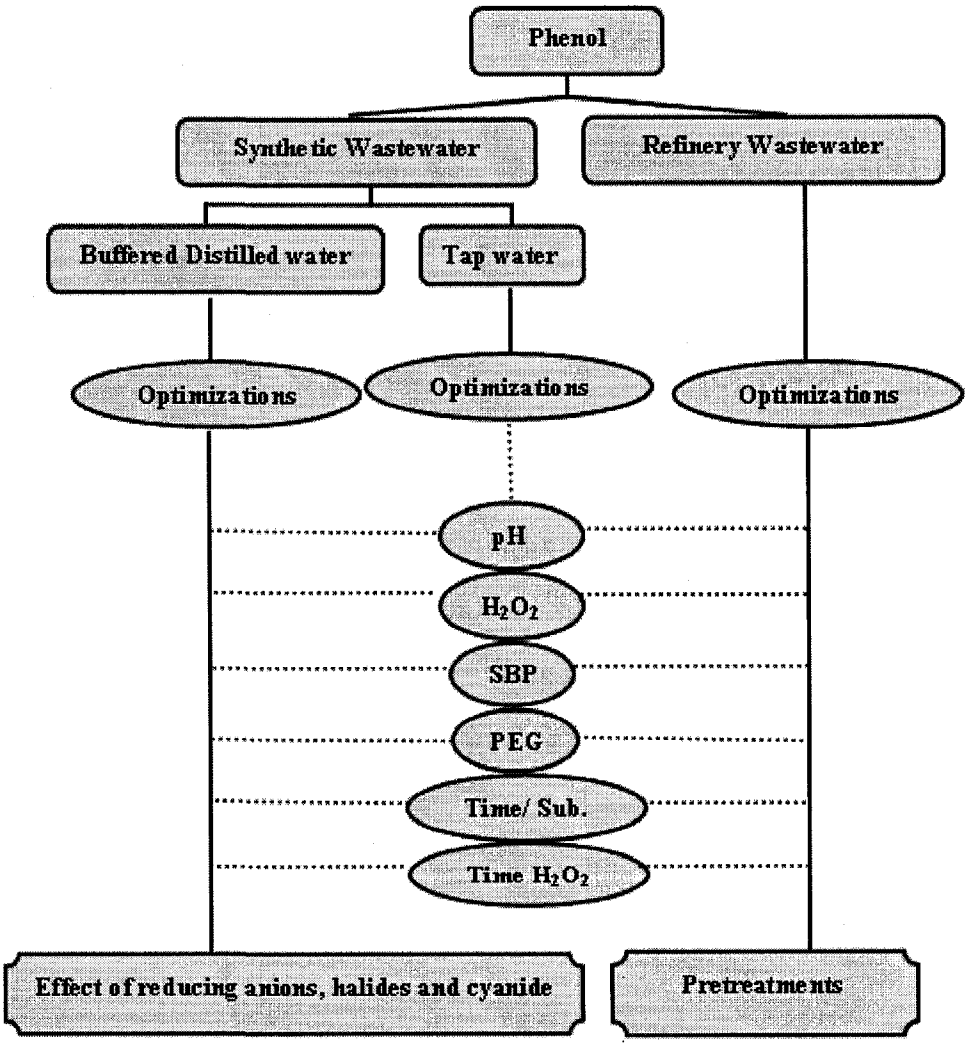


Figure 4.48: Conversion of 2-MBT over time; **Conditions:** 1.0 mM 2-MBT and 40.0 mM phosphate buffer pH 7.1; **Test method:** HPLC
—▲— 1.0 U/mL SBP and 1.0 mM H_2O_2 —■— 3.0 mM H_2O_2 with no SBP

4.3 Phenol (Synthetic and Refinery Wastewaters) Results

The optimal conditions required for the enzymatic treatment of ~1.0 mM phenol in synthetic and refinery (R) wastewater (Scheme 4.3) were conducted in this section. The effect of reducing anion, halides and cyanides on removal of phenol in synthetic wastewater was also investigated. In addition, a pretreatment method will be proposed for more efficient enzymatic treatment. Table 4.5 demonstrate the initial conditions (pH and phenol concentrations) of the refinery samples and the enzyme optimization results.



Scheme 4.3: Analysis approach for phenol

4.3.1 Optimum pH

Synthetic wastewater: Previous research with SBP and phenol in synthetic wastewater had shown an optimum pH of 6.0-8.5 [5]. A similar broad range with SBP has also been reported with a maximum hydrogen peroxide consumption rate at pH 6.4 [26].

Refinery wastewater samples: experiments were conducted to investigate the effect of pH on the enzymatic treatment for five wastewater samples, collected at different times. The pH range studied was 3.5-9.5. All tests were conducted under stressed SBP condition in which the amount of SBP present limited the reaction to substantially less than full conversion, resulting in clearer optimum observation. The hydrogen peroxide concentration was kept at the optimum level for the specific wastewater sample (Table 4.5). Similar experiments were run in the presence of PEG $_{3350}$ that was previously found to reduce the SBP concentration required for the phenol conversion [5, 21].

The results in Table 4.5 and Figure 4.49 show that the optimum pH values for the wastewater samples deviated from each other with some having an optimum range of 6.0-7.5 and others 7.5- 9.0. Two different optimum ranges are difficult to explain, but it was observed that all samples were efficiently treated at pH 7.5. In comparing the optimum pH of the five wastewater samples with the synthetic phenol wastewater, similar range of optimum pH was observed. As discussed previously in Section 4.1.1, phenolic compounds such as catechol, resorcinol, chlorophenol (o-, m- and p-), cresol (o-, m- and p-), dichlorophenol (2,4-) and bisphenol A have all showed a similar optimum alkaline-basic pH range [5].

The fluctuation in the wastewater samples, within the optimum pH range, may have resulted from an interaction between the enzyme and various wastewater

constituents. Since full analysis was not done on the refinery samples, such possible interactions are not known.

As shown in Table 4.5, the optimum pH ranges for the wastewater samples was found to be in the same range as the pH rang of wastewater samples. Therefore, control experiments were conducted to investigate the enzymatic treatment without adjusting pH by buffers. The results show a similar or better phenol conversion was achieved without adjusting the pH of the wastewater samples when compared to the buffer-adjusted samples. Hence, it can be speculated that the buffering capacity did not increase the enzyme efficiency. As a result of this study, all optimization experiments were run under the original sample pH without buffer adjustment.

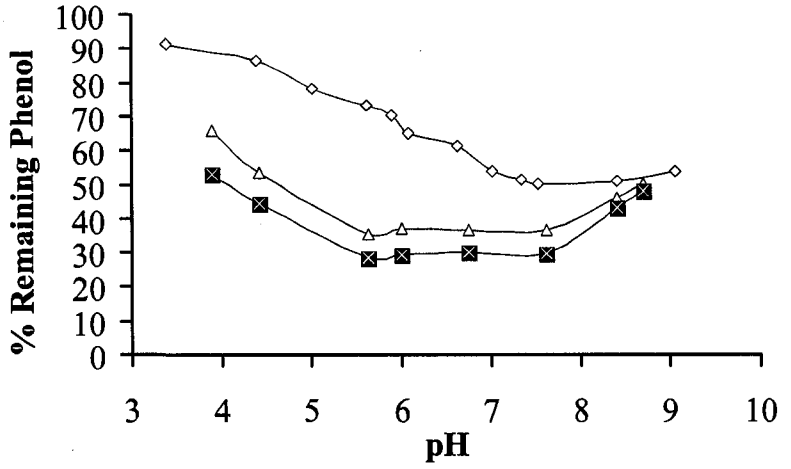


Figure 4.49: pH optimization for the enzymatic conversion of phenol in wastewater; **Conditions:** pH optimization for SBP using 0.4 U/mL for R-1, 0.5 U/mL for R-4 both with optimum H_2O_2 (Table 4.5); **Test method**: HPLC

4.3.2 Optimum hydrogen peroxide to substrate ratio

Synthetic wastewater: previous research with partially purified SBP and phenol in synthetic wastewater has shown an optimum hydrogen peroxide to be 1.2 mM for 1.0 mM phenol [5]. In the present study, using crude SBP, the ratio increased to 1.5 mM for 1.0 mM phenol conversion in synthetic water and to 2.0 mM in tap water (Figure 4.50). According to the peroxidase reaction mechanism, for every one mole of hydrogen peroxide being consumed, two moles of the phenol are converted to aromatic radicals [8]. As previously discussed in Section 4.1.2, the higher demand of hydrogen peroxide can result from some of the dimers products being substrates for the enzyme [16), 19)] and from the catalase ability of SBP to convert hydrogen peroxide to water [8].

Refinery wastewater samples: the refinery samples showed even higher demand for the hydrogen peroxide, 2.5 to 3.8 mM (Table 4.5; Figure 4.50). The increased peroxide demand could be due to several factors. Firstly, sufficient hydrogen peroxide was needed for the molar equivalent to phenol and any other compounds, such as cresols, that were competing for the enzyme, including the generated intermediates. Secondly, there may be reducing agents or ions present that consumed some hydrogen peroxide. Thus, excess hydrogen peroxide can overcome this problem; however, high hydrogen peroxide concentration can cause enzyme inhibition as discussed in Section 4.1.2. In such situations, hydrogen peroxide may have to be fed continuously into the system or added stepwise. When comparing the enzyme inhibition resulting from excess hydrogen peroxide, refinery samples were less susceptible to inhibition than synthetic and tap water samples (Figure 4.50).

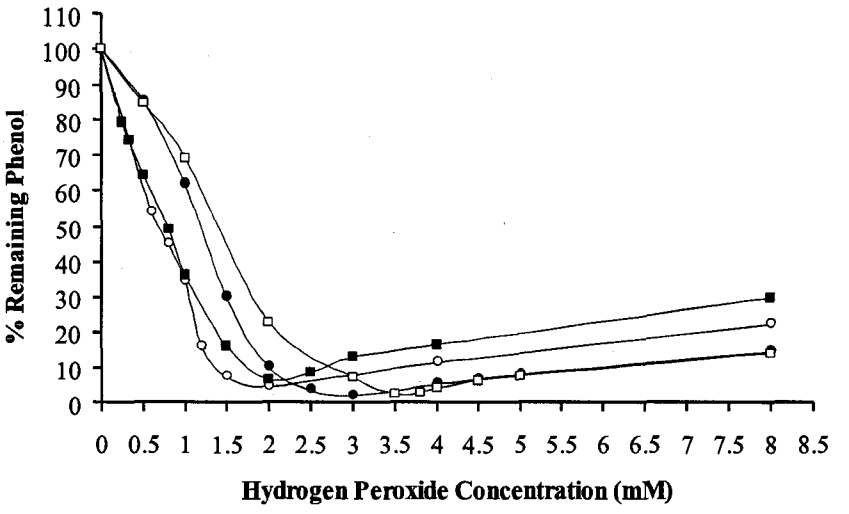


Figure 4.50: Effect of hydrogen peroxide concentration on the enzymatic conversion of phenol; **Conditions:** pH was adjusted to optimum value for synthetic and tap water but the R samples had no pH adjustment. Included are aqueous samples of synthetic, tap and the best and worst real (R) sample excluding sample 1 due to low initial concentration, optimum SBP concentrations (Table 4.5); **Test Method**: HPLC

—○— Synthetic —■— Synthetic in tap water —□— R3 —●— R5

4.3.3 Minimum SBP concentrations and PEG effect

Synthetic wastewater: previous research with partially purified SBP and phenol in synthetic wastewater has shown that 0.9 U/mL of SBP was sufficient for 95% conversion of 1.0 mM phenol. The presence of PEG_{3350} (50mg/L) has been shown to reduce the SBP requirement by 33% [5]. In the present study with crude SBP, the minimum SBP concentration required for 95% conversion of 1.0 mM phenol was found to be 1.25 U/mL with a similar PEG_{3350} effect. In tap water, the SBP concentration required increased to 1.5-1.75 U/mL with a reduction in the PEG_{3350} effect to 15-20%.

Refinery wastewater samples: the refinery samples showed 1.2- to 1.8-fold higher enzyme concentration requirement for the optimal conversion of phenol as

compared to the synthetic ones over the same reaction period (Figure 4.51; Table 4.5). The higher SBP requirement may be due to several factors. Firstly, it may be due to other aromatic compounds that were competing for the enzyme. Some of the samples showed presence of cresols up to 0.12 mM. Such low concentrations would not alone account for this large jump in optimum enzyme concentration. Secondly, the presence of inhibitory molecules may have caused the enzyme deactivation. Lastly, it may have result from other reducing compounds present that consumed the available oxidant.

PEG_{3350} showed no effect on three samples (R-2, 4 and 5; Table 4.5). With the refinery sample (R-1) only 5-15% improvement in the phenol removal and SBP reduction was observed. When the hydrogen peroxide was not limiting and the enzyme concentration was reduced, it was apparent that inactivation of the enzyme occurred, suggesting an alternative mode of inactivation than co-precipitation with products.

After enzymatic treatment of phenol, the absorption maxima for the aqueous solution were in the visible range at 424 nm due to its brownish color. However, products formed with SBP were large flocs that settled in minutes leaving it with approximately 20% of the color produced.

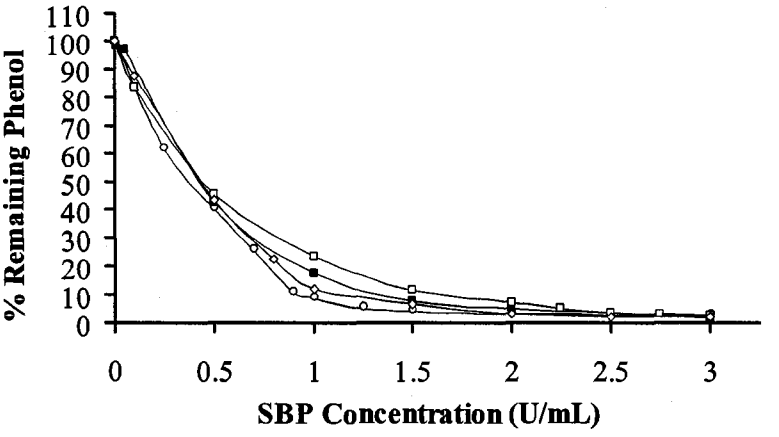


Figure 4.51: Minimum SBP concentration required for 95% conversion of phenol; **Conditions:** 1.0 mM phenol with optimum H_2O_2 and pH (Table 4.5) and 3 h reaction time. Included are aqueous samples of synthetic, tap, and the best and worst real (R) samples excluding sample 1 due to low initial concentration; **Test Method**: HPLC
—○— Synthetic —■— Synthetic in tap water —□— R3 —◇— R4

4.3.4 Consumption of the aromatic substrates over time

The phenol conversion was monitored during the first 3.0 hours of the reaction. The SBP was found to be fast in oxidizing the substrate at its optimal activity (Figure 4.52; Table 4.5). The time required for 95.0% conversion of the phenol was found to be 30-90 minutes (Table 4.5). Peroxidases in general are known to be faster in oxidizing the substrate than other enzymes but, at the same time, they become inactivated more rapidly. The inactivation may be due to the phenoxyl radicals that react with the active site of the enzyme or due to co-precipitation of the enzyme by the product formed or it may be due to suicide inactivation pathways involving hydrogen peroxide [19] as discussed in Section 4.1.2.

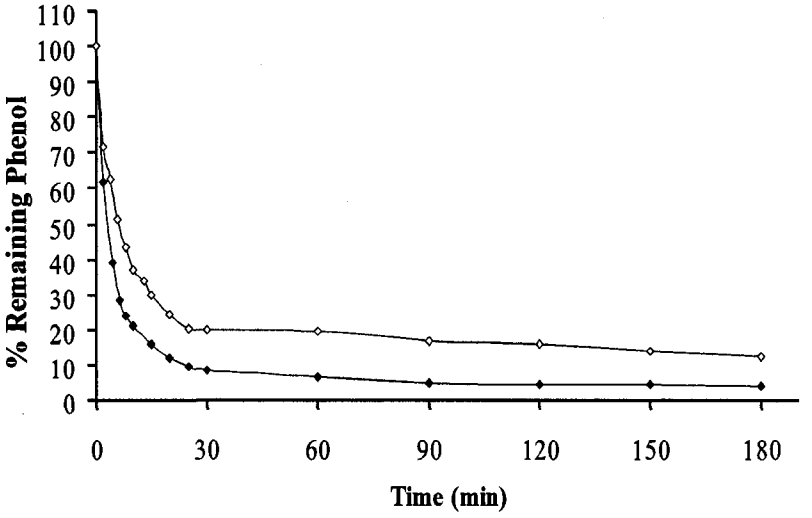


Figure 4.52: Conversion of phenol over time; **Conditions:** Monitoring phenol removal over 3 h reaction for R-2 at two different enzyme concentrations with optimum H_2O_2 (Table 4.5). **Test Method**: HPLC
—◆— R2 (2.0 U/mL) —◇— R2 (1.0 U/mL)

Table 4.5: Phenol results

Sample #	Initial [Phenol] (mM)	Initial pH of Sample	Enzyme Optimization Nominal (U/mL)	Optimum pH Range (buffered)	Optimum Hydrogen peroxide (mM)	Monitoring the reaction time (min)	PEG Effect (%)	Monitoring H_2O_2 Consumption (min)
Syn.	1.0	N.A.	1.25	6.0	1.5	ND	30	ND
Tap	1.0	6.2-6.8	1.5-1.75	N.A.	2	ND	15-20	ND
1	0.856	6.76	1	5.5-7.5	2	30	5-15	150-180
2	1.03	7.79-8.14	2	7.7-8.7	3.8	45-60	No effect	180
3	1.0	7.67	2.25	6.2-7.5	3.5	45-60	5-10	180
4	0.954	8-8.68	1.5	7.9-9	3.25	90	No effect	60
5	0.961	7.31	2	7.2-8.2	2.5	90	No Effect	180

4.3.5 Pretreatment of Refinery Samples

Since the refinery samples consistently required more enzyme than the synthetic samples, the rate of oxidant consumption was investigated. Hydrogen peroxide removal was monitored over time both in synthetic and real samples with no enzyme. It was observed that even in absence of enzyme, some of the samples consumed up to 30.0 mM hydrogen peroxide while the control consumed 5-10% of its optimum concentration in three hours.

Figure 4.53 demonstrates the real sample consumed about of 4.0 mM hydrogen peroxide over 5h. A second aliquot of equal concentration was added after 24.0 h and it too was consumed at a similar rate. Based on this observation, the hydrogen peroxide demand was determined in each real sample and it was found that samples 2-4 consumed 10-30 mM of hydrogen peroxide while the samples 1 and 5 consumed less than 5.0 mM. The elevated levels of hydrogen peroxide demand may be due to several unknown compounds competing for oxidant. However, the enzyme had out-competed the unknowns for the hydrogen peroxide consumption since the phenol concentration was still reduced below 95% in the first hour (Figure 4.52).

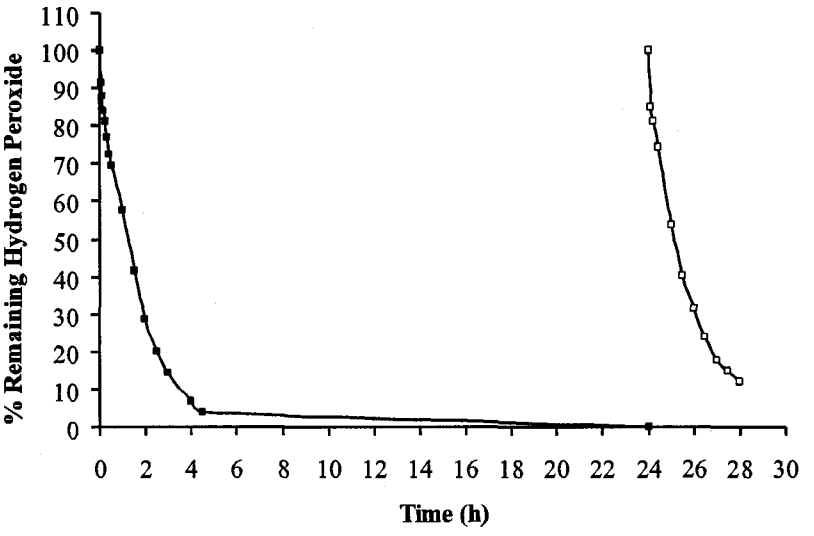


Figure 4.53: Hydrogen peroxide consumption by sample R2 over time; **Conditions:** Consumption of H_2O_2 on R-2 with two step addition and in absence of enzyme; **Test methods:** hydrogen peroxide color test

—■— Aliquot 1 (4.0 mM) —□— Aliquot 2 (4.0 mM)

A pretreatment was implemented in order to reduce enzyme and hydrogen peroxide demands in the real samples. Hydrogen peroxide was added to the real samples until no further consumption of the oxidant was occurring. Figure 4.54 shows Sample 2 pretreated with both 10 mM and 3.8 mM hydrogen peroxide. When pretreatment with 10 mM hydrogen peroxide was followed by the enzyme treatment, the required concentrations of hydrogen peroxide and enzyme were reduced to the same level as required by the synthetic samples. When pretreatment with only 3.8 mM hydrogen peroxide was followed by the enzymatic treatment, the hydrogen peroxide and enzyme concentrations were reduced from the non-pretreated samples but still had greater hydrogen peroxide-to-substrate ratios and enzyme concentration demands than the synthetic samples.

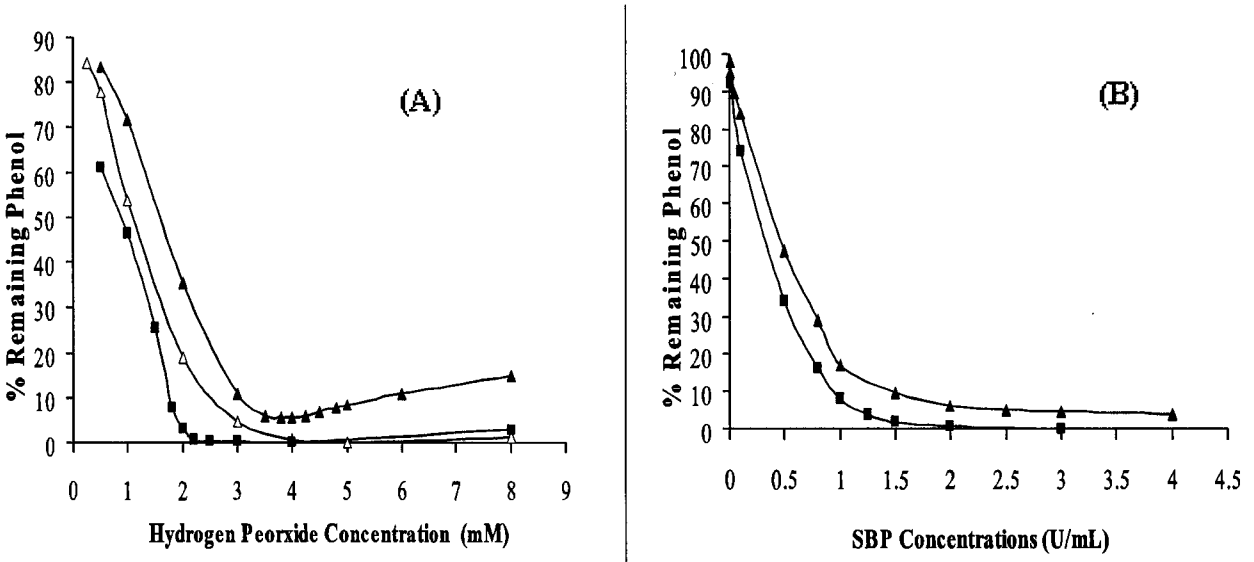


Figure 4.54: Pretreatment of refinery sample R2 with H_2O_2; **Conditions**: **(A)** H_2O_2 optimization with 2.0 U/mL SBP with and without pretreatment. **(B)** SBP optimization with and without pretreatment, H_2O_2 at 3.8 mM; **Test method**: HPLC
—▲— R2 No Pretreatment —△— Pretreatment (3.5 mM) —■— Pretreatment (10 mM)

4.3.6 Influence of Reducing Anions, Halides, Cyanide and Metals Determination

Since the refinery samples required higher amount of hydrogen peroxide and SBP, experiments were conducted to investigate a variety of dissolved matter that might be present in the petrochemical effluent to have caused such an effect.

Reducing Anions: Previous studies on HRP and phenol had demonstrated that reducing anions, such as sulfite and thiosulphate, can have a negative effect on the conversion of phenol by out-competing the enzyme for the oxidant [27]. It was also demonstrated that significant improvement on the phenol conversion was noticed in the presence of sufficient hydrogen peroxide for the reducing ion reactions. It was hypothesized that the rate of inactivation of the enzyme would decrease as a result of the reducing ions reaction products that react with the oxidized phenolic species and thus neutralize their inactivating capability [27]. In the present study, with crude SBP, it was

observed that none of the reducing anions tested showed significant inactivation directly on the phenol removal when adequate hydrogen peroxide was present (Figure 4.55). Although, in the absence of SBP, both 200mg/L thiosulphate and sulfite consumed 80% and 54% of the same initial hydrogen peroxide concentrations, respectively. Since the phenol conversion was not affected, it is speculated that the rate of hydrogen peroxide consumption by SBP was higher than that with the reducing ions. It was noticed that when thiosulphate was used with SBP, the color of the reaction changed from brownish (424 nm) to reddish (474 nm) with little or no visible precipitate. The change in color indicates different set of products made.

Halides: Previous studies on HRP and phenol had demonstrated that halides, such as 200 mg/L of iodide, resulted in an increase in the hydrogen peroxide to substrate ratio but were able to achieve > 95% removal of phenol with the same enzyme concentration [27]. The present study with the crude SBP showed similar results with iodide consuming 31% and bromide consuming 15% of the hydrogen peroxide as compared to the control consuming 12% (without SBP), but no significant changes were observed in phenol conversion in the presence of SBP (Figure 4.55).

Cyanide: the plastic industry uses cyanide in the synthesis of various condensation and polymerization reactions and it is often released in petrochemical effluents [28]. Previous studies on HRP and phenol had demonstrated that cyanide inhibited the HRP [27]. Similar behavior was observed with SBP in this study also (Figure 4.55). The cyanide is believed to diffuse into the distal cavity of the enzyme and coordinate directly to the iron (III), resulting in the change of the coordination from 5 to 6

and inhibiting the enzyme [8]. The refinery samples were tested for the presence of cyanide and no cyanide was found.

Metals Determination: In previous investigation peroxidases had shown sensitivity to certain metals such as Cd(II) and Fe(III) and Mn(II) [29, 27]. In the present study, the refinery samples were tested for Fe(III), Pb(II), Cu(II), Mg(II) , Zn(II) and Cd(II) by using atomic adsorption equipment. The concentrations of the tested metals were in the same concentration ranges as those in tap water, i.e. below 0.5 mg/L. Hence, it is concluded that the SBP inhibition had not result because of the metals.

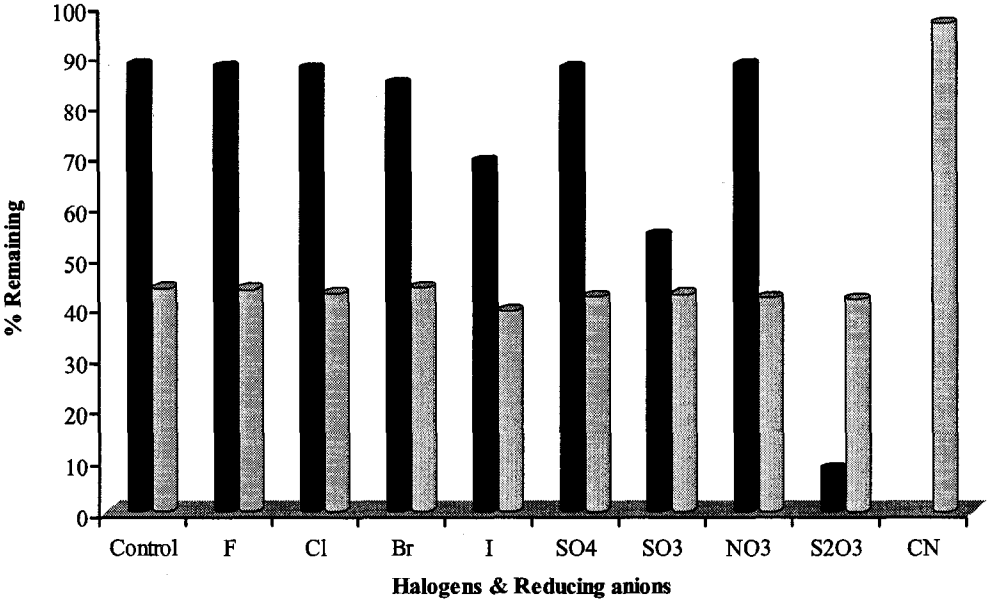


Figure 4.55: Effect of halides & reducing anions; **Conditions:** Effect of halides and reducing anions made from their sodium salts at 200 mg/L. Stress conditions SBP 0.5 U/mL and 1.2 mM H_2O_2 for 1.0 mM phenol ratio; **Test Method**: HPLC
■ Hydrogen Peroxide/No SBP ▨ Phenol with SBP

4.3.7 Comparing SBP to Laccase

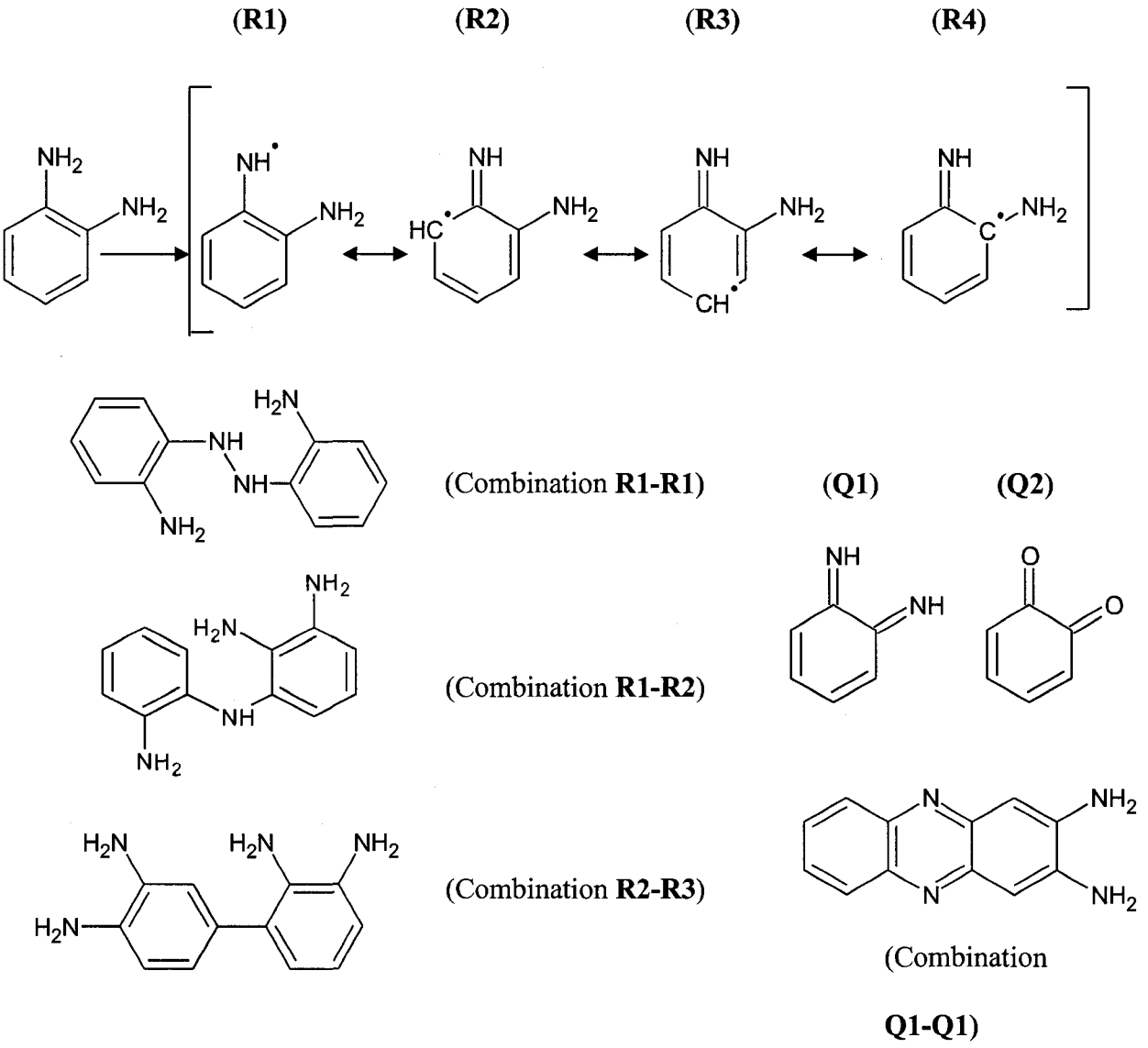
Experiments were conducted to investigate the effect of laccase (SP504) on the conversion of phenol [2] in the refinery samples. The following points summarize the comparison between the performance of SBP and Laccase enzyme:

1. SBP showed a broad pH range (6.0 -9.0) whereas laccase required a narrow pH range (5.6-6.0) [2]. No pH adjustment was required for the refinery sample treatments in the presence of SBP, whereas laccase required pH adjustments for all samples.
2. Higher SBP concentrations (10-fold) were required for the phenol conversion when compared to laccase based on the nominal activity value [2]. It should be noted that SBP is cheaper than laccase). More than 95% of phenol was converted with SBP in all the samples. However, laccase could convert only 90% in the same time duration of 3.0 hours (R-2, 3, 4 and 5). 95% conversion of phenol with laccase required a longer period of time.
3. SBP required hydrogen peroxide for oxidizing the substrate, whereas laccase used only oxygen. Higher hydrogen peroxide may be needed in real applications for can oxidizing other compounds present in the effluent.
4. Laccase was slower in converting the phenol [2] but was capable of retaining its activity whereas SBP was faster at converting phenol but was more susceptible to inactivation.
5. SBP was more resistant to hydrogen peroxide inactivation when compared to other peroxidases [1, 4, 18]. SBP was capable of out-competing most reducing anions and halides for oxidant, while laccase in the presence of fluoride ions showed a significant reduction in phenol conversion.

6. Both enzymes showed inactivation in presence of cyanide [2]. Hence, if cyanide is present in refinery waste, pretreatments will be required.
7. None of the heavy metals seemed to be present in high enough concentrations in any of the real samples to alter the conversion of phenol.
8. To account for the greater enzyme demand of refinery wastewaters we can only conclude that other substances in the matrix are adsorbing some of the free enzyme, affecting the stability of the enzyme, or forming a reversible inhibitor complex with the enzyme, none of which can be confirmed without further investigation[2].

4.4 Radical Coupling to Form Dimers

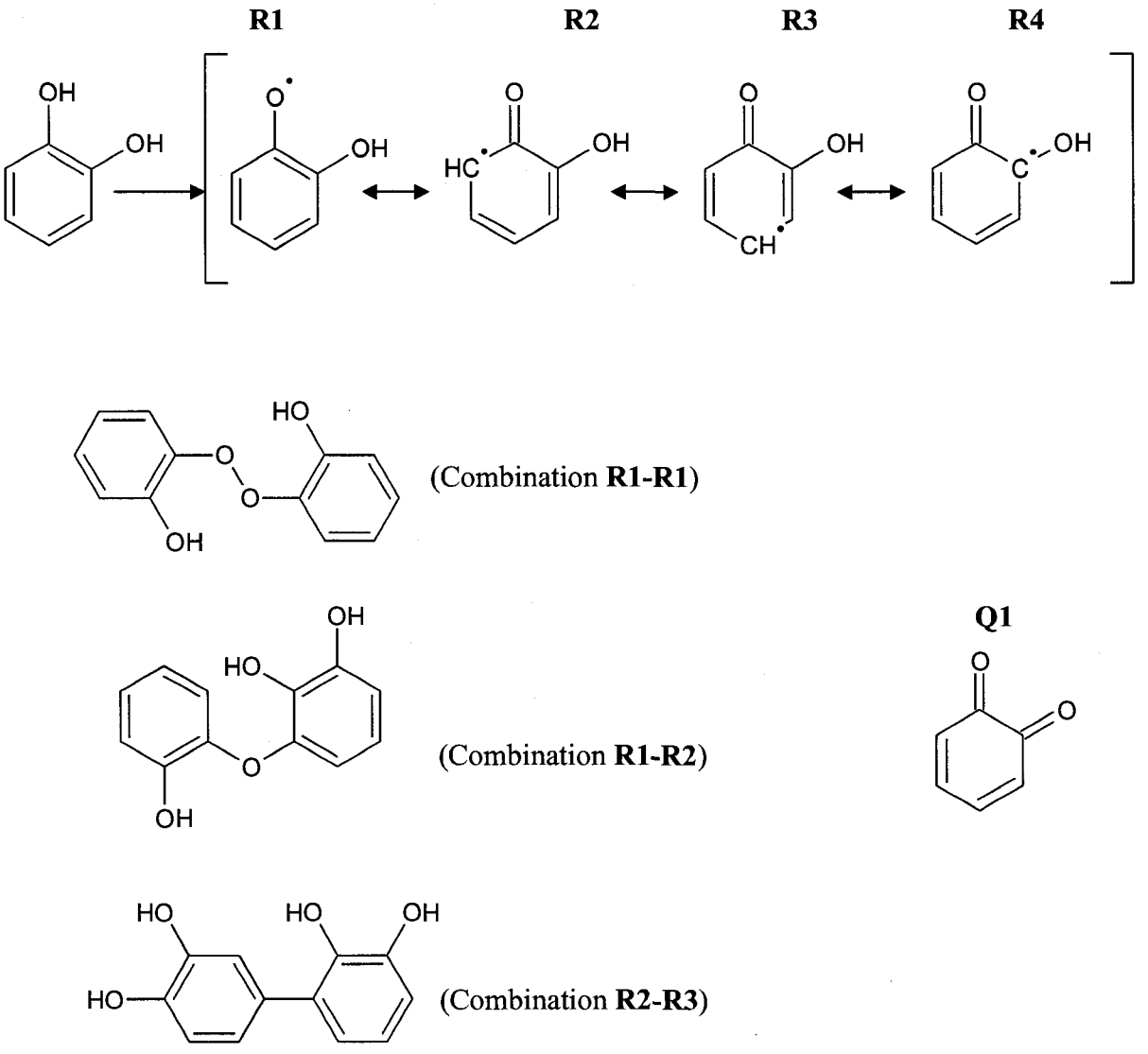
Schemes 4.4-4.7 show the possible structure of the dimers from the coupling of SBP-generated aryldiamines, aryldiols, MBT and phenol radicals. Scheme 4.4 demonstrates four resonance (R1-R4) possibilities from the enzymatic oxidation of o-PD and one quinone (Q1) possible structure that can be transferred to o-benzoquinone (Q2). The coupling of these radicals can be though nitrogen-nitrogen bond (combination R1-R1), nitrogen-carbon bond (combination R1-R2) or carbon-carbon bond (combination R2-R3). Other combinations of nitrogen-carbon bond and carbon-carbon bond are possible through ortho or para positions coupling. Also, the quinone (Q1) coupling can form two nitrogen-carbon bonds (combination Q1-Q1).



Scheme 4.4: Structure of the possible resonance contributions and dimers for o-PD

Scheme 4.5 demonstrates four resonance (R1-R4) possibilities from the enzymatic oxidation of catechol and one quinone (1Q) possible structure. The coupling of these radicals can be though oxygen-oxygen bond (combination R1-R1), oxygen-carbon bond (combination R1-R2) or carbon-carbon bond (combination R2-R3). Other combinations

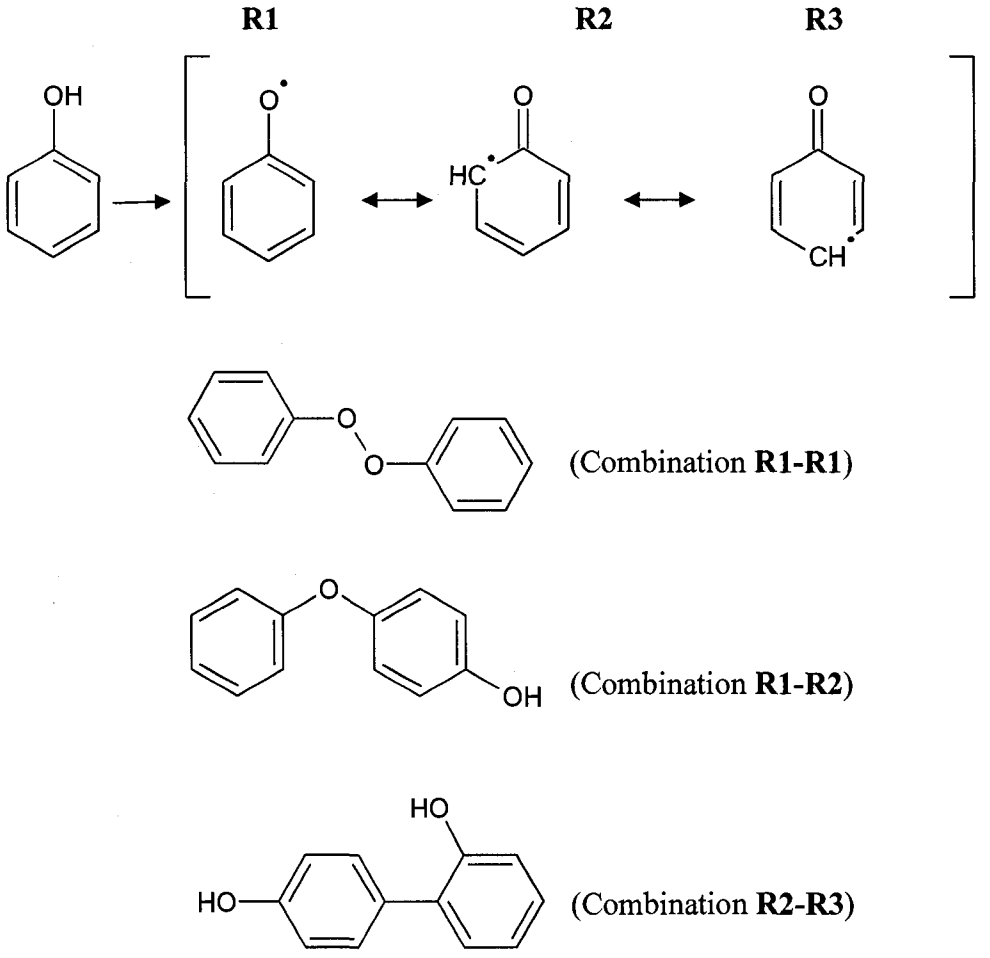
of oxygen-carbon bond and carbon-carbon bond are possible through ortho or para positions coupling.



Scheme 4.5: Structure of the possible resonance contributions and dimers for catechol

Scheme 4.6 demonstrates three resonance (R1-R3) possibilities from the enzymatic oxidation of phenol. The coupling of these radicals can be though oxygen-oxygen bond (combination R1-R1), oxygen-carbon bond (combination R1-R2) or carbon-

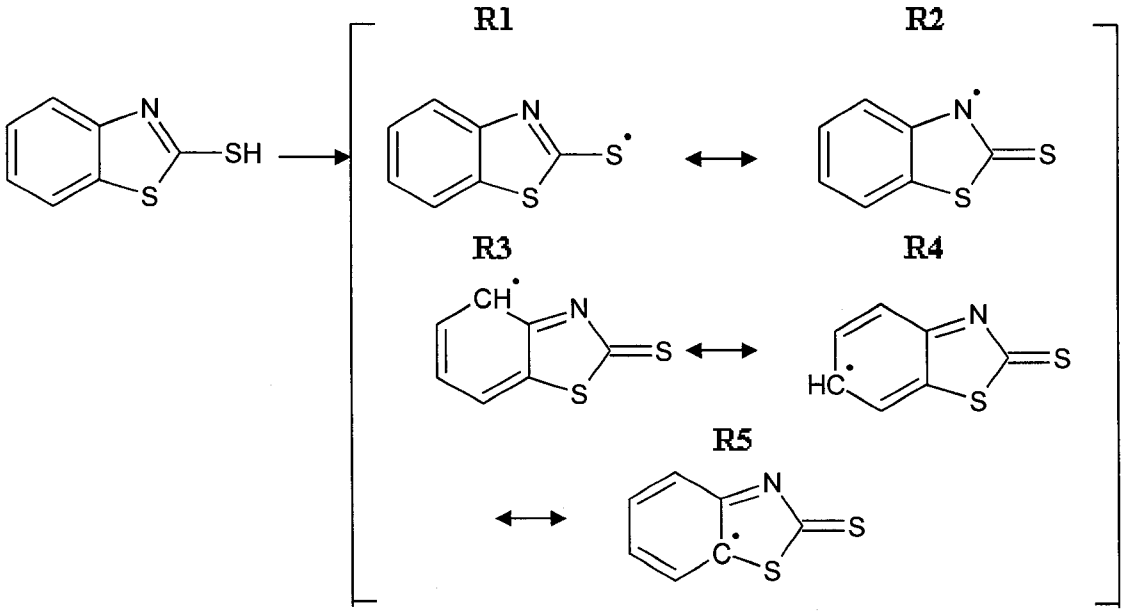
carbon bond (combination R2-R3). Other combinations of oxygen-carbon bond and carbon-carbon bond are possible through ortho or para positions coupling.

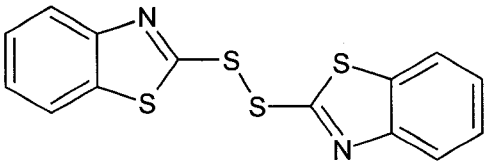


Scheme 4.6: Structure of the possible resonance contributions and dimers for phenol

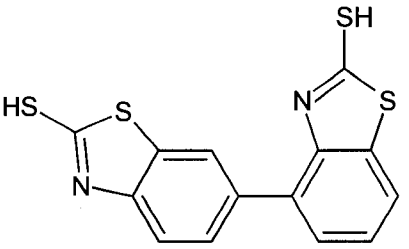
Scheme 4.7 demonstrates five resonance (R1-R5) possibilities from the enzymatic oxidation of 2-MBT. The coupling of these radicals can be though sulphur-sulphur bond (combination R1-R1), sulphur-carbon bond (combination R1-R3), sulphur-nitrogen bond (combination R1-R2), carbon-carbon bond (combination R3-R4), carbon-nitrogen bond

(combination R2-R4) and nitrogen-nitrogen bond (combination R3-R4). Many other combinations are possible with similar bond formation.

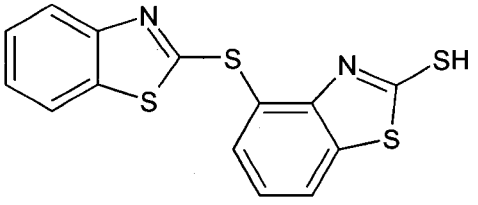




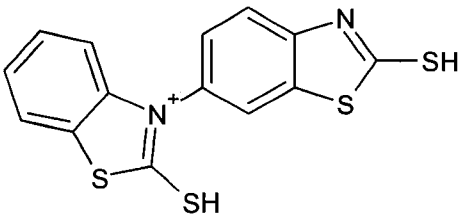
(Combination **R1-R1**)



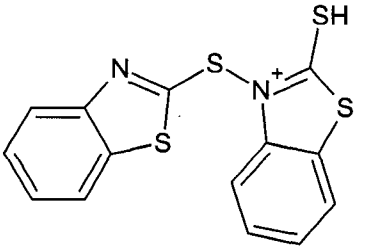
(Combination **R3-R4**)



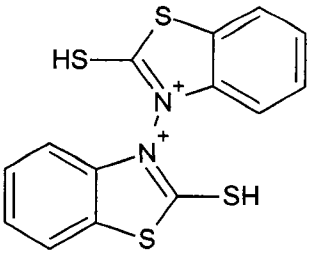
(Combination **R1-R3**)



(Combination **R2-R4**)



(Combination **R1-R2**)



(Combination **R2-R2**)

Scheme 4.7: Structure of the possible resonance contributions and dimers for 2-MBT

4.5 Sources of error

In every experiment, the combination of systematic and random errors can affect the accuracy and reliability of the results. Systematic errors are due to analytical techniques and instruments, whereas random errors are due to personal carelessness. All experiments were run in duplicate or triplicate. The averages of the data are presented in the graphs. For the enzymatic optimization, the standard deviation was between 0.1 and 2.2% when test were run on the same day. The standard deviation had increased up to ~7% when the average of the data was calculated based on trials on three different days. This may have resulted due to several factors such as temperature, stirring level, chemical stock preparation, SBP activity loss, etc. For the removal of polymeric color products, the standard deviation was between 0.1 and 5% when tested on the same day. Another major source of error was due to the reaction of some dimers of phenylenediamine with the colorimetric reagent (TNBS) which led to errors in the amount of initial substrate remaining in solution. Experiments were frequently reproduced in order to assess the validity of values obtained.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Crude SBP has been shown to be a suitable enzyme for the peroxidase-catalyzed polymerization of aryldiamines (o-PD, m-PD and p-PD), aryldiols (catechol, resorcinol and hydroquinone), 2-MBT and phenol. The maximum conversion of these substrates is highly dependent on the optimal conditions which were substrate-specific. The aryldiamines required a lower SBP concentration than the aryldiols for 95% conversions of the substrates. PEG_{3350} shows an insignificant effect on the conversion of phenylenediamines and aryldiols but was effective for 2-MBT in protecting the SBP from inhibition. The removal (not conversion) > 90% of the aryldiamine-derived polymeric products was achieved using SDS alone. The removal > 90% of the catechol- and resorcinol-derived polymeric products was achieved using alum alone, while only 60% removal of the hydroquinone-derived polymeric products was achieved using alum.

Crude SBP was effective in catalyzing the oxidation of phenol in refinery wastewater without pH adjustment. The refinery samples showed a higher demand for hydrogen peroxide and SBP than synthetic wastewater. PEG_{3350} shows an insignificant effect on the conversion in the refinery samples. Pretreatment with hydrogen peroxide was found to be effective in reducing the SBP demand required by the enzymatic reaction in the refinery samples.

5.2 Future Work

The results of these experiments have shown that soybean peroxidase is a practical alternative to other enzymes that have been previously studied. In order to implement the enzymatic method of treatment to full-scale industrial application, several aspects must be considered: (1) cost analysis to determine the economic feasibility of applying crude SBP in wastewater treatment; (2) An environmental impact assessment to determine the fate and toxicity of the soluble and insoluble polymeric products; (3) Characterization of the polymeric products in terms of size and structure and the capability of such polymers to co-precipitate the enzyme; (4) characterization of the bond formation between the radicals and the enzyme; (5) Implication of higher molecular mass of PEG or other additives to protect the enzyme from inactivation; (6) Investigating other flocculating/coagulating agents in the polymeric products; (7) Implementation of the enzymatic treatment in continuous-flow reactor to ensure it is industrially feasible; (8) Characterization of the wastewater matrix in order to understand the enzymatic reaction and the possible need for a pretreatment method.

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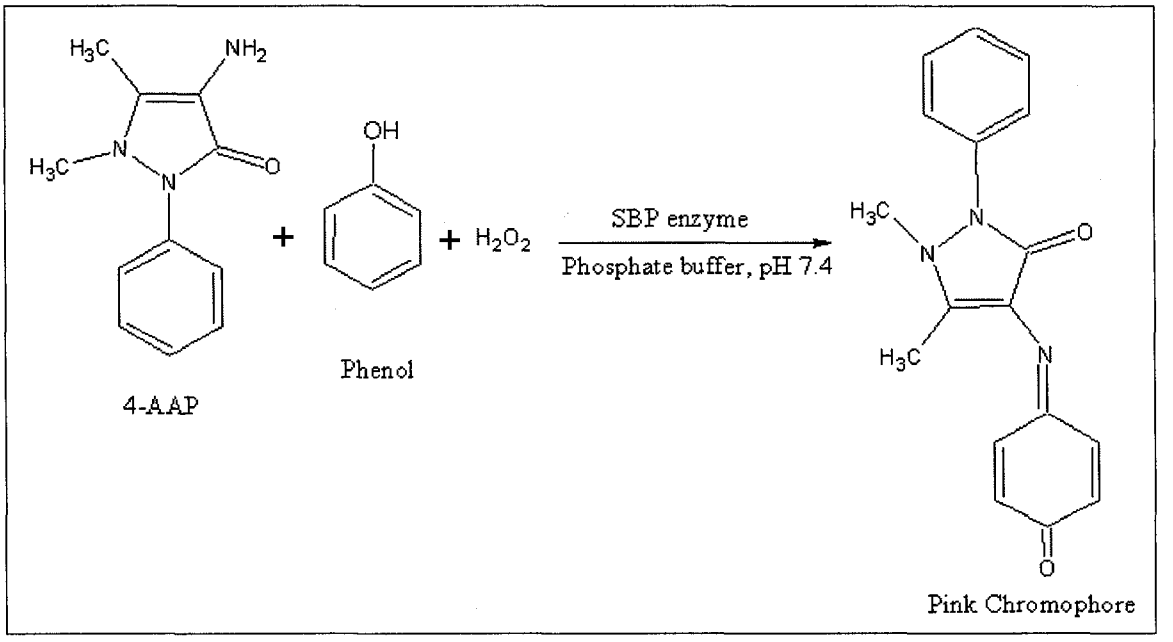
APPENDICES

APPENDIX A

Colorimetric Assay for Soybean Peroxidase Activity

Test Reaction

Scheme A-1 demonstrates the reaction of phenol and 4-AAP in the presence of hydrogen peroxide with SBP as catalyst. The product of such reaction generates a pink chromophore, λ_{max} 510 nm.



Scheme A-1: SBP activity test reaction

Test Conditions

- Instrument: UV-Vis.
- Wavelength: The absorbance measurements were at a wave length of 510 nm.
- Test Time: Measurements time duration were for 35 seconds at 5 second interval reading.
- Reagents: In a 50.0 mL volumetric flask:
    - 100.0 µL of 100.0 mM of H_2O_2.
    - 25.0 mg of 4-AAP.

- 5.0 ml of 10x concentrate (100.0 mM phenol; 0.5 M phosphate buffer (pH 7.4))
- Dilute to 50.0 mL with distilled water.

- Test Procedure: In a 1.0 mL cuvette:

Blank

- 950 µL of the reagent.
- 50 µL of water.

Sample

- 950 µL of the reagent.
- 50 µL of dilute SBP.

(The SBP dilution were made (between 20 x to 100 x))

Data Calculation Procedure:

The enzyme activity was measured based on the initial rate of color formation. The initial rate was measured by calculating the change of absorbance over time (the slope of the line) taking into account the dilution factor of reaction and the extinction coefficient of the product. The enzyme activity calculations are summarized in the following:

1. The rate of change in absorbance:

$$\text{Rate} = \Delta A_{510} / \Delta t = \quad \Delta A_{510}/s$$

2. The rate is converted from $\Delta A_{510}/s$ to $\Delta A_{510}/min$.
3. The change in concentration using $A_{510} = \varepsilon \Delta c l$

$$\Delta c = A / \varepsilon l \quad \text{where } \varepsilon = 6.0\ mM^{-1}\ cm^{-1} \text{ and } l = 1.0cm$$

4. Δc will be in mM/min, since this all happened in 1mL cuvette then

mM//min x 1ml = μmol/min

Note 1μmol/min = 1U where U is the catalytic activity of the enzyme.

5. Considering the dilution factor :

   = dilution *x* (1000μL (total volume in cuvette)/ 50μL (added enzyme))

6. The activity of the enzyme (U/mL) was calculated by multiplying the activity of the enzyme in cuvette by the dilution factor.

The crude SBP used in this study had an activity of ~ 70.0 U/mL. Figure A-1 illustrate the change of absorbance as a function of time for SBP.

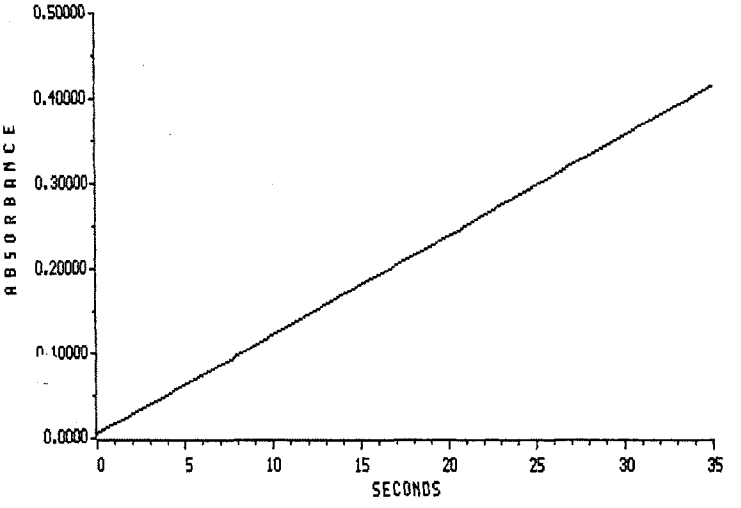


Figure A-1: Enzyme activity plot

APPENDIX B

Preparing SBP solution from dry solid crude SBP

The SBP solutions were prepared in the following order:

- The crude solid SBP stored at -15°C was allowed to warm to room temperature (Note: its preferable to not keep the crude SBP at room temperature for a long time in order to prevent minor activity loss)
- The concentration of enzyme solution usually prepared was in the range 10-20mg of the crude SBP per mL of 0.2mM phosphate buffer.
- The enzyme solutions were allowed to stir gently on a stir plate (Note: vigorous stirring can cause enzyme to lose its activity). The prepared enzyme might not be a true solution even after along time of stirring.
- The enzyme solutions were filtered using a gravity filtration for purification (Note: it's preferable to gravity filter the enzyme twice).
- The enzyme solution was then stored at a temperature of 4°C.

The activity of the enzyme varied depending on how fresh is the solution. The enzyme activity was found to be slightly higher for the not-microfiltered enzyme than the one microfiltered. This was due to the presence of some micro-solid particles that participate in the test reaction.

APPENDIX C

SBP Specification

The purity of the protein solution (not the enzyme) was determined using the RZ (ReinheitsZahl/ reference) value. The RZ value measures the hemin content of the SBP but not the enzyme activity. The purity number or the RZ value was the ratio of the maximum absorbance of the hemin content at 403 nm to the aromatic amino acids at 275 nm. A well prepared pure SBP has an RZ value between ranges of 2.0-3.7 at a pH of 7.4. In this study, the RZ value was measured in the maximum absorbance at 404 nm and 276 nm as shown in figure C-1. The RZ value was found to be 0.748 ± 0.1 at pH 7.4; the low RZ value can result from other proteins absorbing at 275 nm in the crude enzyme.

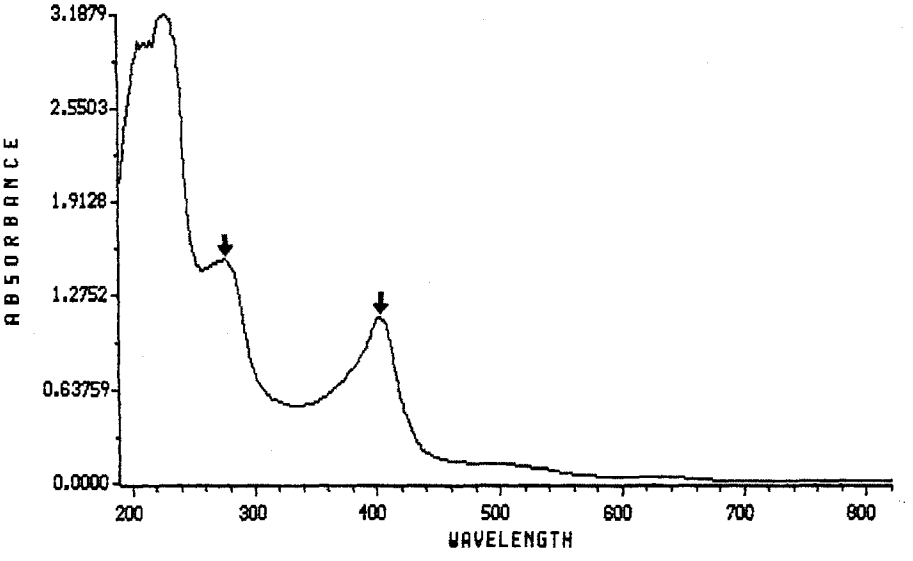


Figure C-1: RZ direct absorbance plot

Figure C-2 illustrates the effect of separating the hemin group from the protein by adjusting the pH to 2.0 using HCl and then centrifuge where the hemin will be in the supernatant. The RZ value was found to be 0.111 ± 0.1. We would accept that the RZ value would be nearly zero but this could results from other inorganic Fe^{3+} hemoprotein contaminated that absorbs at 403 nm (Reference).

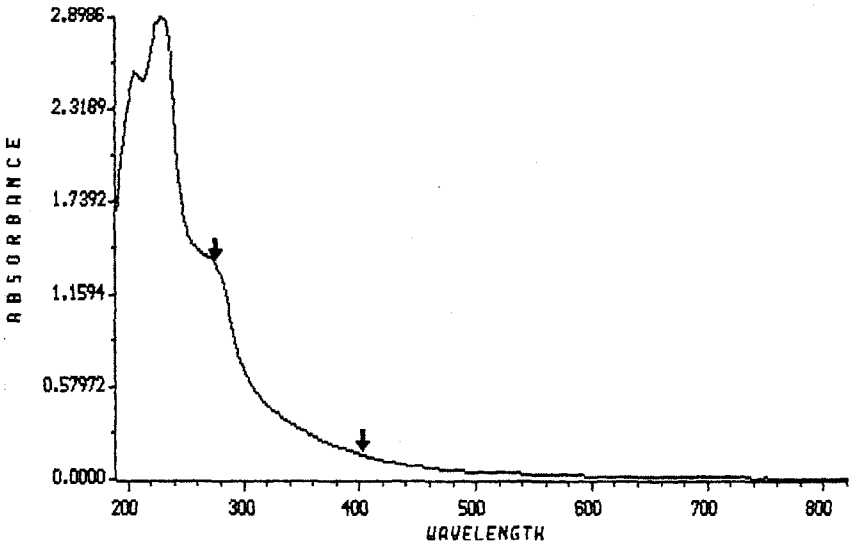


Figure C-2: RZ direct absorbance plot after pH adjustment

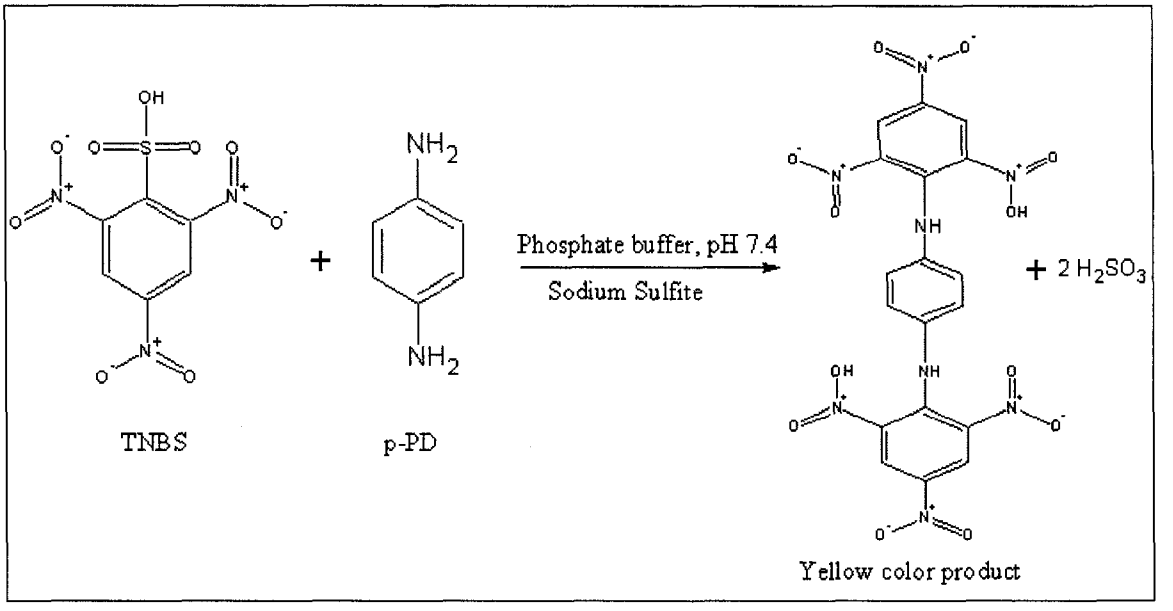
In this study, the enzyme solutions were ~14.0 mg of crude SBP per mL that has ~70.0 U of active enzyme per mL according to the enzyme activity color test. The activity of the SBP solution was also determined by measuring the amount of iron using an A.A. spectrophotometer. Using the mass/molar proportional ratio, the activity of SBP was found to be 49.0± 0.5 U of active enzyme per 1mL. The activity using this method was lower compared with the activity using color test; this could result from other interferences present in the crude enzyme affecting the A.A. spectrophotometer reading.

APPENDIX D

Colorimetric Assay for Aromatic Amines

Test Reaction

Scheme D-1 demonstrates the reaction of TNBS and p-PD in presence of sodium sulfite at pH 7.4. The product of such reaction generates a yellow chromophore. The color intensity generated is directly proportional to the concentration of the aromatic compound.



Scheme D-1: TNBS test reaction

Test Conditions for the sample:

- Instrument: UV-Vis.
- Wavelength: Depending on the substrate begin tested.
- Time for color development: Depending on the substrate being tested.
- Test Procedure: In a 1.0 mL cuvette:

Blank

  - 700 µL of distilled water
  - 100 µL of 0.2 M phosphate buffer (anilines pH 7.4).

- 100 µL of 20 mM sodium sulfite.
- 100 µL of 10 mM TNBS

Sample

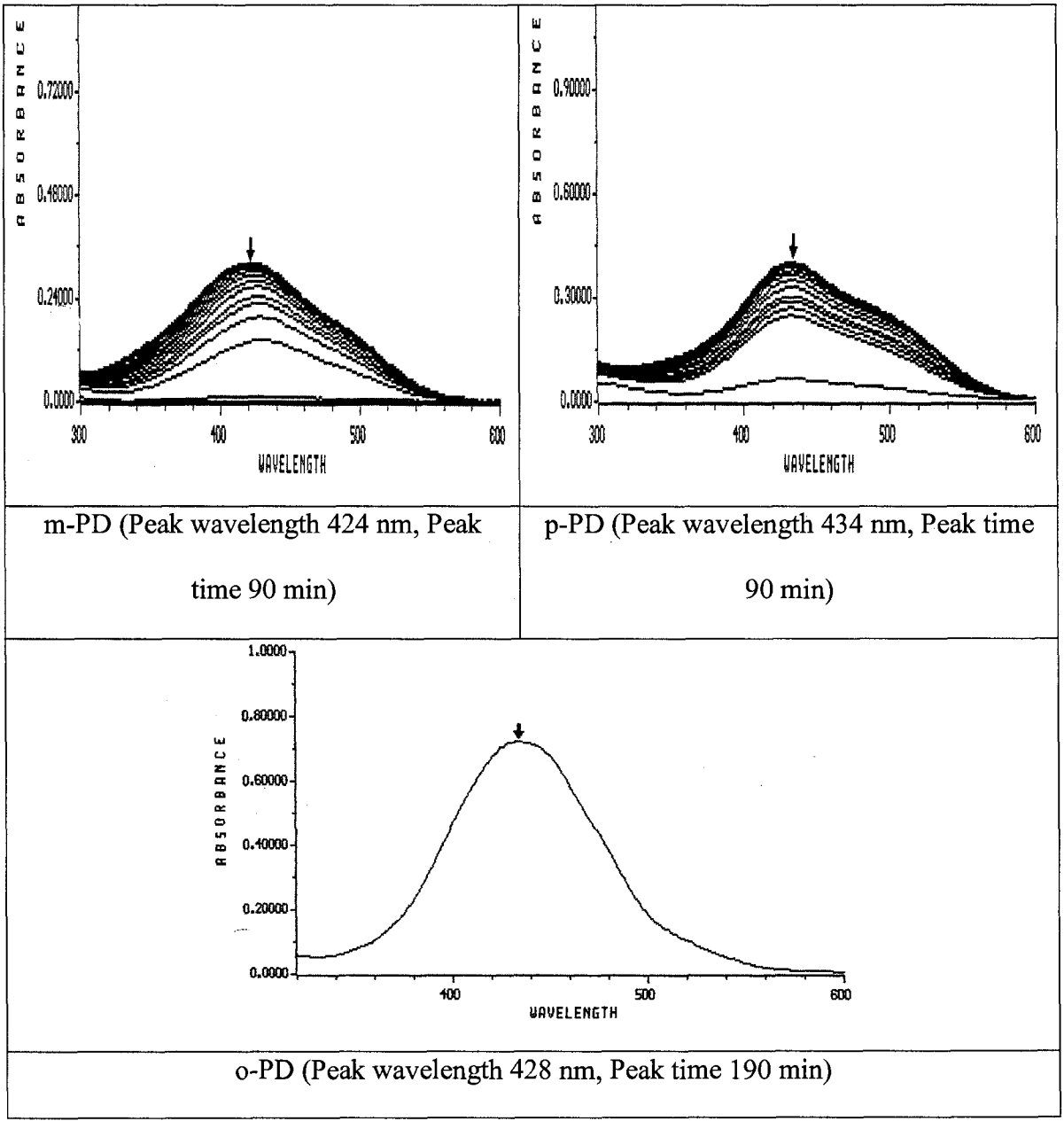
- 0-600 µL of distilled water
- 100-600 µL of aromatic aryl amines.
- 100 µL of 0.2 M phosphate buffer (anilines pH 7.4).
- 100 µL of 20 mM sodium sulphite.
- 100 µL of 10 mM TNBS

Test Conditions for determining the peak wavelength and peak time:

Experiments were conducted to investigate the color product peak wavelength and the time taken for full color development. The peak wavelength and peak time various depending on what aryl amine (o-PD, m-PD and p-PD) compound being tested.

- Instrument: UV-Vis.
- Test mode: Overlay mode (over time)
- Wavelength range tested: The absorbance measurements were monitored over a wavelength range of 300 to 600 nm.
- Time duration: The samples were monitored in a regular intervals (5-10 min intervals) until the maximum absorbance is reached.

Figure D-1 and table D-1 illustrates the peak wavelength and peak time for o-PD, m-PD and p-PD.



m-PD (Peak wavelength 424 nm, Peak time 90 min)

p-PD (Peak wavelength 434 nm, Peak time 90 min)

o-PD (Peak wavelength 428 nm, Peak time 190 min)

Figure D-1: Plots for TNBS peak wavelength and peak time

Table D-1: Optimum TNBS assay conditions			
Compound	Wavelength (nm)	Time for color development (min)	Extinc. Coeff. ($L.mol^{-1}.cm^{-1}$)
o-PD	428	190	21,479
m-PD	424	90	38,165
p-PD	434	90	34,963

Once the peak time and the wavelength were determined, standard curves were plotted and used to determine unknown concentrations of substrate.

Standard Curve of o-PD:

The standard curve of o-PD is shown in Figure D-2. The wavelength was at 428 nm. The concentration range was between 0.005 to 0.055 mM. The equation of the best fit line was $y = 21.749x - 0.0553$ and R^2 value was 0.9979.

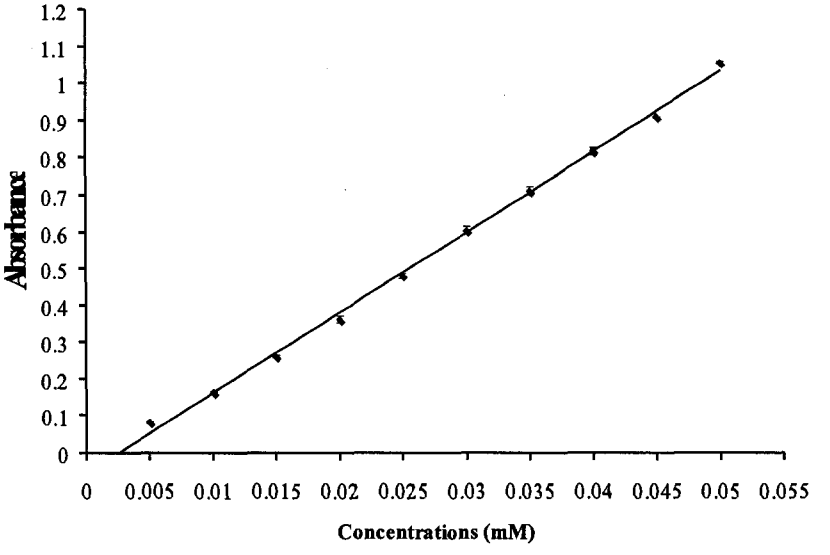


Figure D-2: Colorimetric assay standard curve of o-PD

Standard Curve of m-PD:

The standard curve of m-PD is shown in figure D-3. The wavelength was at 424 nm. The concentration range was between 0.005 to 0.03 mM. The equation of the best fit line was $y = 38.165x - 0.0081$ and R^2 value was 0.9992.

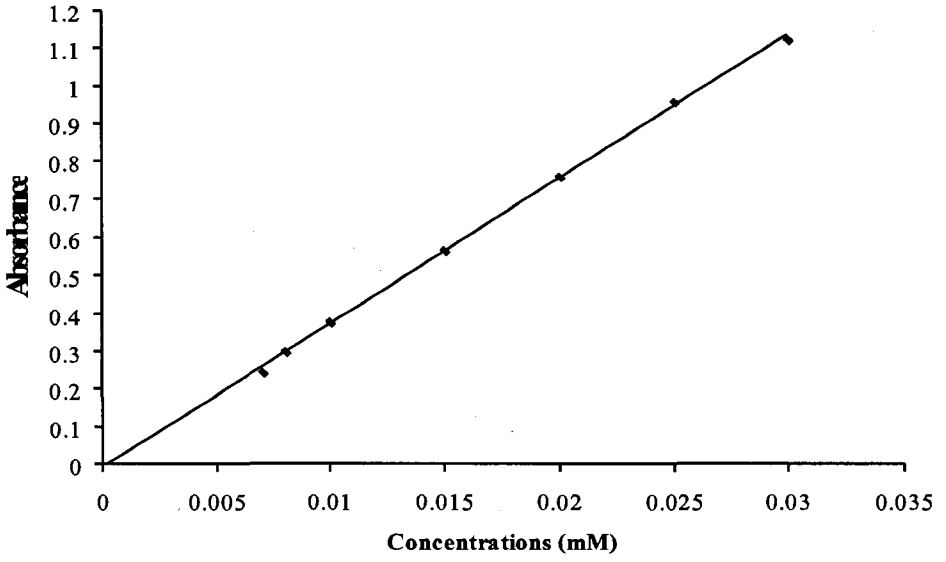


Figure D-3: Colorimetric assay standard curve of m-PD

Standard Curve of p-PD:

The standard curve of p-PD is shown in figure D-4. The wavelength was at 434 nm. The concentration range was between 0.005 to 0.04 mM. The equation of the best fit line was $y = 34.963x - 0.0502$and R^2 value was 0.999.

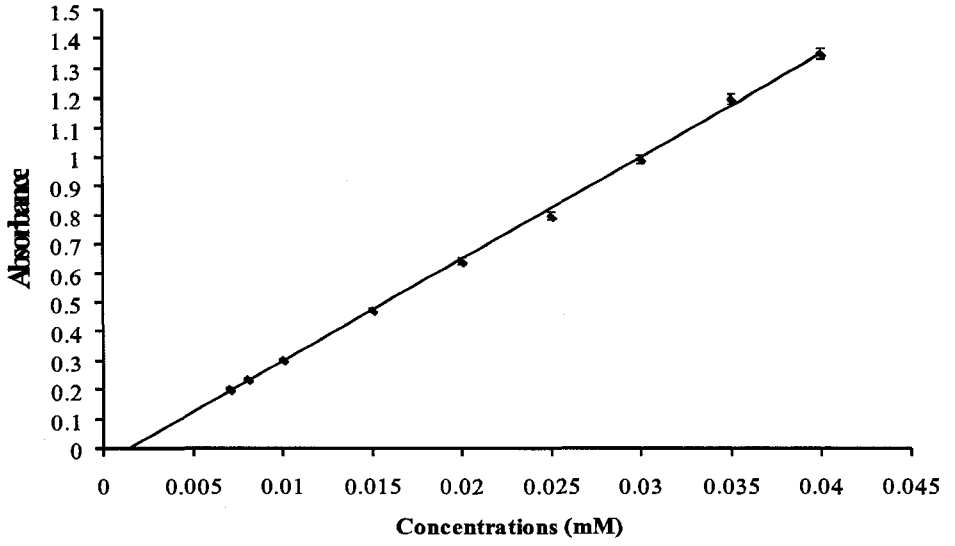


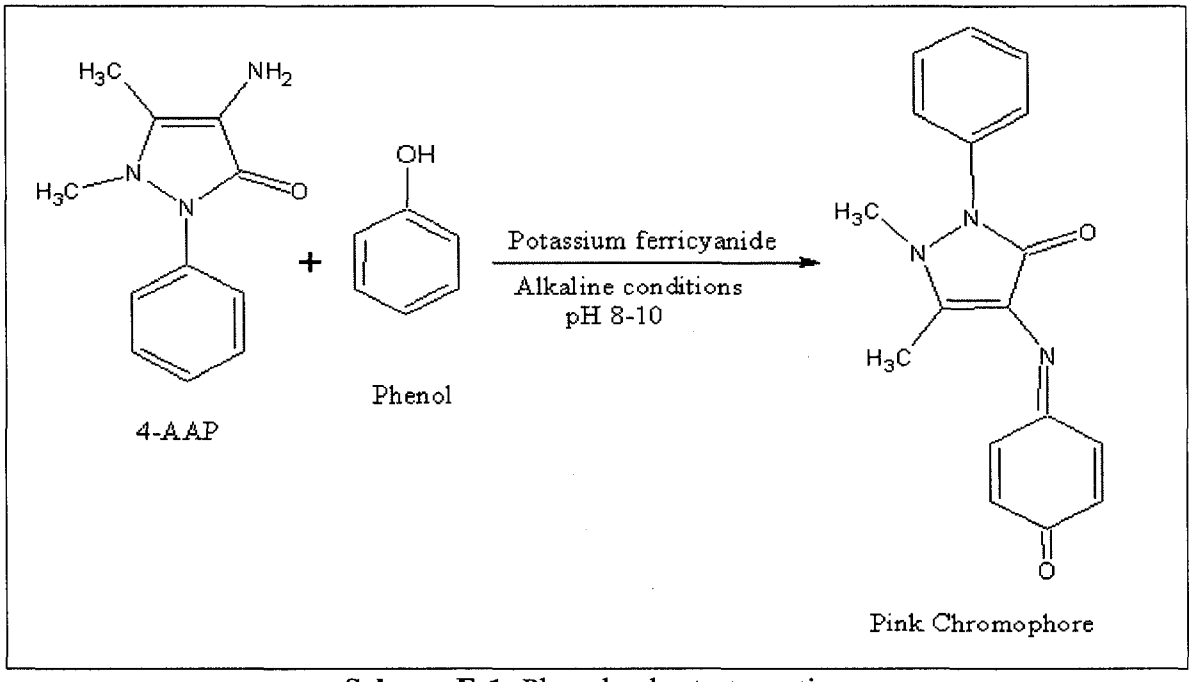
Figure D-4: Colorimetric assay standard curve of p-PD

APPENDIX E

Colorimetric Assay for Aromatic Phenols

Test Reaction

Scheme E-1 demonstrates the reaction of phenol and 4-AAP in presence of Potassium ferricyanide at alkaline conditions of pH 8-10. The product of such reaction generates a pink chromophore. The color intensity generated is directly proportional to the concentration of the phenolic compound.



Scheme E-1: Phenol color test reaction

Test Conditions

- Instrument: UV-Vis.
- Wavelength: The absorbance measurements were at a wave length of 510 nm.
- Time for color development: approximately 60 seconds is enough for completer color development.
- Reagents:
  - 20 mM 4-AAP in 0.25 M $NaHCO_3$.

In a 50.0 mL volumetric flask:

- 0.2033 g of 4-AAP.
- 12.5 mL of 1.0 M $NaHCO_3$.
- Dilute to 50.0 mL with distilled water.

➢ 83.4 mM $K_3Fe(CN)_6$ in 0.25 M $NaHCO_3$.

In a 50.0 mL volumetric flask:

- 1.373 g of $K_3Fe(CN)_6$.
- 12.5 mL of 1.0 M $NaHCO_3$.
- Dilute to 50.0 mL with distilled water

- Test Procedure: In a 1.0 mL cuvette:

Blank

- 800 µL of distilled water
- 100 µL of 20.0 mM 4-AAP.
- 100 µL of 80.0 mM $K_3Fe(CN)_6$.

Sample

- 0-800 µL of distilled water
- 100-800 µL of aromatic phenol.
- 100 µL of 20.0 mM 4-AAP.
- 100 µL of 80.0 mM $K_3Fe(CN)_6$.

Standard Curve of Phenol:

The standard curve of phenol is shown in Figure E-1. The wavelength was at 510 nm. The concentration range was between 0.005 to 0.04 mM. The equation of the best fit line was $y = 12.82x + 0.0186$ and R^2 value was 0.9999.

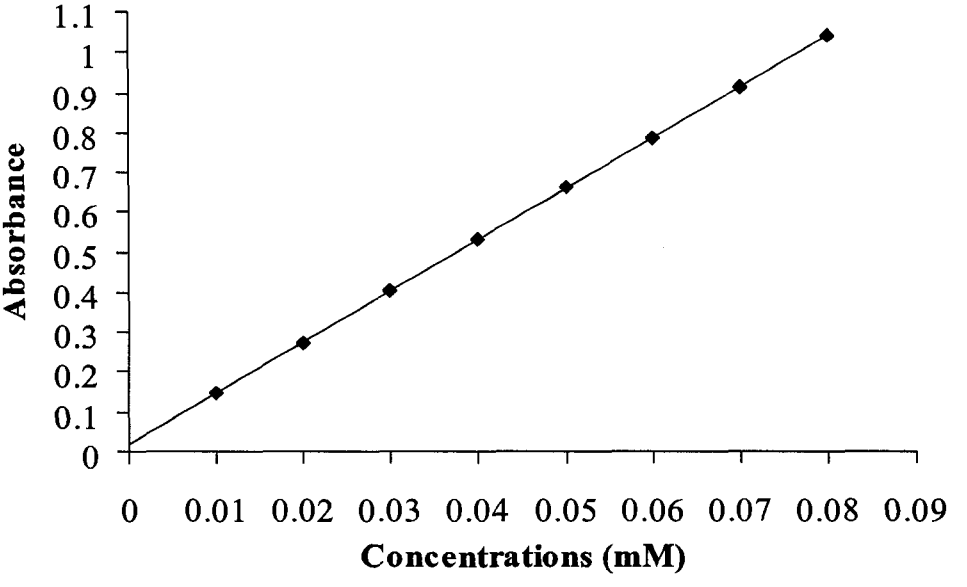


Figure E-1: Colorimetric assay standard curve of phenol

APPENDIX F

Colorimetric Assay for Hydrogen Peroxide

Test Reaction

The test reaction of hydrogen peroxide assay is similar to SBP activity color test reaction in which phenol and 4-AAP reacts in presence of limiting hydrogen peroxide under concentrate ARP as an enzyme catalyst. The product of such reaction generates a pink chromophore. (See Appendix A)

Test Conditions

- Instrument: UV-Vis.
- Wavelength: The absorbance measurements were at a wave length of 510 nm.
- Time for color development: approximately 1-2 minutes was enough for completer color development.
- Reagents: In a 20.0mL volumetric flask:
    - 0.25 mL of Novo ARP liquid concentrate.
    - 51.0 mg of 4- AAP (12.5mM).
    - 10.0 ml of 10x concentrate (100.0 mM phenol; 0.5 M phosphate buffer (pH 7.4)
    - Dilute to 20.0 mL with distilled water.
- Test Procedure: In a 1.0 mL cuvette:

Blank

- 200 µL of the reagent.
- 800 µL of distilled water.

Sample

- 200 µL of the reagent.
- 100-800 µL of hydrogen peroxide
- 0-800 µL of distilled water.

(The SBP dilution were made (between 20 x to 100 x))

Standard Curve of Hydrogen Peroxide:

The standard curve of hydrogen peroxide is shown in Figure F-1. The wavelength was at 510 nm. The concentration range was between 0.01 to 0.2 mM. The equation of the best fit line was $y = 5.1415x - 0.0207$and R^2 value was 0.9971.

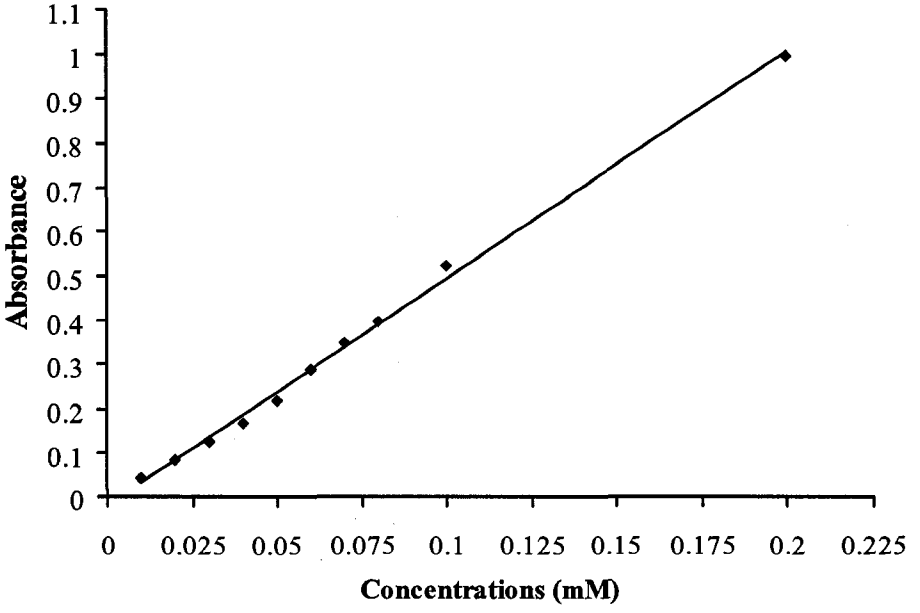


Figure F-1: Colorimetric assay standard curve of hydrogen peroxide

APPENDIX G

Direct Absorbance Standard Curves

Direct Absorbance Standard Curve of o-PD:

The direct absorbance wavelength of o-PD was 290 nm (Figure G-1).

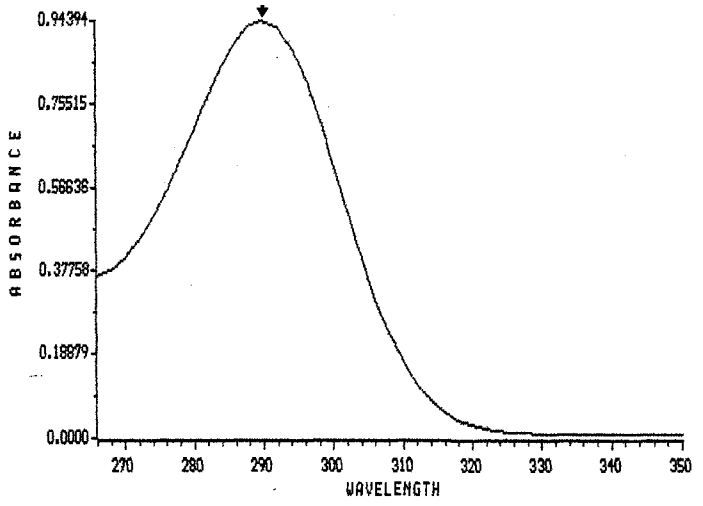


Figure G-1: Direct absorbance wavelength of o-PD

The standard curve of o-PD is shown in Figure G-2. The wavelength was at 290 nm. The concentration range was between 0.05 to 0.40 mM. The equation of the best fit line was $y = 2.7907x - 0.0132$ and R^2 value was 0.9995.

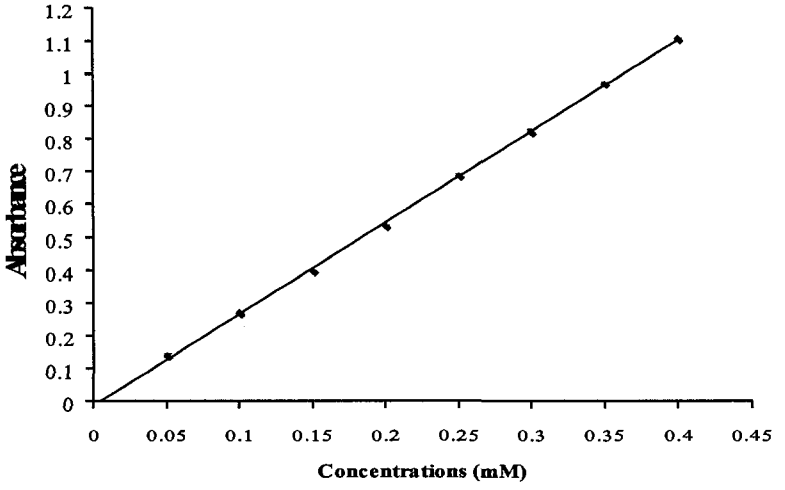


Figure G-2: Direct absorbance standard curve plot for o-PD

Direct Absorbance Standard Curve of m-PD:

The direct absorbance wavelength of m-PD was 290 nm (Figure G-3).

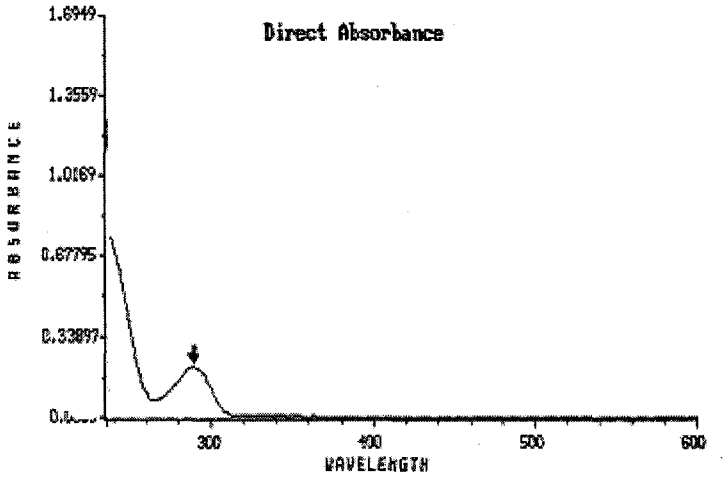


Figure G-3: Direct absorbance wavelength of m-PD

The standard curve of m-PD is shown in figure G-4. The wavelength was at 290 nm. The concentration range was between 0.05 to 0.40 mM. The equation of the best fit line was $y = 2.0532x + 0.005$ and R^2 value was 0.9996.

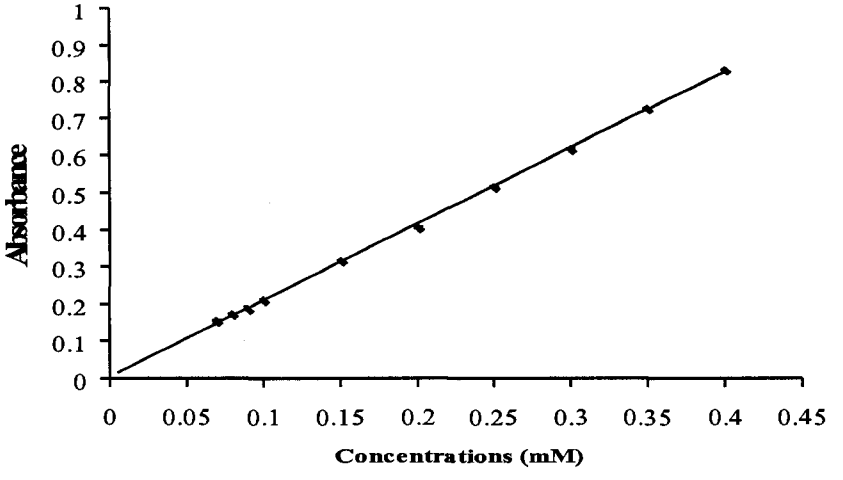


Figure G-4: Direct absorbance standard curve plot for m-PD

Direct Absorbance Standard Curve of p-PD:

The direct absorbance wavelength of p-PD was 304 nm (Figure G-5).

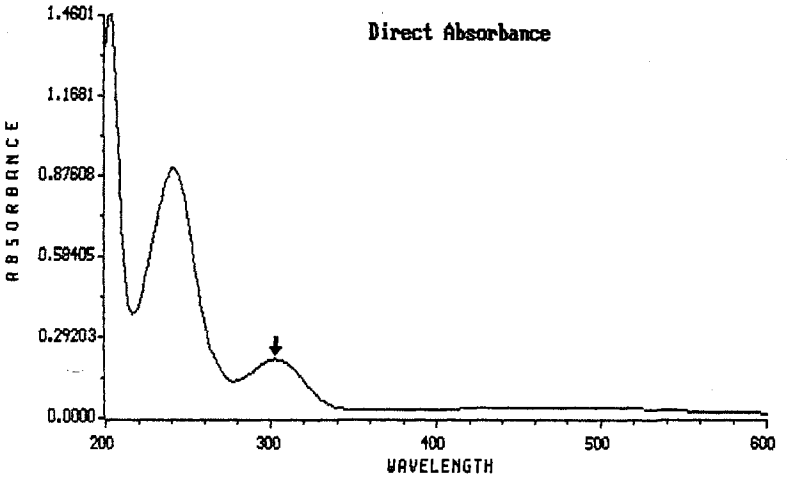


Figure G-5: Direct absorbance wavelength of p-PD

The standard curve of p-PD is shown in figure G-6. The wavelength was at 304 nm. The concentrations rang was between 0.05 to 0.40 mM. The equation of the best fit line was $y = 1.9973x + 0.0421$and R^2 value was 0.9996.

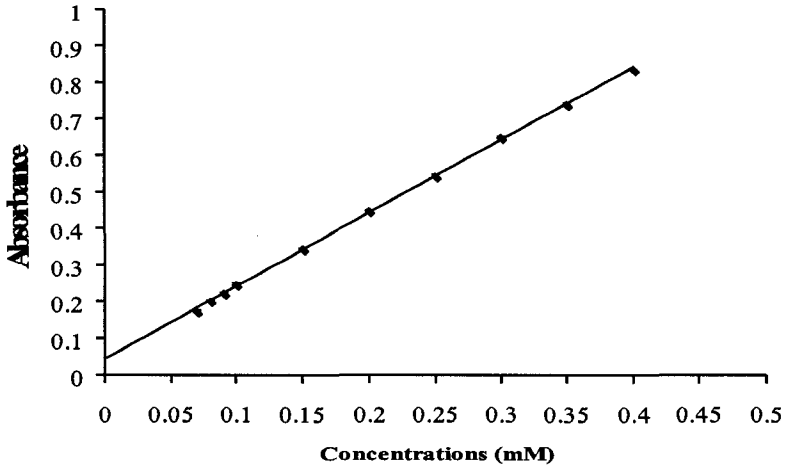


Figure G-6: Direct absorbance standard curve plot for p-PD

Direct Absorbance Standard Curve of Catechol:

The direct absorbance wavelength of catechol was 276 nm (Figure G-7).

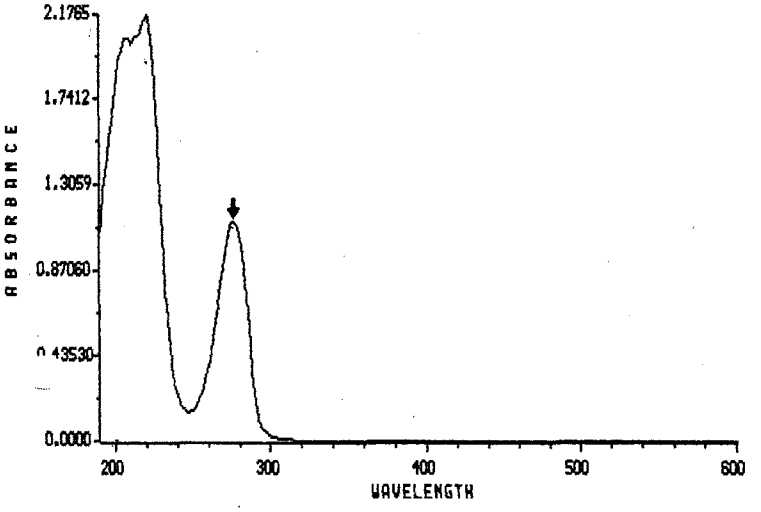


Figure G-7: Direct absorbance wavelength of catechol

The standard curve of catechol is shown in figure G-8. The wavelength was at 304 nm. The concentration range was between 0.05 to 0.5 mM. The equation of the best fit line was $y = 2.2625x + 0.0111$ and R^2 value was 0.9995.

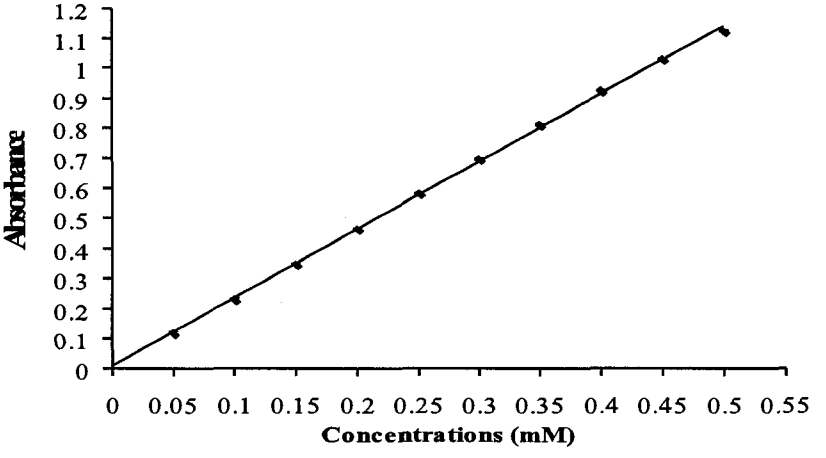


Figure G-8: Direct absorbance standard curve plot for catechol

Direct Absorbance Standard Curve of Resorcinol:

The direct absorbance wavelength of resorcinol was 274 nm (FigureG-9).

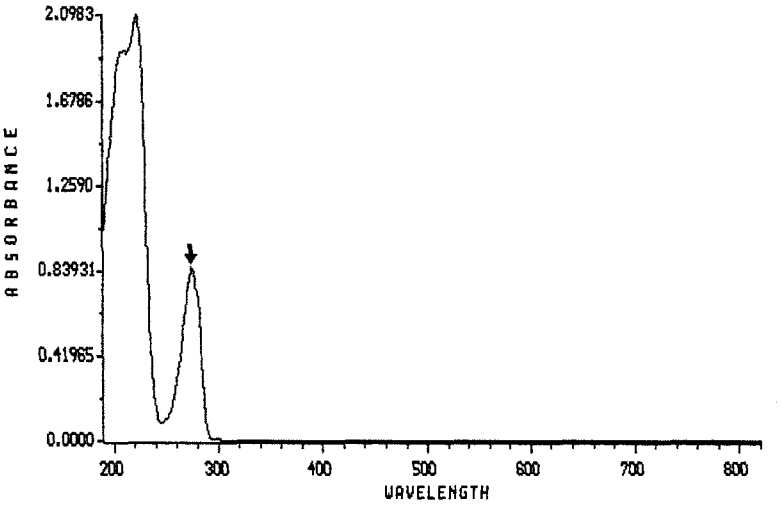


Figure G-9: Direct absorbance wavelength of resorcinol

The standard curve of resorcinol is shown in figure G-10. The wavelength was at 304 nm. The concentration range was between 0.05 to 0.5 mM. The equation of the best fit line was $y = 1.7465x + 0.0018$ and R^2 value was 0.999.

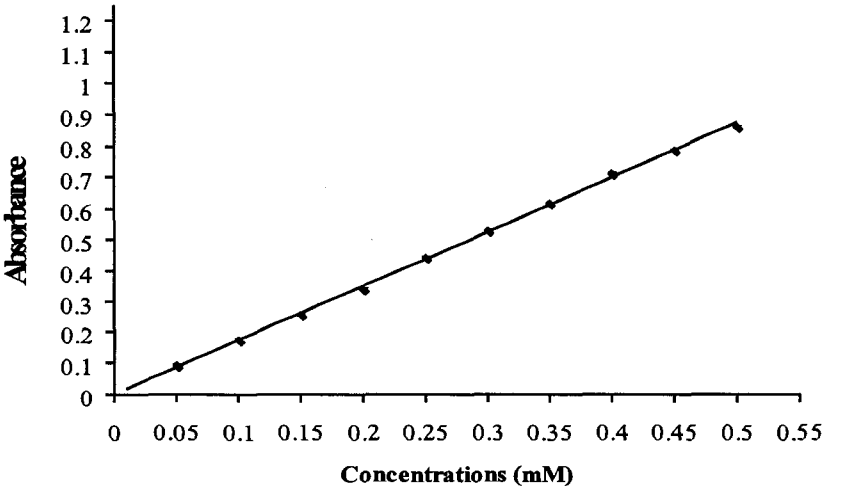


Figure G-10: Direct absorbance standard curve plot for resorcinol

Direct Absorbance Standard Curve of Hydroquinone:

The direct absorbance wavelength of hydroquinone was 290 nm (FigureG-11).

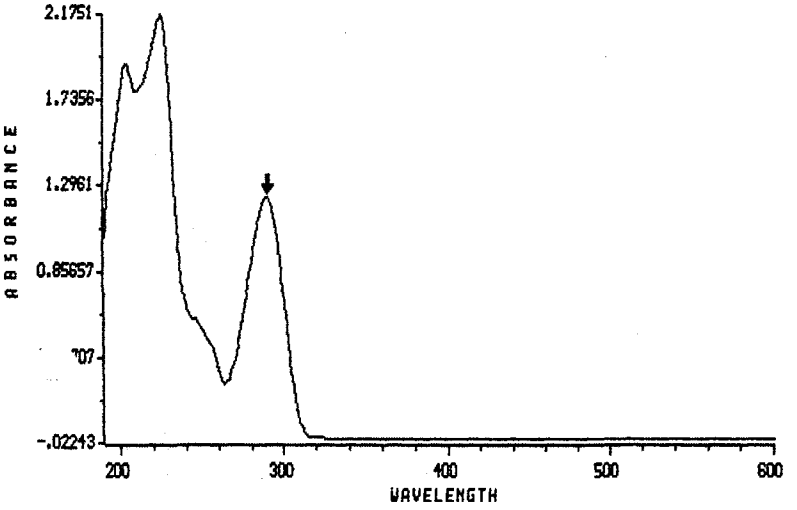


Figure G-11: Direct absorbance wavelength hydroquinone

The standard curve of hydroquinone is shown in figure G-12. The wavelength was at 304 nm. The concentration range was between 0.05 to 0.5 mM. The equation of the best fit line was $y = 2.5473x - 0.0229$ and R^2 value was 0.9992.

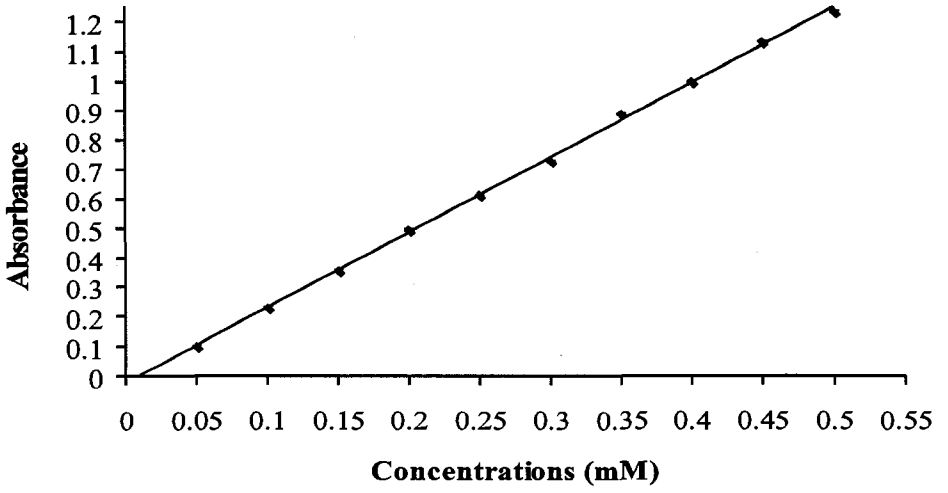


Figure G-12: Direct absorbance standard curve plot for hydroquinone

Direct Absorbance Standard Curve of 2-MBT:

The direct absorbance wavelength of 2-MBT was 310 nm (Figure G-13).

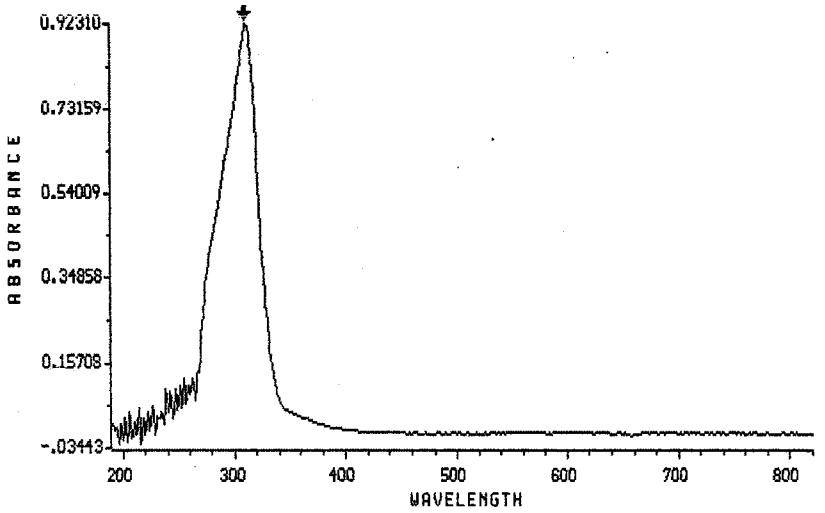


Figure G-13: Direct absorbance wavelength of 2-MBT

The standard curve of 2-MBT is shown in figure G-14. The wavelength was at 304 nm. The concentration range was between 0.01 to 0.05 mM. The equation of the best fit line was $y = 19.692x - 0.0733$ and R^2 value was 0.9999.

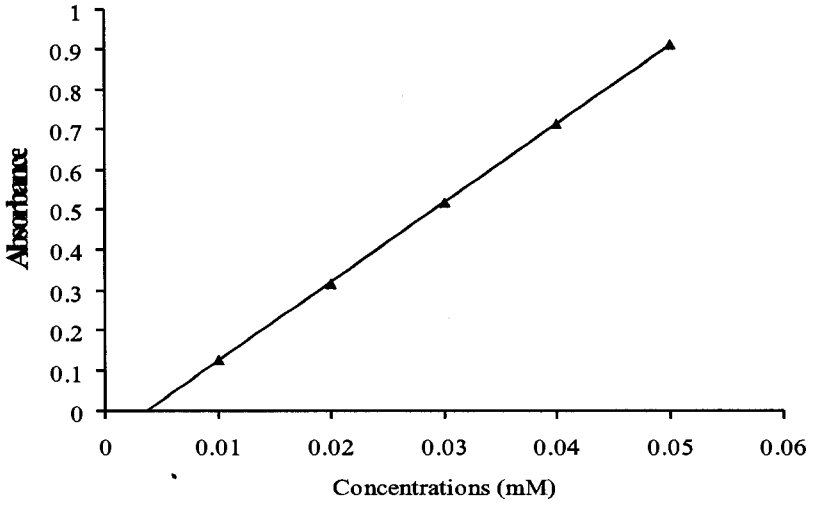


Figure G-14: Direct absorbance standard curve plot for 2-MBT

Direct Absorbance Standard Curve of Phenol:

The direct absorbance wavelength phenol was 276 nm (Figure G-15).

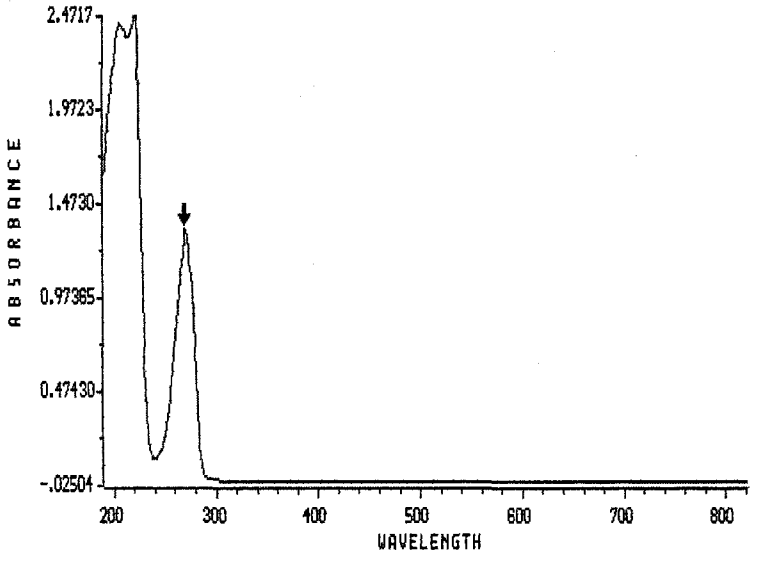


Figure G-15: Direct absorbance wavelength of phenol

The standard curve of phenol is shown in figure G-16. The wavelength was at 276 nm. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line $y = 1.469x + 0.0137$ and R^2 value was 0.9997.

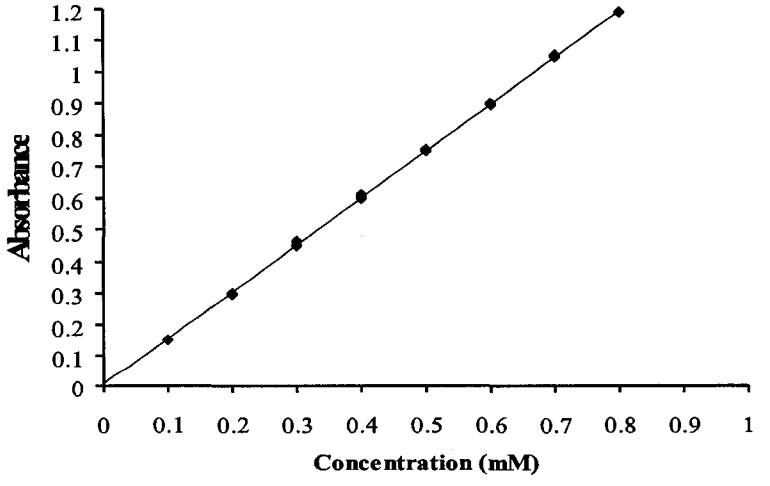


Figure G-16: Direct absorbance standard curve plot for phenol

APPENDIX H

HPLC Standard Curves

HPLC Standard Curve of o-PD:

The HPLC standard curve of o-PD is shown in Figure H-1. The retention time was 1.92 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y= 2875400x+8122.8 and R^2 value was 0.999. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

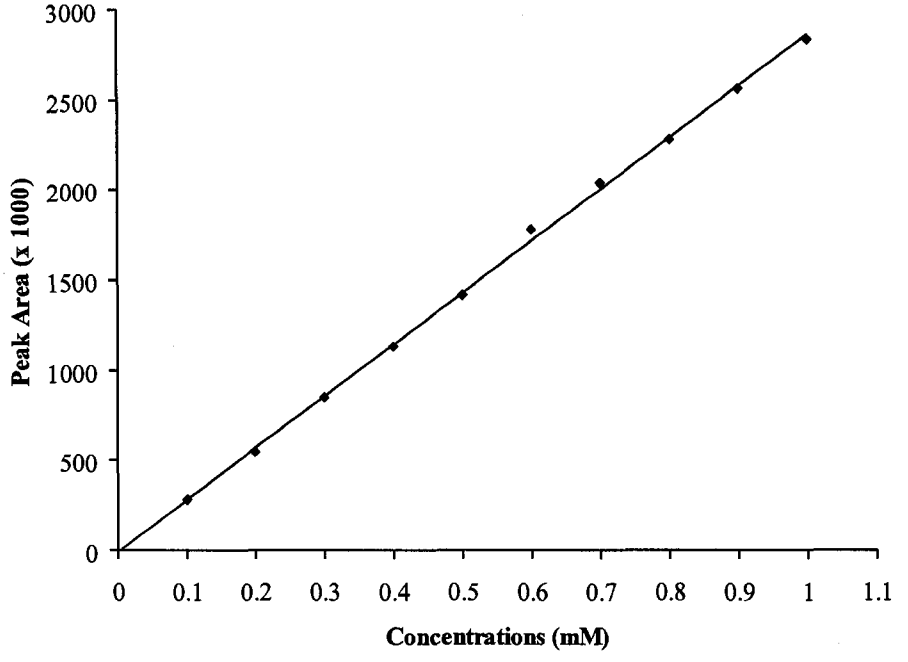


Figure H-1: HPLC standard curve plot for o-PD

HPLC Standard Curve of m-PD:

The HPLC standard curve of m-PD is shown in Figure H-2. The retention time was 1.74 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y = 1219900x + 45101and R^2 value was 0.9999. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

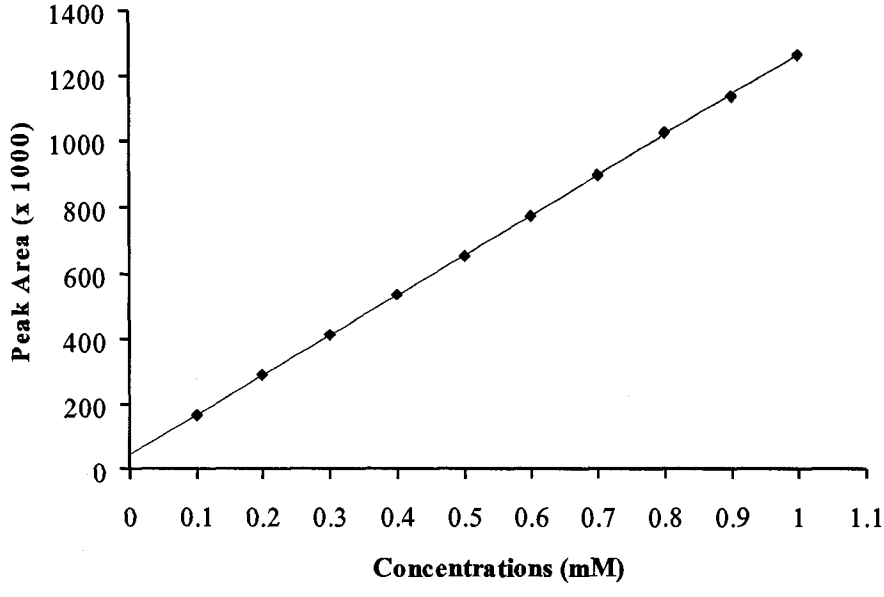


Figure H-2: HPLC standard curve plot for m-PD

HPLC Standard Curve of p-PD:

The HPLC standard curve of p-PD is shown in Figure H-3. The retention time was 1.96 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y = 1284200x + 1891.7and R^2 value was 0.9958. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

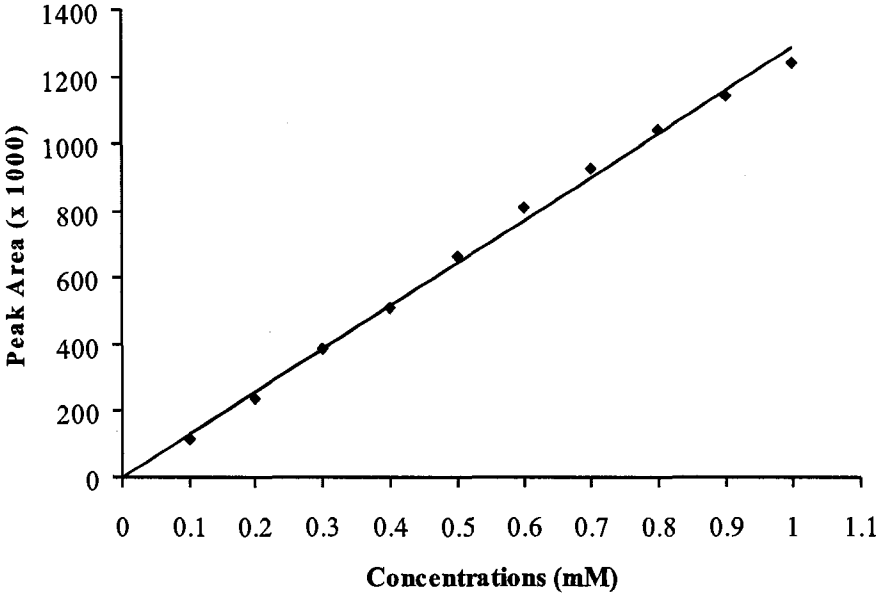


Figure H-3: HPLC standard curve plot for p-PD

HPLC Standard Curve of Catechol:

The HPLC standard curve of catechol is shown in Figure H-4. The retention time was 2.46 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y = 1271800x – 2430 and R^2 value was 0.9987. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

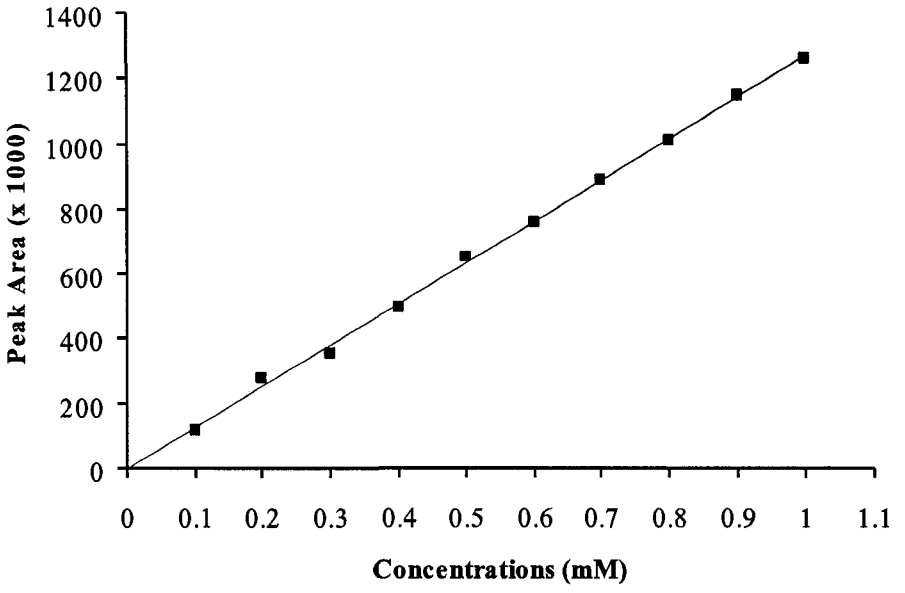


Figure H-4: HPLC standard curve plot for catechol

HPLC Standard Curve of Resorcinol:

The HPLC standard curve of resorcinol is shown in Figure H-5. The retention time was 2.58 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line $y = 1079800x + 86.86$ and R^2 value was 0.9999. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

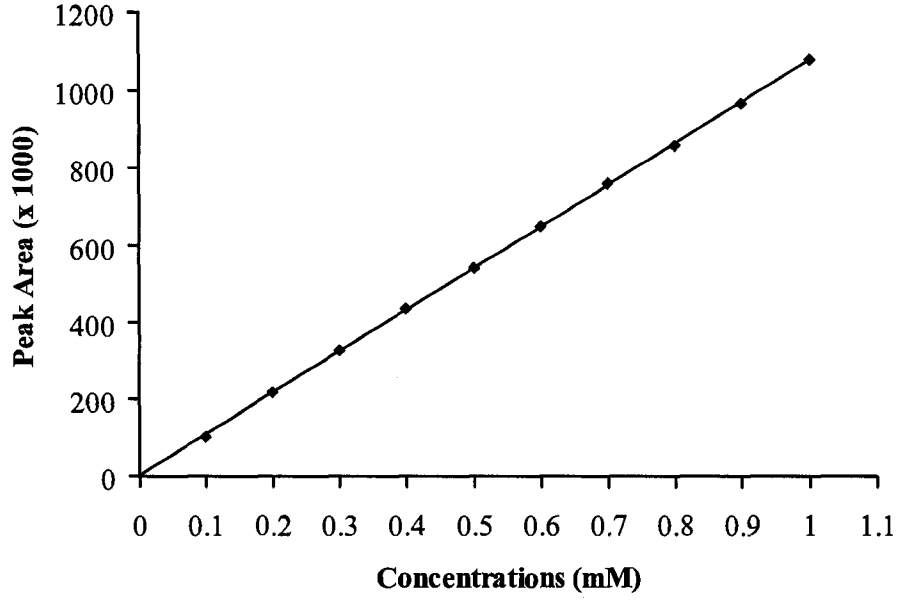


Figure H-5: HPLC standard curve plot for resorcinol

HPLC Standard Curve of Hydroquinone:

The HPLC standard curve Hydroquinone is shown in Figure H-6. The retention time was 2.53 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y = 1437600x - 20691and R^2 value was 0.9971. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

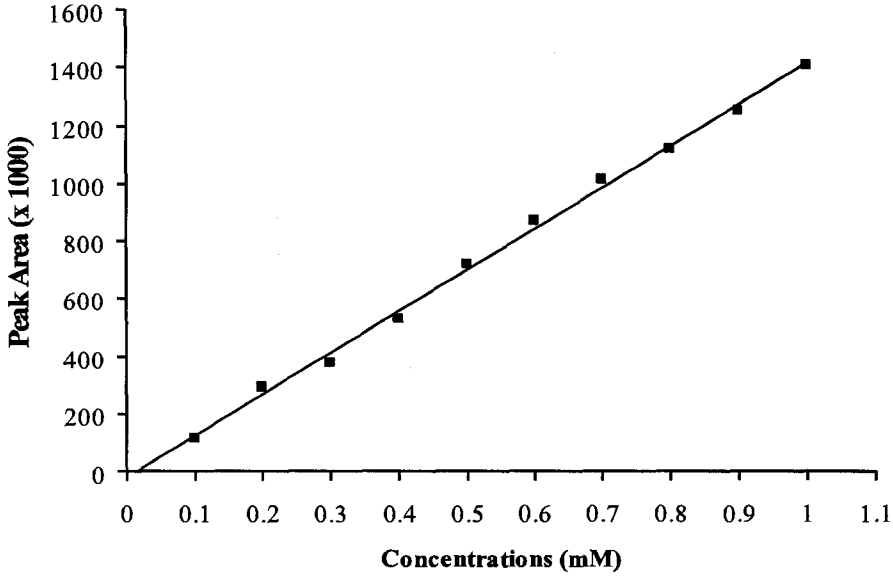


Figure H-6: HPLC standard curve plot for hydroquinone

HPLC Standard Curve of 2-MBT:

The HPLC standard curve of 2-MBT is shown in Figure H-7. The retention time was 2.72 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y= 9836400x+125901 and R^2 value was 0.9998. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

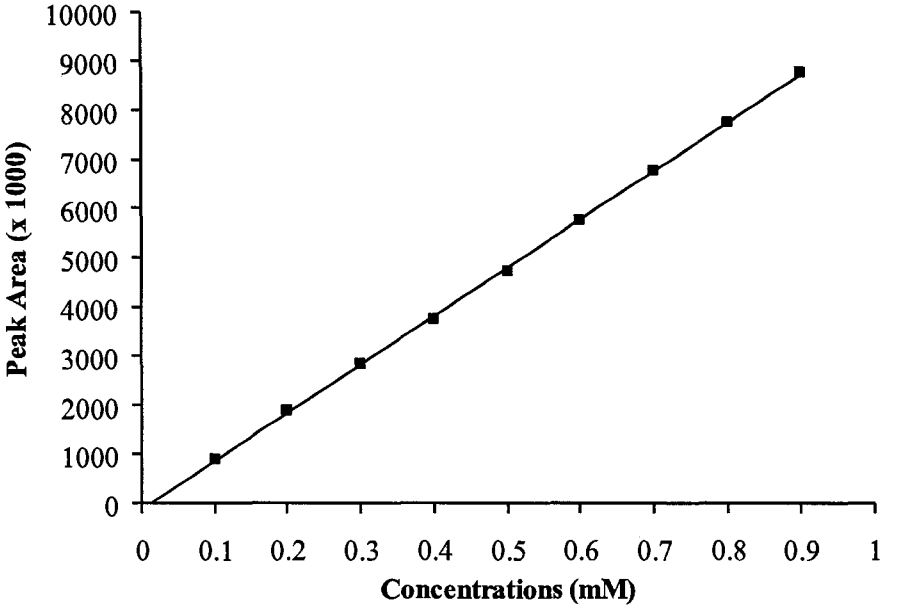


Figure H-7: HPLC standard curve plot for 2-MBT

HPLC Standard Curve of Phenol:

The HPLC standard curve of phenol is shown in Figure H-8. The retention time was 3.85 min. The concentration range was between 0.1 to 1.0 mM. The equation of the best fit line y = 786630x + 7049.9and R^2 value was 0.9993. Table 3.1, summarizes the mobile phase solvents, mobile phase ratio and the absorbance detector set wavelength.

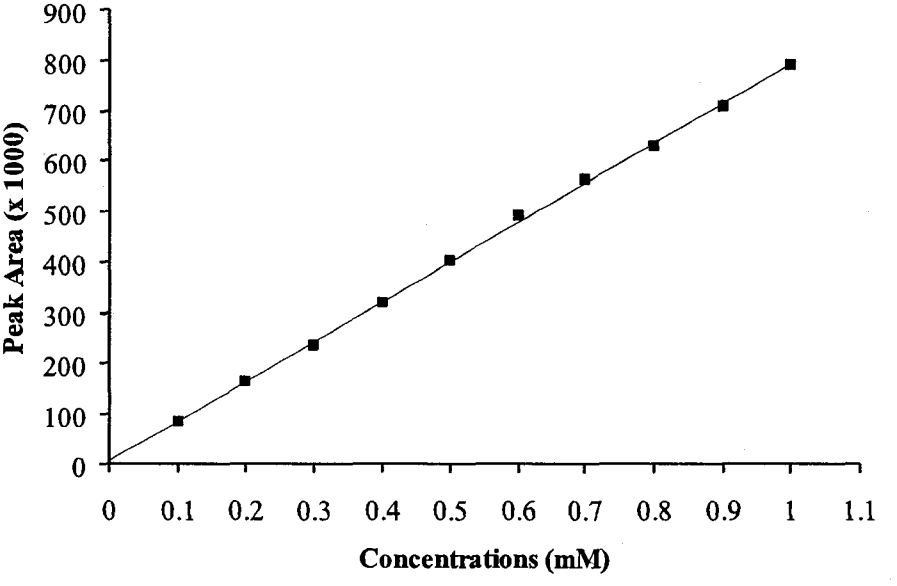


Figure H-8: HPLC standard curve plot for phenol

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