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# Soybean peroxidase catalyzed polymerization and removal of 2,4-dimethylphenol from synthetic wastewater

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# Soybean Peroxidase Catalyzed Polymerization and Removal of 2,4-dimethylphenol from Synthetic Wastewater

by

**Riya Dutta**

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# ABSTRACT

Enzymatic treatment of synthetic wastewater containing 2,4-dimethylphenol (2,4-DMP) was investigated in the presence and absence of polyethylene glycol (PEG) by the enzyme soybean peroxidase. The optimum pH both in the absence and in presence of PEG was 8.0. The optimum [hydrogen peroxide]/ [2,4-DMP] was between 0.9-1.2. A linear relationship existed in presence of PEG between the minimum SBP concentration and initial 2,4-DMP concentrations. In the absence of PEG, a linear relationship did exist at lower substrate concentrations up to 2.0 mM, beyond which the minimum enzyme concentration remained constant and independent of the initial substrate concentration. At lower 2,4-DMP concentrations, there was PEG effect which decreased to almost nil with increase in substrate concentrations. Minimum PEG concentration for 1 mM of 2,4-DMP was found to be 45-50 mg/L. Preliminary kinetic study of the enzyme-catalyzed reaction yielded the values of Michaelis-Menten constants for 2,4-dimethylphenol, in the presence and absence of PEG.

# DEDICATION

To my aunt and uncle "The Mighty Sinhas'"...without your love, inspiration and guidance, I would not have been here in the first place.

To my grandparents, parents and brother.... for seeing me through this long journey...

Last but not the least to Arindam Singha, for teaching me the three Ls of life..... *'love'*, *'laughter'* and *'life itself'*.

Cheers to all of you.....

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Lastly, thanks to those people whose help, support and love made this thesis possible. Special thanks to Katy, Aaron and Moh for letting me eat your brains and vice versa. I cannot think of trading you people for any treasure in this world. Thank you so much.

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# LIST OF ABBREVIATIONS/ACRONYMS

| | |
|---|---|
| AAP | 4-Aminoantipyrene |
| Km | Michaelis–Menten half-saturation constant with respect to 2,4-dimethylphenol (mM) |

CHAPTER 1

# INTRODUCTION

## 1.1 Pollutant

2,4-dimethylphenol (2,4-DMP) has been listed as a priority pollutant in the EPA's list of priority pollutants (Keith and Telliard, 1979). 2,4-DMP is an important raw material for the manufacture of a wide variety of commercial products for industry and agriculture. It has been reported as a suspected cardiovascular or blood toxicant (IRIS), gastrointestinal or liver toxicant (NJFS), kidney toxicant, respiratory toxicant, skin or sense organ toxicant (NJFS).

It has been reported as a high production volume (HPV) chemical that means its production exceeds 1 million pounds annually in the U.S (IRIS). Statistical data available from TRI provides a fair idea about the amount of 2,4-dimethylphenol released in US in the year 2005. In 2005, total on- and off-site release from facilities in all industries was 147,209 lb. Of these releases, total on-site (underground injection, landfills disposal, air release, surface water discharge) disposal was 146,689 lb and 520 lb were transferred off-site (landfills, wastewater treatment plants) for disposal. Data available from TRI shows that the production and use of 2,4-DMP has increased in the U.S. since 1988 when the total on- and off-site release of 2,4-DMP was just 38,674 lb. The European Economic Community (EEC) has enlisted this chemical as dangerous in its Dangerous for the Environment (Nordic Council of Ministers) list. According to data reported by the Tallin Polytechnical Institute (Maazik, 1968), River Purtse discharges about 800 kg of dimethylphenols daily into the Gulf of Finland.

It is under regulatory coverage, which means it is covered by federal environmental laws or by California state laws (because California has unusually effective and innovative laws). 2,4-DMP has been enlisted as one of the priority pollutants in the following regulatory lists in North America :

- Federal Regulatory Program Lists
- Hazardous Constituents (Resource Conservation and Recovery Act)
- Hazardous Substances (Superfund)
- Priority Pollutants (Clean Water Act)
- Registered Pesticides (Federal Insecticide, Fungicide, and Rodenticide Act)
- Toxic Release Inventory Chemicals
- California State Regulatory Program Lists
- Air Contaminants (California Occupational and Safety Health Act)
- California Air Toxics "Hot Spots" Chemicals (Assembly Bill 2588)

## 1.2 Conventional Methods for Treatment of 2,4-DMP

Because of the toxicity of this compound, removal of it from industrial effluent is of great practical significance. Klibanov et al. (1980) listed the currently available methods for removal of phenolic compounds from industrial wastewater and these include solvent extraction, microbial degradation, adsorption on activated carbon, chemical oxidation, membrane processes and irradiation. Physical and chemical treatment processes, including activated carbon adsorption and the various oxidation processes developed in recent years, are typically able to remove organic pollutants to low levels. However, most physicochemical treatment processes are not highly selective in terms of the range

of pollutants removed during treatment. Consequently, such processes are more economical for the treatment of dilute wastewaters and are often used as polishing steps. In chemical oxidation, for example, oxidant dosage increases as the strength of the waste increases or as the required final concentration of the pollutants of concern decreases. Therefore, chemical oxidation processes can become prohibitively expensive for high-strength wastes, even though the target pollutants might only be present in low concentrations (Aitken, 1993).

The standards for discharge of 2,4-DMP are becoming increasingly restrictive; therefore, it is important to find an innovative treatment method that is effective over a wide range of reaction conditions. Research is being conducted to develop an alternative approach to the removal of 2,4-DMP from wastewater by using enzyme-based technology.

## 1.3 Enzyme Treatment

The use of enzymes in waste treatment applications was first proposed in the 1930s (Munnecke, 1976). However, the concept of using an enzyme to remove specific pollutants in waste mixtures was not developed until the 1970s (Aitken, 1993). In recent years, an enzyme-catalyzed polymerization and precipitation process has been explored as a new method for the treatment of aqueous phenolic and anilino molecules (Al-Kassim et al., 1994; Taylor et al., 1998; Mantha et al., 2002), with the bulk of the research done with peroxidases.

Peroxidase-catalyzed oxidative polymerization transforms water-soluble organics into water-insoluble higher polymer compounds without any apparent degradation. Most phenols and anilines were removed from water with more than 99% removal efficiency

(Klibanov et al., 1980). In this treatment strategy, a peroxidase catalyzes the oxidation of phenols and anilines in the presence of hydrogen peroxide (Ibrahim et al., 2001; Mantha et al., 2002). Researchers initially used horseradish peroxidase and then various other peroxidases for removing the toxic organic pollutants from wastewater: Al-Kassim *et al.* (1994) used *Coprinus macrorhizus* peroxidase (CMP), Caza *et al.* (1999) used soybean peroxidase (SBP), Biswas (1999) used crude SBP, while Ibrahim *et al.* (2001) used *Arthromyces ramosus* peroxidase (ARP). Several researchers proposed that instead of peroxidases, laccase, a multicopper oxidase, also might be useful for removing phenolic contaminants from water or wastewater (Bollag *et al,* 1988; Thurston, 1994; Modaressi 2007).

## 1.4 Objectives

Soybean peroxidase was used as a catalyst for the removal of 2,4-dimethylphenol. The objectives of this study included:

- Investigate the feasibility of using soybean peroxidase to catalyze the removal of 2,4-DMP in the concentration range of 0.2-8.0 mM.
- Investigate the effect of additive, polyethylene glycol (PEG), in improving the removal efficiency and its protective effect on the enzyme.
- Optimize of reaction parameters influencing the removal of 2,4-DMP by varying pH, ratio of hydrogen peroxide to substrate, enzyme concentration and PEG concentration.
- Determination of kinetic constants of the enzyme catalyzed reaction.

## 1.5 Scope

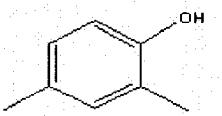
The scope of this study included the following:

- The synthetic wastewater was comprised of 2,4-DMP in buffered solution.
- All reactions were conducted at room temperature (21 ± 3 °C) to remove at least 95 % of substrate.
- The pH of the synthetic of the synthetic wastewater was varied from 4.0 to 9.0.
- Substrate concentrations ranged from 0.2 to 8.0 mM for most parts of the studies.
- Hydrogen peroxide to substrate ratio was varied from 0.9 to 2 in order to investigate the effect of hydrogen peroxide concentration on the reaction.
- Polyethylene glycol (molecular mass = 3350) was used as an additive to protect the enzyme from inactivation.
- The soybean peroxidase enzyme was used as a crude extract.
- Reaction time for all optimization experiments was fixed at 3 hours but the reaction time for kinetic experiments was fixed at 4 minutes.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. 2,4 – dimethylphenol as a Pollutant




2,4-dimethylphenol (C.A.S 105-67-9) is a substituted phenol. It has been also named m-xylenol, 2,4-xylenol, or m-4-xylenol (Weast, 1972). It is a naturally occurring compound, which can be derived from the cresol fraction of petroleum or coal tars by fractional distillation and extraction with aqueous alkaline solutions. (EPA, 1980; Lowry, 1963; Gruse and Stevens, 1942 and Rudolfs, 1953). In its normal state, it exists as a colorless crystalline solid (Weast, 1972, Benet 1974). The commercial product is liquid in form with sweet acrid odor. It exhibits weak acidic properties. The physical and chemical properties, common uses and environmental hazards of 2,4-DMP are discussed below (EPA, 1980; Spectrum laboratories; IRIS; NJFS).

### *2.1.1 Physical and Chemical Properties*

2,4-dimethylphenol has a molecular weight of 122.7 and exists as a colorless , crystalline solid in nature. It has a melting point of 27 to 28 $^{o}$C and a boiling point of 210 $^{o}$C at 760 mm Hg (Weast, 1972; Bennet, 1974; Jordan, 1954). It is slightly soluble in water (solubility: 0.787 g/100 mL) and very soluble in organic solvents such as alcohol and

ether. It exhibits weak acidic properties ($pK_a$ = 10.6), and reacts with aqueous alkaline solutions to form the corresponding salts which are readily soluble in water under alkaline pH (Sober, 1970). The free ortho position (to the hydroxyl group) may be alkylated (Kirk and Othmer, 1964) or halogenated (Rodd, 1952), though such reactions under normal conditions have not been reported. It can also be oxidized to form pseudoquinone, though again the conditions required for this reaction are generally not found in the environment (Rodd, 1952).

**Table 2.1 Physical and Chemical Properties of 2,4-dimethylphenol (ILO, 2007)**

| Property | Information |
|---|---|
| chemical name | 2,4-dimethylphenol |
| Synonym(s) | 1-hydroxy-2,4-dimethylbenzene,<br>2,4-xylenol,<br>4-hydroxy-1,3-dimethylbenzene,<br>4,6-dimethylphenol,<br>m-xylenol |
| Chemical formula | $C_8H_{10}O$ |

**Table 2.1 Physical and Chemical Properties of 2,4-dimethylphenol (continued)**

| **Identification Numbers** | |
|---|---|
| CAS registry | 105-67-9 |
| Caswell No. | 907A |
| EPA Pesticide Chemical Code | 086804 |
| HSDB | 4253 |
| NSC | 3829 |
| RCRA WASTE NUMBER | U101 |
| Molecular Mass | 122.1664 |
| Melting point | 27.5 °C |
| Boiling point | 210.9 °C |
| Density | 0.965 |
| Refractive Index | 1.5447 |
| Flash point | 110 °C |
| Water solubility @ 25 C | Slightly soluble (0.787 g/100 mL) |
| Color/Physical state | Colorless crystals to yellow-brown liquid |
| Henry's Law constant (KoH) @ 25 C | 9.51 X $10^{-7}$ atm-m3/mole |
| Octanol-water partition coefficient (as Log $K_{ow}$) | 2.30 |
| Vapor Pressure @ 25 C | 0.102 mm Hg |

CAS= Chemical Abstract Services; HSDB= Hazardous Substances Data Bank; RCRA= Resource Conservation and Recovery Act;

**Table 2.1 Physical and Chemical Properties of 2,4-dimethylphenol (continued)**

| | |
|---|---|
| pKa dissociation constant @ 25 C | 10.6 |
| Atmospheric OH Rate Constant @ 25 C | 7.15 X $10^{-11}$ cm3/molecule-sec |
| Odor threshold concentration | 0.01 mg/ $m^3$ (in air) ;<br>0.0005-0.4 mg/ $m^3$ (in air)<br>0.4 mg/l (in water) |

### *2.1.2 Common uses of 2,4-dimethylphenol*

2,4-dimethylphenol does not have any direct commercial use, but it is an important raw material for the manufacture of wide variety of commercial products for industry and agriculture. It is used in the manufacture of phenolic antioxidants, disinfectants, solvents, pharmaceuticals, insecticides, fungicides, plasticizers, rubber chemicals, polyphenylene oxide, wetting agents, and dyestuffs and is an additive of lubricants, gasolines and cresylic acid (EPA, 1980).

### *2.1.3 Emission sources of 2,4-dimethylphenol*

2,4-DMP is derived as a complex mixture of phenol and methylated phenol derivatives from fractionation of petroleum or coal tar acids. Commercial cresol and cresylic acids usually contain phenol, the three methylphenols and the dimethylphenols. The presence of this chemical in petroleum fractions and coal tars, along with its use as a raw material for the manufacture of commercial products, proves the fact that it has the potential for both point and non point source of water contamination. This is possible because the disposal of chemical and process wastes from these industries using 2,4-dimethylphenol,

represent feasible modes of entry of this chemical into the environment. Daily use of these products for example pesticide applications, asphalt, roadway runoff and washing of dyed materials, also contribute to the potential of this chemical as a source of water contamination (U.S. EPA, 1975). The compound also occurs naturally in plants, tea, tobacco and cigarette smoke. Maazik, 1968 reported considerable amounts of dimethylphenols are discharged in tar water from shell distillation due to degradation of high molecular weight tars and polymers. Versar, 1975 reported that small quantities of 2,4-dimethylphenol were formed during sewage treatment (biological step) and biological degradation of municipal, biological and industrial wastes.

### *2.1.4 Health Hazards*

It has been reported as a suspected

- Cardiovascular or Blood Toxicant (IRIS)
- Gastrointestinal or Liver Toxicant (NJFS)
- Kidney Toxicant
- Respiratory Toxicant
- Skin or Sense Organ Toxicant (NJFS)

### *2.1.5 Environmental hazards*

Information regarding the concentration, persistence, fate and effects of 2,4-DMP in the environment is limited. The complete biodegradation of 2,4-DMP has been reported as to occur in approximately in two months although the conditions were not stated (Rodd, 1952). A measured steady state bio-concentration factor of 150 was obtained for 2,4-dimethylphenol using bluegills (U.S. EPA, 1978). The skin of humans is considered to be the primary route of occupational exposure to complex mixture containing 2,4-

dimethylphenol. Workers associated with petroleum, coal and coke processing and degreasing operations are affected by exposure to 2,4-DMP.

2,4-DMP is released to the environment as fugitive emissions and in wastewater because of coal tar refining, coal processing and in its use in chemical/plastics manufacturing. When released in water, it degrades principally due to biodegradation with a half-life of hours to days at ambient temperature (Spectrum Laboratories). Adsorption to sediment, particulate matter and soil is moderate with a biodegradation period of 4 days at 19 °C and bio-concentration in fish is not significant. Hauge et al. (1966) established 2,4-DMP to be a ATP blocking agent. They observed the development of vasoconstriction in isolated blood perfuse rabbit lungs. Boutwell and Bosch (1959) reported that 2,4-DMP in high concentrations produced papillomas and carcinomas on the skin of tumor-susceptible mice of the sutter strain.

### *2.1.6 Toxic Release Inventory of 2,4-dimethylphenol (TRI,2005)*

Statistical data available from TRI provides a fair idea about the amount of 2, 4-dimethylphenol released in US in the year 2005. Of these releases, 17,094 lb were air emissions; 1,046 lb were surface water discharges; 128,549 lb was released by underground injection and 520 lb were transferred off-site for disposal. In 2005, total on and offsite release from facilities in all industries was 147,209 lb. These substantial quantities of 2,4-dimethylphenol discharge in environment necessitate the development of an advanced treatment technology.

### *2.1.7 Exposure limits of 2,4-dimethylphenol*

The 48-hour $EC_{50}$ value for acute toxicity to freshwater aquatic life occurs at a concentration as low as 2120 µg/l (EPA, 1980). No data are available regarding the chronic toxicity of 2,4-dimethylphenol to freshwater aquatic life. For controlling

undesirable taste and odor quality of ambient water, EPA has regulated the estimated level to be not more than 400 μg/L. At a concentration of 500,000 μg/L 2,4-dimethylphenol causes complete destruction of chlorophyll of freshwater alga, Chlorella pyrenoidosa, when exposed to more than 48 hours. The odor and taste threshold for 2,4-DMP were determined to be 400 μg/L and 500 μg/L respectively (EPA, 1980).

## 2.2. Treatment methods of 2,4-dimethylphenol

The most common methods for treating 2,4-DMP in industrial effluents are biological treatment, advanced oxidation process (AOP), and adsorption. Biological treatment of wastes containing 2,4-DMP was 94.5 % effective depending upon the chemical oxygen demand. The rate of degradation is 28.2 mg of 2,4-DMP removed per hour by a gram of the initial dry matter of biological inoculums (Pitter and Kucharova-Rosolova, 1974). But 2,4-DMP gets quite moderately adsorbed to soil and sediment, so there is a considerable chance of 2,4-DMP getting adsorbed on sludge and being carried away without being treated in case of biological treatment processes (Pitter and Kucharova-Rosolova, 1974; Spectrum Laboratories; Versar, 1975). Another most common treatment process of 2,4-DMP is by advanced oxidation process (AOP) which generally uses different combinations of oxidation agents, irradiation, and catalysts to generate hydroxyl radicals in sufficient quantity to affect water treatment (Glaze et al., 1987). The typical homogeneous AOP systems used are ozone ($O_3$)/ultraviolet (UV), UV/ $H_2O_2$, Ultrasound (US), US/ $H_2O_2$, US/UV, O3/ $H_2O_2$, $H_2O_2/Fe^{2+}$ (Fenton's reagent) and photo-Fenton (Huang et al., 1993). The efficiency of different advanced oxidation processes for

degradation of 2,4-DMP has been found in the order: $H_2O_2/Fe^{2+}/UV$ > $O_3/US$ > $O_3$ >$O_3/UV$ > $H_2O_2/Fe^{2+}$ > US > $UV/H_2O_2$ > UV (Trapido et al., 1998). The removal rates of some phenols were found to be higher for ozone + UV treatment than ozonation, but the additional cost of UV lamps and energy did not seem to be justifiable (Gurol and Vatistas, 1987). Moreover, quite often the efficiency of AOPs is compound specific (Kuo 1992; Luck et al., 1995; Lin and Peng, 1995). Adsorption by activated carbon has been studied as another alternative for the removal of 2,4-DMP but despite the high adsorption capacity of activated carbon in removing a wide range of dissolved toxic organics from industrial effluents, it is not an economically viable treatment process. Moreover, 30% of the used activated carbon is lost during conventional thermal regeneration processes, and the regenerated carbon has lower adsorption capacity than the original one. Batabyal (1995) studied the adsorption of 2,4-DMP from aqueous solution by coal fly ash. Though fly ash is a low cost starting material, studies have shown that adsorption by fly ash is either irreversible (Khare et al., 1987) or the regeneration of saturated fly ash is not economically feasible. Moreover dumping of spent fly ash will contaminate the ground water. Enzymatic treatment will be an innovative and effective way of treating 2,4-DMP.

## 2.3. Enzymatic Treatment as an option

In 1970, for the first time, the concept of enzyme treatment to remove specific target pollutants was introduced (Aitken, 1993). The enzymes isolated from their parent organisms are preferred over individual organisms containing the enzymes for a number of reasons. The isolated enzymes are highly specific for their substrates, their potency can be better standardized, they are easier to handle and store (Vieth and

Venkatasubramaniam, 1973) and the sludge concentration is easier to control since it does not depend on microbial growth (Bailey and Ollis, 1986). Enzymatic treatment has a number of advantages over conventional methods as listed in next page (Nicell et al., 1993)

- enzymes can act over a broad range of substrates including bio-refractory compounds and also those which are toxic to micro organisms
- they can selectively remove a single class of contaminants
- their response time to shock loadings is pretty quick
- low hydraulic retention time
- their process do not involve biomass growth and acclimation
- their removal process has simpler process control
- they are effective over broad range of pH, temperature and salinity
- they require less oxidant than conventional chemical oxidation methods
- they are equally effective at all substrate concentrations ranging from low to high
- their treatment process involves much milder and less corrosive conditions than compared to chemical oxidation methods

Various aromatic compounds in particular phenols and aromatic amines are present in wastewaters of numerous industries such as coal conversion, petroleum refining, organic chemicals, dyes, paper and pulp mills, resins and plastics. Most of these compounds are priority pollutants since they are toxic and some have been determined to be human carcinogens, therefore the removal of such compounds from industrial aqueous effluent is of great practical significance (Ibrahim et al., 1997). An enzymatic method for the removal of phenols from industrial aqueous effluent has been developed in the past

several years. Klibanov's group (Klibanov et al., 1980, 1983) had proposed a peroxidase-catalyzed method for removal of aromatic compounds. The initial work of Klibanov *et al.* (1980) brought to focus the catalytic removal of phenolic and other aromatic compounds from wastewaters using horseradish peroxidase and hydrogen peroxide. In this method, peroxidase enzymes catalyze the oxidation of phenol with hydrogen peroxide, generating phenoxy radicals (Walsh, 1979). These radicals diffuse from the active site of the enzyme into the solution and react non-enzymatically (see Chapter 2.4) to eventually form higher oligomers and polymers, which can be removed from wastewater by sedimentation or filtration (Klibanov et al., 1983; Nicell et al., 1992). It was found that enzymatic treatment was effective over a wide range of pH, temperatures and phenol concentrations (Klibanov et al., 1983). The main drawback of enzyme treatment is the need for the large amount of enzyme since HRP can be inactivated during the reaction. In order to minimize such inactivation, Nakamoto and Machida, 1992 had suggested adding compounds such as polyethylene glycol (PEG) to decrease the adsorption of polymers onto the enzyme active site. They also postulated that the hydroxyl end groups of PEG bond with the hydrogen bonding sites on the polymeric products. It had been demonstrated that the addition of PEG greatly reduces the amount of enzyme required, thus increasing the cost effectiveness (Wu et al., 1993, 1997, 1998). Wu et al. (1998) confirmed the earlier suggestion (Nakamoto and Machida, 1992) that PEG acts as a sacrificial polymer, i.e. PEG combins with the polymerization products formed during the reaction, because it has a higher partition affinity with the polymer products than peroxidases. This enables less enzyme interaction with polymer products.

HRP is by far the most researched peroxidase. However, due to its high cost, much effort has been devoted to find a suitable alternative. As a result, a number of peroxidase

enzymes obtained from different sources like plant, animal and microbial sources have been investigated for the treatment of aromatic phenols and amines in wastewater. Al-Kassim et al. (1993, 1994) used *Arthromyces ramosus* and *Coprinus macrorhizus*, Caza et al., 1999 and Biswas, 1999, used crude soybean peroxidase while several researchers have used laccase as another alternative for removing phenolic contaminants from wastewater (Bollag et al, 1988; Thurston, 1994; Modaressi, 2005). The advent of new technology for the production, isolation and purification of enzymes has made the use more competitive. These advances are leading to the development of new commercial applications of enzymes (Tsutsumi et al., 2001).

The scope of enzymatic treatment is broad at the same time it has limitations. It works effectively for the treatment of aromatic compounds containing the phenolic group, e.g., phenol, phenols substituted with halo, alkyl, aryl, alkoxyl and aryloxyl groups, as well as aromatic compounds containing amino groups, e.g. aniline and analogously substituted anilines such as 4-chloroaniline and diphenylamine. The enzymatic treatment is not effective on aromatic compounds containing a nitro group even if they are phenolic, aniline compounds, or hydrocarbon aromatic compounds like benzene and toluene. However, biological treatment is a widely accepted treatment method, but compounds, as phenol is toxic to microbes at a concentration as low as 2 mg/L and cannot be treated. Moreover, enzymes can effectively treat biorefractory compounds like diphenylamine. The most important challenge of enzymatic treatment is the tendency of the enzyme to be susceptible to inactivation, which in turn increases the cost of the enzymatic treatment.

## 2.4. Soybean Peroxidase (SBP) as the enzyme

Soybean peroxidase is extracted from the seed coat of soybean, belongs to the enzyme class oxidoreductase. It belongs to the family of class III secretory plant peroxidases (Welinder et al., 1992) that can oxidize a wide variety of organic and inorganic substrates using hydrogen peroxide (Dunford, 1999). SBP is a 37 kD glycoprotein and exists as a single peroxidase in the seed coat of soybean. (Gillikin and Graham, 1991). Studies indicate that the amino acid sequence of SBP shows 57% similarity that of with horseradish peroxidase isoenzyme C (HRPC), another well-known member of this class of peroxidases (Henriksen et al., 2007). The potential applications of SBP are considerable because of high thermal stability (McEldoon and Dordick, 1996) and a high reactivity and stability at low pH (McEldoon et al., 1995; Nissum et al. 2001).

One of the major challenges associated with peroxidase-catalyzed phenol removal is the tendency of the enzyme to be susceptible to inactivation by various side reactions of the treatment process (Buchanan and Nicell, 1997), thus increasing the cost of the enzymatic treatment process (Cooper and Nicell, 1996). The problem of enzyme cost can be managed by using a less expensive source of enzyme. Since the seed coat of the soybean is a byproduct of the soybean food industry, SBP has the potential of being a cost-effective alternative to HRP for wastewater treatment.

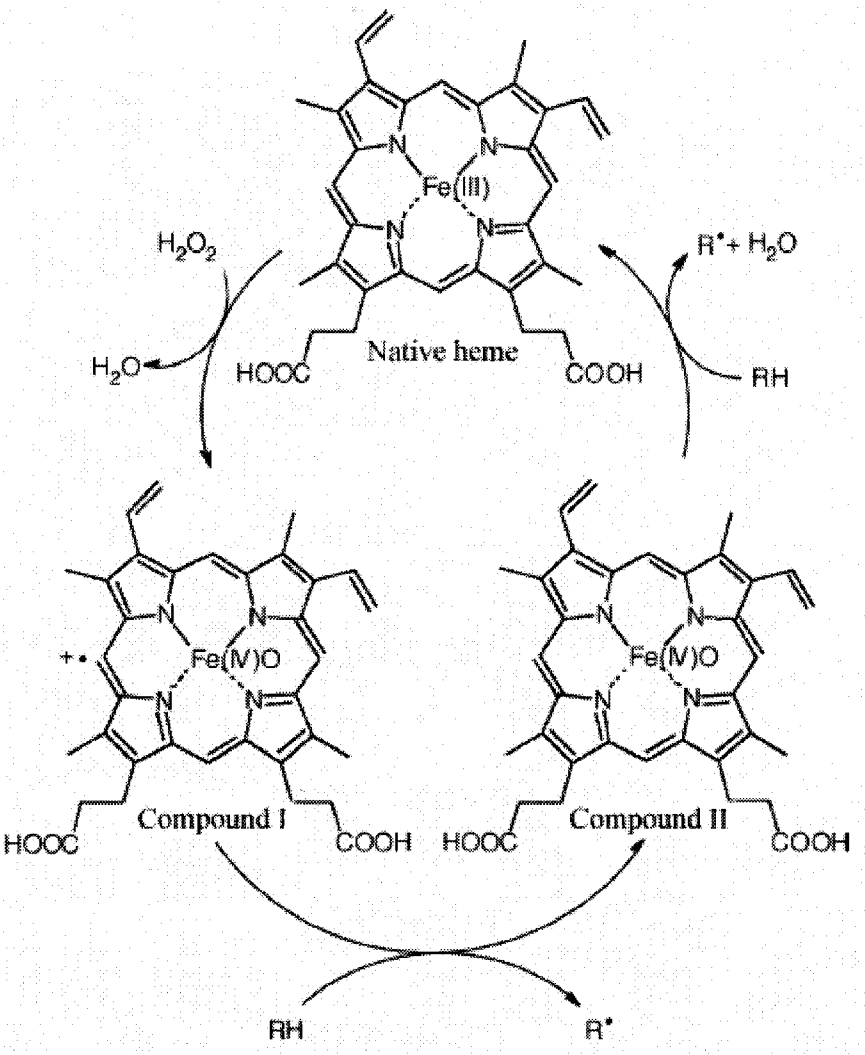



**Figure 2.1:** Catalytic cycle of heme-containing peroxidases in the presence of hydrogen peroxide (Torres *et al.*, 2003)

### *2.4.1 Mechanism of SBP catalyzed reaction*

Figure 2.1 illustrates the steps of an enzyme catalytic cycle occurring at the active site of heme peroxidases. Heme peroxidases have a common active site that contains very similar prosthetic groups. The prosthetic group for all plant peroxidases is

ferriprotoporphyrin IX. Ferriprotoporphyrin IX is made up of four pyrrole rings joined by methane bridge with iron (III) in the centre of the molecule (Dunford, 1999).

Most biochemical reactions involve more than one substrate. The general form of such a reaction would be a two substrate, two-product reaction, which is also known as bi-bi reactions. For enzyme catalyzed two substrate reactions, there are two kinds of mechanisms. One of them is the non- sequential reaction mechanism, in which the first product is produced and released from the enzyme before the second substrate is bound (Palmer, 1995). This mechanism is called ping-pong bi-bi mechanism (Figure 2.2).

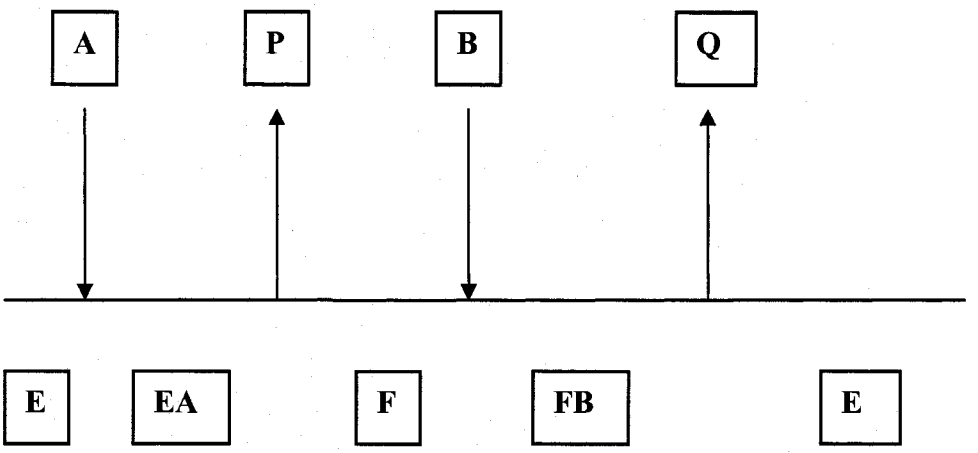



**Figure 2.2:** Ping-pong mechanism

Peroxidase follows a modified ping-pong mechanism (Dunford, 1999). The conventional ping-pong mechanism is reversible which indicates that there is a finite upper limit for rate of reaction. The rate-limiting step depends on the degree of substrate saturation. Substrate binding is the rate-limiting step for lower substrate concentrations (Equation

2.1), but when the enzyme is saturated with substrate, product release is the rate-limiting step (Equation 2.2) (Dunford, 1999).

$$E+A \rightleftharpoons EA \qquad (2.1)$$

$$FB \rightleftharpoons Q+E \qquad (2.2)$$

Peroxidase kinetics and ping-pong kinetics are generally similar with the exception that the former is irreversible. For an irreversible enzyme reaction, the enzyme and its substrate must collide and form some type of encounter complex where the rate at which the complex reacts is much faster than the rate at which they dissociate and reverts to the initial reactants. The enzyme-substrate complex is short-lived and as fast as the substrate encounters the enzyme, the complex will be formed and immediately dissociate into product. Hence, the rate of reaction seems to have no upper limit and larger the concentration of reactants the faster is the reaction. The observed irreversibility means that the back reaction to initial reactant's rate is not measurable. As the substrate concentrations increase, eventually either saturation will occur or some other process such as enzyme inactivation may occur (Palmer, 1995; Dunford, 1999). The peroxidase reaction mechanism involves a change in the oxidation state of an iron atom located in the heme at the centre of the catalytic site of the enzyme (Dunford, 1999).

The mechanism can be best described as a sequence of steps, which have been shown below

$$E + H_2O_2 \xrightarrow{k_1} Ei + H_2O \qquad (2.3)$$

$$\xrightarrow{k_2}$$

$$Ei + AH_2 \rightarrow Eii + AH^{\bullet} \qquad (2.4)$$

$$\xrightarrow{k_3}$$

$$Eii + AH_2 \rightarrow E + AH^{\bullet} + H_2O \qquad (2.5)$$

The steps comprising one entire catalytic cycle can be explained as follows:

I. Native enzyme (E) reacts with peroxide, which in turn oxidizes it to form a compound designated as Ei (Compound I). Ei is two electrons above the native enzyme state and carries the peroxide oxygen.

II. Ei then oxidizes an aromatic molecule ($AH_2$) in its active site to release a free radical $AH^{\bullet}$ into the solution. During this step, Ei is reduced to a compound, which is designated as Eii (Compound II).

III. Eii oxidizes another aromatic molecule ($AH_2$) to release a free radical $AH^{\bullet}$ and itself gets converted back to the native state (E).

In the case of phenol as the aromatic donor, one-electron oxidation and loss of a proton lead to the resonance-stabilized phenoxyl radical which has four contributors as follows: in Figure 2.3.

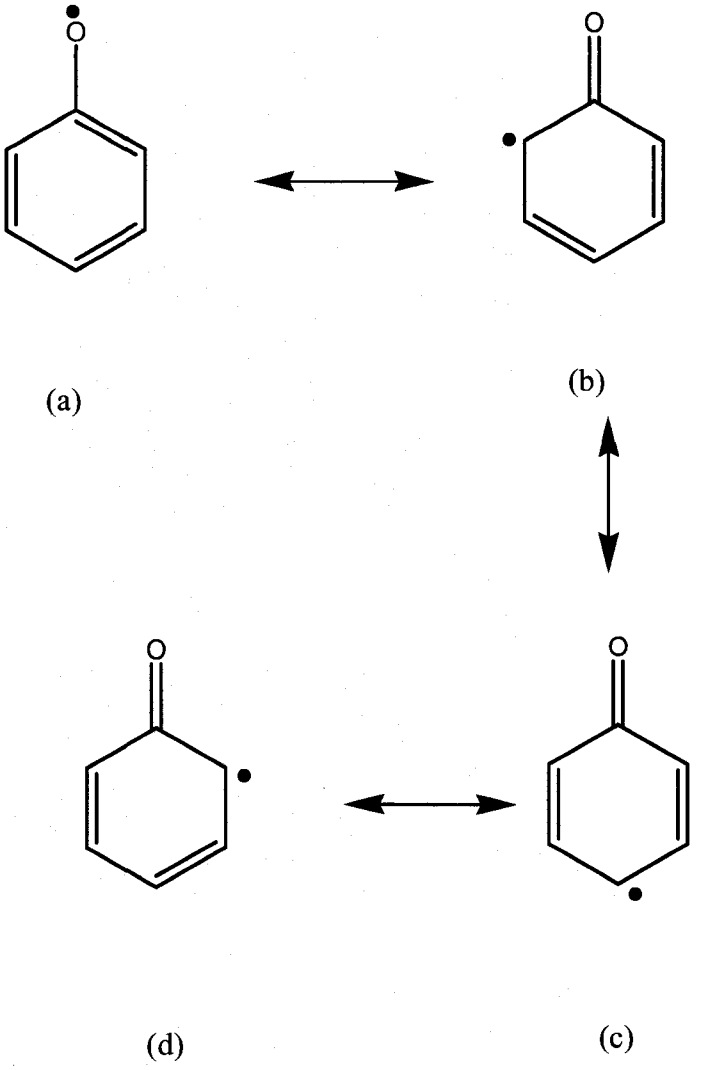



**Figure 2.3:** Resonance contributors of phenol radical

The two free radicals released from the enzyme catalytic site, diffuse into the solution and combine non-enzymatically to form a dimer. As seen, from Figure 2.3 the radical has unpaired electron density on the oxygen atom as well as the *ortho-* and *para-* ring carbons. Thus when radicals couple it is through these positions. With parent phenol, the five stable dimers are given in Figure 2.4 (Yu et al., 1994; Dec and Bollag, 1994).

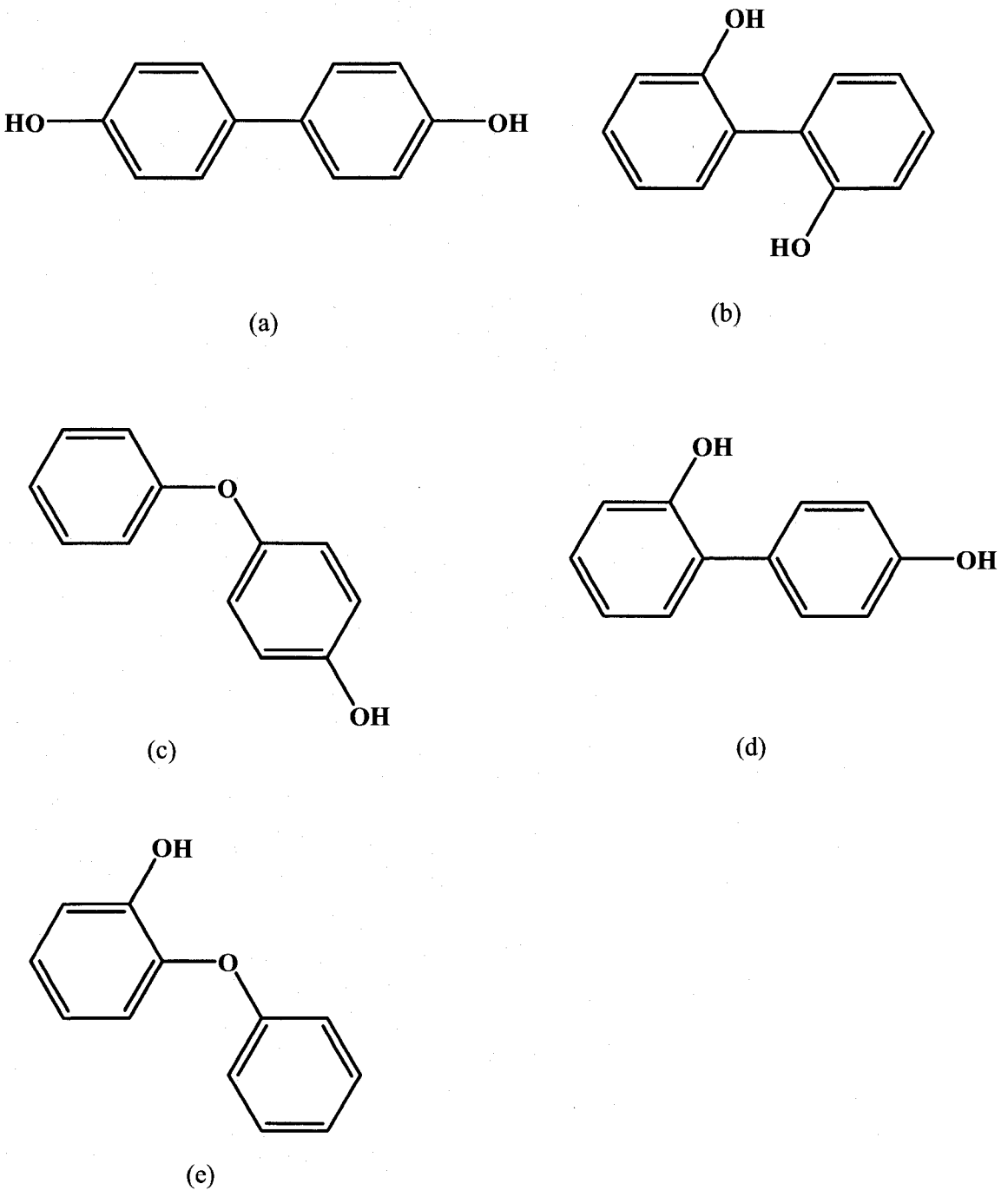



**Figure 2.4:** Possible stable dimers produced in phenol coupling (Yu et al., 1994)

Of these compounds, the peroxide is thought to be unstable and not turn up in the product distribution. The remaining dimers are all phenolic in at least one position and, if they remain in solution, are susceptible to further enzymatic cycles to be elaborated to trimers, tetramers and beyond with the same pattern of reactivity. This will continue until the oligomers/polymers formed reach their solubility limit and precipitate. With respect to the phenolic compound of interest in this study, 2,4-dimethylphenol, further discussion is given in Chapter 4.7.

### *2.4.2 Mechanism of SBP inactivation*

The main setback of enzyme application is that they suffer from inactivation during the reaction. The known ways of enzyme inactivation are:

(1) Inactivation due to phenoxyl radical – according to Klibanov et al., 1983, a permanent inactivation can result from the attachment of a free radical to the active centre of the enzyme, forming a bond at or near the active site. Such a bond may block the active site and upset the critical geometric configuration of the enzyme, thus reducing the enzyme activity.

(2) Inactivation due to end-product polymer – Nakamato and Machida (1992) proposed a parallel theory. They argued that if the peroxidase inactivation occurred due to interaction between the enzyme's active site and phenoxyl radical, it would be difficult or impossible to reduce the amount of enzyme required. They suggested that the apparent enzyme inactivation is by end product polymer, which adsorbs enzyme molecules and hinders the access of substrate to the enzyme's active site.

(3) Inactivation due to peroxide known as suicide inactivation, under conditions of 'excess' $H_2O_2$, peroxide being the suicide substrate (Arnao et al., 1990; Baynton et al.,

1994). Based on works presented by Arnao et al., 1990 and Nakajima and Yamazaki, 1987, horseradish peroxidise undergoes mechanism-based or suicide inactivation. This happens when either in the absence of reductant substrate or in the presence of excess hydrogen peroxide.

Depending upon $H_2O_2$ concentration, inactivation of the enzyme occurs by either or combination of two possible pathways, one reversible and another irreversible (Arnao et al., 1990 a; 1990b; Nakajima and Yamazaki, 1987). Nicell and Wright (1997) in their study observed that the inactivation curves of remaining activity versus time exhibited a rapid phase (0-60 seconds) which was hydrogen peroxide concentration-dependent, and a slow phase characterized by a gradual loss of enzyme activity that was neither time-dependent nor hydrogen peroxide concentration-dependent.

At low $H_2O_2$ concentrations (below 1mM), in the absence of phenol, the inactivation process is predominantly a reversible inactivation pathway leading to Compound III, Eiii, which likely accounted for the observed rapid inactivation, according to

$$Eii + H_2O_2 \rightarrow Eiii + H_2O \qquad (2.6)$$

Compound III was catalytically inactive but its formation did not represent a terminal inactivation of peroxidase since Compound III decomposed to native peroxidase according to

$$Eiii \rightarrow E_N + O^-_2 + H^+ \qquad (2.7)$$

The return to the native state was sufficiently slow that once the Compound III was formed, the enzyme is severely hampered in carrying out the catalytic oxidation of aromatic substrates. Therefore, any accumulation of enzyme in the compound III state represented loss in catalytical efficiency (Dunford 1999, Nicell & Wright 1997).

At higher hydrogen peroxide concentrations, another pathway can lead to irreversibly inactivated intermediate, Compound P-670 (Dunford 1999, Villalobos & Buchanan 2002, Nicell & Wright 1997).

$$Eiii + rS \rightarrow E_i + rS^{\cdot} \qquad (2.8)$$

$$Ei + H_2O_2 \rightarrow P_{670} \qquad (2.9)$$

Baynton et al. (1994) concluded that the overall inactivation process comprised of both reversible and irreversible processes and exhibited a second order inactivation rate constant of $0.023 \pm 0.005$ $mM^{-1}.s^{-1}$ at pH 7.4 and 25 °C.

In the presence of both hydrogen peroxide and phenol fixed at 0.5 mM concentrations, the inactivation process of HRP was found to be irreversible, time- and phenol concentration dependent process and also mechanism based with a second order inactivation rate constant of $0.019 \pm 0.004$ $mM^{-1}.s^{-1}$ (Baynton et al., 1994). In their study, it was also concluded that a competition was established between two catalytical pathways: the catalase Compound III forming pathway and the suicide inactivation pathway (formation of inactive enzyme).

## 2.5. Application of SBP in wastewater treatment

The use of SBP for environmental and industrial applications was first implemented by Johnson et al. (1992). They extracted SBP from soybean seed hulls and used it for the preparation of phenolic resins. For this study, SBP was used both as a slurry of hulls in water solution and as a partially purified enzyme and their efficiencies were compared.

Pokora and Johnson (1993) carried on the work with three different forms of SBP – slurry in water, on a solid support or directly in the ground form. In this study, SBP was used for

oxidizing organic and heavy metal contaminants in wastewater. In their study they compared the cost of treatment of waste water with SBP and HRP. It was found that rough estimation of costs for the treatment of 1 $m^3$ of wastewater containing 1 $mmol/dm^{-3}$ phenols with soybean seed-hulls was US $ 0.51 whereas the cost of treating the same with medium purity – industrial grade of HRP was US $ 57.60 (Ibrahim et al., 2001).

Wright and Nicell (1996) characterized a medium purity industrial grade SBP for the removal of aqueous solution of phenolic compounds. SBP was efficient between a pH range of 3 and 9, being most effective between pH 6 and 9 when used to treat phenolic compounds. SBP's capacity to retain its catalytical ability over a wide range of temperatures was found to be pH dependent. At room temperature (around 25 °C), it was very stable at neutral and alkaline conditions.

Peroxidases have wide variety of uses that range from indicators in the food processing industries to catalysts for the removal of phenolic products from wastewater (Thompson, 1987). Most of the peroxidases like HRP do not remain active under adverse conditions such as high temperature above 65 °C (Miland et al., 1996). This limits their applicability in the field of bioremediation of industrial wastewaters on a large scale. Other limitations include the cost of purification of the enzyme. SBP has an unusually high thermal stability, being active at 70 °C (McEldoon and. Dordick, 1996).

The availability and the low commercial value of soybean seed hulls along with the ease of extraction of SBP from the seed hulls make it an excellent alternative for the bioremediation of phenolic compounds. In their study, Flock et al. (1999) compared the efficiency of SBP seed hulls with that of purified SBP in removing phenol and 2-chlorophenol using hydrogen peroxide. Since in this study SBP was found to be readily extracted by soaking the seed-hulls in water or buffered solutions, they were directly used

in the reactor and proved more effective. This success was attributed to the possible capacity of the seed-hulls to absorb polymerized products that absorb the active enzyme and also the slow leeching of SBP from the hulls in the reactor minimizes inactivation of the enzyme from the free radicals. Moreover, it was found that removal of phenol with purified SBP was not much effective since the enzyme was subjected to inactivation by the free radicals, hydrogen peroxide or the polymerized products. Bassi et al. (2004) further studied the direct application of soybean seed-hull extracts for the bioremediation of phenolic wastes in their work. It was found that the activity of the crude enzyme was increased along with an increase in temperature. Hence, it can be concluded that SBP retains it catalytic ability at elevated temperatures whether it is in high purity or in the form of a crude extract (Biswas, 1999).

Taylor et al.(1996) provided a comparison of HRP and microbial peroxidase to SBP for treatment of phenols and reported that SBP was an effective alternative. This work was followed by a comparative cost analysis of phenols treated individually and separately by SBP, HRP and microbial peroxidase (Taylor et al., 1996, 1998).

Caza et al. (1999) showed the versatility of this enzyme by treating a wide spectrum of phenolic compounds (phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, o-cresol, m-cresol, p-cresol, 2,4-dichlorophenol and Bisphenol A) with medium purity, industrial grade SBP.

Kinsley and Nicell (2000) did further work in removing phenol with medium purity-industrial grade SBP. In this study, the relationship between the protective effect of PEG on SBP and different molecular weights of PEG were analyzed. It was found that the effectiveness of PEG increased with its molecular weight, with the minimum effectiveness at molecular weight of 1000 and below and the maximum at 35,000 g/mol.

Wilberg (2002) studied the efficiency of low purity SBP (LP-SBP) in catalyzing an aqueous solution of phenol. It was found that minimum LP-SBP concentrations for 95 % phenol removal were 1.7 times lower than that required by Kinsley and Nicell (2000) using medium purity SBP.

Kennedy et al. (2002) extensively optimized the conditions affecting the treatment of 2,4-dichlorophenol with soybean peroxidase. An interesting phenomenon was observed that in the absence of PEG at optimum pH, the removal efficiency of various concentrations of 2,4-DCP (50-500 mg/l) was found to be zero-order within the SBP range 0.001 to 0.1 U/mL.

Knutson et al. (2005) studied the enzymatic bio-bleaching of two recalcitrant paper dyes – stilbene dye and methine dye with horseradish peroxidase, soybean peroxidase and laccase with a mediator (ABTS). Both the peroxidases were more effective at chromophore removal compared to laccase, but SBP was found to be more effective especially for the methine dye. Moreover, SBP proved to be effective at bio-bleaching over a wide range of pH from pH 4.0 to pH 8.0 and did not require PEG to enhance its activity.

Patapas et al. (2007) did a comparative study on the efficiencies of *Arthromyces ramosus* peroxidase (ARP) and SBP in removing dinitrotoluenes via reduction with iron to diaminotoluenes (DAT) and then subsequent peroxidase-catalyzed oxidative polymerization. It was observed that SBP was less susceptible to inactivation by the lower pH values than ARP. The enzyme requirement for 95 % DAT removal was much lower in case of SBP compared to ARP. SBP did not exhibit a significant activity reduction in the presence of excess hydrogen peroxide, but was found to have a higher hydrogen peroxide demand compared to ARP. This stability towards hydrogen peroxide was attributed to

greater catalase activity of SBP than ARP. No PEG effect was found with either enzyme in treating DAT, suggesting that protectiveness of PEG is substrate-dependent.

Steevensz et al. (2007) studied the comparison of the oxidative coupling of aqueous phenol catalyzed by laccase SP504 and by soybean peroxidase (SBP) on synthetic and refinery wastewater samples. SBP was found to be robust to pH with a broad optimum range from 6.0-8.5 with synthetic samples. A pH of 7.5 was found to be optimum for all the refinery samples. Without pH adjustment, in the refinery samples the conversion of initial phenol by SBP was as good as or better than the buffered synthetic samples. With the pH of most refinery wastewaters being slightly alkaline, the above-mentioned characteristics of SBP can be advantageous for its industrial uses. In this study with SBP, it was observed that none of the reducing anions (sulfite and thiosulfate) presented showed significant inactivation directly on the phenol removal when adequate hydrogen peroxide was present. The crude SBP used in this study was able to achieve > 95% removal of phenolics with the same enzyme concentration in the presence of iodide and bromide but with a higher hydrogen peroxide to substrate requirement. Both laccase and SBP showed inactivation in the presence of cyanide, and even a large amount of $H_2O_2$ in case of SBP could not out-compete the cyanide for binding to the active site.

## 2.6. Kinetics of Enzyme catalyzed Reaction

### *2.6.1 Enzyme as a catalyst*

A catalyst accelerates a chemical reaction without changing its extent and can be removed unchanged from among the end products of the reaction. It has no thermodynamic effect on the reaction it is catalyzing i.e. the amount of free energy liberated or taken up when a reaction has been completed will be the same whether a catalyst is present or not.

A catalyst acts by reducing the energy of activation. The catalyst or part of it combines with the reactants to form a different transition state that is much more stable and of lower energy compared to that involved in the uncatalyzed reaction. Figure 2.5 shows the free energy changes, both in an uncatalyzed and catalyzed reaction (Palmer, 1995).

The simplest mechanism of catalysis can be accounted as follows. For a single substrate reaction, the substrate binds with the active site of the enzyme to form a relatively stable enzyme-substrate complex. The catalytic reaction eventually takes place with the formation of another unstable transition state complex from which the product is released (Eq 2.10).

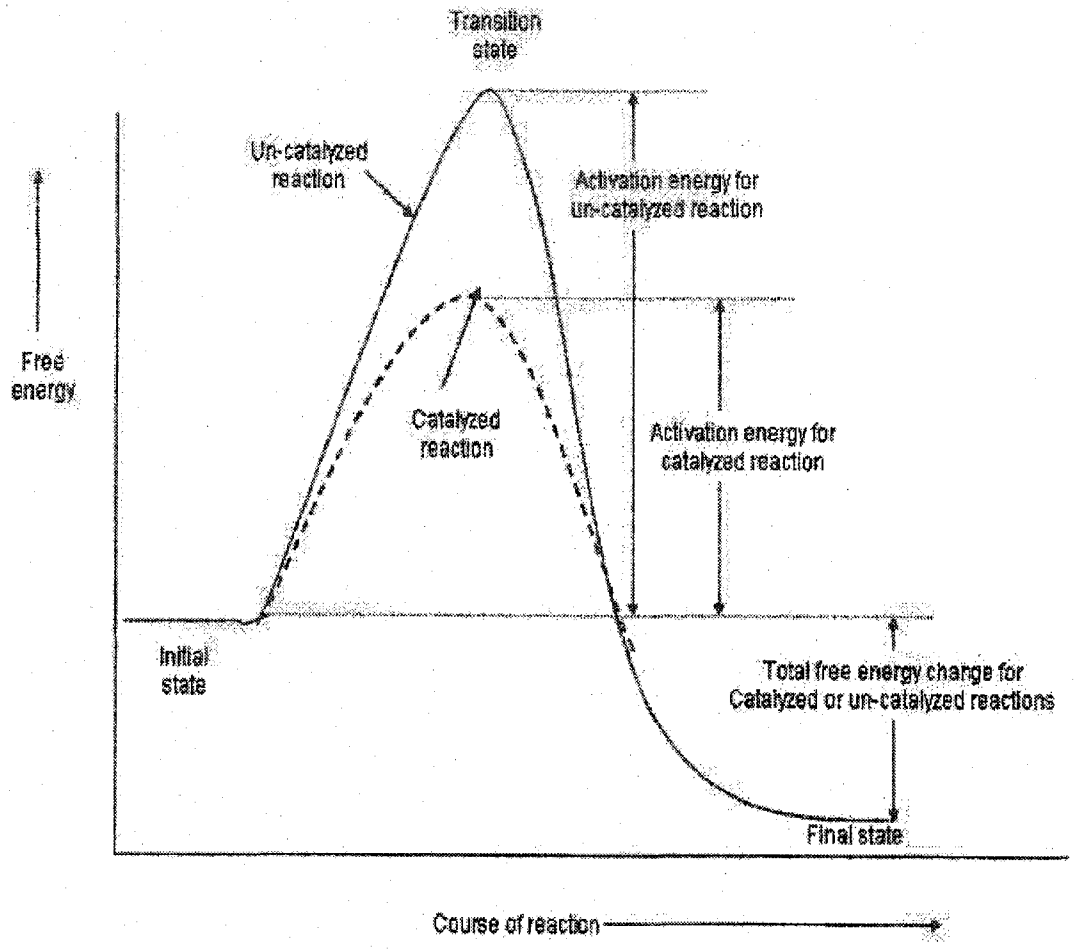



**Figure 2.5:** Free energy changes for an energetically favorable reaction, showing the effects of a catalyst.

More complicated enzyme catalyzed mechanisms involve additional transition states and/or reactive intermediates and or complexes as shown in Eq. 2.10 below .The free energy profile of this type is illustrated in Figure 2.6 (Palmer 1995).

For a reaction of this form

$$E + S \underset{}{\overset{k_1}{\rightarrow}} ES \overset{k_2}{\rightarrow} E + P \qquad (2.10)$$

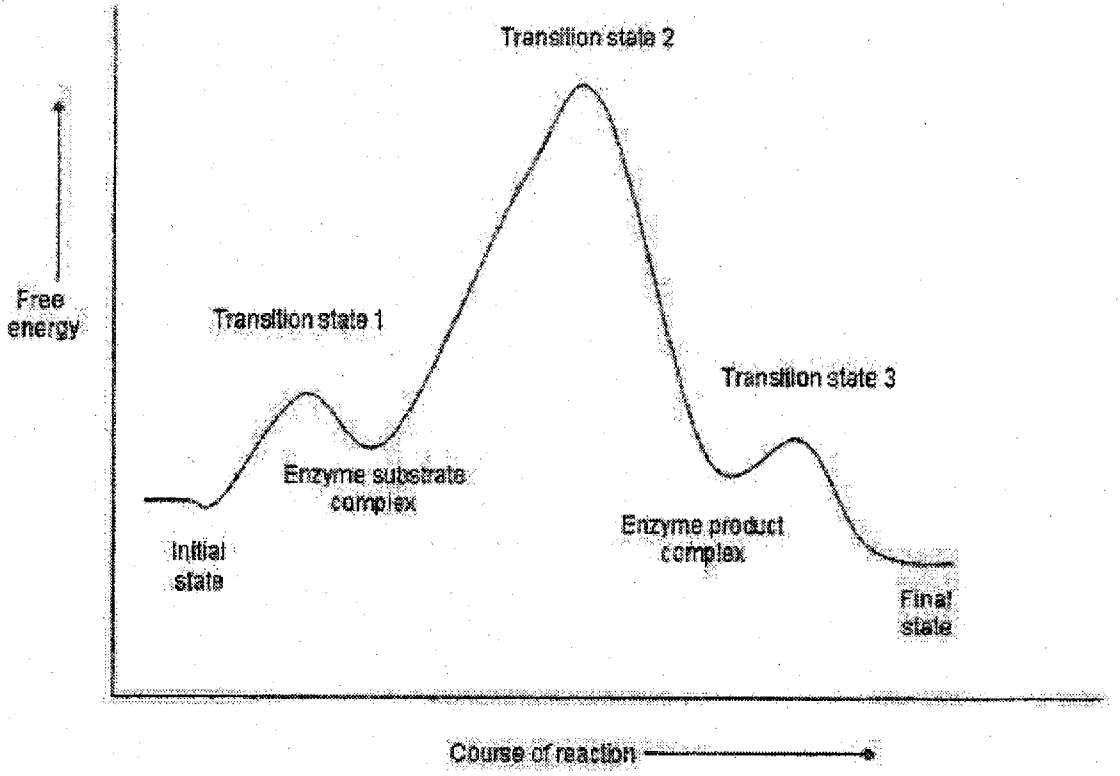



**Figure 2.6:** The Free Energy Profile of an Enzyme Catalyzed Reaction involving the formation of enzyme-substrate and enzyme-product complex (Palmer, 1995)

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad (2.11)$$

(Where ES → EP is the rate-limiting step)

The overall reaction rate is given by $k_2$ [ES] and the best estimation of $k_2$ comes from $V_{max}$, since $V_{max} = k_2 [E_0]$

Where, E = Enzyme

S = Substrate

P = Product

ES = Enzyme-Substrate Complex

EP = Enzyme-Product Complex

$V_{max}$ = Limiting rate of reaction

$k_1$ $k_2$ $k_3$ are reaction rate constants

### *2.6.2 Use of initial velocity to investigate the kinetics of enzyme catalyzed reaction*

For any reaction starting with reactants only, a graph as shown in Figure 2.7 can be plotted by measuring the disappearance of substrate or appearance of product with time.

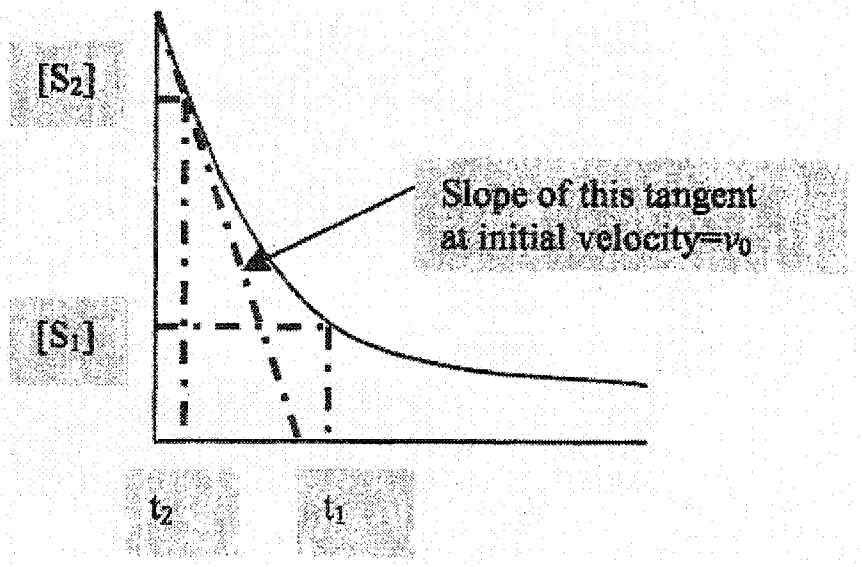



**Figure 2.7:** A typical progress curve for substrate concentration

The rate of reaction at any time t is the slope of the curve at that point. This may be constant for a little time at the start of the reaction and then decreases with the decreasing concentration of the reactant(s) as the reaction proceeds, finally falling to zero. Either at this point, all the reactants have been converted into products or more commonly, a chemical equilibrium has been setup with the rate of the forward reaction now being equal to the rate of the back reaction.

The initial velocity ($v_o$) of the reaction is the reaction rate at t = 0 and may be determined by drawing a tangent to the graph as shown in Figure 2.7. From the tangent,

$$v_o = \frac{[S_2] - [S_1]}{t_2 - t_1}$$

The importance of initial velocity is that it is a kinetic parameter determined for the reaction in a situation, which can be easily specified. At time, t = 0, the concentrations of each reactant are known from the amounts actually added which would not be true at any other point during the reaction. Moreover, enzymes are often unstable in solution, so the restriction of investigations to $v_o$ determinations give the best chance of avoiding errors caused by the loss of enzyme activity with time. Lastly, since there are no products present at t = 0, no back reaction will be taking place.

### *2.6.3 Enzyme Saturation*

The most distinguishable kinetic feature of the enzyme is that it shows saturation. Nearly all enzyme-catalyzed reactions with single substrate or multiple substrates where one substrate is kept constant show first-order dependence of rate on substrate concentration at lower substrate concentration and with the increase in substrate concentration, instead of increasing indefinitely, the rate of reaction

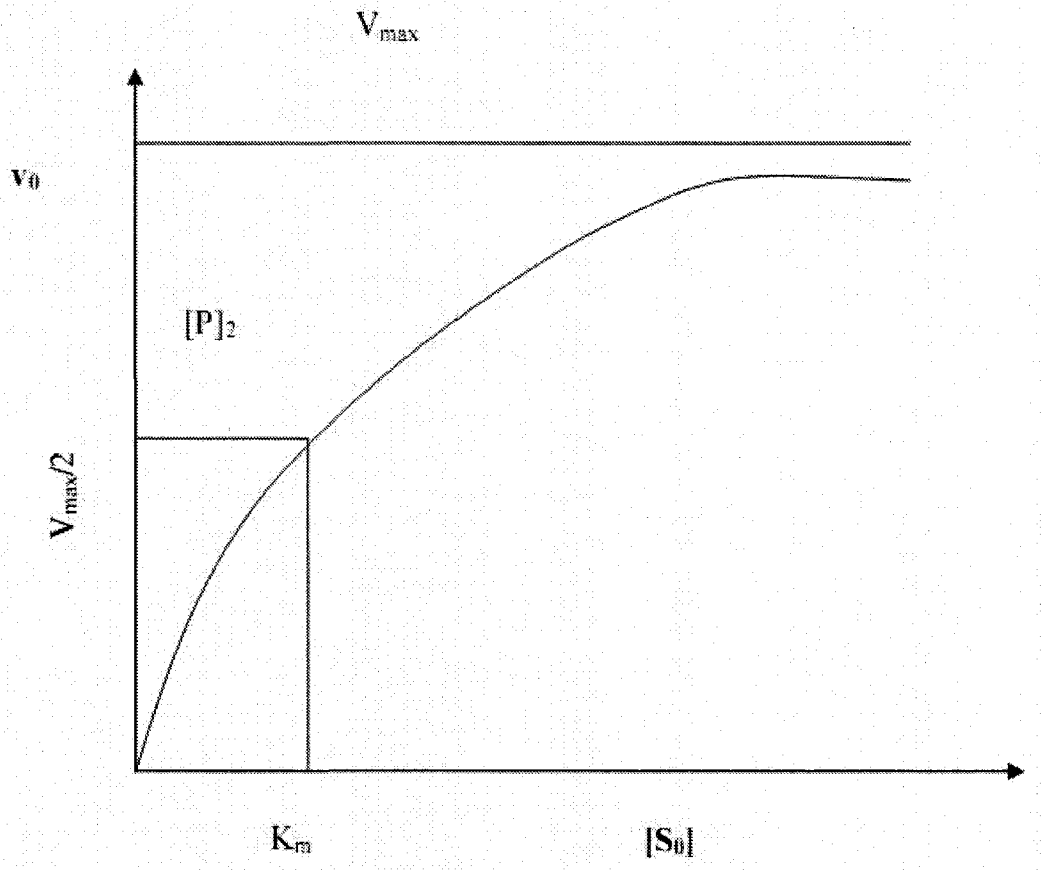



**Figure 2.8:** Initial velocity against initial substrate concentration at constant initial enzyme concentration for a single substrate enzyme catalyzed reaction

becomes zero-order with respect to substrate. In Figure 2.8 the initial rate denoted by $v_o$, is plotted against substrate concentration $[S_o]$. Initial rate $v_o$ has been shown to reach the limiting rate $V_{max}$ with significant increase in substrate concentration $[S_o]$. However, most of the times the curve does not reach the limit at any finite concentration of substrate and rather remains far from it at even higher concentration that can be realistically achieved (Cornish-Bowden, 1988).In those cases, a substrate concentration ranging from 0.2 $K_m$ to 2 $K_m$ is chosen and $V_{max}$ is statistically calculated.

### *2.6.4 Michaelis-Menten Equation for single substrate steady state kinetics*

The simplest equation for a single substrate enzyme catalyzed reaction where one substrate-binding site per enzyme exists, can be shown as below

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightarrow}} E + P \qquad (2.12)$$

Kinetic model to explain the relationship between $v_o$ and $V_{max}$ was proposed by Michaelis and Menten. In their model, it was assumed that equilibrium between enzyme, substrate and enzyme-substrate complex was instantly set up and maintained and the breakdown of this complex to products was too slow to disturb this equilibrium. Moreover, it was further assumed by Michaelis and Menten that the substrate was usually present in a much greater concentration than the enzyme.

Figure 2.6 showed that for many single-substrate and pseudo single-substrate enzyme catalyzed reactions, there exists a hyperbolic relationship between initial velocity, $v_o$ and initial substrate concentration $[S_o]$ so that

$$v_o = \frac{V_{max}\ [S_0]}{[S_0] + K_m} \qquad (2.13)$$

Where

$v_o$ = initial velocity

$V_{max}$ = limiting initial velocity at saturation

$K_m$ = Michaelis-Menten constant.

$[S_o]$ = initial substrate concentration

For enzyme reactions following Michaelis-Menten type equation, when $v_o = V_{max}/2$, the substrate concentration becomes numerically equal to $K_m$.

For substrate concentration, Km » [So],

$$v_o = \frac{V_{max}\ [S_0]}{[S_0] + K_m} = \frac{V_{max}\ [S_0]}{K_m} \quad \alpha \quad [S_0] \qquad (2.14)$$

and the reaction follows a first-order kinetics.

When the substrate concentration, [So] is equal to $K_m$ , the reaction is of mixed order and $v_o = V_{max}/2$. Finally, for higher substrate concentration [So] »Km., the reaction is independent of the substrate concentration and it follow zero-order kinetics. Then the equation becomes

$$v_o = \frac{V_{max}\ [S_0]}{[S_0] + K_m} = \frac{V_{max}\ [S_0]}{[S_0]} = V_{max} \qquad (2.15)$$

### *2.6.5 Two-substrate Enzyme catalyzed Reaction*

Most biochemical reactions involve more than one substrate. The general form of such a reaction would be a two substrate, two-product reaction that is also known as bi-bi reactions. For enzyme catalyzed two substrate reactions there are two kinds of mechanisms, one of them is described below:

#### 2.6.5.1 Ping-pong mechanism

In ping-pong mechanism or double displacement mechanism, the first product is produced and released from the enzyme before the second substrate is bound. The Michaelis-Menten equation takes the form:

$$v_o = \frac{V_{max}\ [A]\ [B]}{[A]\ [B] + K_{mB}\ [A] + K_{mA}[B]} \qquad (2.16)$$

Where,

$K_{mA}$, $K_{mB}$ = Michaelis -Menten constants, for substrates A and B respectively

$v_o$ = initial reaction rate

$V_{max}$ = limiting initial velocity or maximum velocity at saturation

When concentration of any one of the substrates A or B is kept constant, Eq. 2.16 becomes very similar to one substrate Michaelis-Menten equation:

$$v_o = \frac{V^{app}_{max}\ [S_0]}{[S_0]\ + K^{app}_m} \qquad (2.17)$$

$V^{app}_{max}$, $K^{app}_m$ are apparent values, as long as the concentration of one substrate remains constant, and can be obtained from Michaelis- Menten plots.

### *2.6.6 Enzyme Inhibition*

Substances that cause enzyme-catalyzed reactions to proceed more slowly when they are present in the reaction mixture are called inhibitors and the phenomenon is called inhibition. The inhibition of an enzyme may be reversible or irreversible. The classical reversible inhibitions are of three types: 1) Competitive Inhibition, 2) Uncompetitive Inhibition and 3) Mixed Inhibition.

### 2.6.6.1 Substrate Inhibition

A special case of uncompetitive inhibition is substrate inhibition, which occurs at high substrate concentrations in about 20% of all known enzymes (e.g. invertase is inhibited by sucrose) (London South Bank University). Uncompetitive inhibition is most commonly encountered in multi-substrate reactions where the inhibitor is competitive with respect to one substrate (e.g. $S_2$) but uncompetitive with respect to another (e.g. $S_1$) (Palmer, 1995) . The key feature of these uncompetitive inhibitors is they are incapable of binding to free enzyme. This occurs when the inhibitor binds to a site which only becomes available after the substrate ($S_1$) has bound to the active site of the enzyme i.e. the inhibitors can only bind to the enzyme-substrate complex (Palmer,1995).

Substrate inhibition is primarily caused by more than one substrate molecule binding to an active site meant for just one, often by different parts of the substrate molecules binding to different subsites within the substrate binding site (Department of Biological Sciences, University of Paisley). If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction, at high substrate concentrations. It may be modelled by the following scheme

$$E+S_1 \underset{}{\overset{k_1}{\rightleftharpoons}} ES_1 + S_2 \overset{k_2}{\rightleftharpoons} ES_1S_2 \overset{k_p}{\rightleftharpoons} \text{Product} \qquad (2.18)$$

$$E+S_1 \overset{k_1}{\rightleftharpoons} ES_1 + I \overset{k_I}{\rightleftharpoons} ES_1I \rightleftharpoons \text{No Product} \qquad (2.19)$$

For substrate inhibition, $I = S_2$

$$v_o = \frac{V^{app}_{max}\ [S_0]}{[S_0]\ + K^{app}_{m}} \tag{2.20}$$

$$v_o = \frac{V_{max}}{1 + [S_0]/K_I} \tag{2.21}$$

# CHAPTER 3

# MATERIALS AND METHODS

The experimental procedures and analytical techniques used in the study are presented in this chapter.

## 3.1 Materials

Soybean peroxidase (E.C. 1.11.7, Industrial Grade lot #18541NX) was obtained from Organic Technologies (Coshocton, OH). The enzyme was stored at -15 °C. A sub-stock solution was prepared from it and was stored at 4 °C. The specific activity of the aqueous soybean peroxidase (SBP) solution was determined using the Soybean peroxidase activity assay described later in this chapter.

2,4-dimethylphenol (2,4-DMP) was purchased from Aldrich Chemical Co. (Milwaukee,WI). It has a purity of 98% and contained cresols as impurities. The 2,4-DMP stock solution was stored at 4 °C.

Catalase (E.C. 1.11.1.6, lot # 120H706) from bovine liver was purchased from Sigma Chemical Company Inc. (St. Louis, MO).

Polyethylene Glycol (PEG) was purchased from Sigma Chemical Co.(St. Louis, MO). For this study, PEG having molecular mass of 3350 g/mol was used.

Hydrogen peroxide (30% w/v) was supplied by BDH Inc. (Toronto, ON).

Analytical grade monobasic and dibasic sodium phosphate was purchased from BDH, Toronto, ON. All other chemicals used for this study were of analytical grade and were purchased from Fisher Scientific, Fair Lawn, NJ and BDH, Toronto, ON.

Plastic syringes were purchased from Becton Dickinson & Co, Clifton, NJ. For coarse filtration, Whatman (No 42) filter paper was used. For microfiltration, 0.2 μm HT Tuffryn membrane filters from Gelman Labs (Mississauga, ON) were used.

## 3.2 Equipment

Absorbance was measured using a Hewlett Packard Diode Array Spectrophotometer (model 8452A) equipped with Vectra ES/12 computer (λ range of 190-820 nm and 2 nm resolution) A quartz cuvette with 10 mm path length was purchased from Hellma Limited, Concord, ON, to measure the absorbance.

For standardization of the chemicals, high performance liquid chromatography (HPLC) was carried out on a system obtained from Waters Co, Milford, MA. It had a model 2487 dual wavelength absorbance detector, model 1525 binary HPLC pump and model 717 auto-sampler. A Symmetry $C_{18}$ (5 μm, 4.6 X 150 mm) column was used for this study. The Waters System was operated by BREEZE software.

The statistical analysis of kinetic parameters was done using the software Sigma Plot 10.0.

The pH was measured by using an I.Q. Scientific Instruments Inc EA940 pH meter with a stainless steel micro pH probe (PH15-SS) obtained from London Scientific (London, ON).

## 3.3 Experimental Procedure

Batch reactors were set up to study the effect of pH, hydrogen peroxide [$H_2O_2$] concentration, enzyme concentration and PEG concentration over a fixed reaction period. Experiments were conducted at room temperature, approximately 22 °C. The main aim of the experimental design was to achieve at least 95% conversion of 2,4-DMP by optimizing pH, [$H_2O_2$ /substrate ] ratio, enzyme concentration and PEG concentration.

Batch reactors of volumes 20 ml or 100 ml were used for this study. They contained a buffered solution of 2,4-DMP,with or without PEG and required amount of enzyme. $H_2O_2$ was added to initiate the reaction and the batch reactors were kept open and were mixed vigorously using Teflon-coated stir bars and a magnetic stirrer.

After an appropriate reaction period, a known volume of reaction mixture from the batch reactor was quenched with sufficient amount of catalase (stock 0.5 mg/ml) to stop the reaction. Samples were filtered using 0.2 μm HT Tuffryn membrane filters and analyzed for residual 2,4-DMP by HPLC.

### *3.3.1 pH*

The first parameter, which was optimized, was pH. 20 ml batch reactors were used for all optimization experiments. The reaction mixture consisted of buffer, 2,4-DMP, SBP, and $H_2O_2$. Substrate concentration in all the batch reactors was maintained at 1 mM. pH was optimized under two different concentrations of SBP enzyme – 0.01 U/ml & 0.05 U/ml. Effect of pH was also monitored in the presence and absence of PEG. When present, PEG concentration in the batch reactor was 400 mg/l. pH optimization was done by varying the pH within a range of 4.0 to 9.0 using different buffer solutions. The reaction time in all cases was three hours.

### *3.3.2 Hydrogen Peroxide to substrate molar ratio*

The effect of [$H_2O_2$] to [substrate] molar ratio was studied at the optimal pH obtained in the previous experiments to achieve 95% 2,4-DMP conversion in a three hour reaction period. The ratio was varied between 0.5 to 2 at three different substrate concentrations of 1, 3& 5 mM. PEG was present at a concentration of 400 mg/L.

### *3.3.3 Soybean peroxidase Concentration*

The enzyme stock was prepared and stored at 4 °C. Whenever a new stock was prepared, the activity was measured using the SBP activity assay.

The effect of enzyme concentration was studied at the optimum pH and optimum [$H_2O_2$] to [substrate] molar ratio obtained in the previous experiments to achieve 95% 2,4-DMP conversion in three hour reaction period. A range of substrate concentrations, 0.2-8.0 mM, was tested both in the presence and in absence of PEG. SBP concentrations in the batch reactors varied from 0.08 U/mL to 0.6 U/mL as when required. A concentration of 400 mg/L of PEG was used to determine the minimum enzyme concentration in the presence of PEG.

### *3.3.4 PEG Concentration*

PEG concentration was optimized at the optimum pH, optimum [$H_2O_2$] to [substrate] molar ratio obtained in the previous experiments. The optimization was done under stressed enzyme conditions. A range of substrate concentrations, 1-5 mM was tested. Stock solution of PEG $_{3350}$ was prepared at 4g/L. PEG concentration in the batch reactors varied from 0-150 mg/L.

### *3.3.5 Kinetics of Enzymatic Reaction*

The initial rates of 2,4-DMP conversion were measured at various concentrations of 2,4-DMP ranging from 0.5 to 13 mM with an enzyme concentration of 0.1 U/mL at pH 8.0 in 100 mL open batch reactors at room temperature of 22 ± 2°C. Initial [$H_2O_2$] to [substrate] ratio was maintained at 1.2. The total reaction time for the kinetic study was 4 minutes. These experiments were conducted by taking 1 mL of the sample from the reaction mixture at predetermined time and halting the reaction by adding the sample to a high dose of catalase.

## 3.4 Analytical Techniques

### *3.4.1 Soybean peroxidase activity assay*

The soybean peroxidase activity colorimetric assay utilized the coupling of phenol to 4-AAP with hydrogen peroxide as the oxidant. This assay was run at pH 7.4. In the alkaline environment, 4-AAP performed an electrophilic attack on the para-position of phenolic compounds forming an intermediate compound, which was further oxidized by hydrogen peroxide to a pink color quinoneimine chromophore absorbing at 510 nm. This test determines the activity of peroxidase enzyme by monitoring the appearance of color in the sample.

$$\text{AAP} + \text{phenol} + H_2O_2 \longrightarrow \text{Pink chromophore}$$

(Wavelength max 510 nm)

The assay mixture consists of 10 mM phenol, 2.4 mM 4-AAP and 0.2 mM hydrogen peroxide in 50 mM phosphate buffer of pH 7.4. 950 µl of this reagent and 50 µl of dilute

enzyme were mixed for a final volume of 1.0 ml and monitored at 510 nm, 25°C. The rate is measured by calculating the change in absorbance over change in time (the slope of the line produced), which is proportional to the rate of hydrogen peroxide consumption. This in turn is used to calculate activity by taking into account the dilution of the sample and the extinction coefficient (molar absorptivity) of 6000 $M^{-1}$ $cm^{-1}$. Enzyme activity was determined in units of catalytic activity per milliliter (U $ml^{-1}$), with 1.0 U equal to the amount of enzyme required to convert 1.0µmol of hydrogen peroxide/minute (Ibrahim et al. 2001). The enzyme activity is calculated as follows

- The rate of change in absorbance between 0 and 20 s is calculated:

$$Rate = \frac{\Delta A}{\Delta t}$$

- Activity in the cuvette (U/ml) $= \frac{\Delta A \times 1.0mL}{(6.0mM^{-1}cm^{-1})(1.0cm)}$ x D

  Where,

  $\Delta A$ = change in absorbance per minute

  1.0 = total volume in the cuvette (mL)

  6.0 = milli molar extinction coefficient ($mM^{-1}cm^{-1}$)

  D = dilution factor

### *3.4.2 2,4-DMP concentration assay*

HPLC was used to measure the concentration of 2,4-DMP. The lowest concentration of 2,4-DMP detected by HPLC is 0.02 mM. Peak areas were measured for different concentrations of 2,4-DMP, 0.2-15 mM at 278 nm (60:40 = acetonitrile: 0.1 % acetic acid

was used for elution). A standard curve was developed from the data obtained and was used to measure the concentrations of 2,4-DMP in sample solution.

### *3.4.3 Buffer preparation*

Buffers were prepared according to Gomori (1955). The pH values ranged from 4.0-9.0 for this study. Acetic acid and sodium acetate buffers were used for the pH range from 3.0-5.5. Monobasic and dibasic sodium phosphate buffers were used for pH 5.6-8.0. For higher pH, (8.2-9.0) bicarbonate buffers were used.

# CHAPTER 4

# RESULTS AND DISCUSSION

The conversion of 2,4-dimethylphenol from solution catalyzed by the enzyme soybean peroxidase has been studied. The enzymatic reaction is dependent on various parameters such pH, hydrogen peroxide concentration, enzyme concentration, PEG concentration and reaction kinetics. Hence, it was necessary to optimize the important parameters that could affect the enzymatic treatment process. The reaction parameters optimized were pH, hydrogen peroxide concentration and enzyme concentration. The experiments were designed to achieve a conversion of at least 95 % of the initial 2,4-DMP in the solution. The protective effect of PEG on the enzyme was also studied for 2,4-DMP conversion.

In addition, experiments to understand the mechanism and kinetics of soybean peroxidase were done. Michaelis-Menten constants for 2,4-DMP in the presence and absence of PEG were obtained.

## 4.1 Effect of pH

The catalytic activity of an enzyme during a reaction can be highly dependent on the ionization state of some of its amino acid residues conserved for catalytic efficiency. The characteristics of these ionisable side chains in turn are dependent upon pH of the surroundings in which they are reacting. This means enzyme activity is usually pH-dependent. Besides, at extremes of pH, the structure of the enzyme protein may be disrupted and the protein itself denatured (Palmer, 1995). Hence, the effect of pH on

enzyme activity should be studied to find the pH range over which the enzyme functions at its best with respect to specificity constant ($V_{max}/K_m$). It is so because specificity constant takes into account both the $V_{max}$, which represents the turnover number of the enzyme, and $K_m$, which represents the affinity of the enzyme for the substrate.

Wright and Nicell (1999) studied the impact of pH on SBP activity by varying the pH of enzyme-activity assay buffer. They found that SBP was active over a wide range of reaction pH with a maximum activity at pH 6.4 with > 90% of the maximum activity observed between pH 5.7 and 7.0 and > 10% between pH 3.0 and 9.0. Wright and Nicell (1999) also tested the stability of SBP by incubating it at 25 °C as a function of pH and time. They found that in acidic buffer, SBP undergoes time dependent inactivation whose rate and extent depend on the pH of the incubation mixture. For example at pH 2 and 3, SBP undergoes first-order inactivation, which leads to zero activity within two and four days respectively. Inactivation at pH 5.0 was found to be biphasic; SBP incubated in either neutral or alkaline buffers were found to be comparatively stable for over 20 days of incubation.

pH optimization for the enzyme-catalyzed conversion of 2,4-DMP was determined by varying the pH within a range of 4.0-9.0. Different buffers were prepared to obtain each pH as required. Acetate buffers were used for the pH range of 4.0-5.5, phosphate buffers were used for pH 6.0-8.0 and bicarbonate buffers were used for the pH range of 8.0-9.0. Initial substrate concentration for all the experiments was 1 mM. pH was optimized both in the presence and absence of PEG under stress/stringent enzyme condition, in which the low amount of enzyme present limited the reaction to substantially below full conversion so as to clearly see the effect of pH. PEG when present was in excess at a concentration of 400 mg/L. To determine the effect of enzyme concentration, pH optimization was also

done by varying enzyme concentration from 0.01 U/mL to 0.05 U/mL in presence of PEG. In each batch reactor, the initial substrate and enzyme concentrations were kept constant to ensure that the conversion of 2,4-DMP in each case was dependent on pH. The reaction time was kept at three hours.

The results are shown in Figure 4.1 and 4.2. Figure 4.1 shows an alkaline pH of 8.0 to be the optimum. Other studies with SBP found similar optimum pH ranges with phenolic compounds (Caza et al., 1998; Wright and Nicell, 1999; Kennedy et al., 2002; Steevensz et al., 2007). Caza et al., 1998 found that SBP was robust to pH with an optimum pH range from 6.0-8.0 for synthetic samples containing a range of phenolic compounds. Steevensz et al., 2007 similarly also found a global pH optimum of 7.5 for the treatment of refinery samples containing phenol with SBP. The catalytic mechanism of plant peroxidases is dependent on two key residues, which include the distal histidine-42 as the proton acceptor from hydrogen peroxide and the distal arginine-38 as a charge stabilizer (Dunford 1999). It is thought that SBP should work at pH values below six due to the abnormally low $pK_a$ of the distal histidine, which is 3.2 (Nissum et al. 2001). This was observed in a previous study, which compared the conversion of 2,4- and 2,6-diaminotoluene (DAT) by using both ARP and the SBP. As expected, due to the lower $pK_a$ of the distal histidine, SBP was less susceptible to inactivation by the lower pH values than ARP and also an optimum pH of 5.2 was observed for both the isomers in case of SBP (Patapas et al. 2007). This was not observed in this study and the only explanation for the higher pH optima that can be made is perhaps due to the change in $pK_a$ of the distal histidine due to the unique microenvironment of the active site.

Previous work of Kennedy et al., 2002 showed that the presence of PEG shifted the optimum pH from 8.2 to 6.2 when 2,4-dichlorophenol was treated with SBP. The same

was not observed here in this study. Figure 4.2 shows that PEG did have effect on conversion efficiency of 2,4-DMP compared to that in absence of PEG (Figure 4.1) but did not change the optimum pH, though the optimum range became broader, from 7.5 to 8.6. It can also be seen that different enzyme concentrations did not change the optimum pH. Wright and Nicell (1999) did not observe the same. In their study, it was found that lower SBP concentration shifted the optimal pH of phenol conversion reaction from 6.0 to 9.0. No possible reason could be given for this shift in optimum pH.

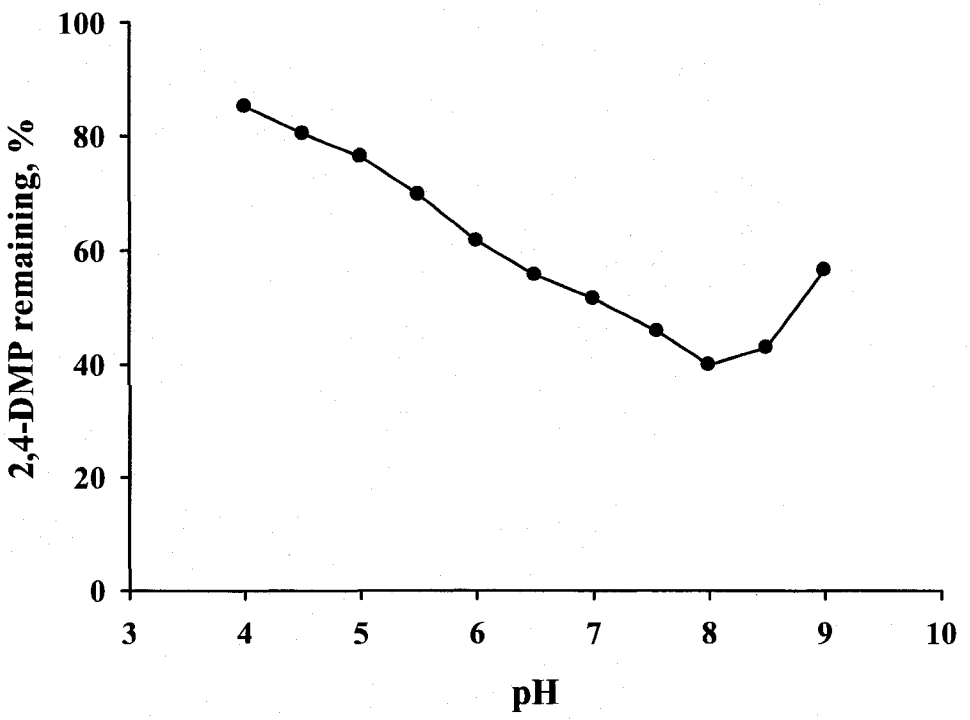



**Figure 4.1:** Effect of pH on 2,4-DMP conversion in the absence of PEG

Conditions: 1 mM 2,4-DMP in batch reactor, buffered with 50 mM acetate buffer for the pH range of 4.0 to 5.5; 50 mM phosphate buffer for the pH range of 6.0 to 8.0 and 50 mM bicarbonate buffer for pH > 8.0, after 3 hour reaction with 0.05 U/mL soybean peroxidase.

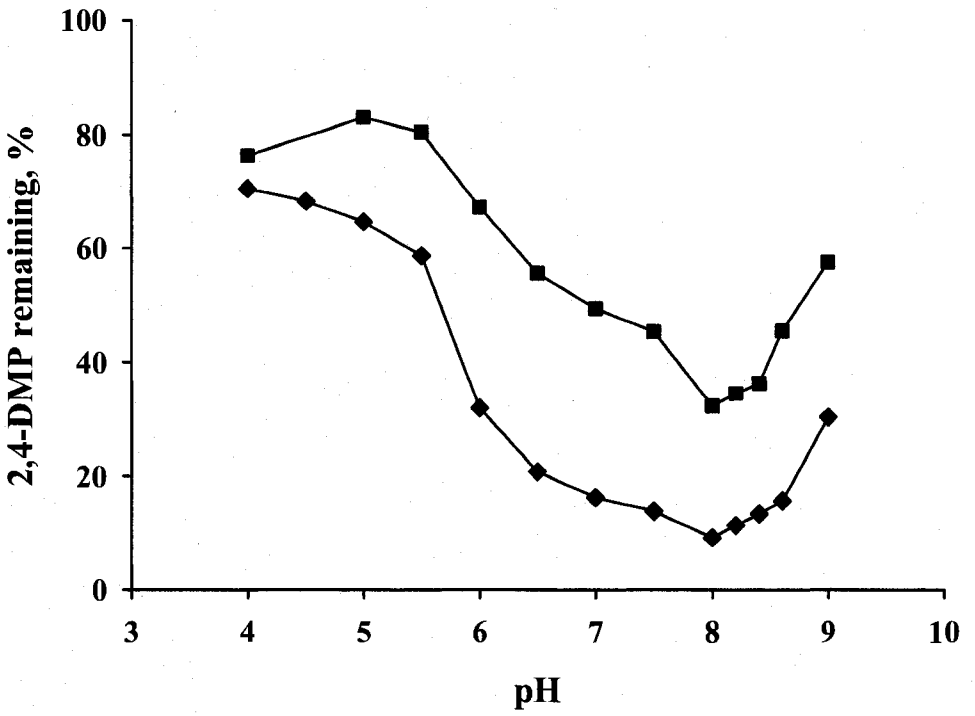



**Figure 4.2:** Effect of pH on 2,4-DMP conversion as a function of Soybean Peroxidase activity in the presence of PEG

Conditions: 1 mM 2,4-DMP in batch reactor, buffered with 50 mM acetate buffer for the pH range of 4.0 to 5.5; 50 mM phosphate buffer for the pH range of 6.0 to 8.0 and 50 mM bicarbonate buffer for pH > 8.0, after 3 hour reaction with 0.01 or 0.05 U/mL Soybean Peroxidase.

## 4.2 Effect of Hydrogen Peroxide Concentration

Stoichiometrically, for every mole of hydrogen peroxide consumed, 2 moles of 2,4-dimethylphenol are converted, provided the resulting dimer is insoluble in water (Dunford, 1999).

$$H_2O_2 + 2\,AH_2 \rightarrow 2\,AH^{\bullet} + 2\,H_2O \qquad (4.1)$$

However, Yu et al., 1994 reported that many of the soluble dimmers (p,p'-biphenol, o,o'-biphenol, o,p'-biphenol, p-phenoxyphenol and o- phenoxyphenol) formed during phenol oxidation, were much better substrates of the enzyme than phenol itself and their conversion to higher polymers will require more hydrogen peroxide. Hence, this increases the optimal [$H_2O_2$] to [substrate] molar ratio above the stoichiometric ratio. In the limit of an infinite polymer, the expected stoichiometry would be 1 mole of hydrogen peroxide per mole of phenolic group. In addition, peroxidases exhibit catalase activity, which is the reduction of hydrogen peroxide to water outside of the normal enzymatic cycle (Dunford, 1999). This too can add to the hydrogen peroxide demand, but is dependent on enzyme concentration in solution.

Experiments were performed to determine the effects of the initial molar ratio between hydrogen peroxide ($H_2O_2$) and 2,4-DMP on the conversion efficiency of DMP at the optimal pH. This effect was studied at 1, 3 and 5 mM 2,4-DMP concentrations. The [$H_2O_2$] to [substrate] molar ratio was varied from 0.5 to 2 in the presence of limiting enzyme concentrations but sufficient PEG in order to determine the minimum ratio required to achieve 95% conversion . The reactions were given sufficient time of 3 hours (Wu, 1997) to go to completion. The results of these experiments are shown in Figures 4.3 to 4.5 for different substrate concentrations.

Figure 4.3, represents effect of $[H_2O_2]$/[substrate] on conversion efficiency of 1 mM 2,4-DMP. It can be seen, that the optimum range lies between 0.9 -1.2. Beyond ratio 1.2, the residual concentration of 2,4-DMP increased slowly with an increase in ratio. Catalase activity has been reported as the main pathway by which enzymes protect themselves from peroxide inactivation (Hiner et al., 2002). Greater catalase activity of SBP compared to other enzymes could be the reason for its significant stability in presence of excess hydrogen peroxide (Patapas et al., 2007).

With 3 mM (Figure 4.4) and 5 mM (Figure 4.5) 2,4-DMP concentrations the optimum range remained the same, but rapid inactivation of the enzyme was observed with increase in ratio beyond 1.2. No reason is advanced to account for this phenomenon.

The optimum $[H_2O_2]$ to [substrate] molar ratio for most phenolic compounds treated with SBP lies in the range of 0.6-1.2 and they also showed a definite optimum point unlike that found here (Caza et al., 1999). However, the presence of broad optimum range matched the findings of Wu et al., 1997 with HRP. Controlling the amount of hydrogen peroxide is very important for optimal operating conditions. Since hydrogen peroxide is required to convert the enzyme to Compound I, an insufficient concentrations of peroxide can hinder the overall activity of the enzyme (Dunford, 1999). Again, an excess of hydrogen peroxide will reduce activity by converting the enzyme to the catalytically slow Compound III (Nicell and Wright, 1997; Dunford 1999). Both the trends can be observed in Figures 4.3 to 4.5 as the amount of residual 2,4-DMP increased below and above the optimal $[H_2O_2]$ to [substrate] molar ratio.

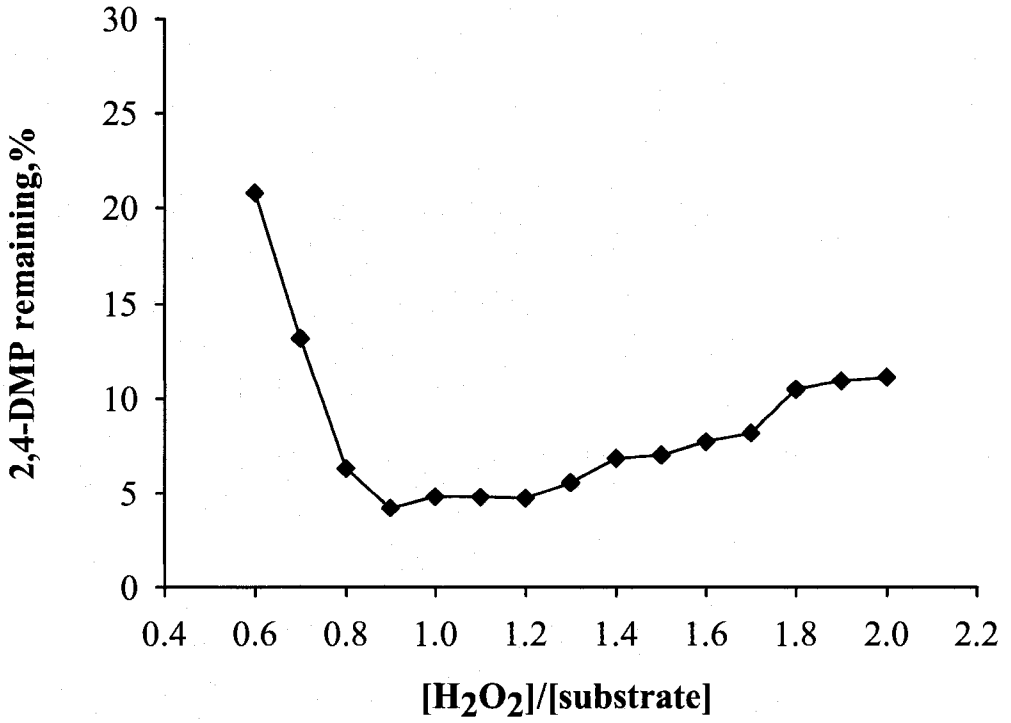



**Figure 4.3:** Effect of $[H_2O_2]$/ [substrate] on 1 mM 2,4-DMP conversion in the presence of PEG

Conditions: 1 mM 2,4-DMP treated with 0.05 U/mL Soybean Peroxidase in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, after 3 hour reaction time, and PEG at 400 mg/L.

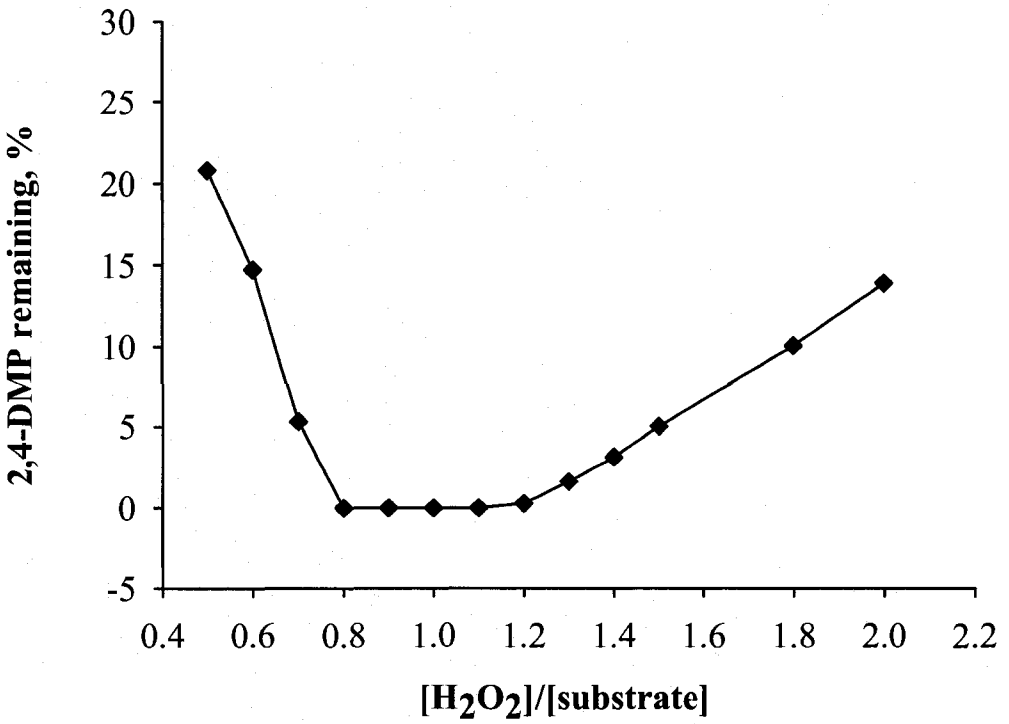



**Figure 4.4:** Effect of $[H_2O_2]$/ [substrate] on 3 mM 2,4-DMP conversion in the presence of PEG

Conditions: 3 mM 2,4-DMP treated with 0.09 U/mL Soybean Peroxidase in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, after 3 hour reaction time, and, PEG at 400 mg/L.

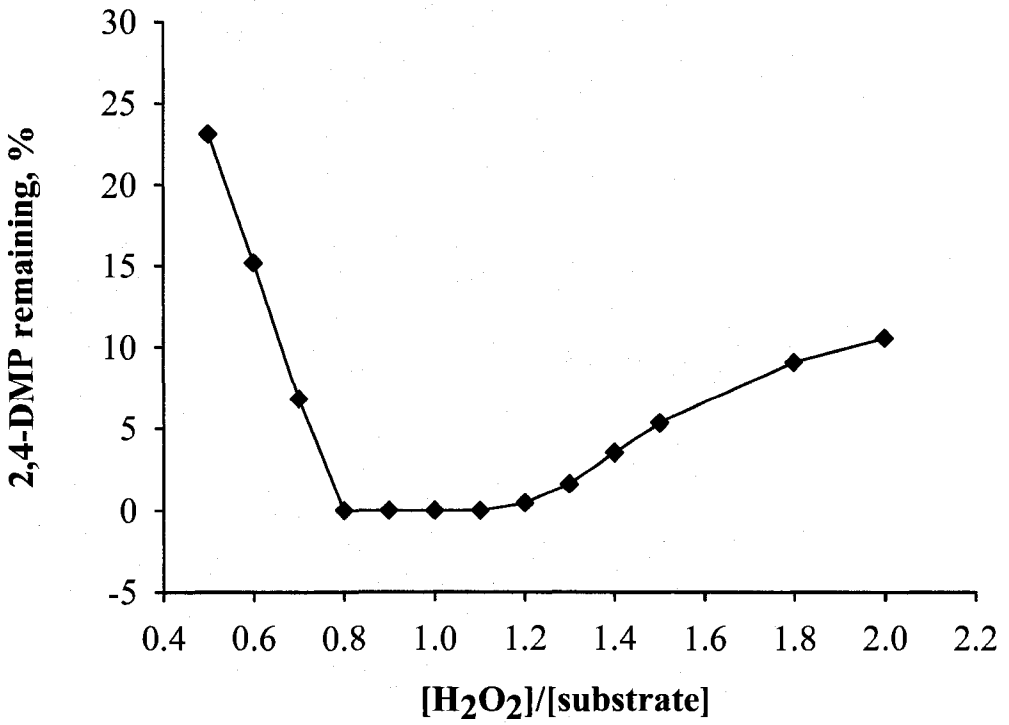



**Figure 4.5:** Effect of $[H_2O_2]$/ [substrate] on 5 mM 2,4-DMP conversion in the presence of PEG

Conditions: 5 mM 2,4-DMP treated with 0.15 U/mL Soybean Peroxidase in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, after 3 hour reaction time, and , PEG at 400 mg/L when present.

## 4.3 Effect of Enzyme (SBP) Concentration

One of the major challenges associated with peroxidase catalyzed phenol conversion is the prohibitive cost of the enzyme (Ibrahim et al., 2001; Cooper and Nicell, 1996) as explained before. Hence, optimization or rather minimization of the enzyme concentration is necessary for the enzymatic treatment process to be economically viable.

### *4.3.1 In the absence of PEG*

Minimum enzyme concentration required for 2,4-DMP conversion was determined at the optimum pH and optimum [$H_2O_2$] to [substrate] molar ratio found during the previous experiments. A range of substrate concentrations, 0.2-8.0, mM was examined to determine the minimum enzyme concentration required to achieve 95 % substrate conversion. A three hour reaction period was chosen for this study. SBP concentration was varied from 0.01 U/mL to 1.2 U/mL as required in each case.

Figures 4.6 to 4.15 represent the effect of enzyme concentration on 2,4-DMP conversion over a range of substrate concentrations, 0.2-8.0 mM. The enzyme concentration that yields 95 % or more 2,4-DMP conversion was chosen as the desired minimum enzyme concentration. Table 4.1 summarizes the minimum enzyme requirement for the reaction conditions considered.

Figure 4.16, a linear plot between the initial 2,4-DMP concentration and the minimum enzyme concentration required for 95% conversion shows, that minimum enzyme concentration increased with an increase in substrate concentration over the range of 0.2-2 mM. As expected a linear relationship exists between the enzyme concentrations and initial substrate concentrations treated. This result matches with results found in previous

studies: Ibrahim et al. (2001) with phenol and ARP; Ghioureliotis (1997) with phenol and SBP; Modaressi et al. (2005) with Bisphenol A and laccase.

Beyond 2 mM substrate concentration, the minimum enzyme concentration required remained the same with increase in substrate concentration as shown in Figure 4.17. Moreover, in Figures 4.12-4.15, it can be seen that for each specific enzyme concentration, approximately the same amount of 2,4-DMP was removed no matter what the initial 2,4-DMP concentration was. Such similar trends were obtained by Kennedy et al. (2002) in their study of treatment of 2,4-dichlorophenol with SBP. It was concluded that at these substrate concentrations, the SBP reaction reaches zero-order and becomes independent of substrate concentration. The possible reason would be that these substrate concentrations tested are well above the half-saturation concentration ($K_m$) of SBP (Kennedy et al., 2002). Because of this, an attempt has been made later to investigate the kinetic parameters of this particular reaction.

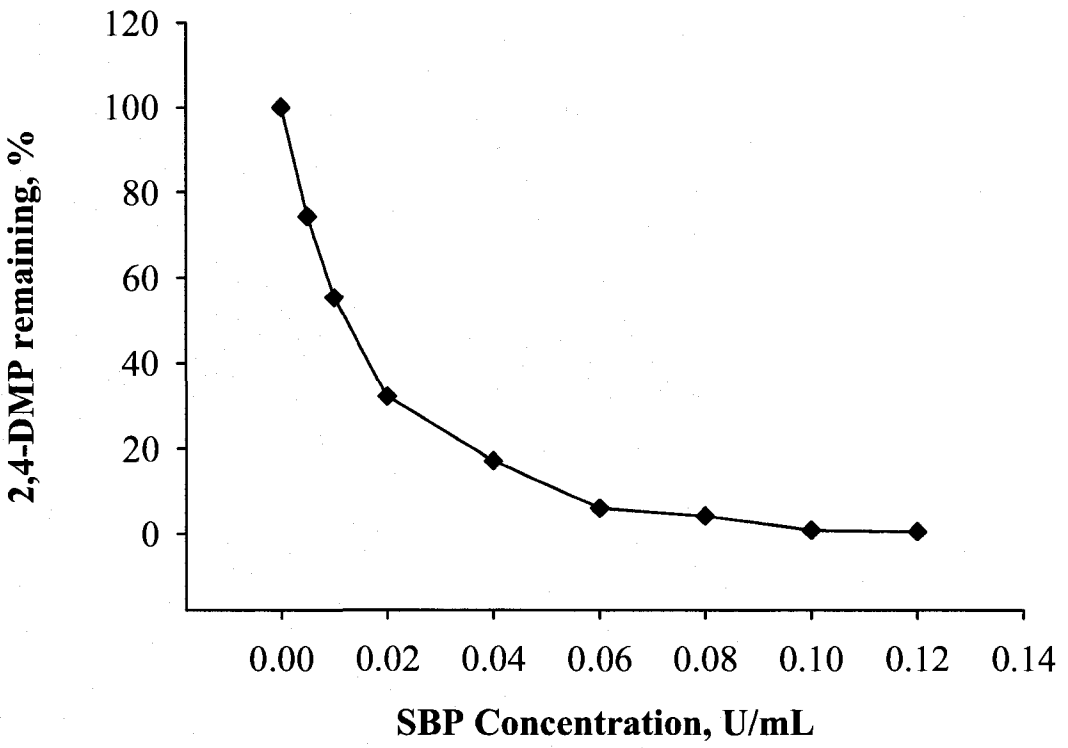



**Figure 4.6:** Effect of enzyme concentration on conversion of 0.2 mM 2,4-DMP in the absence of PEG

Conditions: 0.2 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time.

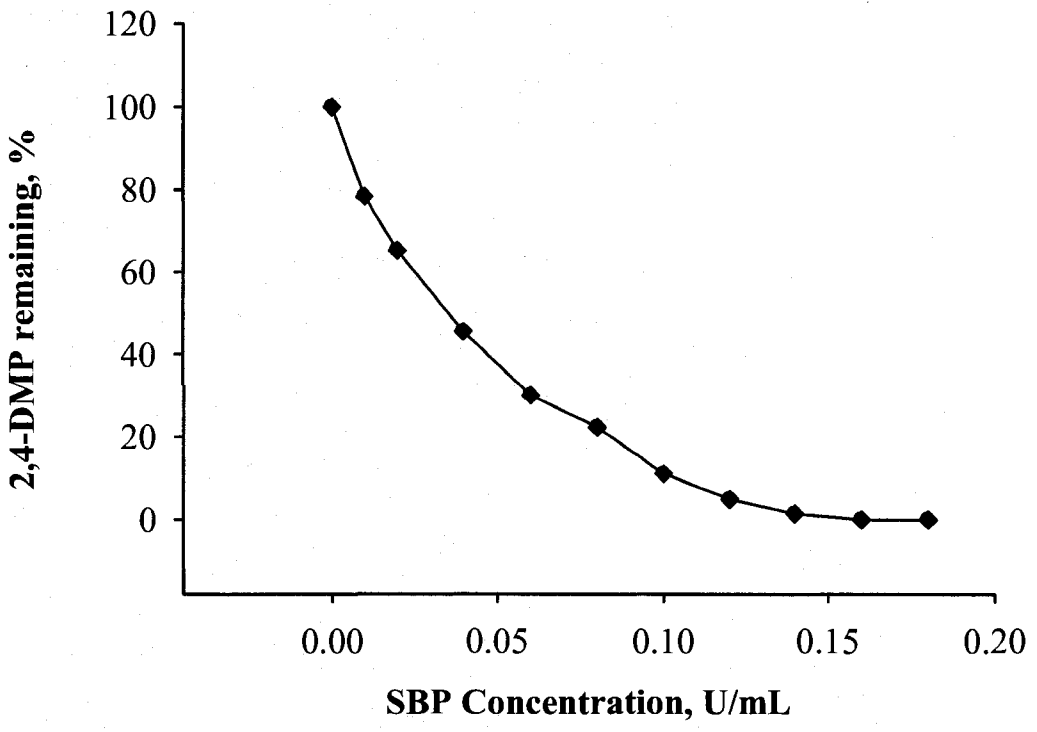



**Figure 4.7:** Effect of enzyme concentration on conversion of 0.4 mM 2,4-DMP in the absence of PEG

Conditions: 0.4 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time.

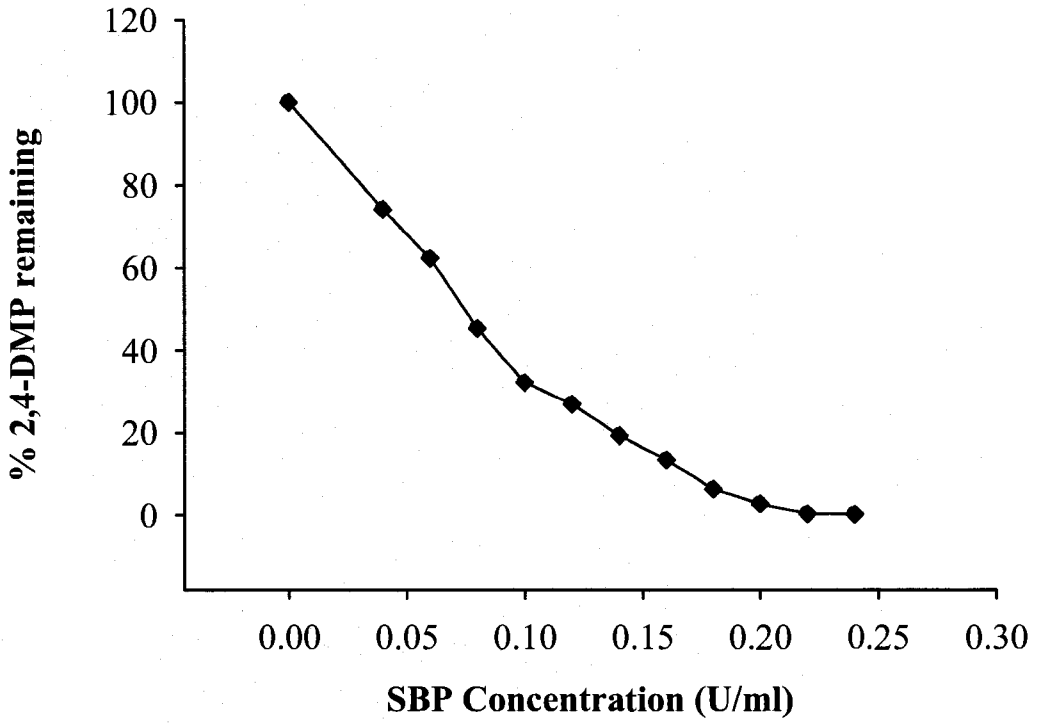



**Figure 4.8:** Effect of enzyme concentration on conversion of 0.6 mM 2,4-DMP in the absence of PEG

Conditions: 0.6 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time.

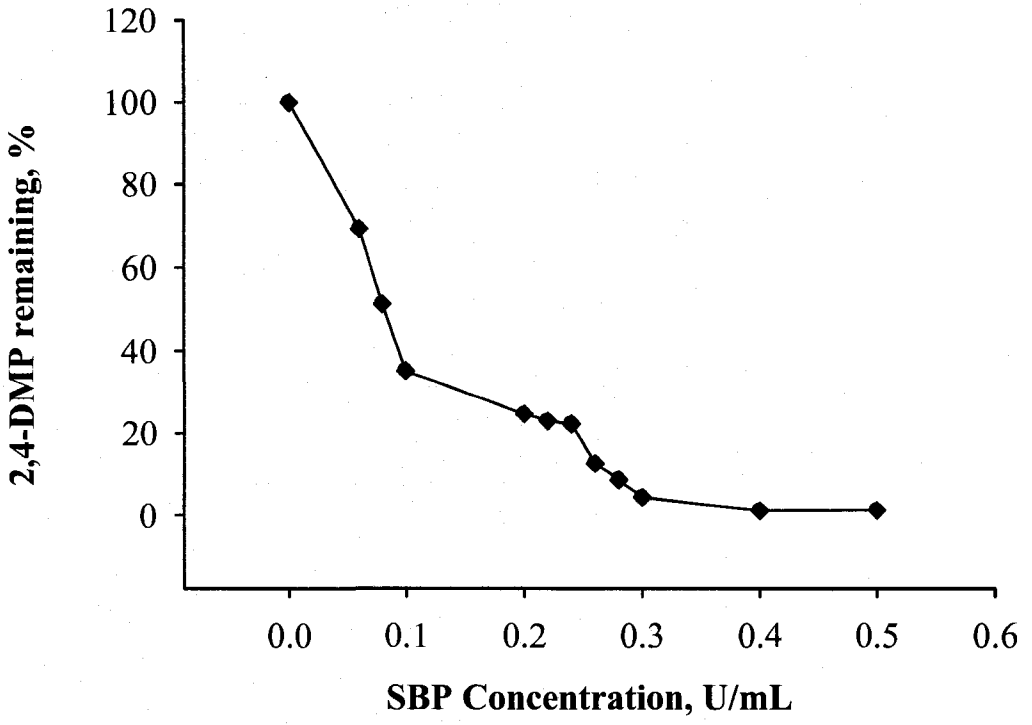



**Figure 4.9:** Effect of enzyme concentration on conversion of 1 mM 2,4-DMP in the absence of PEG

Conditions: 1 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time

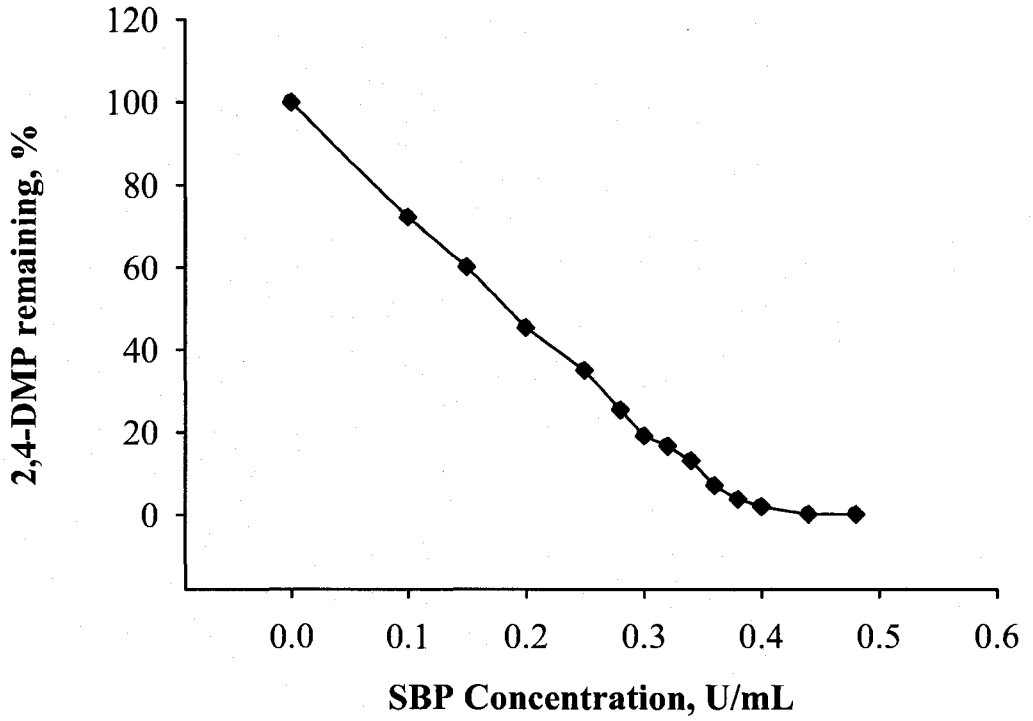



**Figure 4.10:** Effect of enzyme concentration on conversion of 1.5 mM 2,4-DMP in the absence of PEG

Conditions: 1.5 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time

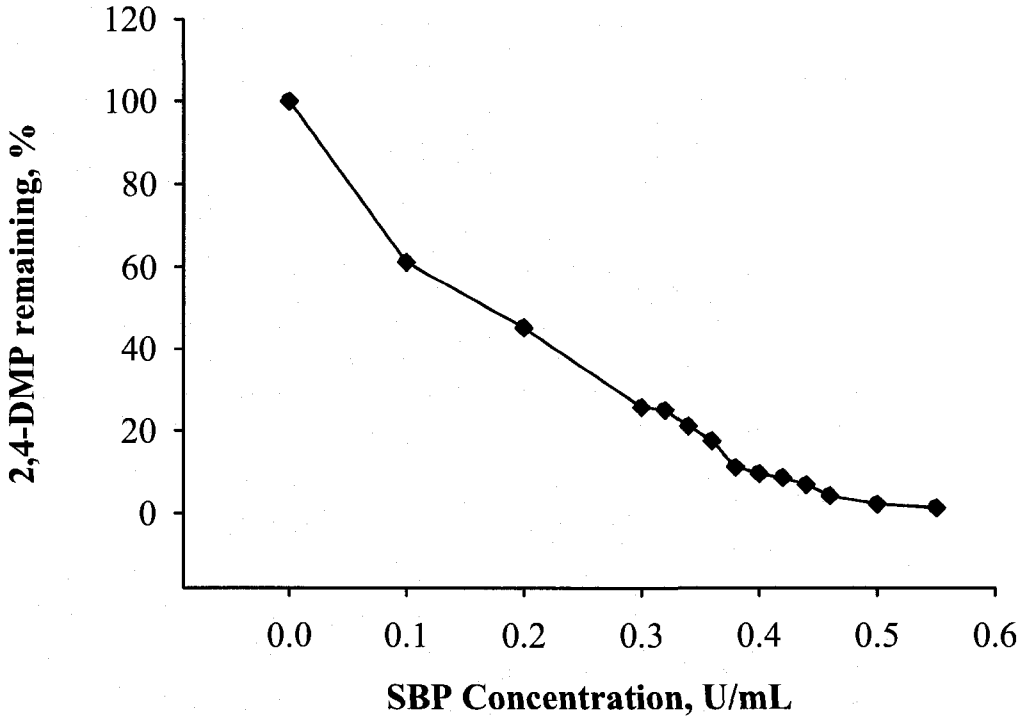



**Figure 4.11:** Effect of enzyme concentration on conversion of 2 mM 2,4-DMP in the absence of PEG

Conditions: 2 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time

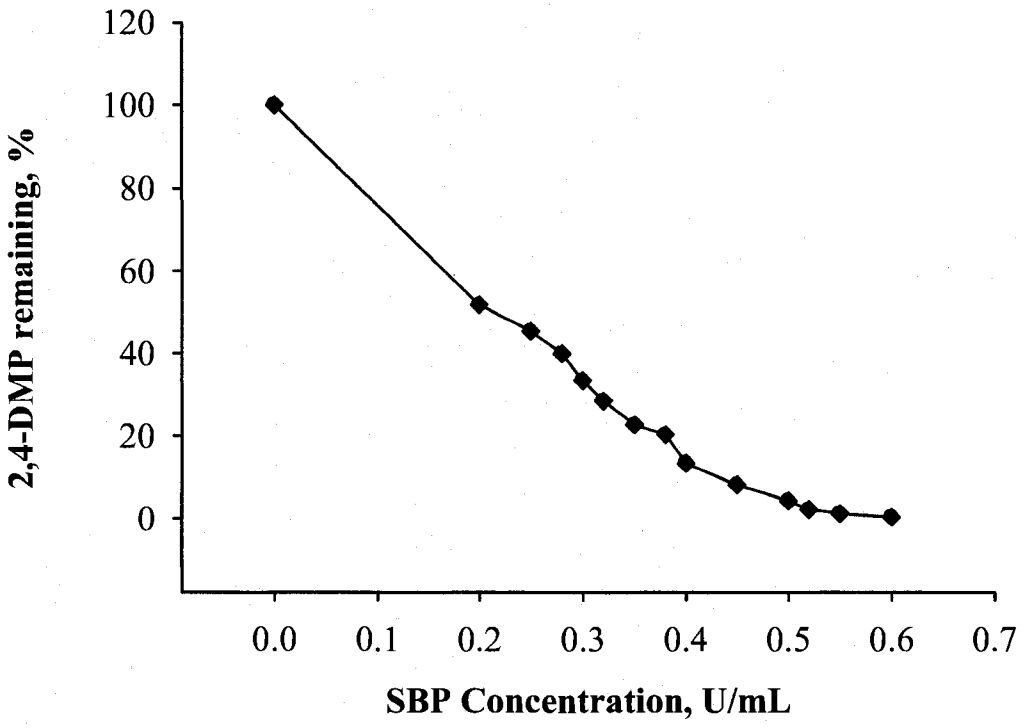



**Figure 4.12:** Effect of enzyme concentration on conversion of 3 mM 2,4-DMP in the absence of PEG

Conditions: 3 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time

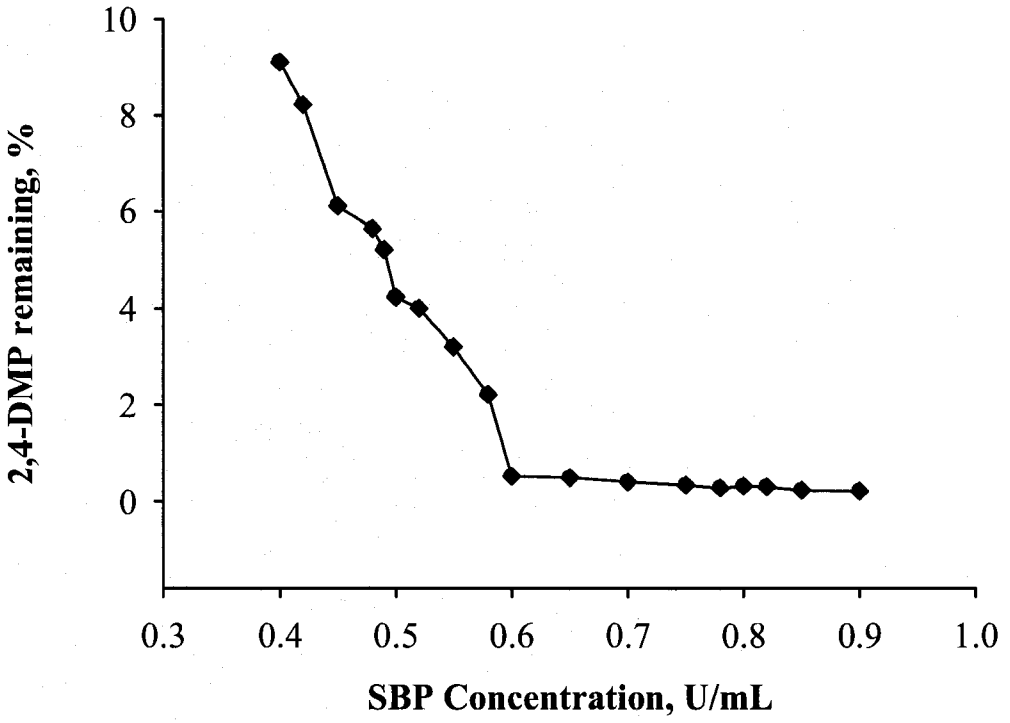



**Figure 4.13:** Effect of enzyme concentration on conversion of 5 mM 2,4-DMP in the absence of PEG

Conditions: 5 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 3 hour reaction time

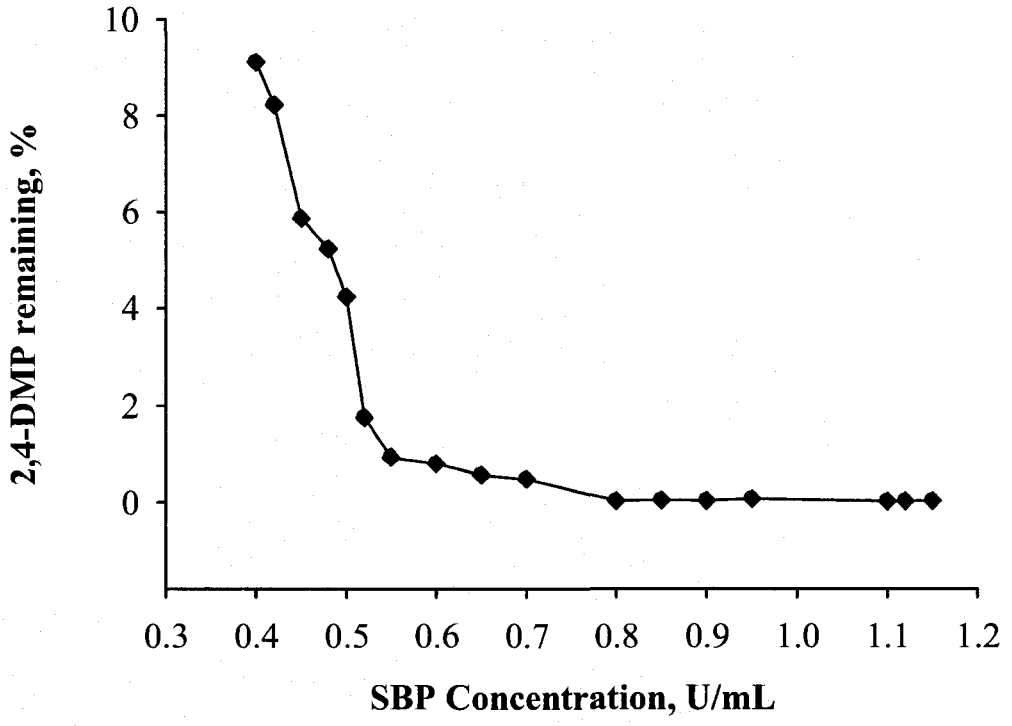



**Figure 4.14:** Effect of enzyme concentration on conversion of 7 mM 2,4-DMP in the absence of PEG

Conditions: 7 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time

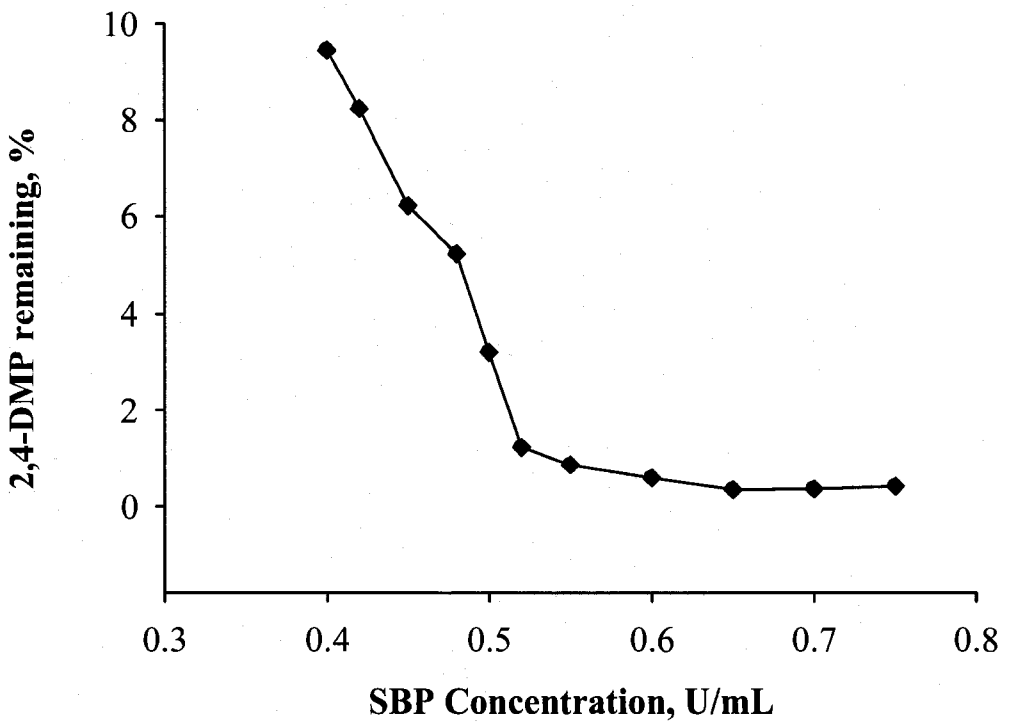



**Figure 4.15:** Effect of enzyme concentration on conversion of 8 mM 2,4-DMP in the absence of PEG

Conditions: 8 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time

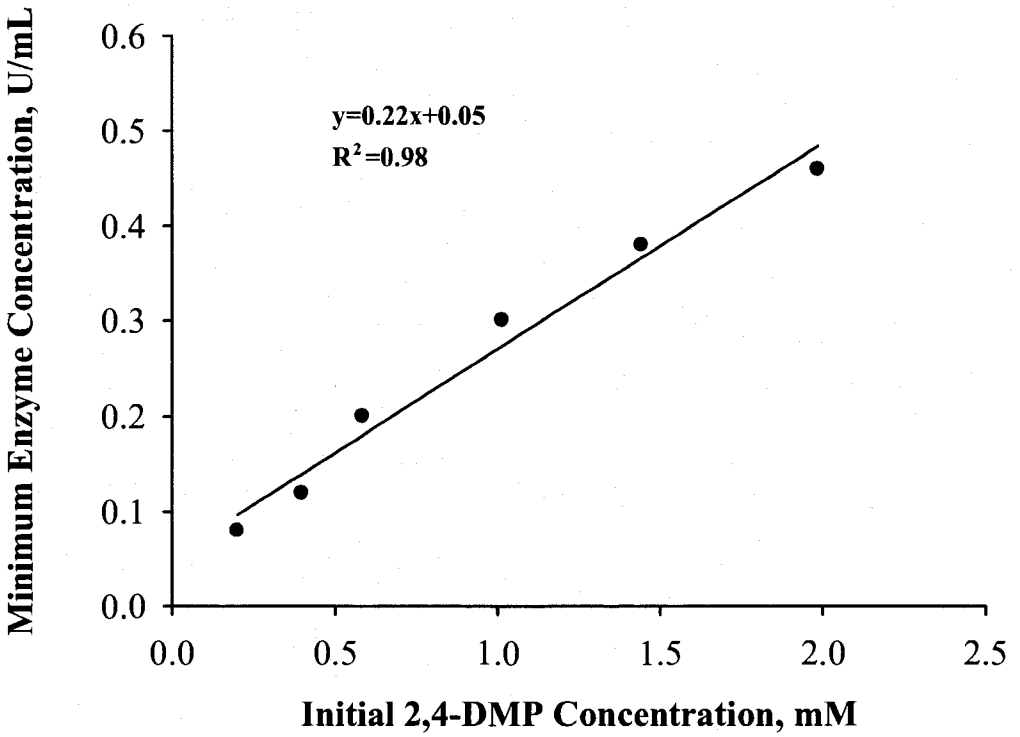



**Figure 4.16:** Linear relationship between minimum enzyme concentration required for 95 % substrate conversion and initial 2,4-DMP concentration (0.2-2 mM) in the absence of PEG (Figures 4.6-4.11)

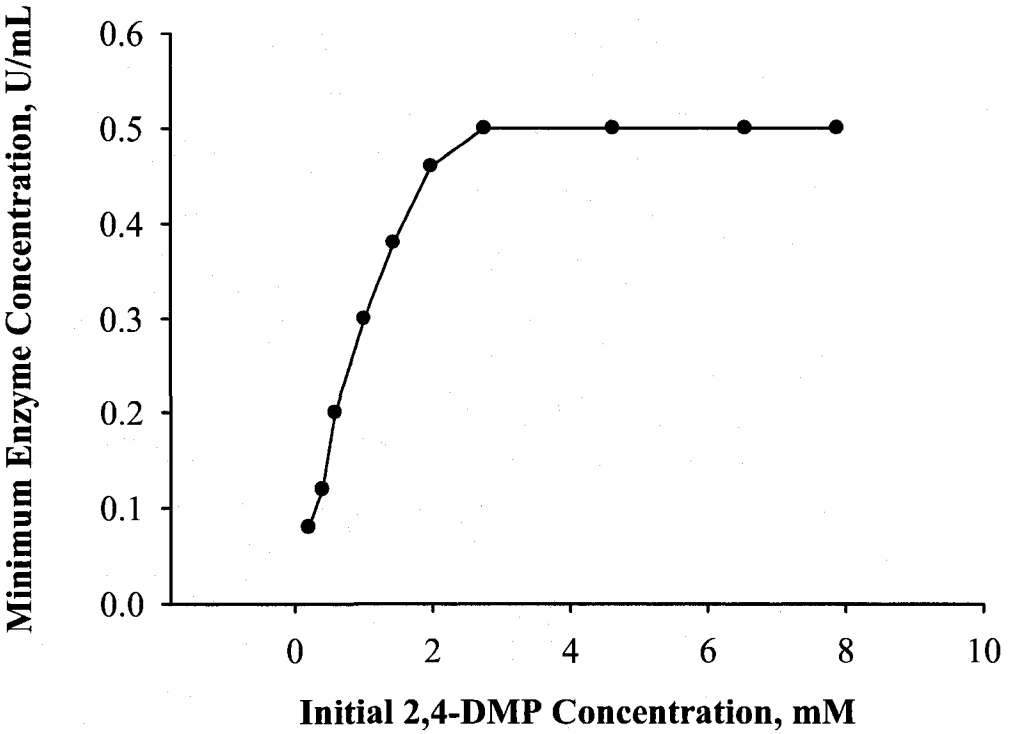



**Figure 4.17:** Relationship between minimum enzyme concentration, required for 95 % substrate conversion and initial 2,4-DMP concentration (0.2-8 mM) in the absence of PEG

**Table 4.1: Minimum Enzyme Concentration in absence of PEG**

| Initial Substrate concentration, mM | Minimum Enzyme concentration, U/mL |
|---|---|
| 0.2 | 0.08 |
| 0.4 | 0.12 |
| 0.6 | 0.2 |
| 1 | 0.3 |
| 1.5 | 0.38 |
| 2 | 0.46 |
| 3 | 0.5 |
| 5 | 0.5 |
| 7 | 0.5 |
| 8 | 0.5 |

### *4.3.2 In the presence of PEG*

Minimum enzyme concentration required for the conversion of 2,4-DMP in presence of PEG, was determined at the optimum pH and optimum [$H_2O_2$] to [substrate] molar ratio found during the previous experiments. A range of substrate concentrations, 1-8 mM, was examined to determine the minimum enzyme concentration required to achieve 95% substrate conversion. A three hour reaction period was chosen for this study. SBP concentration was varied from 0.05 U/mL to 0.55 U/mL as required in each case. PEG was present in excess at a concentration of 400 mg/l to ensure maximum protection of the enzyme.

Figures 4.18 to 4.22 represent the effect of enzyme concentration on 2,4-DMP conversion over a range of substrate concentrations, 1-8 mM. Table 4.2 summarizes the minimum enzyme requirement for the reaction conditions considered.

Figure 4.23 shows that a linear relationship exists between minimum enzyme concentrations, required for 95% conversion of 2,4-DMP, and initial substrate concentration as expected from previous studies (Modaressi et al., 2005; Ibrahim et al., 2001; Kinsley and Nicell, 2000). The effect of PEG on enzyme performance was determined by plotting the minimum enzyme concentration, required in the presence and absence of PEG, versus 2,4-DMP concentration (Figure 4.24). It can be seen from Figure 4.24 that though there is a considerable "PEG effect" at lower substrate concentrations (ratio of the slopes of the linear portions is 4.4), the effect steadily decreased with an increase in substrate concentration and almost became nil at the highest substrate concentration. More investigation needs to be done to explain this particular phenomenon.

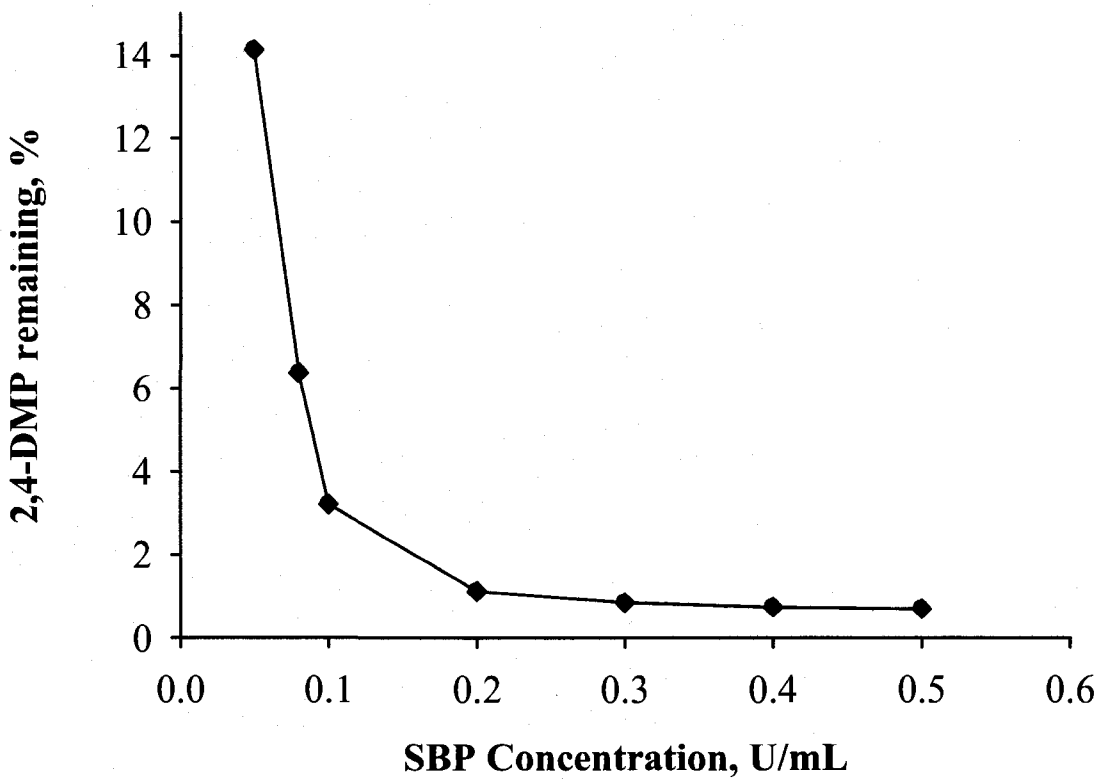



**Figure 4.18:** Effect of enzyme concentration on conversion of 1 mM 2,4-DMP in presence of PEG

Conditions: 1 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 3 hour reaction time. PEG at 400 mg/L

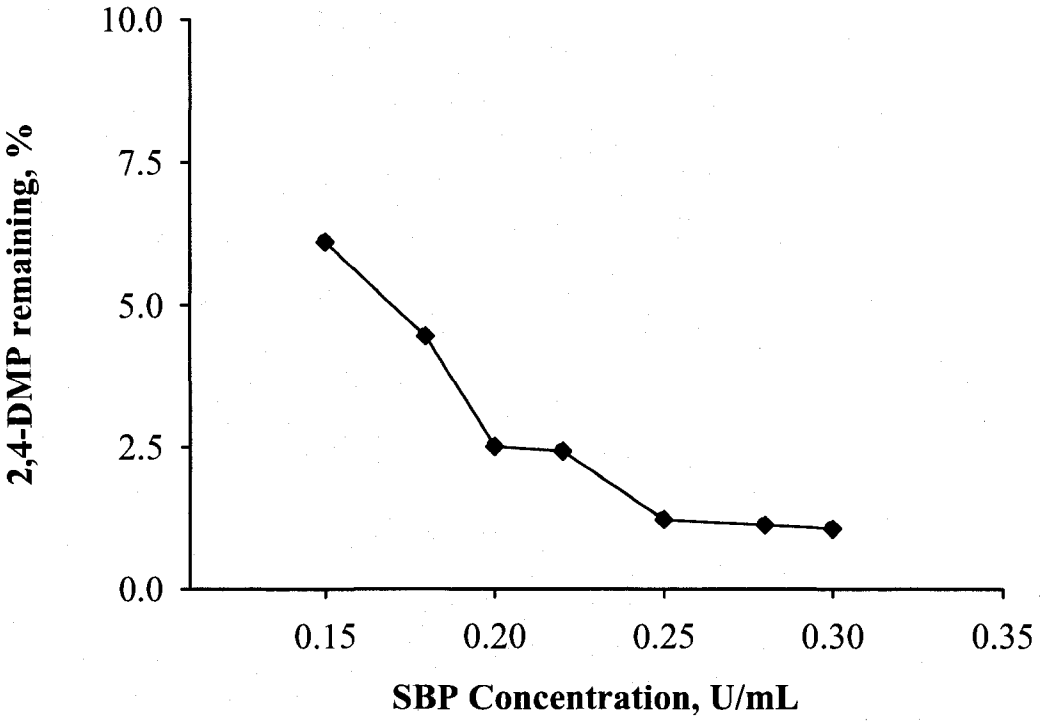



**Figure 4.19:** Effect of enzyme concentration on conversion of 3 mM 2,4-DMP in presence of PEG

Conditions: 3 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 3 hour reaction time. PEG at 400 mg/L

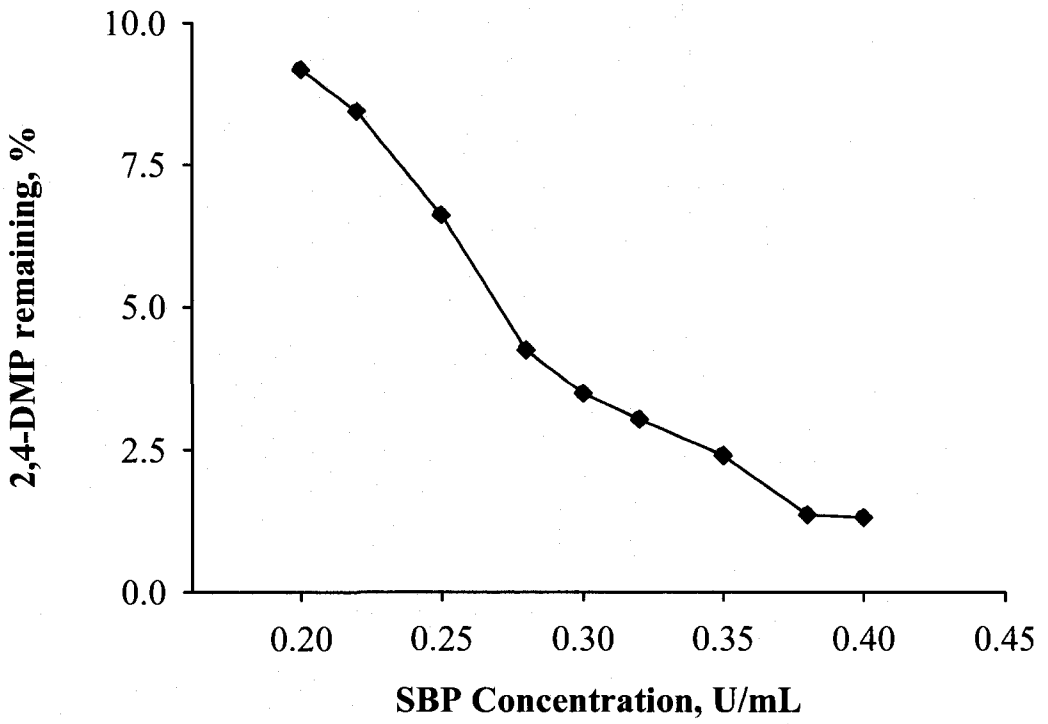



**Figure 4.20:** Effect of enzyme concentration on conversion of 5 mM 2,4-DMP in presence of PEG

Conditions: 5 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 3 hour reaction time. PEG at 400 mg/L.

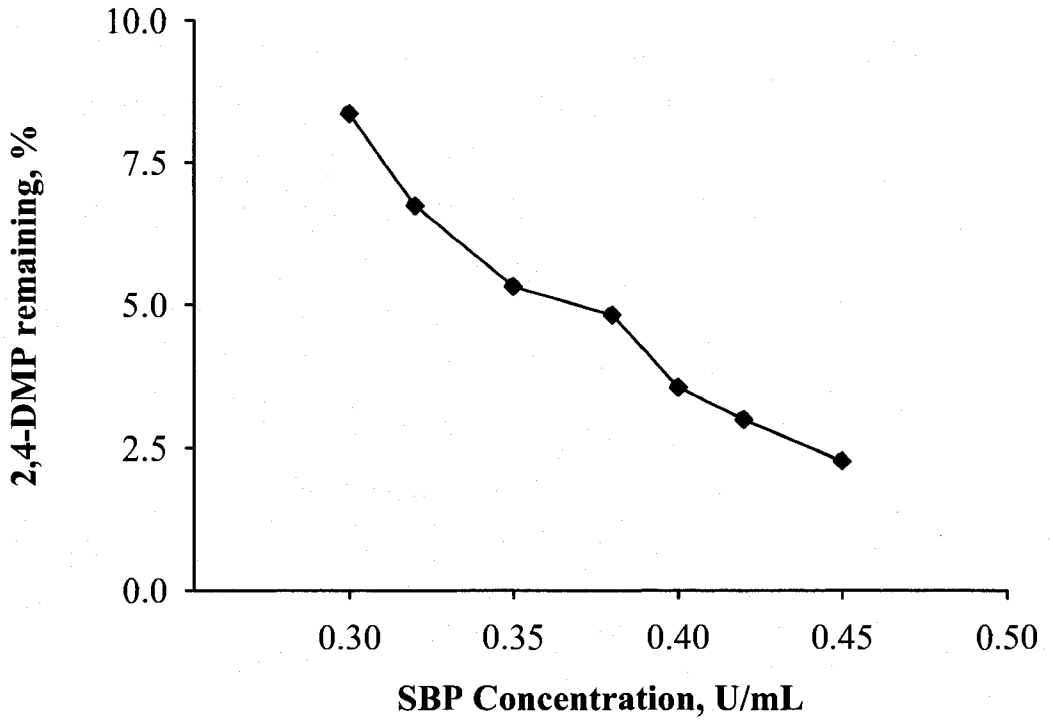



**Figure 4.21:** Effect of enzyme concentration on conversion of 6 mM 2,4-DMP in the presence of PEG

Conditions: 6 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 3 hour reaction time. PEG at 400 mg/L.

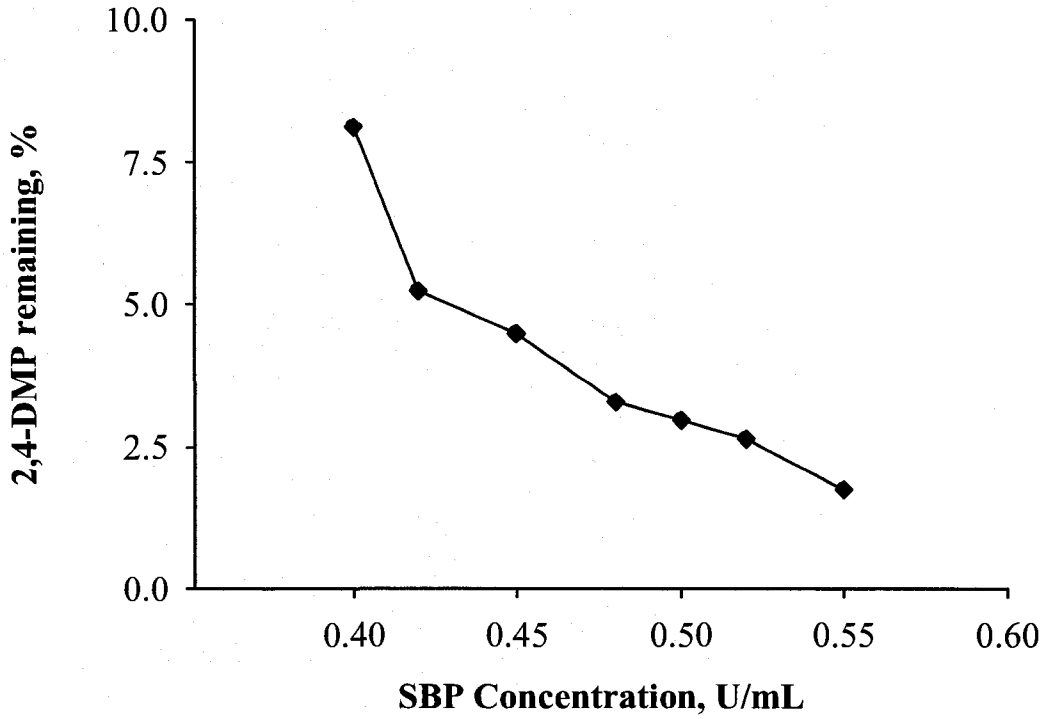



**Figure 4.22:** Effect of enzyme concentration on conversion of 8 mM 2,4-DMP in the presence of PEG

Conditions: 8 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 3 hour reaction time. PEG at 400 mg/L..

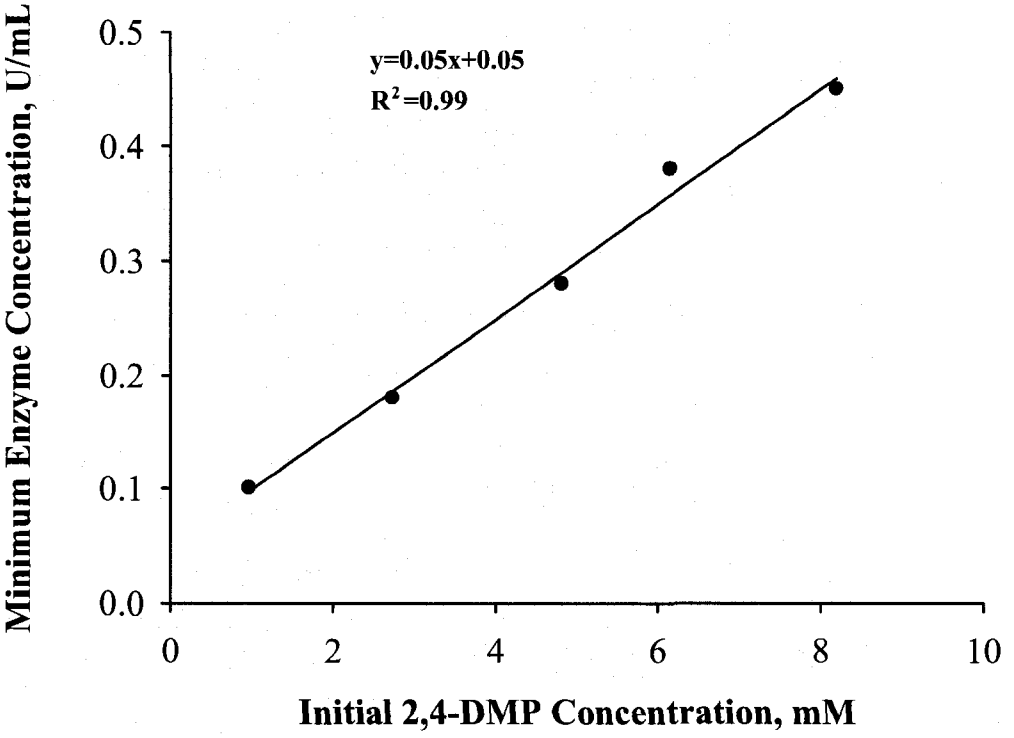



**Figure 4.23:** Linear relationship between minimum enzyme concentrations required for 95 % substrate conversion and initial 2,4-DMP concentrations (1-8 mM) in presence of PEG

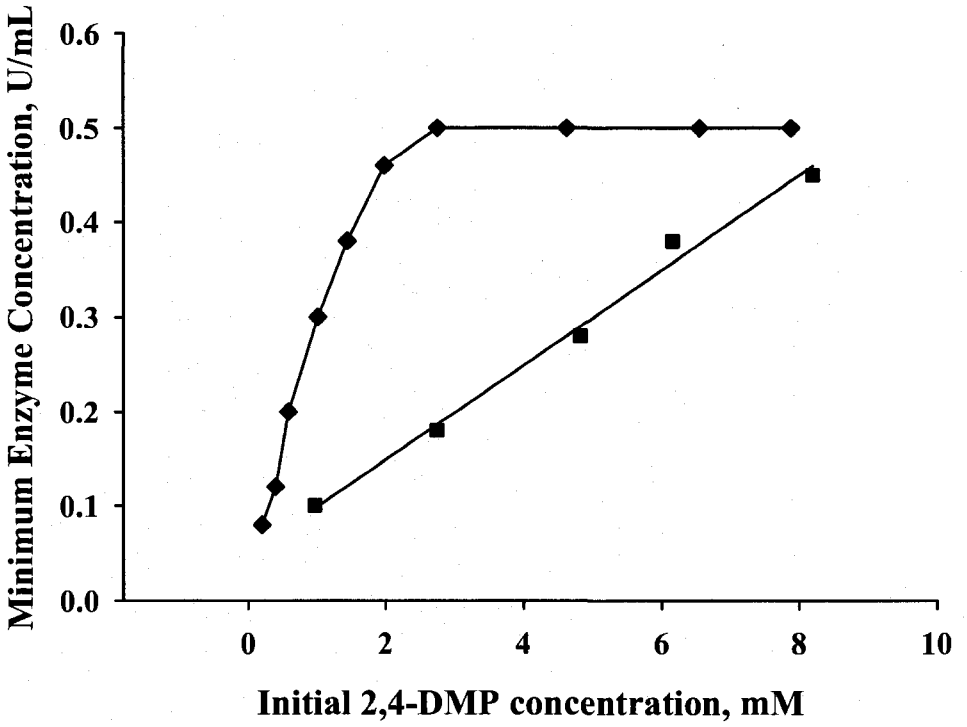



**Figure 4.24:** Comparison of effect of initial substrate concentrations on required minimum enzyme concentrations in absence and presence of PEG

**Table 4.2: Minimum Enzyme Concentration in presence of PEG**

| Initial Substrate concentration, mM | Minimum Enzyme concentration, U/mL |
|---|---|
| 1 | 0.1 |
| 3 | 0.18 |
| 5 | 0.28 |
| 6 | 0.38 |
| 8 | 0.45 |

## 4.4 Effect of Polyethylene Glycol (PEG) Concentration

Polyethylene glycol (PEG) is a synthetic additive. Wu et al., 1997 established that among several additives, PEG was the best one since it significantly reduced the HRP requirement for treatment of phenol and had no negative overdose effect shown by other additives like gelatin and polyelectrolyte. They also found out that at minimum PEG concentration, there was little PEG remaining in solution after completion of reaction. Kinsley and Nicell, 2000 also studied residual PEG remaining in solution and determined that COD increases rapidly with increasing residual PEG concentration. Because of this, optimum PEG concentrations should be determined in order to minimize residual PEG.

The effectiveness of PEG as a function of its concentration was determined. A series of experiments were conducted to optimize the minimum PEG concentration required to protect the enzyme at optimum pH and $[H_2O_2]$ to [substrate] ratio determined earlier. This effect was studied at 1, 3 and 5 mM 2,4-DMP. PEG concentration was varied from 0 to 150 mg/l in presence of limiting enzyme conditions to see the effect of PEG clearly. The reactions were given sufficient 3 hours time to go to completion. The results of these experiments are shown in Figures 4.25 to 4.27 for different substrate concentrations. From the percent 2,4-DMP conversion found at each PEG concentration, it could be observed that beyond 45-50 mg/L PEG concentration, there was hardly 0.5 % more substrate conversion. Beyond this concentration, excess PEG neither increased nor decreased the conversion efficiency. The same result was found at all the three substrate concentrations. Because of this, the minimum PEG concentration required for enzyme protection was chosen to be between 45-50 mg/L. Previous studies (Modaressi et al., 2005; Kinsley and Nicell, 2000; Ibrahim 1998) showed a definite linear relationship

between initial substrate concentrations and minimum PEG concentrations, which mean that with an increase in substrate concentration, the minimum PEG concentration required also increases. Nevertheless, the same did not happen in this study as a linear relationship could not be established.

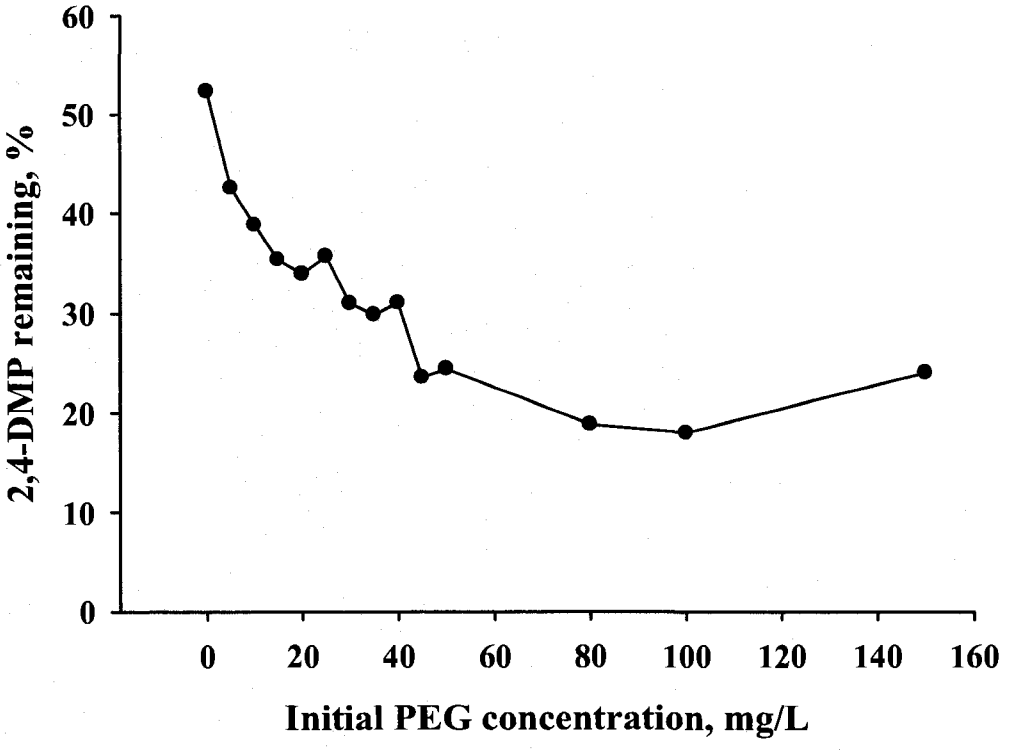



**Figure 4.25:** Effect of PEG concentration on 1 mM 2,4-DMP conversion

Conditions: 1 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and after 3 h reaction time with 0.05 U/mL soybean peroxidase

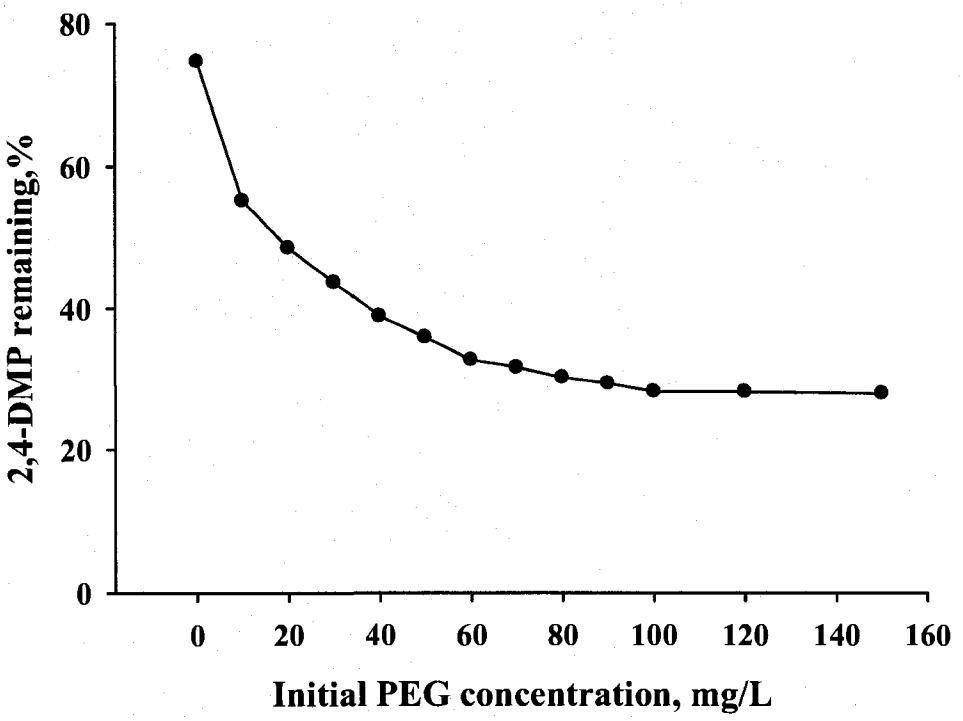



**Figure 4.26:** Effect of PEG concentration on 3 mM 2,4-DMP conversion

Conditions: 3 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and after 3 h of reaction time with 0.09 U/mL soybean peroxidase

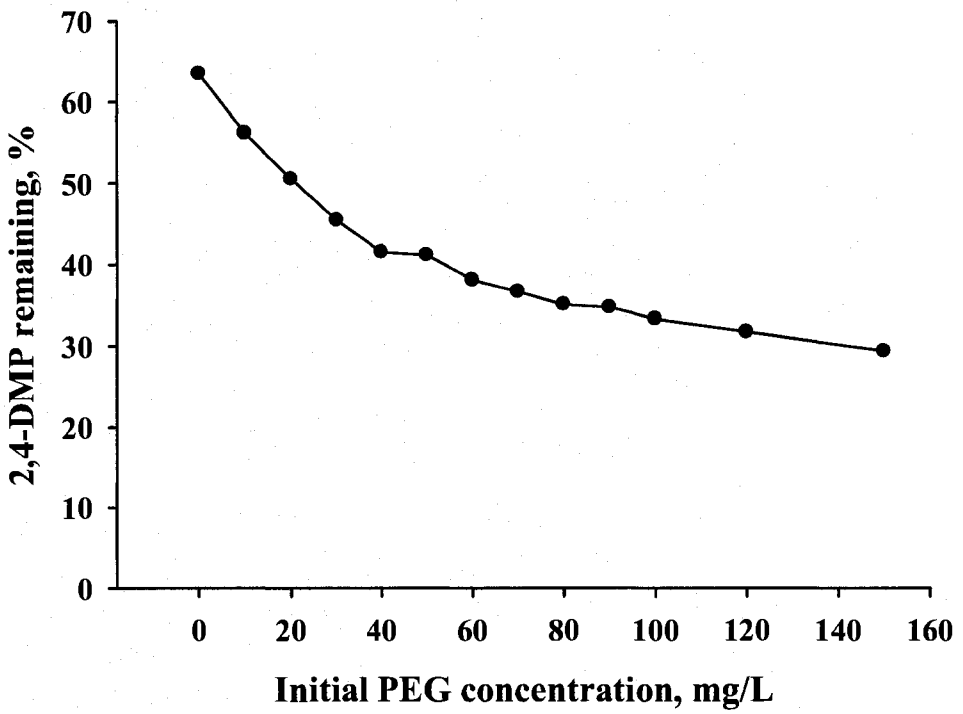



**Figure 4.27:** Effect of PEG concentration on 5 mM 2,4-DMP conversion

Conditions: 5 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and after 3 h reaction time with 0.14 U/mL soybean peroxidase

## 4.5 Progress Curve for 2,4-DMP Conversion

Progress curves of percent 2,4-DMP remaining versus time for two substrate concentrations were determined under optimum reactor conditions without PEG over a three hour reaction period. At selected time intervals, 1 mL of the samples were withdrawn from the batch reactor and 0.2 mL of catalase (stock 0.5 mg/mL) was added to each sample to stop the reaction. Then it was filtered and the filtrate was used for HPLC analysis to determine the percent conversion.

Figures 4.28 and 4.29 represent progress curves of 2,4-DMP conversion for 1mM and 5 mM substrate concentrations, respectively. Though the desired 95% conversion was almost achieved within the first 20 minutes, the reaction was allowed to proceed for 3 hours. The 3 h time frame is important from a practical operational point of view as a lag time is required to give the dimers, trimers and higher polymers enough time to polymerize non-enzymatically and precipitate out of the solution (Kennedy et al., 2002). Figures 4.30 to 4.32 show the chromatograms of progress of the enzyme-catalyzed conversion of 5 mM 2,4-DMP and formation of byproducts with time. Figure 4.31 represents the chromatogram of the enzyme-catalyzed reaction at $20^{th}$ minute and its scale has been blown to 10 times of the original scale of Figure 4.30. Figure 4.31 shows the presence of numerous soluble intermediate dimers and trimers formed during the reaction at that time. Figure 4.32 represents the chromatogram of the enzyme-catalyzed reaction at the end of 3 hours and it has been blown to 15 times of the original scale. Figure 4.32 when compared to Figure 4.31 shows a comparatively clearer chromatogram with the disappearance of all intermediate products and with the presence of very few products.

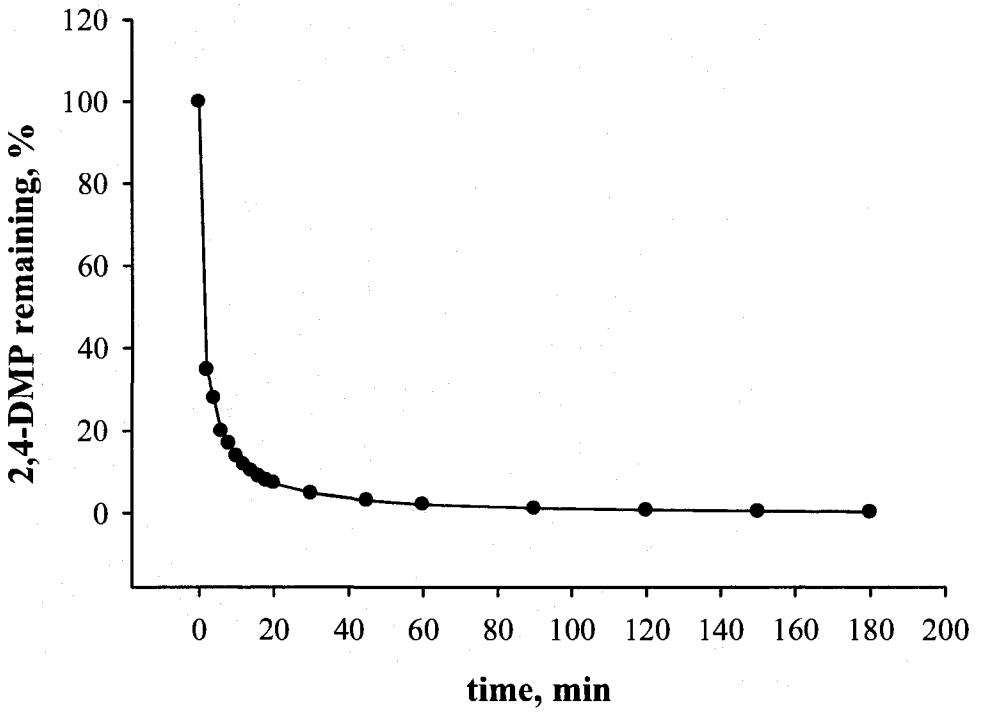



**Figure 4.28:** Progress curve for percent conversion of 1 mM 2,4-DMP catalyzed by SBP at room temperature in the absence of PEG.

Conditions: 1 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, treated with 0.3 U/mL soybean peroxidase.

Further experiments were conducted to justify the time chosen for enzymatic reaction. Figure 4.33 represents effect of enzymatic concentration on conversion of 3 mM 2,4-DMP in a reaction period of 1 hour in the presence of PEG. The minimum concentration of SBP required to achieve 95 % conversion or more is 0.3 U/mL whereas it can be seen from Figure 4.34 the same requires 0.18 U/mL of enzyme in a reaction period of 3 hours. As the reduction in the cost of enzyme is the main aim of the enzymatic treatment process, it is justifiable to compromise on reaction time to save on enzyme cost.

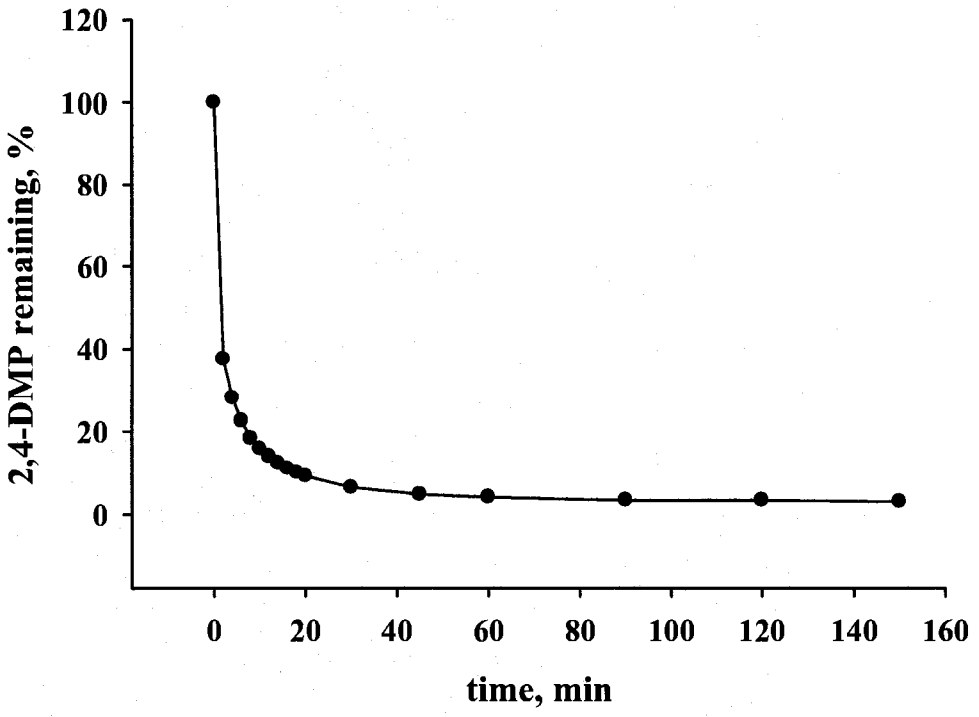



**Figure 4.29:** Progress curve for percent conversion of 5 mM 2,4-DMP catalyzed by SBP at room temperature in the absence of PEG.

Conditions: 5 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, treated with 0.5 U/mL soybean peroxidase.

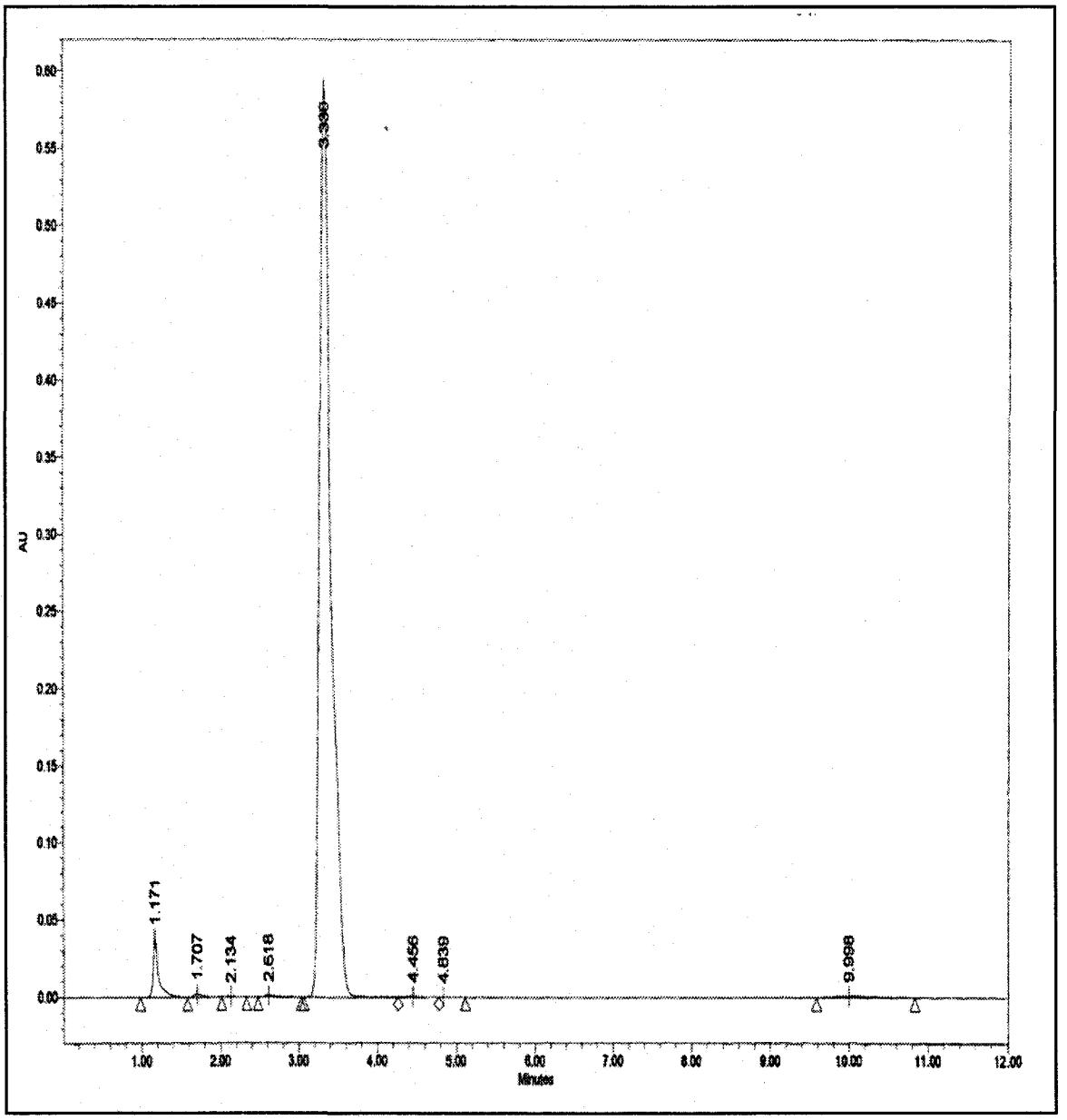



**Figure 4.30:** Chromatogram of enzyme catalyzed conversion of 5 mM 2,4-DMP at $0^{th}$ minute

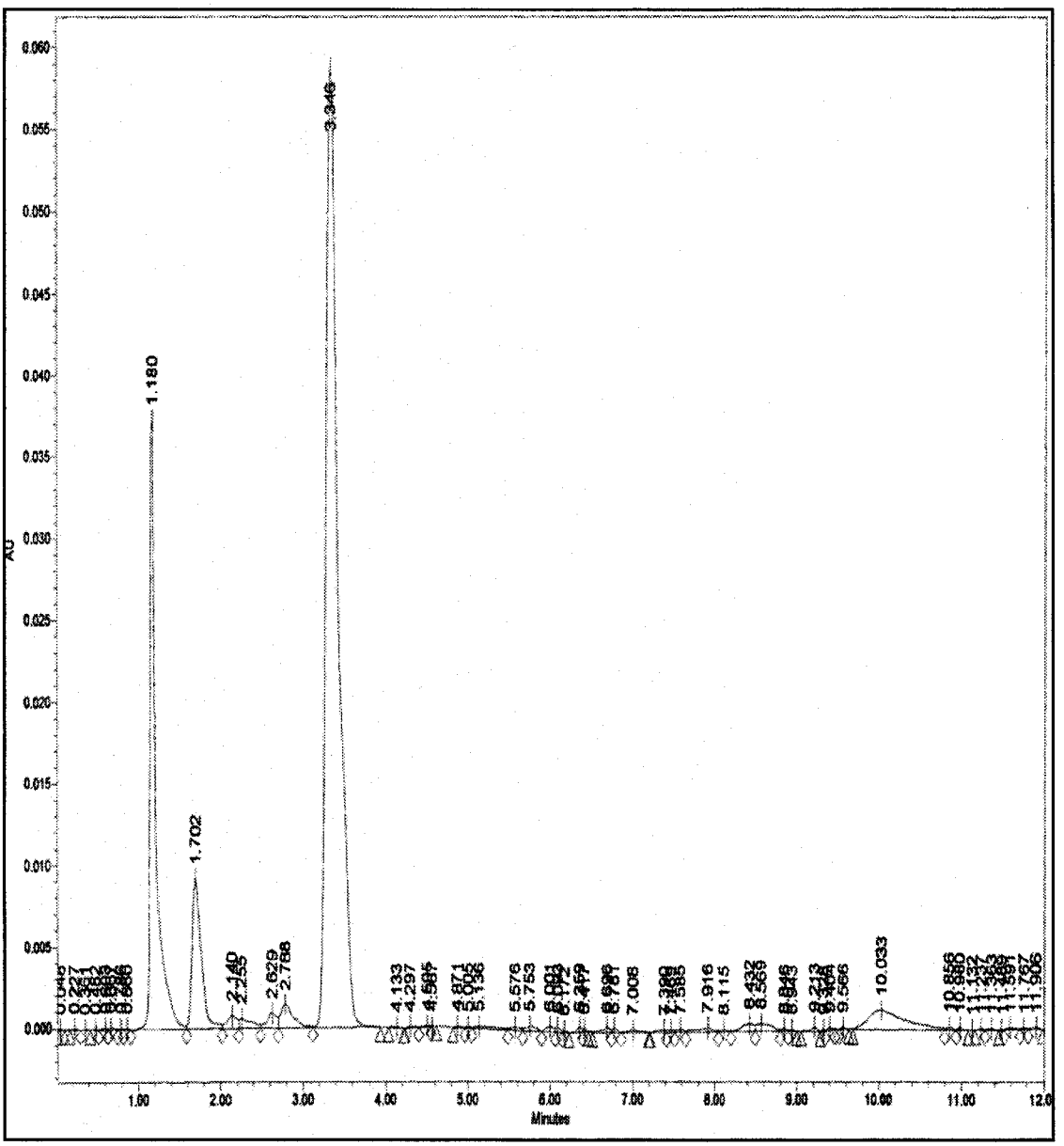



**Figure 4.31:** Chromatogram of enzyme catalyzed conversion of 5 mM 2,4-DMP at 20$^{th}$ minute

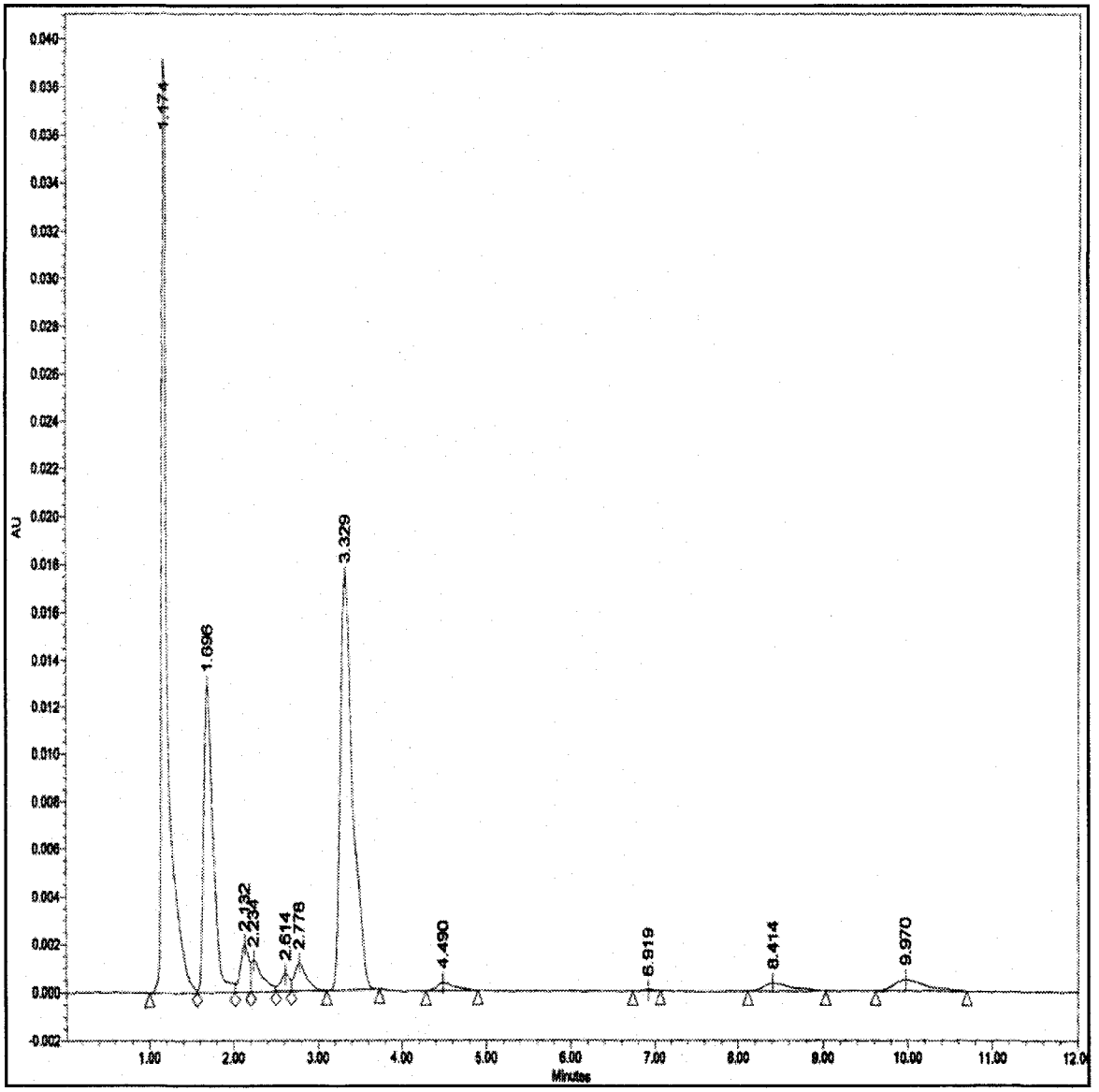



**Figure 4.32:** Chromatogram of enzyme catalyzed conversion of 5 mM 2,4-DMP at 180$^{th}$ minute

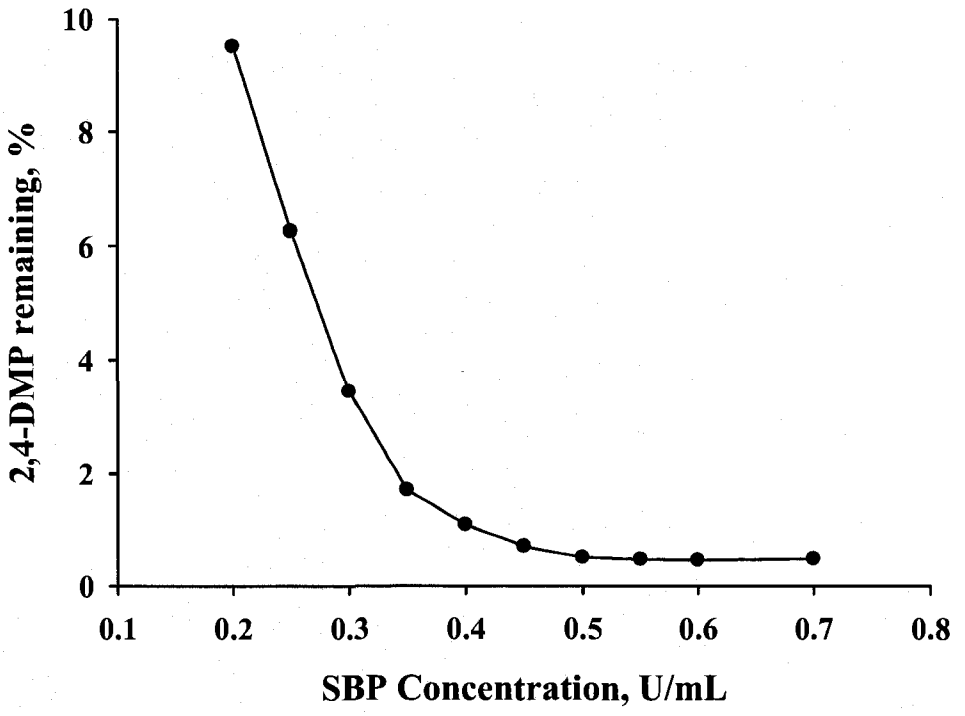



**Figure 4.33:** Effect of enzyme concentration on conversion of 3 mM 2,4-DMP in presence of PEG in 1 hour

Conditions: 3 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a [$H_2O_2$] to [substrate] ratio of 1.2 and 1 hour reaction time with PEG at 400 mg/L

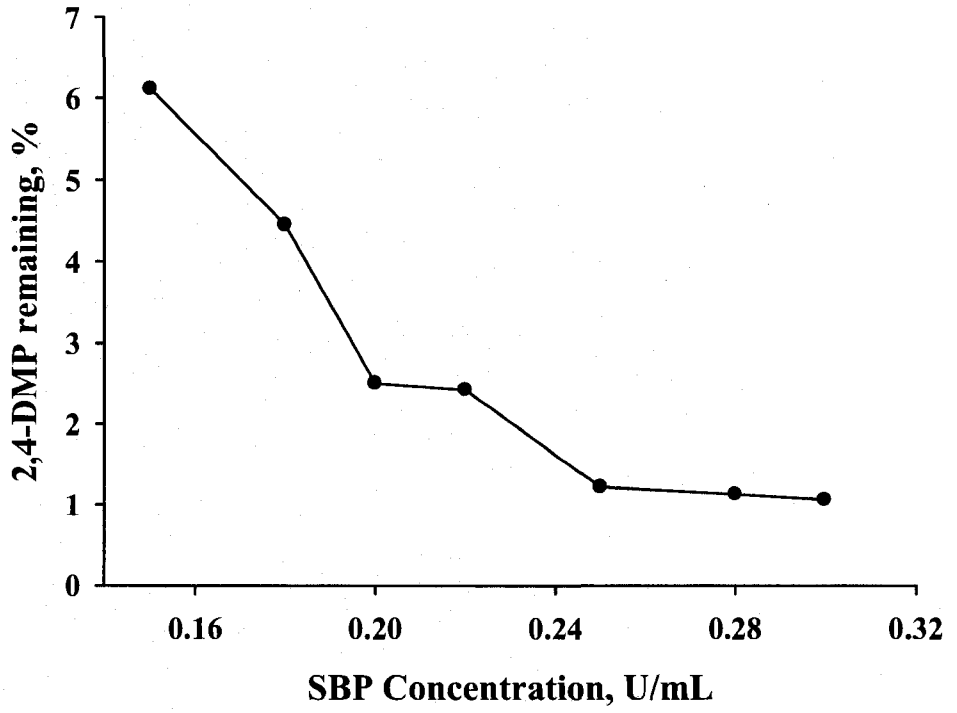



**Figure 4.34:** Effect of enzyme concentration on conversion of 3 mM 2,4-DMP in the presence of PEG in 3 hours.

Conditions: 3 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, with a $[H_2O_2]$ to [substrate] ratio of 1.2 and 3 hour reaction time, PEG at 400 mg/L.

## 4.6 Investigation of Kinetic Behavior of SBP

In this study, initial velocity ($v_o$) were determined to investigate the kinetics of enzyme-catalyzed reaction. Since the enzymes are often unstable in solution, the investigations of $v_o$ gave the best chance to avoid errors caused either by loss of enzyme activity with time or by-product inhibition (Palmer, 1995). The Michaelis-Menten model was applied to determine the kinetic constants, $K_m$ and $V_{max}$ of the SBP catalyzed reaction in the presence and absence of PEG.

The disappearance of 2,4-DMP from the reaction mixture during initial 4 minutes of reaction was used to determine the initial velocity of the enzyme-catalyzed reaction of 2,4-DMP and hydrogen peroxide. These experiments were conducted by taking 1 mL of the sample from the reaction mixture at predetermined time and halting the reaction by adding the sample to a high dose of catalase.

### *4.6.1 Michaelis-Menten Constants for 2,4-DMP with SBP in the absence of PEG*

Two series of experiments were carried out:

Series 1: Varying the concentrations of 2,4-DMP and hydrogen peroxide. 2,4-DMP concentration ranged from 0.5-13 mM and the ratio between substrate concentration and hydrogen peroxide concentration was kept constant at 1.2.

Series 2: Varying the concentration of 2,4-DMP from 1 to 15 mM and using a constant hydrogen peroxide concentration of 1 mM.

Both sets of experiments were carried out at various enzyme concentrations between 0.001 and 0.1 U/mL. Finally based on the data collected at each concentration, an enzyme concentration of 0.1 U/mL was chosen as appropriate for the kinetic experiments.

#### 4.6.1.1 Series 1

The initial rates of 2,4-DMP conversion were measured at various concentrations of 2,4-DMP ranging from 0.5 to 13 mM with an enzyme concentration of 0.1 U/mL at pH 8.0 in open batch reactors at room temperature of 22 ± 2°C. Initial $[H_2O_2]$/[substrate] ratio was maintained at 1.2.

The experiments were run under the above-mentioned conditions and the values of initial velocity were obtained by using polynomial fit of third order (Figure 4.35). The values of $v_o$ in Table 4.3 show an increase up to a substrate concentration of 5 mM, beyond which there is a sharp and steep decrease in the initial velocities. This is attributed to probable inhibition of the enzyme (0.1 U/mL) by high concentrations of $H_2O_2$ present at higher substrate concentrations since $[H_2O_2]$/[substrate] was maintained at 1.2. Thus, it was decided not to use these results of Series 1 for determining the kinetic constants of the enzyme.

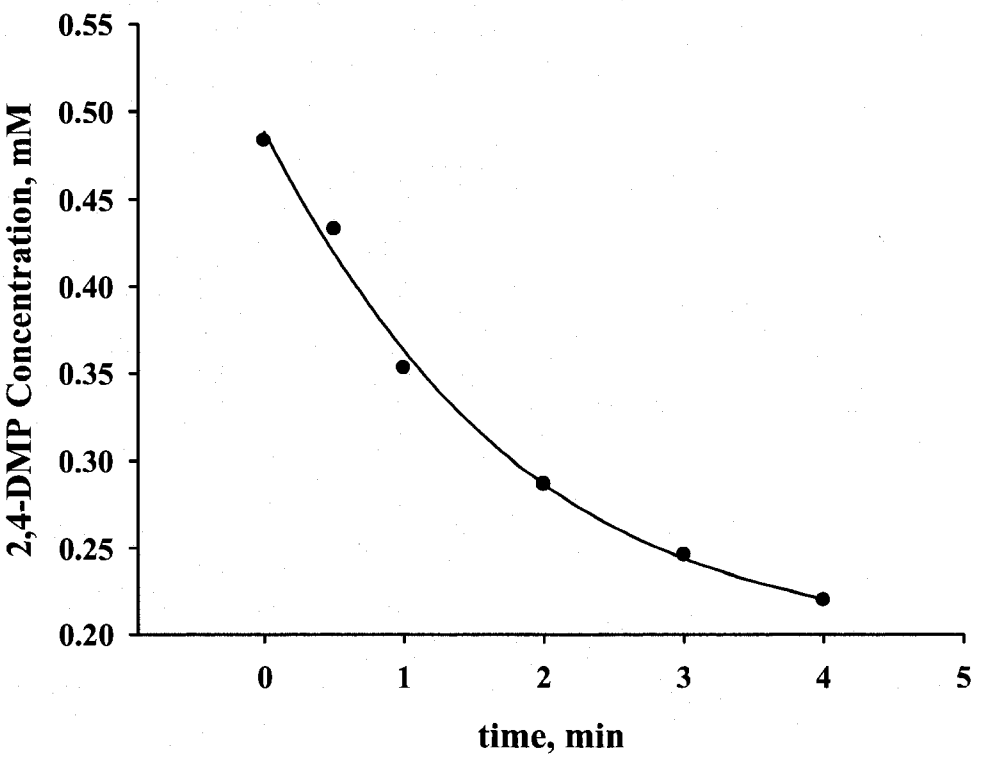



**Figure 4.35**: Time progress curve of conversion of 2,4-DMP for the measurement of initial velocity of SBP-catalyzed reaction under following conditions : 0.5 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, treated with 0.1 U/mL soybean peroxidase and with a $[H_2O_2]$ to [substrate] ratio of 1.2. The initial velocity measured, $v_o$ = 0.1541 mM/min.

■ Remaining 2,4-DMP Concentration, mM

—— Best fit to 3 $^{rd}$ order polynomial

**Table 4.3:** Initial velocities of SBP catalyzed 2,4-DMP conversion (under conditions given in legends in Figure 4.35

| 2,4-DMP concentration, mM | Initial Velocities, mM/min |
|---|---|
| 0.48 | 0.15 |
| 0.99 | 0.38 |
| 2.93 | 0.93 |
| 4.81 | 1.40 |
| 6.62 | 1.02 |
| 8.10 | 0.54 |
| 13.22 | 0.39 |

#### 4.6.1.2 Series 2

The Michaelis-Menten model has been developed with respect to a single substrate enzyme-catalyzed reaction with one substrate-binding site per enzyme and involving the formation of a single intermediate complex. Such reactions are rare. However, the requirement for a single-substrate reaction can be taken to include pseudo single-substrate reactions and also variations with respect to one substrate in a multi-substrate reaction, provided the other substrates are kept at a constant concentration (Palmer, 1995). Thus for this set, a constant concentration of $H_2O_2$ was used at all DMP concentrations. The $K_m$ of $H_2O_2$ has been found, in previous studies to be around 0.1 mM (Bodalo *et al.*, 2007). Thus, by choosing a $H_2O_2$ concentration of 5 to 10 times its $K_m$, a near-saturating value, during the first four minutes of the reaction, which was the time used for initial velocity studies in this case. Initial velocity studies were run at two different constant hydrogen

peroxide concentrations, 0.5 and 1 mM. Based on the data collected at each $H_2O_2$ concentration, 1mM hydrogen peroxide was chosen as appropriate for the kinetic experiments.

The initial rates of 2,4-DMP conversion were measured at various concentrations of 2,4-DMP 1 to 15 mM, with an enzyme concentration of 0.1 U/mL at pH 8.0 and in open batch reactors at room temperature of 22 ± 2°C.

The experiments were first run under the above-mentioned conditions and the values of initial velocity were obtained by using polynomial fit of third order (Figure 4.36). The values of $v_o$ show an increase up to a substrate concentration of 10.3 mM, beyond which there was a very gradual decrease in the initial velocities (Table 4.4). This is attributed to possible substrate inhibition of the enzyme by 2,4-DMP at this high concentration. Phenol at a concentration of 15 mM inhibits the enzyme ARP, because of which the initial velocity falls beyond this concentration (Ibrahim, 1998). Hence, only the data up to a substrate concentration of 10.3 mM were considered. The experiments were again run in triplicates for 2,4-DMP concentrations, 1-10.3 mM. The plot of initial rate ($v_o$) vs. 2,4-DMP concentration in Figure 4.37 follows a hyperbolic pattern as expected for Michaelis-Menten kinetics. Non-linear regression analysis of data yielded the values of Michaelis-Menten constants, $K_m = 11.6 \pm 2.0$ mM and $V_{max} = 4.58 \pm 0.47$ mM/min.

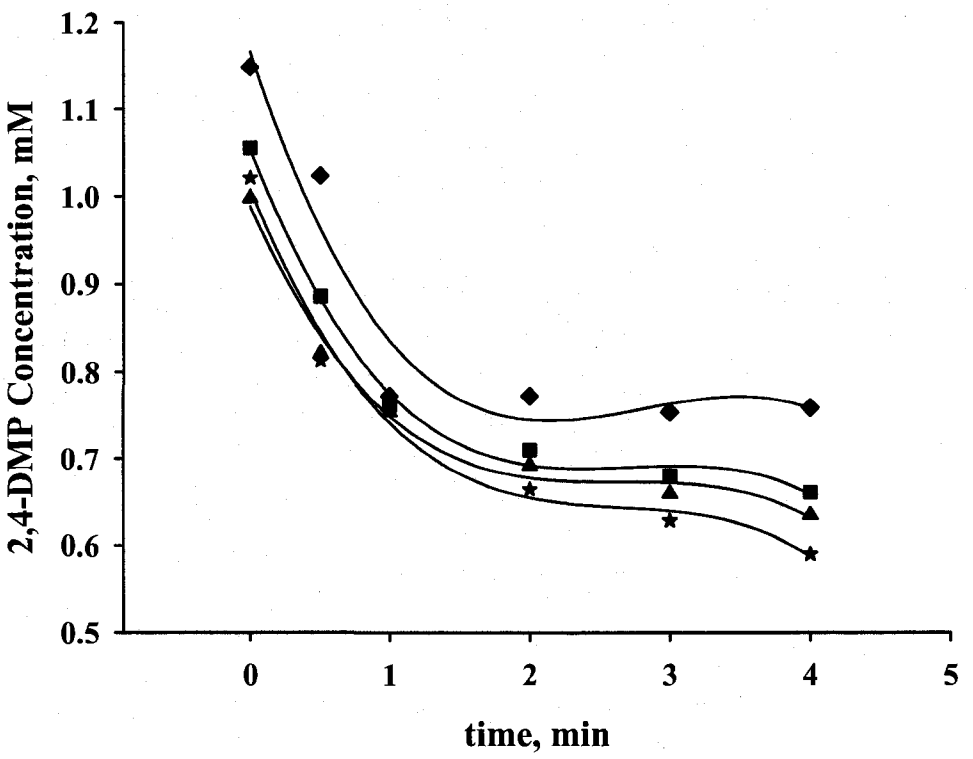



**Figure 4.36**: Progress curves of conversion of 2,4-DMP for the measurement of initial velocity of SBP-catalyzed reaction under following conditions : 1.0 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, treated with 0.1 U/mL soybean peroxidase and with a $H_2O_2$ concentration of 1 mM. The average initial velocity measured, $v_o$ = 0.4147 mM/min.

◆ Remaining 2,4-DMP Concentration, mM (Run 1)

▲ Remaining 2,4-DMP Concentration, mM (Run 2)

★ Remaining 2,4-DMP Concentration, mM (Run 3)

■ Remaining 2,4-DMP Concentration, mM (average)

—— Best fit to 3 $^{rd}$ order polynomial

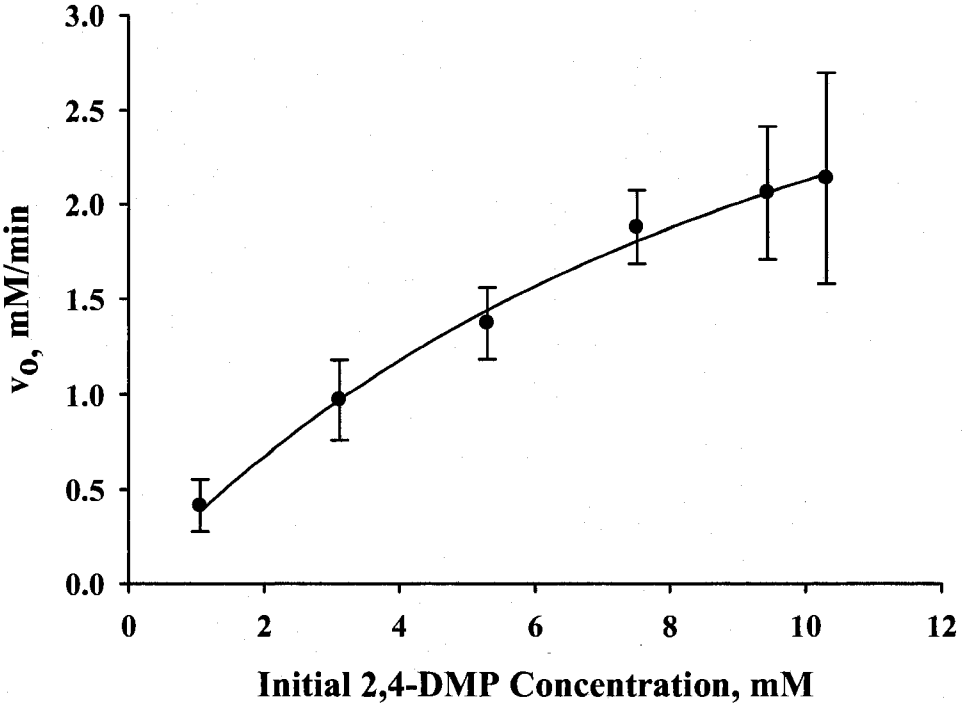



**Figure 4.37**: Fit to Michaelis-Menten Equation of initial velocity of enzyme-catalyzed 2,4-DMP reaction vs. initial 2,4-DMP concentration in absence of PEG

Conditions: 1-10.3 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer after 4 minute enzyme treatment with SBP at 0.1 U/mL. Fitted kinetic parameters are: $V_{max} = 4.58 \pm 0.47$ mM/min, $K_m = 11.6 \pm 2.0$ mM

**Table 4.4:** Initial velocities of SBP catalyzed 2,4-DMP conversion (under conditions given in legend to Figure 4.36)

| 2,4-DMP concentration, mM | Initial Velocities, mM/min | | | |
|---|---|---|---|---|
| | Run 1 | Run 2 | Run 3 | Average |
| 1.06 | 0.49 | 0.36 | 0.39 | 0.42 |
| 3.11 | 1.09 | 0.88 | 0.94 | 0.97 |
| 5.29 | 1.43 | 1.44 | 1.25 | 1.37 |
| 7.51 | 1.98 | 1.78 | - | 1.88 |
| 9.44 | 2.29 | 1.95 | 1.94 | 2.06 |
| 10.31 | 2.44 | 1.88 | 2.10 | 2.14 |
| 11.5 | - | - | - | 2.09 |
| 12.09 | - | - | - | 2.08 |
| 13.06 | - | - | - | 2.01 |
| 13.79 | - | - | - | 1.94 |
| 14.70 | - | - | - | 1.76 |
| 15.29 | - | - | - | 1.71 |

### *4.6.2 Michaelis-Menten Constant for 2,4-DMP with SBP in the presence of PEG*

The effect of PEG on the Michaelis-Menten constants is investigated here. The initial rates of 2,4-DMP conversion were measured at various concentrations of 2,4-DMP 1 to 15 mM, with an enzyme concentration of 0.1 U/mL at pH 8.0 and in open batch reactors at room temperature of 22 ± 2°C. Initial $H_2O_2$ concentration was maintained at 1mM in all the batch reactors.

The experiments were first run under the above-mentioned conditions and the values of initial velocity were obtained by using polynomial fit of third order (Figure 4.38). The values of $v_o$ show an increase up to a substrate concentration of 10.5 mM, beyond which there was a very gradual decrease in the initial velocities (Table 4.5). This is attributed to possible substrate inhibition of the enzyme by 2,4-DMP at this high concentration. Hence, only the data of substrate concentrations, 1-10.5 mM were considered. The experiments were again run in triplicates for 2,4-DMP concentrations, 1-10.5 mM. The plot of initial rate ($v_o$) vs. 2,4-DMP concentration in Figure 4.39 follows a hyperbolic pattern as expected for Michaelis-Menten kinetics. Non-linear regression analysis of data yielded the values of Michaelis-Menten constants, $K_m$ = 15.85 ± 9.83 mM and $V_{max}$ = 6.72 ± 2.76 mM/min.

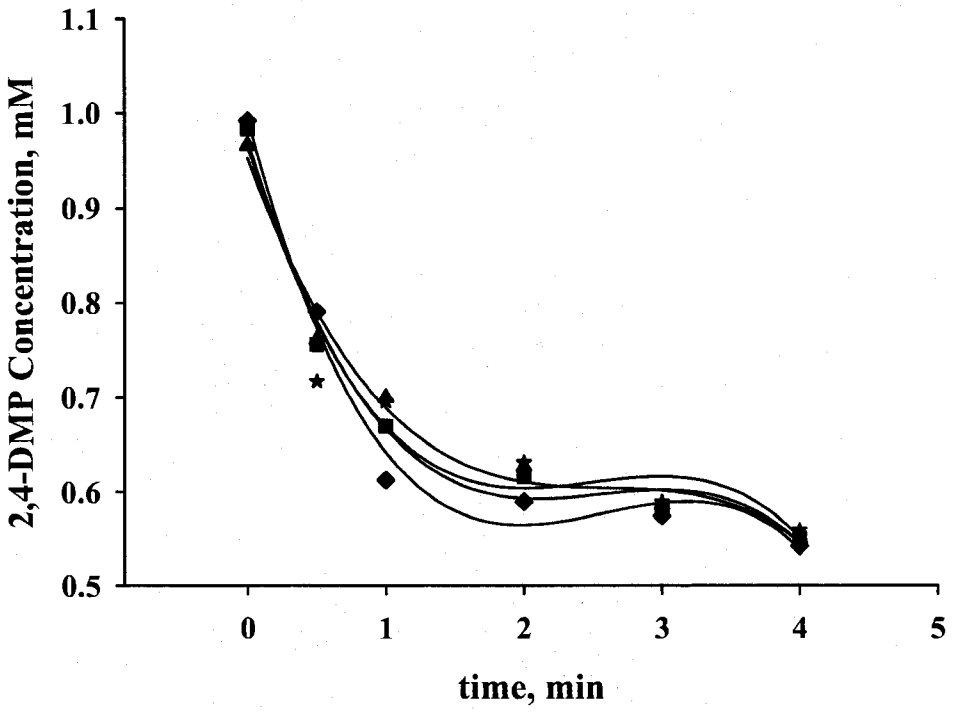



**Figure 4.38**: Progress curves of conversion of 2,4-DMP for the measurement of initial velocity of SBP-catalyzed reaction under following conditions : 1.0 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer, treated with 0.1 U/mL soybean peroxidase, $H_2O_2$ of 1 mM concentration and PEG at 400 mg/L. The average initial velocity measured, $v_o$ = 0.4684 mM/min.

◆ Remaining 2,4-DMP Concentration, mM (Run 1)

▲ Remaining 2,4-DMP Concentration, mM (Run 2)

★ Remaining 2,4-DMP Concentration, mM (Run 3)

■ Remaining 2,4-DMP Concentration, mM (average)

—— Best fit to 3 $^{rd}$ order polynomial

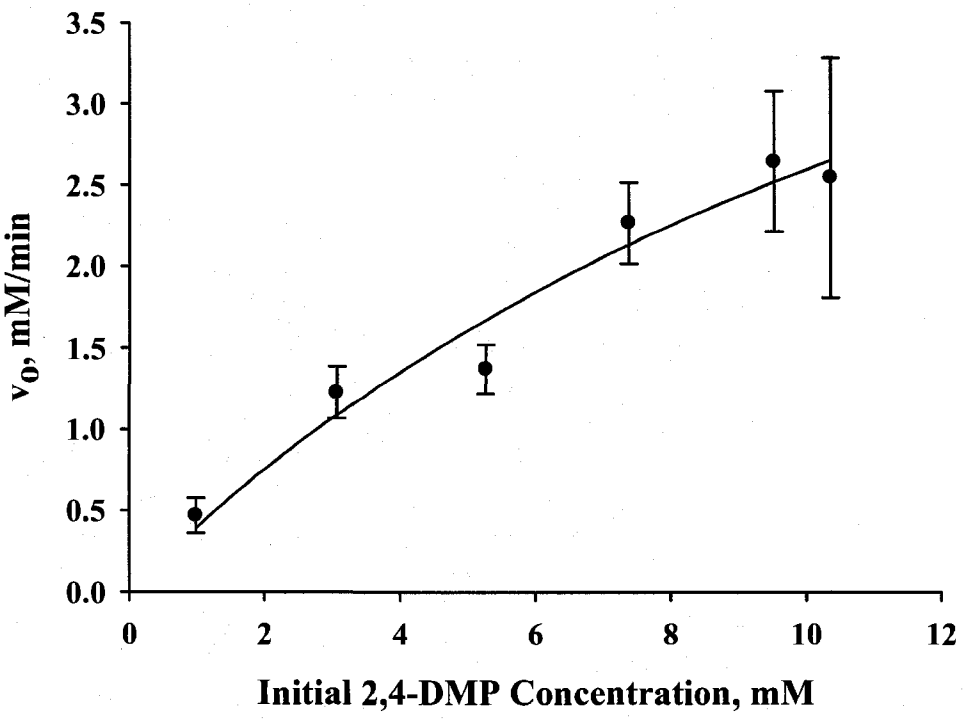



**Figure 4.39**: Fit to Michaelis-Menten Equation of initial velocity of enzyme-catalyzed 2,4-DMP reaction vs. initial 2,4-DMP concentration in presence of PEG

Conditions: 1-10.35 mM 2,4-DMP in batch reactor, buffered at pH 8.0 with 50 mM phosphate buffer after 4 minute enzyme treatment with SBP at 0.1 U/mL and PEG at 400 mg/L. Fitted kinetic parameters are: $V_{max} = 6.72 \pm 2.76$ mM/min, $K_m = 15.85 \pm 9.83$ mM

**Table 4.5:** Initial velocities of SBP catalyzed 2,4-DMP conversion (under conditions given in legends in Figure 4.38)

| 2,4-DMP concentration, mM | Initial Velocities, mM/min | | | |
|---|---|---|---|---|
| | Run 1 | Run 2 | Run 3 | Average |
| 0.98 | 0.55 | 0.40 | 0.46 | 0.47 |
| 3.07 | 1.36 | 1.15 | 1.18 | 1.23 |
| 5.27 | 1.43 | 1.44 | 1.25 | 1.37 |
| 7.38 | 2.06 | 2.13 | 2.61 | 2.27 |
| 9.52 | 2.95 | 2.34 | - | 2.65 |
| 10.35 | 2.07 | 2.47 | 3.10 | 2.55 |
| 12.44 | - | - | - | 2.5 |
| 13.41 | - | - | - | 2.19 |
| 13.61 | - | - | - | 2.1 |
| 14.1 | - | - | - | 1.69 |
| 14.77 | - | - | - | 2.19 |

**Table 4.6:** Kinetic characteristics of SBP for 2,4-DMP substrate and Hydrogen peroxide co-substrate

| Conditions | Km (mM) | Vmax (mM/min) | Vm/Km |
|---|---|---|---|
| Absence of PEG | 11.6 ± 2.0 | 4.58 ± 0.47 | 0.40 |
| Presence of PEG | 15.85 ± 9.83 | 6.72 ± 2.76 | 0.42 |

If Tables 4.4 and Table 4.5 are compared then it can be seen that the initial velocities in both cases were almost same until a concentration of 5 mM, beyond which PEG increased the initial velocities at each substrate concentration. At the same time, it can also be seen that with inclusion of PEG, the triplicate data at each substrate concentration were less consistent compared to that found in absence of PEG.

Enzyme specificity, $V_{max}/K_m$, is equal to 0.40 and 0.42 in the absence and presence of PEG, respectively. It can be seen from Table 4.6 that there was no PEG effect on the kinetic parameters. This is because the estimation of kinetic parameters was done by initial velocity studies. As initial velocities are measured at time, t = 0, so there should not be any PEG Effect on the kinetic constants during that time of the reaction.

Theoretically, initial rate $v_o$ has been shown to reach the limiting rate $V_{max}$ with significant increase in substrate concentration $[S_o]$. However, most of the times the curve does not reach the limit at any finite concentration of substrate and rather remains far from it at

even higher concentration that can be realistically achieved (Cornish-Bowden, 1988). In those cases, a substrate concentration ranging from 0.2 $K_m$ to 2 $K_m$ is chosen and $V_{max}$ is statistically calculated from the fit of initial rate data to the Michaelis-Menten equation. Unfortunately, in this case, it was impossible to achieve that range of substrate cocnentrations because of probable substrate inhibition beyond 10.3 mM. Therefore, experiments were conducted within the substrate concentration range of 1-10.3 mM and the data have been fitted to Michaelis-Menten equation to generate the values of kinetic parameters. This can be a possible reason why there is a high standard deviation in the kinetic parameter values especially in presence of PEG (Table 4.6).

## 4.7 Possible Products for 2,4-DMP Radical Coupling

Formation of dimers may provide valuable information on the preferred reaction pathways and hence from that we can know the structure of possible reaction products. Enzymatic oxidation of 2,4-DMP will occur at the OH group and the unpaired electron may delocalize by resonance, as shown in Figure 4.40.

The pattern of reactivity discussed in Chapter 2.4.1 applied to the 2,4-dimethylphenoxyl radical leads to 10 dimer isomers of two main types: those of thermodynamic stability like that of the starting 2,4-DMP (in Figure 4.41 below), and those of much lower thermodynamic stability (in Figure 4.42 below). The dimers in Figure 4.41 are more stable because aromaticity has been retained in both rings. The first dimer in Figure 4.42 is an inherently peroxide. The rest of the compounds shown in Figure 4.42 are less stable because a quaternary centre has disrupted the aromaticity of one of the rings. Formation of these compounds cannot be ruled out but is thought to be less likely.

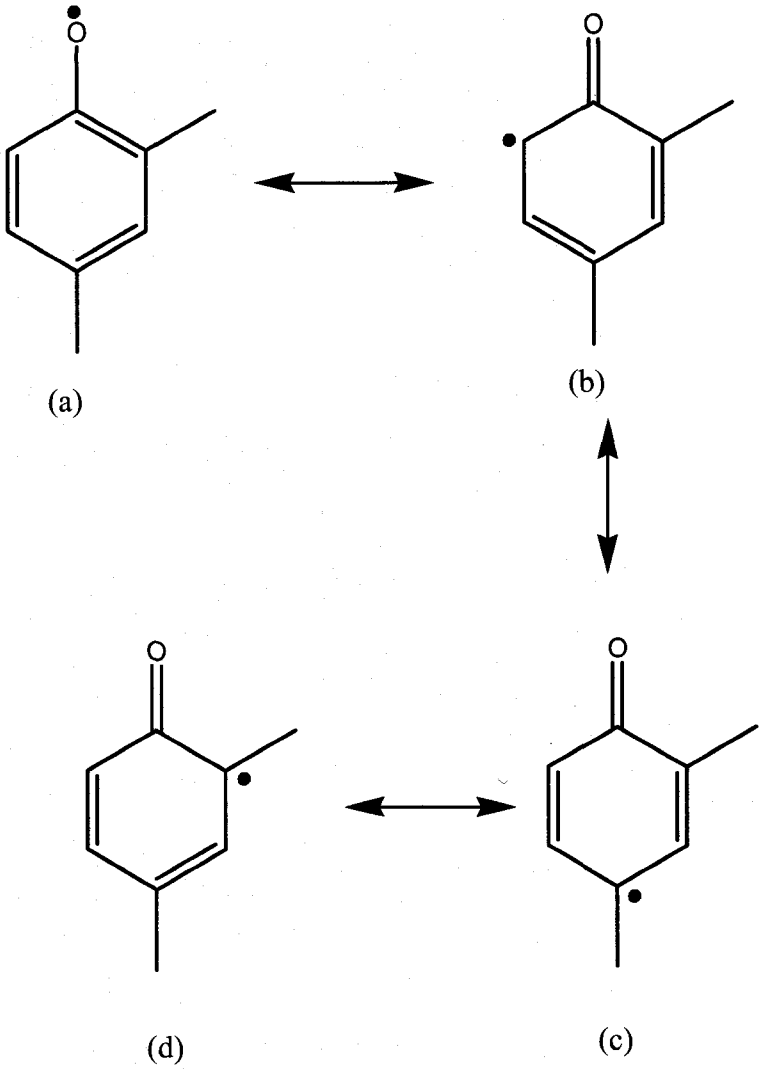
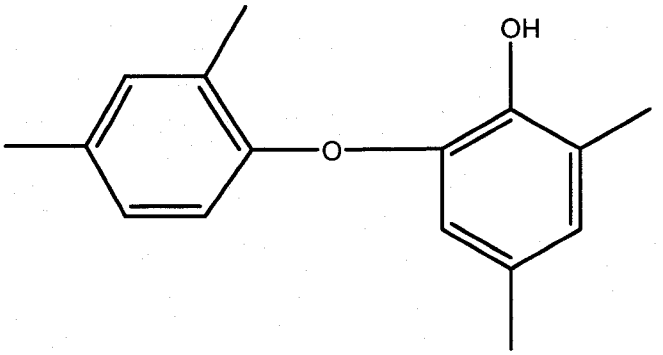
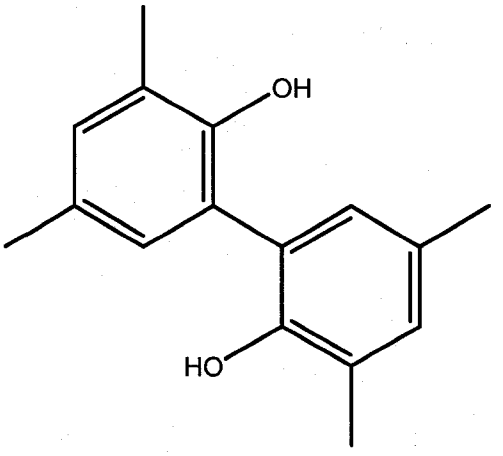



**Figure 4.40:** Resonance contributors of the 2,4-DMP radical

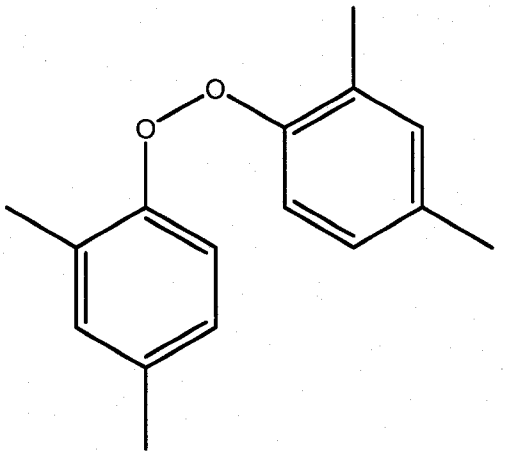



Reaction between contributors (a) and (b)

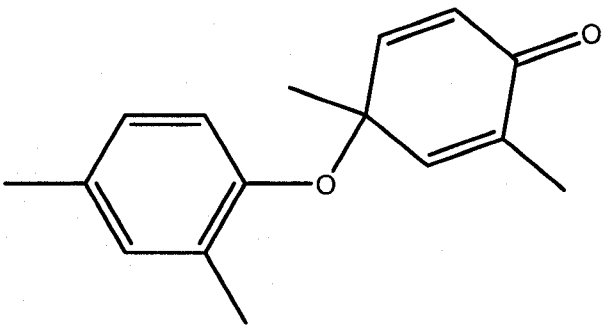



Reaction between contributors (b) and (b)

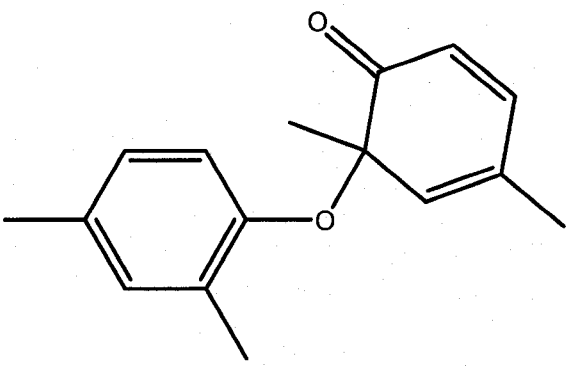
**Figure 4.41:** Stable dimer products of SBP catalyzed 2,4-DMP conversion



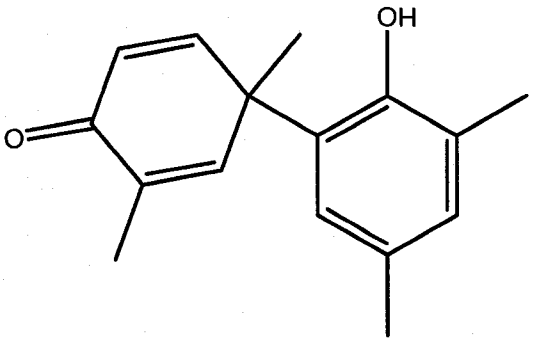

Reaction between contributors (a) and (a)




Reaction between contributors (a) and (c)

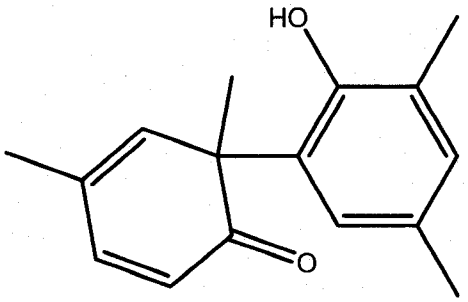



Reaction between contributors (a) and (d)

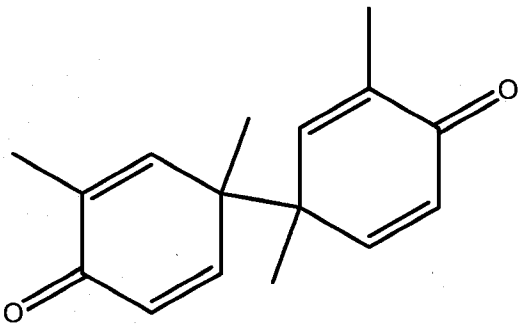



Reaction between contributors (b) and (c)

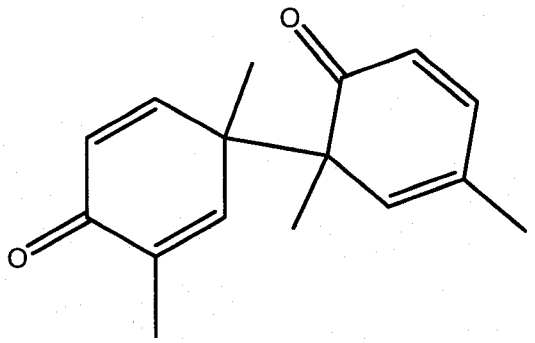
**Figure 4.42(a):** Unstable dimer products of SBP catalyzed 2,4-DMP conversion



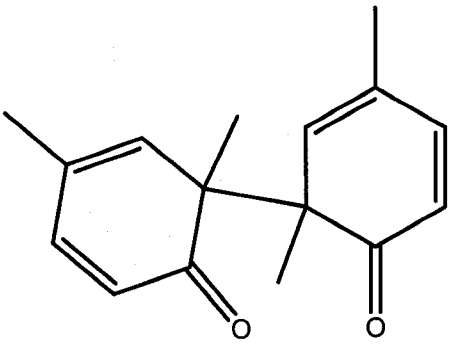

Reaction between contributors (b) and (d)




Reaction between contributors (c) and (c)




Reaction between contributors (c) and (d)




Reaction between contributors (d) and (d)

**Figure 4.42(b):** Unstable dimer products of SBP catalyzed 2,4-DMP conversion

# CHAPTER 5

# CONCLUSIONS & RECOMMENDATIONS

## 5.1 Summary

A number of batch experiments were carried out under different operating conditions to optimize the enzyme-catalyzed reaction and to have an understanding of the basic kinetics guiding this process. The following conclusions are drawn from the results of the experiments:

### *5.1.1 Process Optimization*

All experiments were designed to achieve a conversion of at least 95% of the initial 2,4-dimethylphenol (2,4-DMP) concentration while using soybean peroxidase (SBP). The reaction parameters optimized were pH, hydrogen peroxide concentration, soybean peroxidase concentration, PEG concentration and reaction time.

5.1.1 The optimum pH for 2,4-DMP conversion by SBP, both in presence and absence of PEG was 8.0. Inclusion of PEG increased the enzyme's tolerance in the pH range above the optimum.

5.1.2 The optimum hydrogen peroxide to 2,4-DMP ratio was found to be in the range 0.9-1.2. The enzyme showed tolerance to increase in hydrogen peroxide concentration beyond the optimum at lower 2,4-DMP concentrations but the same tolerance was not observed at higher substrate concentrations.

5.1.3 Minimum SBP concentrations required for more than 95 % conversion of 2,4-DMP concentrations ranging from 1.0 to 8.0 mM, followed a linear relationship, in presence of PEG,

$$y = 0.05\ x + 0.05$$

where x = initial 2,4-DMP concentration (mM) and y = minimum SBP concentration (U/mL).

However, the relationship between the minimum SBP concentration required and initial 2,4-DMP concentration, in absence of PEG, was linear only in the range of 0.2-2.0 mM,

$$y = 0.22\ x + 0.05$$

Above 2.0 mM 2,4-DMP, the minimum SBP concentration required was 0.5 U/mL irrespective of initial 2,4-DMP concentration. When the linear relationships in both cases are compared, it is seen that, under similar conditions of pH and temperature, the minimum SBP concentration required in absence of PEG was 4.4 times that required when PEG was present.

5.1.4 The minimum effective PEG concentration required for 1,3 and 5 mM of 2,4-DMP was found to be 45-50 mg/L.

5.1.5 Progress curves were observed for 1 and 5 mM 2,4-DMP concentrations. The enzyme catalyzed reaction was completed in the first 20 minutes; but further analysis of the reaction chromatograms showed that the reaction required extra time for the dimmers, trimers and higher polymers to polymerize non-enzymatically and precipitate out of the solution specially at higher substrate concentrations of 2,4-DMP.

### *5.1.2 Process Kinetics*

5.1.3 One substrate kinetic studies at 1.0 mM $H_2O_2$ and 1 to 10.3 mM 2,4-DMP concentration yielded the following values for the apparent Michaelis-Menten constants:

In the presence of PEG: $V_{max} = 6.72 \pm 2.76$ mM/min, $K_m = 15.85 \pm 9.83$ mM

In the absence of PEG: $V_{max} = 4.58 \pm 0.47$ mM/min, Km = $11.6 \pm 2.0$ mM

5.1.4 The initial velocities in the absence and presence of PEG remained almost the same up to substrate concentrations of 5 mM, beyond which PEG increased the initial velocities at each substrate concentration. The enzyme specificity, $V_{max}/K_m$, was equal to 0.40 and 0.42 in the absence and presence of PEG, respectively, which shows that the two parameters did not change on addition of PEG. It was also observed that in the presence of PEG, the data were less consistent as those in absence of PEG.

## 5.2 Conclusions

In this study, it has been found that after a particular substrate concentration, the minimum enzyme concentration becomes independent of initial 2,4-DMP concentration, which means SBP can be used more efficiently at higher substrate concentrations and this fact can be used for bio-remediation purposes. Moreover, as the "PEG effect" at higher concentrations becomes less significant, the cost of additional chemicals like PEG can be saved. Because of all these findings, the potential of SBP in the field of bioremediation should be seriously explored.

## 5.3 RECOMMENDATIONS

To ensure that the SBP catalyzed polymerization process is economically and industrially viable, further improvements are needed. The major cost for the enzymatic process is the cost of enzyme itself, so investigation needs to be done to increase the lifetime and stability of the enzyme in solution.

From previous studies (Wu et al. 1996, 1997), it can be seen, that the addition of PEG considerably reduces the amount of enzyme required. This study shows that at lower 2,4-DMP concentration, a considerable PEG effect exists which gradually reduces and finally becomes nil at the highest substrate concentration. Research is required to explain this and also to develop a model for the dependence of the protective effect of PEG on initial substrate concentration.

While determining the kinetic constants of SBP catalyzed 2,4-DMP removal it has been assumed that the enzyme suffers substrate inhibition at higher substrate concentrations and thus only the range of data with increasing initial velocities were taken and simple Michaelis-Menten model was fitted to it. However, a complex Michaelis-Menten model needs to be developed which will take into account the mechanism of substrate inhibition. The possibility of other inhibitory effects should be studied, e.g. by hydrogen peroxide on reaction rate and if so it would need to be incorporated in the model.

To ensure that the enzymatic treatment process is industrially viable, it needs to be implemented on a continuous scale. As a result, development of a continuous flow reactor is essential. The development of this process should also include application to real wastewater matrices.

The main objective of enzymatic treatment is to be an environmentally friendly treatment alternative. The enzymatic process involves the formation and precipitation of polymeric by-products from solution. Ghioureliotis and Nicell (2000) have determined the toxicity of soluble byproducts of peroxidase-catalyzed polymerization of phenolic compounds. Similarly, investigation is required to determine whether these new products are more toxic than the starting monomer pollutants. Therefore, it is very important to do product identification to understand the nature and toxicity of soluble and insoluble by-products.

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# APPENDICES

# Appendix A

# Soybean Peroxidase Activity Assay

**1. General:**

The purpose of enzyme activity assay is to determine the amount of active enzyme present in the solution. The soybean peroxidase activity colorimetric assay utilized the coupling of phenol to 4-AAP with hydrogen peroxide as the oxidant. This assay was run at pH 7.4. This test determines the activity of peroxidase enzyme by monitoring the appearance of color in the sample.

$$AAP + phenol + H_2O_2 \longrightarrow \text{Pink chromophore}$$

(Wavelength max 510 nm)

**2. Reagents**

10 mM phenol

2.4 mM 4-AAP

0.2 mM hydrogen peroxide

50 mM of phosphate buffer of pH 7.4.

**3. Procedure**

In a semi-micro cuvette,

950 µl of the reagent

50 µl of dilute enzyme

The above two were mixed for a final volume of 1.0 ml and monitored at 510 nm, 25°C. Immediately, after the addition of the sample, shake the cuvette and then place it in the spectrophotometer to monitor the rate by calculating the change in absorbance over change in time (the slope of the line produced), which is proportional to the rate of hydrogen peroxide consumption.

**4. Calculation**

The enzyme activity is calculated as follows

- The rate of change in absorbance between 0 and 20 s is calculated:

$$Rate = \frac{\Delta A}{\Delta t}$$

- Activity in the cuvette (U/ml) $= \frac{\Delta A \times 1.0mL}{(6.0mM^{-1}cm^{-1})(1.0cm)}$ x D

  Where,

  $\Delta A$ = change in absorbance per minute

  1.0 = total volume in the cuvette (mL)

  6.0 = micro molar extinction coefficient ($mM^{-1}cm^{-1}$)

  D = dilution factor

Enzyme activity was determined in units of catalytic activity per milliliter (U $ml^{-1}$), with 1.0 U equal to the amount of enzyme required to convert 1.0µmol of hydrogen peroxide/minute (Ibrahim et al. 2001). Figure A-1 represents the change in absorbance with time while measuring the enzyme activity.

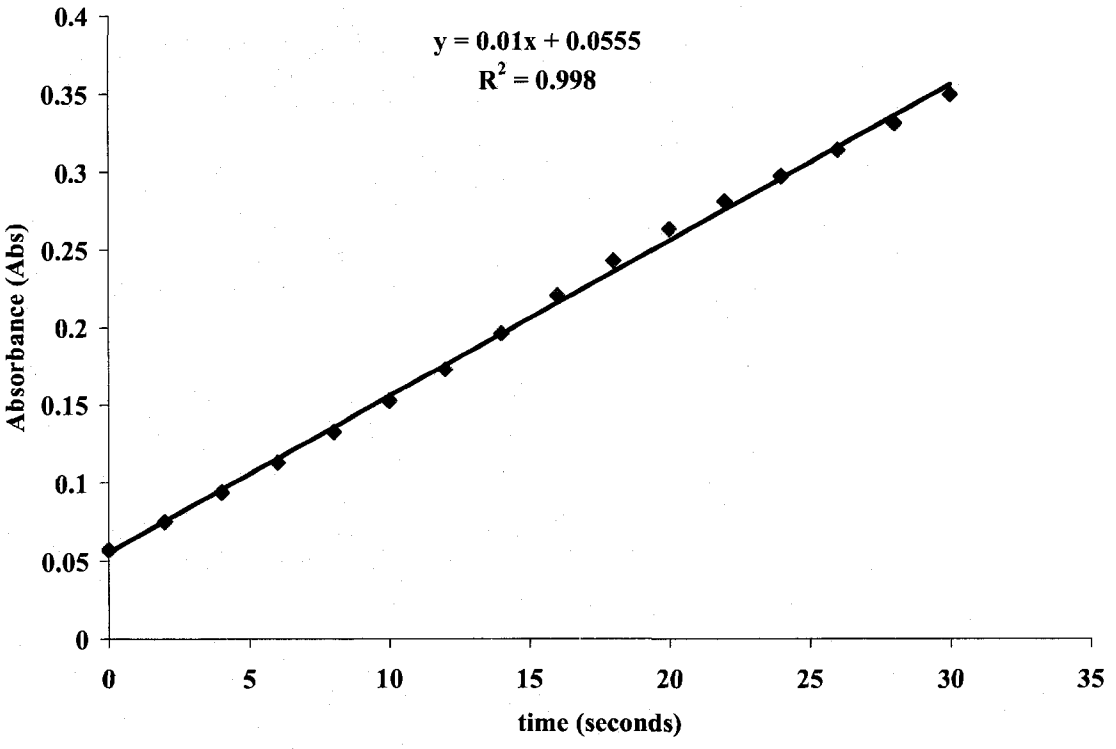


**Figure A-1:** Enzyme activity standard calibration curve

# Appendix B

# Standard curve for 2,4-dimethylphenol

## 1. General

HPLC was used to identify and quantify 2,4-DMP. A standard curve was prepared which was used to determine the concentration of 2,4-DMP in the reaction and product.

## 2. HPLC standard Curve

Different known concentration of 2,4-DMP ranging from 0.02-15 mM were prepared. The samples were run in HPLC for 12 minutes. Peak areas are measured for these concentrations of 2,4-DMP, 0.2-15 mM at 278 nm (60:40 = acetonitrile: 0.1 % acetic acid was used for elution). A standard curve was developed from the data obtained and was used to measure the concentrations of 2,4-DMP in sample solution.

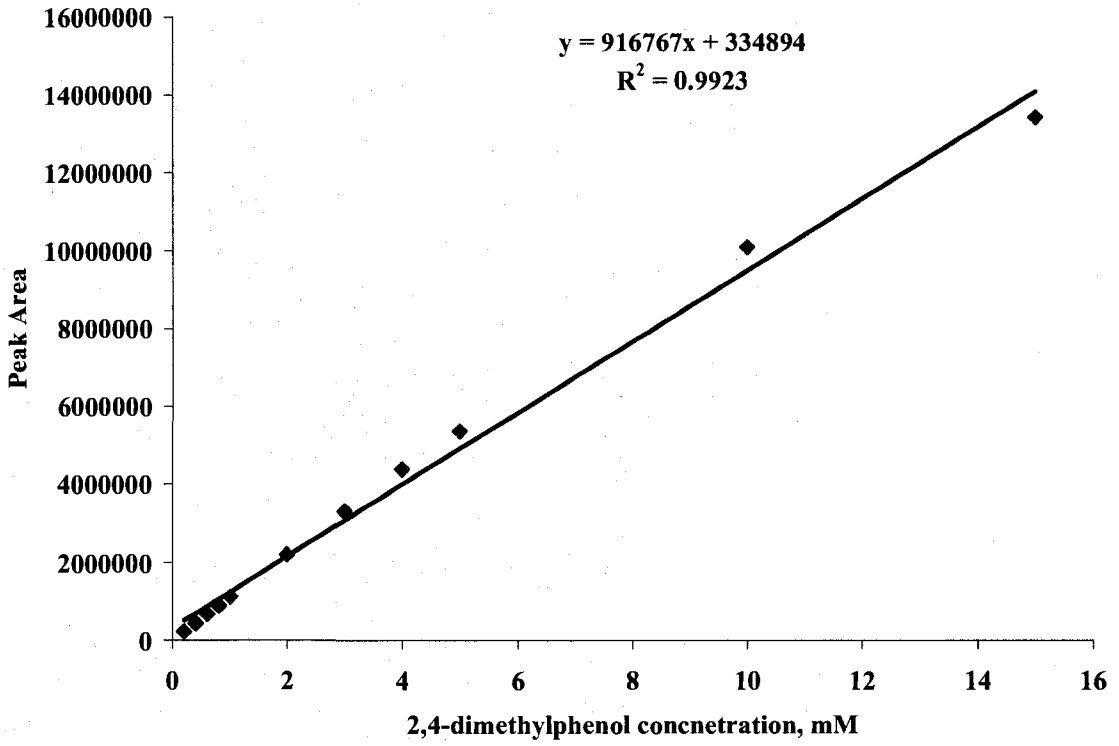


**Figure B-1:** HPLC Standard curve for 2,4-DMP

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