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## **NOTE TO USERS**

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**REMOVAL OF PHENOLIC COMPOUNDS FROM SYNTHETIC  
WASTEWATER USING SOYBEAN PEROXIDASE**

by

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**A thesis submitted to the  
College of Graduate Studies and Research through  
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in partial fulfillment of the requirements for the  
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at the University of Windsor**

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## ABSTRACT

Soybean peroxidase (SBP), an acidic peroxidase which is isolated from the hulls of the bean, catalyzes the oxidation of various aqueous aromatic compounds in the presence of hydrogen peroxide. The polymers formed during the reaction have a lower solubility than their monomeric precursors, and are readily precipitated from solution.

Experiments were conducted to investigate the efficiency of using SBP to remove several different phenolic compounds from unbuffered synthetic wastewater. All tests were carried out in continuously stirred batch reactors. The phenol derivatives studied included parent phenol, chlorinated phenols and methyl phenols. The optimum conditions to achieve at least 95% removal of the phenolic compounds were determined for the following parameters: pH, SBP dose in the absence and presence of polyethylene glycol (PEG), hydrogen peroxide to substrate ratio, and PEG dose.

Experimental results showed that SBP efficiently removed aromatic compounds from wastewater in the presence of hydrogen peroxide. An increase in the hydrogen peroxide to substrate ratio beyond the optimum resulted in enzyme inactivation, which reduced the substrate removal efficiency. The optimum pH for different phenolic compounds ranged from 5.5 to 8. For each substrate, the optimum enzyme dose varied significantly with the lowest being 0.015 U/mL for bisphenol A and with the highest being 0.60 U/mL for phenol. The studies showed that PEG reduced the amount of SBP required for 95% removal of the substrate by up to 60 times, as was the case for bisphenol A. For all substrates, except p-cresol, an increase in PEG dose beyond the optimum did not significantly increase or decrease the removal efficiency.

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## **1. INTRODUCTION**

Phenolic compounds are discharged in the wastewater streams of various industries such as coal conversion, wood preservation, metal casting, and pulp and paper manufacturing. Most of these compounds are toxic and have been classified as hazardous pollutants. Phenols in water have special adverse effects since as little as 0.005 mg/L of phenol will cause objectionable tastes and odours when it combines with chlorine to form chlorophenols (Lanouette, 1977). The development of proper technology for the removal of phenolic compounds from wastewater is considered to be a top priority due to the increasingly stringent effluent discharge requirements. The present treatment methods for removing aromatic compounds from wastewater, including chemical oxidation, adsorption on activated carbon and solvent extraction, suffer from many drawbacks. The introduction of an enzyme based treatment technology by Klibanov *et al.* (1980) is expected to be a feasible alternative to the conventional treatment methods.

### **1.1 Enzyme Background**

Using enzymes to remove toxic pollutants from wastewater is not a new concept; however, extensive research on the treatment of phenolic wastewater using enzymes has begun only recently. All enzymes are proteins, which are large molecules consisting of amino acid units joined in series. These biological catalysts increase the rate of the chemical reaction which is taking place (Palmer, 1981). The substrate of an enzyme is defined as the reactant of an enzyme-catalyzed reaction. The region which contains the binding and catalytic sites is termed as the active site of the enzyme.

The enzyme used in this study is a peroxidase, called soybean peroxidase (SBP). Peroxidases oxidize reduced compounds in the presence of hydrogen peroxide. Several other peroxidases have been studied, with horseradish peroxidase (HRP) being the most extensively researched to date. Although the SBP enzyme has not been used in research as much as HRP, it is considered to be a suitable alternative to the other peroxidases. Previous studies in our labs (Al-Kassim *et al.*, 1995) indicated that SBP is effective in removing several different phenolic compounds from wastewater. This process produces a minimal amount of waste and is cost competitive with the other peroxidases that have been studied.

## **1.2 Wastewater Treatment**

An enzymatic method for treating phenolic compounds in wastewater has been developed, which uses peroxidases in the presence of hydrogen peroxide to form phenoxy radicals. These radicals couple to form larger oligomers which are practically insoluble in water and can be easily separated by filtration or sedimentation. Phenolic compounds come from a variety of sources and this method has many advantages over conventional treatment processes.

### **1.2.1 Sources of Phenolic Compounds**

Phenolic compounds are produced from a variety of industries. Phenol was widely used in the 19th century as an antiseptic and local anaesthetic. Today, the largest single use of phenol is in the production of phenolic resins, and next is its use in the production of caprolactam (an intermediate in the production of nylon 6), and in the production of bisphenol A (which is mainly used in the production of phenolic resins).



Phenolic resins are used as a binding material in insulation materials, chipboard, paints and casting sand foundries (World Health Organization, 1994). Phenols are used in the manufacture of paint and varnish removers, lacquers, rubber, ink, illuminating gases, tanning dyes, perfumes and soaps. Phenolic wastes are produced during the coking of coal, distillation of wood, and the operation of gas works and oil refineries.

Chlorinated phenols are mainly used as intermediates in the production of other chemicals, in the synthesis of dyes, pigments, phenolic resins, pesticides and herbicides and as a wood preservative. The isomer 2-chlorophenol is used as an intermediate for fire-retardant varnishes, for cotton fabric treatment to provide rot resistance, and as an ingredient in coal processing. The compound 4-chlorophenol is used as an intermediate for higher chlorophenols, dyes and fungicides and 2,4-dichlorophenol is an ingredient of antiseptics (World Health Organization, 1989).

Cresols are used as solvents, disinfectants, and in the production of fragrances, antioxidants, dyes, pesticides, resins, and as wood preservatives. Cresols are contained in crude oil, coal tar, and flyash from coal and wood combustion (World Health Organization, 1995). The isomer o-cresol is used as a chemical intermediate for deodorizing and odour enhancing compounds and pharmaceuticals. The compound p-cresol is mainly used in the formulation of antioxidants for lubricating oil and motor fuel, rubber, and polymers. The isomer m-cresol is used in the manufacture of explosives and in the production of herbicides and insecticides.

Since phenolic compounds are produced and used in a number of different industrial processes, treatment of these wastewater streams is of great importance. The enzymatic treatment can either be used as a pre-treatment in conjunction with methods already in place or to replace conventional methods.

### 1.2.2 Conventional Methods

The decision on a waste treatment process selection comprises the evaluation of many factors, such as effluent requirements and economic feasibility. Table 1.1 lists the current methods used for treating phenolic wastes.

**Table 1.1: Conventional Treatment Technologies for Phenolic Compounds (Nicell, 1991)**

<b>Recovery Systems</b>	Countercurrent extraction Pulsed column extraction
<b>Physical/Chemical Treatment Systems</b>	Chlorine oxidation Chlorine dioxide oxidation (as sodium chlorite) Ozone oxidation Hydrogen peroxide oxidation (Fenton's reagent) Potassium permanganate oxidation Incineration Hydrocarbon stripping and combustion Photocatalytic oxidation Activated carbon adsorption Landfilling Coagulation
<b>Biological Treatment Systems</b>	Biooxidation pond Aerated lagoon Stabilization pond Oxidation ditch Trickling filter Activated sludge Rotating biological contactors

These conventional methods suffer from such serious drawbacks as incomplete removal, high cost, formation of hazardous by-products and applicability to a small concentration range. Biological treatment, for example, is dependent on the health and activity of the microbial population. These microorganisms require sufficient food and oxygen, and stable environmental conditions, including pH and temperature, if they are to

maintain optimum efficiency (Lanouette, 1977). Hence, with these shortcomings, alternative methods are desirable.

### **1.2.3 Use of Enzymes**

Enzymes are often preferred over intact organisms since the isolated enzymes act with greater specificity, which allows specific groups of pollutants to be targeted. The enzymes are easier to handle and store than microorganisms, and enzyme concentration is not dependent on bacterial growth rates (Nicell *et al.*, 1995).

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

- application to a broad range of compounds;
- action on, or in the presence of, many substances which are toxic to microbes;
- operation at both high and low concentrations of contaminants;
- operation over wide temperature, pH and salinity ranges;
- no shock loading effects;
- no delays associated with acclimatization of biomass;
- reduction in sludge volume (no biomass generation);
- better defined system with simpler process control.

The same group also discussed the following potential advantages of an enzyme based treatment over chemical/physical processes:

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

Klibanov *et al.* (1980) developed an enzymatic method for removing over 30 different phenols and anilines from wastewater using horseradish peroxidase (HRP).

They noted certain reaction parameters which were of significant importance, such as pH, hydrogen peroxide concentration and peroxidase concentration. Their results indicated that extremely large amounts of HRP were required and, therefore, this proposed method of treatment did not seem very promising due to the high cost of the enzyme. Continued research showed that a great reduction in enzyme requirements could be achieved by using additives such as gelatin and polyethylene glycol (Nakamoto *et al.*, 1992). Wu Y. *et al.* (1997) compared different additives in the removal of phenolic compounds and concluded that polyethylene glycol (PEG) was the best additive and that the amount of horseradish peroxidase required decreased significantly with an increase in PEG dose. Although HRP efficiently removed phenolic compounds from wastewater, it is unlikely ever to become a commodity suitable for this process due to the vagaries of root cultivation and processing (Taylor *et al.*, 1996). All previous studies were conducted in buffered solutions.

### **1.3 Objective**

The objective of this study was to optimize the reaction parameters, in unbuffered tap water, to achieve at least 95% removal of several different aromatic compounds by using soybean peroxidase (SBP).

### **1.4 Scope**

The scope of this study included:

- (i) Aromatic compounds studied were: parent phenol, 2-, 3-, 4-chlorinated phenols, o-, m-, p-cresols, 2,4-dichlorophenol, 4,4'-Isopropylidenediphenol

(referred to hereafter as bisphenol A). The chemical structures of these compounds are shown in Appendix D.

- (ii) Parameters optimized included: pH, SBP enzyme dose in the presence and absence of polyethylene glycol (PEG), molar ratio of hydrogen peroxide dose to substrate dose, and PEG dose.
- (iii) Effect of coprecipitation of two substrates on the optimum enzyme dose.
- (iv) Effect of PEG on the optimum enzyme dose.

## **2. LITERATURE REVIEW**

### **2.1 Peroxidase Background**

Selectivity for the removal of certain compounds may be important in order to meet increasingly strict regulatory criteria or to facilitate further treatment. If toxic compounds are removed selectively, the bulk of the organic material in wastewater may be treated biologically, therefore reducing overall treatment costs. Enzymatic treatment represents one method by which selective removal of pollutants may be accomplished (Aitken, 1993).

The applicability of an enzyme based treatment technology for the removal of aromatic compounds in an aqueous mixture has been studied by many researchers. Klibanov *et al.* (1980) were first to propose a horseradish peroxidase (HRP) method, which was used to remove over 30 different phenols and aromatic amines from water with removal efficiencies for some pollutants exceeding 99%. The removal efficiency is the percentage of the chemical removed from solution under specified conditions. Table 2.1 provides a complete list of the compounds studied by Klibanov and co-workers as summarised by others. The parameters which were investigated included reaction time, pH, hydrogen peroxide dose and enzyme dose. The experiments indicated that treatment for 3 hours resulted in 99.8% removal efficiency. One compound, *o*-chlorophenol, was chosen for further investigation and the results were as follows: there was a broad maximum in removal efficiency between pH 6 and 8; removal efficiency increased upon increasing the hydrogen peroxide concentration (H<sub>2</sub>O<sub>2</sub>) until it reached a maximum; with an increase in peroxidase concentration there was an increase in the removal efficiency.

**Table 2.1: Enzymatic Removal of Aromatic Amines and Phenols from Water by Horseradish Peroxidase (Nannipieri and Bollag, 1991)**

<b>Compound</b>	<b>Removal Efficiency (%)</b>	<b>H<sub>2</sub>O<sub>2</sub> (mM)</b>	<b>pH</b>
Benzidine	99.9	1	5.5
3,3'-Dimethoxybenzidine	99.9	1	5.5
3,3'-Diaminobenzidine	99.6	1	5.5
3,3'-Dichlorobenzidine	99.9	1	5.5
3,3'-Dimethylbenzidine	99.6	1	5.5
1-Naphthylamine	99.7	5	5.5
2-Naphthylamine	98.3	5	5.5
5-Nitro-1-naphthylamine	99.6	5	5.5
N,N'-Dimethylnaphthylamine	93.2	5	5.5
Phenol	85.3	1	4.0
2-Methoxyphenol	98.0	1	5.5
3-Methoxyphenol	98.6	1	5.5
4-Methoxyphenol	89.1	1	7.0
2-Methylphenol	86.2	1	4.0
3-Methylphenol	95.3	1	4.0
4-Methylphenol	85.0	1	5.5
2-Chlorophenol	99.5	1	7.0
3-Chlorophenol	66.9	1	7.0
4-Chlorophenol	98.7	1	5.5
2,3-Dimethylphenol	99.7	1	4.0
2,6-Dimethylphenol	82.3	1	5.5
Aniline	72.9	1	7.0
4-Chloroaniline	62.5	1	5.5
4-Bromoaniline	84.5	1	5.5
Fluoroaniline	86.4	1	7.0
1,3-Diaminophenol	98.6	1	7.0
Diphenylamine	80.5	1	7.0
1-Naphthol	99.6	1	4.0
2-Nitroso-1-naphthol	98.9	1	4.0
4-Phenylphenol	99.9	1	4.0
4-Hydroxyquinoline	99.8	1	7.0

Although HRP is the peroxidase that has been most researched, other peroxidases, such as *Coprinus macrorhizus* peroxidase (CMP), *Arthromyces ramosus* peroxidase (ARP), and soybean peroxidase (SBP) have proven to be very effective in removing phenolic compounds from water (Al-Kassim *et al.*, 1994, 1993; McEldoon *et al.*, 1995).

Plant peroxidases possess a wide substrate specificity and, in the presence of hydrogen peroxide, can oxidize a large variety of aromatic compounds (McEldoon *et al.*, 1995). These compounds are oxidized by hydrogen peroxide through an iron atom located at the catalytic site of the enzyme. The peroxidatic reaction mechanism is shown below (Dunford, 1991):



In this catalytic cycle, the native enzyme (E) is oxidized by hydrogen peroxide to an active intermediate referred to as Compound I (E<sub>i</sub>). Compound I accepts an aromatic compound (AH<sub>2</sub>) into its active site. The aromatic compound is oxidized, resulting in the release of a free radical (• AH) back into the solution. The enzyme is now in the Compound II (E<sub>ii</sub>) state and oxidizes another aromatic compound, releasing a second free radical into solution. This final step returns the enzyme to its original state, thus completing the catalytic cycle.



The overall enzymatic reaction is as follows:



The free radicals formed during this cycle diffuse from the active center of the enzyme into solution where they combine to form polymers with reduced solubility that tend to precipitate out of solution. The polymers which remain in solution can be oxidized again resulting in the formation of a larger polymer, which in turn has a further reduced solubility. In the removal of phenol, the radicals formed are phenoxy radicals that could couple with each other to generate tetramers (Yu, *J et al*, 1994).

Although Reactions (1) to (3) dominate in an aqueous mixture of enzyme, hydrogen peroxide and aromatic substrate, there are some side reactions that also occur which are believed to be responsible for the inactivation and inhibition of the enzyme (Nicell *et al*, 1993). It has been reported by Arnao *et al*. (1990) that once the enzyme is in the Compound II state, it can be oxidized by excess hydrogen peroxide to form Compound III ( $\text{E}_{\text{iii}}$ ) according to the following equation:



The return to the native enzyme is extremely slow, and therefore once in the Compound III form, the enzyme is sufficiently ineffective in carrying out the oxidation of aromatic substrates.

## **2.2 Use of Peroxidase in Wastewater Treatment**

Selection of a waste treatment process involves the evaluation of many parameters, such as: pollutants required to be removed, permit requirements for disposal, economic feasibility, and the potential to form toxic by-products which need subsequent treatment. Since standards for the discharge of phenolic compounds are becoming more strict, there is a demand for improved methods of treatment.

There are many advantages of an enzyme based treatment over conventional biological and chemical/physical treatments which have been discussed by Nicell *et al.* (1995). In most instances, physicochemical treatment processes are not very selective in terms of the number of pollutants removed during treatment; therefore, such processes are more economically feasible for the treatment of dilute wastewaters. Chemical oxidation, for example, can become very expensive for high strength wastes, although the targeted pollutants might have a low concentration. Activated sludge is commonly used to reduce organic load in municipal and industrial wastewaters; however, it has difficulty in removing toxic pollutants to low levels (Aitken, 1993).

Although the enzymatic method has many advantages over conventional treatment technologies, it has a significant disadvantage: the relatively short catalytic lifetime of the enzyme. The short catalytic lifetime has been attributed to the inactivation of the peroxidase. Klibanov *et al.* (1983) has suggested that this inactivation occurs during the enzymatic reaction due to the interactions of the phenoxy radicals with the enzyme's active site. Therefore, this enzymatic method has not been considered to be a feasible option for application due to the extremely high cost of the enzyme.

Nakamoto and Machida (1991), on the other hand, have reported that enzyme inactivation is a result of the end-product polymer which adsorbs the enzyme and hinders the access of substrate to the enzyme's active site. They showed that the amount of enzyme could be greatly reduced by adding proteins or hydrophilic synthetic polymers, such as gelatin and polyethylene glycol (PEG). These additives inhibit the interactions between the enzyme and the phenolic polymers.

Wu J. *et al.* (1993) studied the effect of PEG on the minimum horseradish peroxidase (HRP) dose for 95% removal of phenol. They concluded that PEG protected the enzyme activity, and that 1/40 and 1/75 of the original amount of enzyme was required in the presence of PEG, for 1 and 10 mM phenol solutions, respectively. These results were confirmed by Wu Y. *et al.* (1997) where they compared different additives on the removal of phenolic compounds using HRP. The additives selected for the study were PEG, gelatin, and two polyelectrolytes, PERCOL LT24 (cationic) and PERCOL LT20 (non-ionic). Wu Y. and co-workers concluded that polyethylene glycol was better than the other additives for several reasons. They found that excess PEG had no negative effect on the reaction whereas gelatin and the polyelectrolytes actually lowered the removal efficiency and also resulted in not forming particles. The effluent quality of the wastewater which was treated with PEG was better since at minimum PEG dose, there was little PEG left in solution; however, a considerable amount of gelatin remained in solution even at its minimum dose. Gelatin also produced more precipitate than PEG because the minimum gelatin doses were found to be higher than the minimum PEG doses. The results also indicated that the minimum HRP dose required for 95% removal of the aromatic compounds in the presence of PEG was 1/100 less than the original

amount required without PEG for both 2-chlorophenol and 3-chlorophenol (Wu, Y *et al.*, 1993). These reduced amounts of enzyme can make this method more economically competitive with the conventional treatment methods.

Klibanov *et al.* (1983) proposed to remove phenols from coal-conversion wastewaters using HRP. They studied a typical coal-gasification wastewater which contained contaminants other than phenol, such as: ammonia, chloride, cyanide and thiocyanate. The removal efficiency was found to be 97% as compared to 98% for the same phenol concentration but in the absence of the other contaminants. They also found that easily removed phenols aid in the enzymatic precipitation of hard to remove aromatic compounds.

The removal of phenols from a foundry wastewater using HRP was investigated by Cooper *et al.* (1995). These studies were conducted not only to optimize the operating conditions, but also to reduce costs. The economic feasibility of this process rested on reducing the cost of the enzyme. Two approaches were taken to minimize overall treatment costs: (i) evaluate the use of an additive to reduce enzyme requirements; (ii) examine the potential of using a low purity enzyme to achieve equivalent removal at a lower cost. Through experiments, they found that PEG greatly reduced the cost associated with the enzyme. The use of PEG, a relatively inexpensive chemical, reduced the HRP requirements by 1/22 of the original enzyme requirements, which in turn greatly reduced the overall enzymatic treatment cost. Their results also showed that this process was capable of achieving 97-99% removal of total phenols from a foundry wastewater using either purified HRP or a crude HRP extract.

As previously mentioned, it has been suggested that easily removed aromatic compounds aid in the removal of other compounds with lower removal efficiencies (Klibanov *et al.*, 1980). These studies showed that the removal efficiency of phenol increased in the presence of more easily removable compounds. Table 2.2 shows some of these results. Klibanov and co-workers have suggested two explanations, the first one being that phenol has a low reactivity toward peroxidase and is therefore poorly removed. Consequently, the addition of more easily removed compounds increases the yield of free radicals which results in a higher formation of the polymeric products. The second explanation is that phenol is reactive towards peroxidase but the by-products have a low molecular mass and are soluble in water. The addition of compounds with a higher removal efficiency results in the formation of a polymer with a higher molecular mass and therefore precipitates out of solution.

**Table 2.2: Efficiency of the Enzymatic Removal of Phenol in the Absence and in the Presence of Other Compounds (Klibanov *et al.*, 1980)**

<b>Pollutant</b>	<b>Added Compound</b>	<b>Removal Efficiency (%)</b>
Phenol	None	74.6
Phenol	o-Dianisidine	99.7
Phenol	Benzidine	99.5
Phenol	8-Hydroxyquinoline	99.8

These results indicate that more efficient removal of certain pollutants occurs from a mixture of different aromatic pollutants, which is typical of a real wastewater matrix.

### **3. MATERIALS AND METHODS**

#### **3.1 Materials**

Soybean peroxidase (medium purity, 37 purpurogallin units/mg solid) was purchased from Enzymol International Inc., Columbus, OH. Using the enzyme activity assay described in Appendix A (Wu, 1993), the specific activity of the enzyme was determined to be 14 U/mg dry solid. A unit of activity is defined as the number of micromoles of substrate converted per minute at pH 7.4 and temperature 25°C. The enzyme was stored as a dry powder at a temperature of -15°C, while the aqueous soybean peroxidase stock solution was stored in a refrigerator at 4°C.

Catalase (EC 1.11.1.6, 15000 units/mg solid) and polyethylene glycol (with an average molecular mass of 3350 g/gmole) were purchased from Sigma Chemical Co., St. Louis, MO. One unit of catalase decomposes one micromole of hydrogen peroxide per minute at pH 7.0 and temperature 25°C. An aqueous stock solution of catalase was stored at 4°C.

Hydrogen peroxide (30% by mass over volume) was purchased from BDH Inc., Toronto, Ontario. The diluted hydrogen peroxide solutions used in the analysis were prepared daily.

Phenol, chlorinated phenols and methyl phenols, with a purity of 99% or greater, were supplied by Aldrich Chemical Co., Milwaukee, WI. Stock solutions were stored at 4°C.

### **3.2 Analytical Equipment**

The colour absorbance was measured using a Hewlett Packard Diode Array Spectrophotometer Model 8452A (with a wavelength range of 190 to 820 nm and 2 nm resolution), which was controlled by a Hewlett Packard Vectra ES/12 computer. Quartz cells with a 10 mm path length were purchased from Hellma (Canada) Limited, Concord, Ontario. The polystyrene disposable semi-micro cuvettes were purchased from Bio-Rad Laboratories, Hercules, CA.

Samples were centrifuged at 3000 rpm for 30 minutes, using an IEC Centra-8 Centrifuge, supplied by International Equipment Company, USA.

An Expandable Ion Analyzer EA 940, manufactured by Orion Research, was used to measure the pH of the samples. Standard buffer solutions of pH 4.00, 7.00 and 10.00 were purchased from BDH Inc., Toronto, Ontario.

### **3.3 Experimental Procedure**

The experiments in this study were done in batch reactors and were conducted at room temperature, approximately 22°C. This study was designed to achieve at least 95% removal of the aromatic substrate by optimizing the following parameters: pH, PEG dose, SBP dose both with and without PEG, and H<sub>2</sub>O<sub>2</sub> dose.

The batch reactors were glass vials which contained 30 mL of a mixture of aromatic substrate, H<sub>2</sub>O<sub>2</sub>, PEG, SBP enzyme and tap water. The tap water was boiled to remove the chlorine and then it was stirred overnight using a magnetic stirrer and a teflon coated stir bar. The aeration of the tap water was done in order to reintroduce carbon dioxide which raised the buffering capacity of the water solutions otherwise unbuffered.

The components of the sample mixture were added in the following order: aromatic substrate, PEG, SBP enzyme and tap water. Reactions were initiated by adding a known amount of H<sub>2</sub>O<sub>2</sub> to each reactor. For each experiment, there was one control reactor which contained every component as the other reactors except for H<sub>2</sub>O<sub>2</sub>. The control reactor was used to measure the initial amount of the aromatic substrate present in the sample mixture. All of the reactors, including the control reactor, were stirred vigorously for three hours using a magnetic stirrer and teflon coated stir bars. At the end of the reaction period, catalase was added to final concentration of 125 U/mL to stop the reaction and alum was added to a final level of 50 mg/L to the mixture and stirred. The pH of each sample was adjusted to between 6.3 to 7.5 and then stirred again at low speed to allow for the formation of the floc. Approximately 5 mL of each sample was centrifuged, after which the supernatant was analyzed for remaining substrate by the colorimetric method. The following four sections and Figure 3.3.1 describe the procedure used to optimize all of the reaction parameters.

### 3.3.1 pH

The first parameter that was optimized was pH. The reaction mixture consisted of tap water, aromatic substrate, PEG and SBP enzyme. The substrate concentration was 1mM for all of the different phenolic compounds that were tested except for bisphenol-A, which had a total concentration of 0.5 mM in the reactors. The SBP dose used in these experiments was determined from previous studies and the PEG dose in each reactor was 400 mg/L. The pH was then adjusted within the range of 4.00 to 10.00 using concentrated HCl or NaOH.



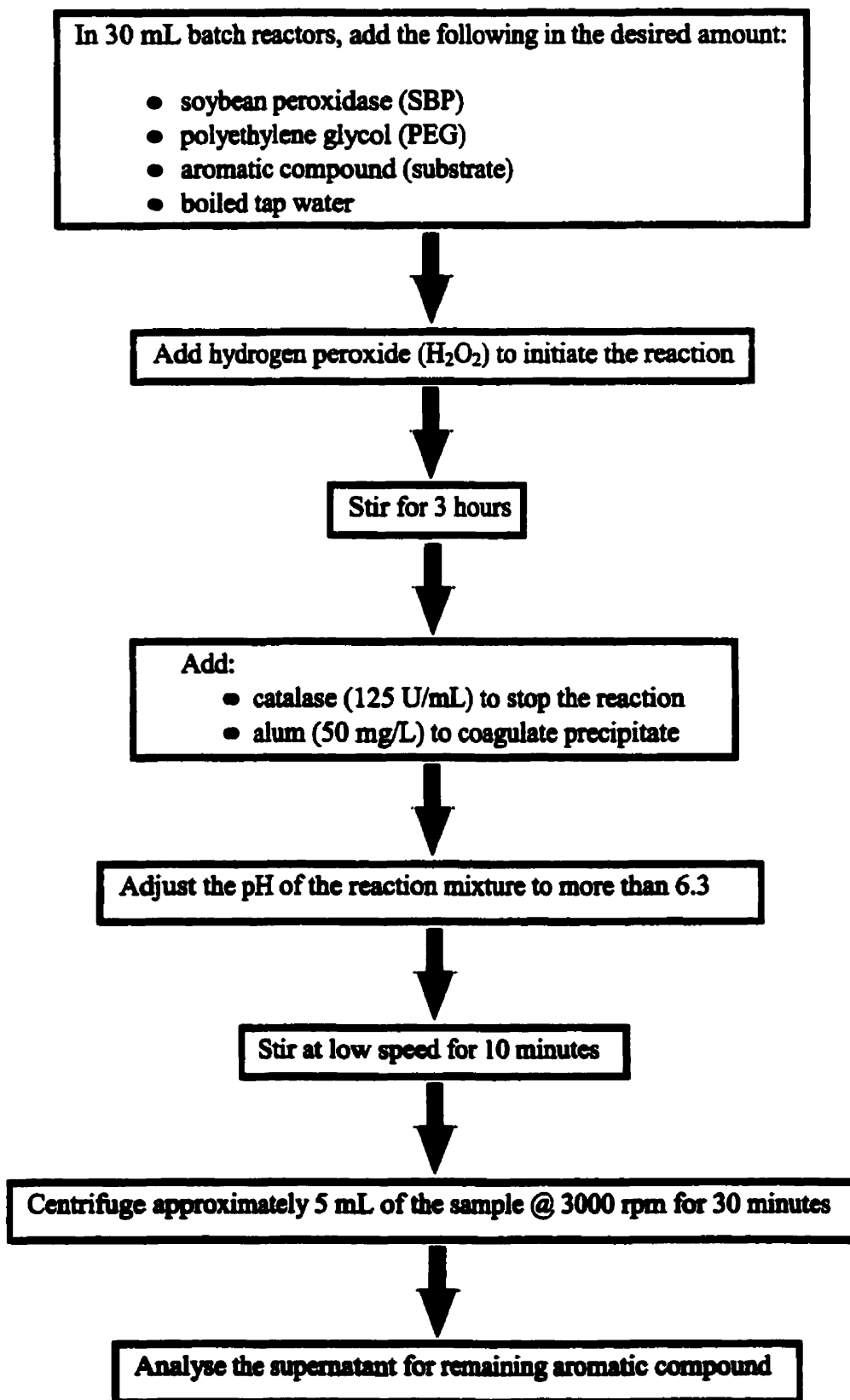


Figure 3.3.1: Flow Chart for Experimental Procedure

### **3.3.2 Peroxidase Dose**

Stock solutions of SBP were prepared from a dry solid and were refrigerated for up to one week at 4°C. Enzyme concentrations are expressed in terms of U/mL, where one unit (U) of activity is the number of micromoles of H<sub>2</sub>O<sub>2</sub> converted per minute at pH 7.4 and a temperature of 25°C.

The optimum SBP dose was determined both in the presence and in the absence of PEG. In one set of experiments, an excess of PEG, 400 mg/L, was added to the reaction mixture, whereas in the other set of experiments, no PEG was added.

These tests were conducted at the optimum pH which was already determined in the previous set of experiments. The H<sub>2</sub>O<sub>2</sub> to substrate ratio was 1.2, which had been determined by other researchers to be the optimum dose. The enzymatic reaction (Section 2.1) indicates that two free radicals are generated for every molecule of peroxide that is consumed. The stoichiometric ratio of peroxide consumed to aromatic molecule precipitated would be 1:2, provided the resulting dimer is completely insoluble in water (Nicell, 1994). Therefore, for 1 mM solution of aromatic compound, theoretically only 0.5 mM of H<sub>2</sub>O<sub>2</sub> would be required instead of the 1 to 1.2 mM that has been observed. This inconsistency between measured and predicted stoichiometry can be explained by the formation of polymers which remain soluble and which react again to form trimers, tetramers or larger polymers which eventually precipitate out of solution (Nicell, 1994). Peroxidase was added in predetermined amounts in order to determine the minimum SBP dose required to remove 95% of the initial substrate concentration.

### **3.3.3 Hydrogen Peroxide Dose**

The next parameter to be optimized was the H<sub>2</sub>O<sub>2</sub> concentration. The amount of hydrogen peroxide which was added to the reactor is expressed as a ratio of millimolar H<sub>2</sub>O<sub>2</sub> to millimolar aromatic compound ( $[H_2O_2]/[Substrate]$ ).

During this set of experiments, the optimum pH and a limiting amount of SBP, as determined previously, were used. Each reactor contained 400 mg/L of PEG as in the previous set of experiments.

### **3.3.4 PEG Dose**

The last parameter to be optimized was PEG dose. A 40 g/L stock solution of PEG was prepared and stored at room temperature. During this final set of experiments, the optimum pH and  $[H_2O_2]/[Substrate]$  were used, and the SBP dose in the reactors was kept at less than the optimum dose determined previously.

## **3.4 Analytical Methods**

### **3.4.1 Aromatic Compound Concentration Assay**

The concentrations of the aromatic compounds were determined by either the direct spectrophotometric method or the colorimetric method. The concentrations of the substrates are expressed as molar quantities, where one millimolar is equal to 94 mg/L of phenol, 128.6 mg/L of chlorinated phenols, 108 mg/L of cresols, 163 mg/L of 2,4-dichlorophenol and 228.3 mg/L of bisphenol-A. The colorimetric method used 4-aminoantipyrine (AAP) and ferricyanide as colour generating substrates when combined

with phenolic compounds. The colour generated at a peak wavelength of 510 nm was directly proportional to the concentration of the aromatic compound. The assay mixture in the plastic cuvette consisted of 100  $\mu\text{L}$  of ferricyanide solution, 100  $\mu\text{L}$  of AAP solution, 200 to 800  $\mu\text{L}$  of aromatic sample, and deionized water to bring the total volume to 1000  $\mu\text{L}$ . A detailed procedure for the colorimetric method can be found in Appendix B. Results show that the colorimetric method could be used for all of the phenolic compounds except p-cresol, in which case the direct spectrophotometric method was used. Therefore, for p-cresol, the calibration curve that is shown in Appendix C is for the direct spectrophotometric method and for all other compounds, only the colorimetric calibration curves are given.

The direct spectrophotometric method was based on the absorbance of ultraviolet (UV) light by phenols. Phenolic compounds absorbed UV light at a maximum wavelength between 270 and 284 nm. In this method, the mixture in the quartz cuvette had a total volume of 1000  $\mu\text{L}$  consisting of the aromatic sample and deionized water.

#### **3.4.2 Peroxidase Activity Assay**

The SBP enzyme activity was measured using the peroxidase activity assay which used phenol, 4-AAP and  $\text{H}_2\text{O}_2$ . This method provided all reagents in excess except for enzyme in order to ensure that the initial rate of reaction was directly proportional to the amount of SBP enzyme present. Enzyme activity is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 25°C.

The assay mixture consisted of 100  $\mu\text{L}$  of 100 mM phenol, 250  $\mu\text{L}$  of 9.6 mM 4-AAP, 100  $\mu\text{L}$  of 2 mM  $\text{H}_2\text{O}_2$ , 500  $\mu\text{L}$  of 100 mM phosphate buffer (pH 7.4), and 50  $\mu\text{L}$  of enzyme sample. Immediately after the addition of the enzyme, the cuvette was shaken and the change in rate of absorbance with time was monitored at a peak wavelength of 510 nm. A detailed description of this method can be found in Appendix A.

### **3.5 Sources of Error**

In any experiment, many errors occur which may affect the reliability of the results. Systematic errors are due to analytical techniques and instruments, whereas random (or human) errors are due to personal carelessness. Calibration curves were done several times and compared to verify accuracy. One set of experiments, the hydrogen peroxide dose series for p-cresol, was repeated 3 times and the results were compared to determine the reliability of the experimental results.

As previously mentioned, the products of the enzymatic reaction are mostly phenols. If a portion of these reaction products remained in solution, they could be absorbed at the same wavelength as the compound being analyzed, and could therefore lead to errors in the amount of initial substrate that actually remained in solution.

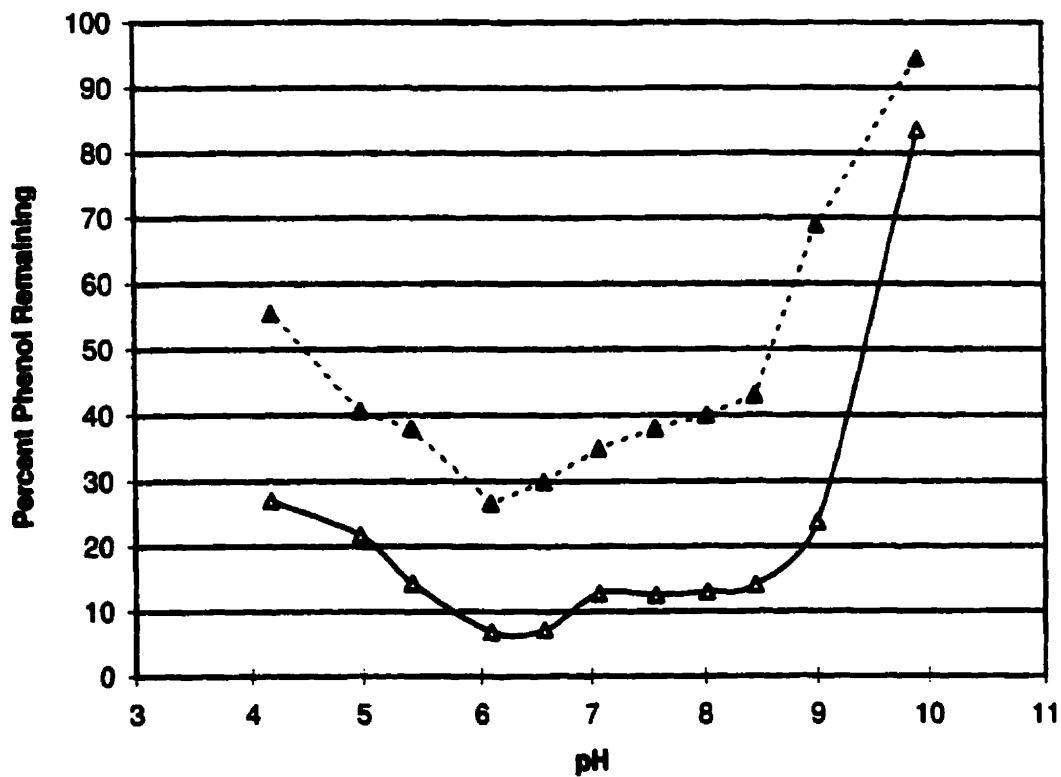
## 4. RESULTS AND DISCUSSION

The experiments in this study were designed to achieve a removal of at least 95% of the initial aromatic substrate concentration that was present in solution. The reaction parameters which were optimized were pH, soybean peroxidase (SBP) dose both in the presence and absence of polyethylene glycol (PEG), hydrogen peroxide to substrate ratio ( $[H_2O_2]/[Substrate]$ ), and PEG dose. The effect of coprecipitation on SBP requirements was also investigated.

### 4.1 pH

The optimum pH was determined for each phenolic compound in the range of 4 to 10. The initial substrate concentration was 1.0 mM for all compounds except for bisphenol A, which had an initial concentration of 0.5 mM. Bisphenol A contains two phenol rings and is not as soluble as the other compounds. In order to get a 1 mM solution, bisphenol A had to be dissolved into solution using one equivalent of NaOH. This bisphenol A solution was a basic solution and when the pH was adjusted to below neutral, the compound precipitated out of solution. In order to ensure that the removal was not due in part to precipitation, a lower initial concentration (0.5 mM) was chosen.

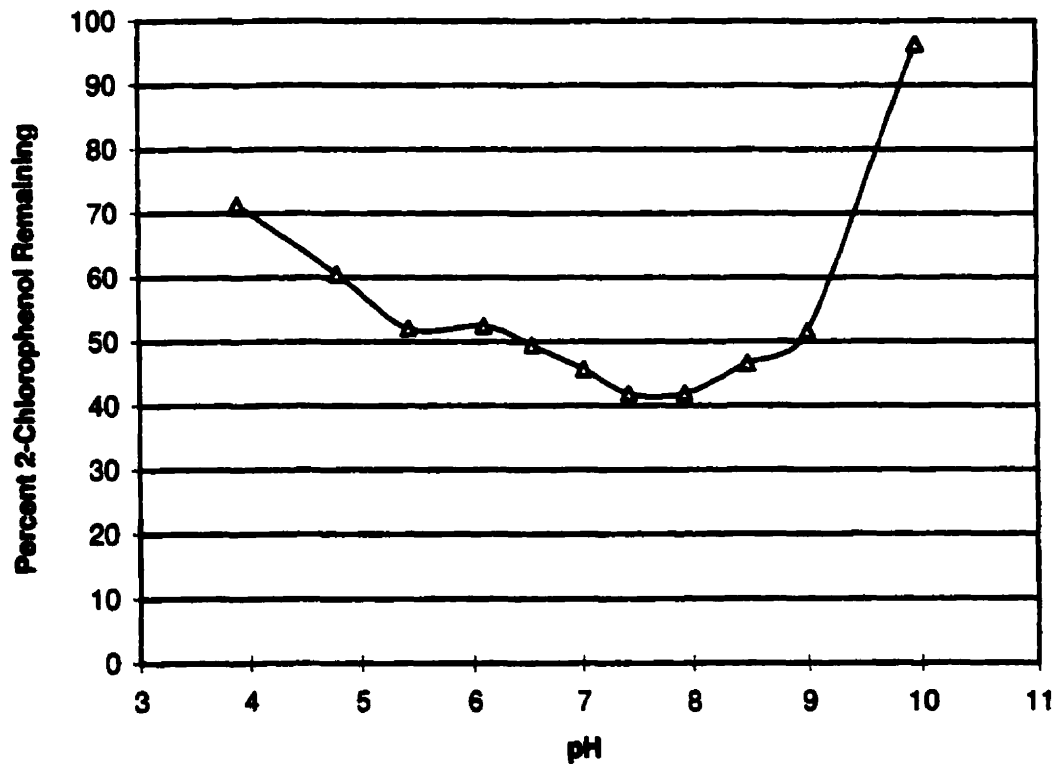
The  $H_2O_2$  to substrate ratio was 1.2 for each experiment and PEG was present in excess at 400 mg/L so that the removal efficiency was only dependent on pH and enzyme dose. The reactions were stopped after 3 hours, which was considered to be sufficient time based on previous experiments (Klibanov *et al.*, 1980). Removal efficiencies as a function of pH are presented in Figures 4.1.1 through 4.1.9. The optimum pH for each aromatic compound is listed in Table 4.1.



—▲— SBP = 0.5 U/mL    - - ▲ - - SBP = 0.3 U/mL

**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$   
 PEG = 400 mg/L

Figure 4.1.1: Effect of pH on the Removal of Phenol

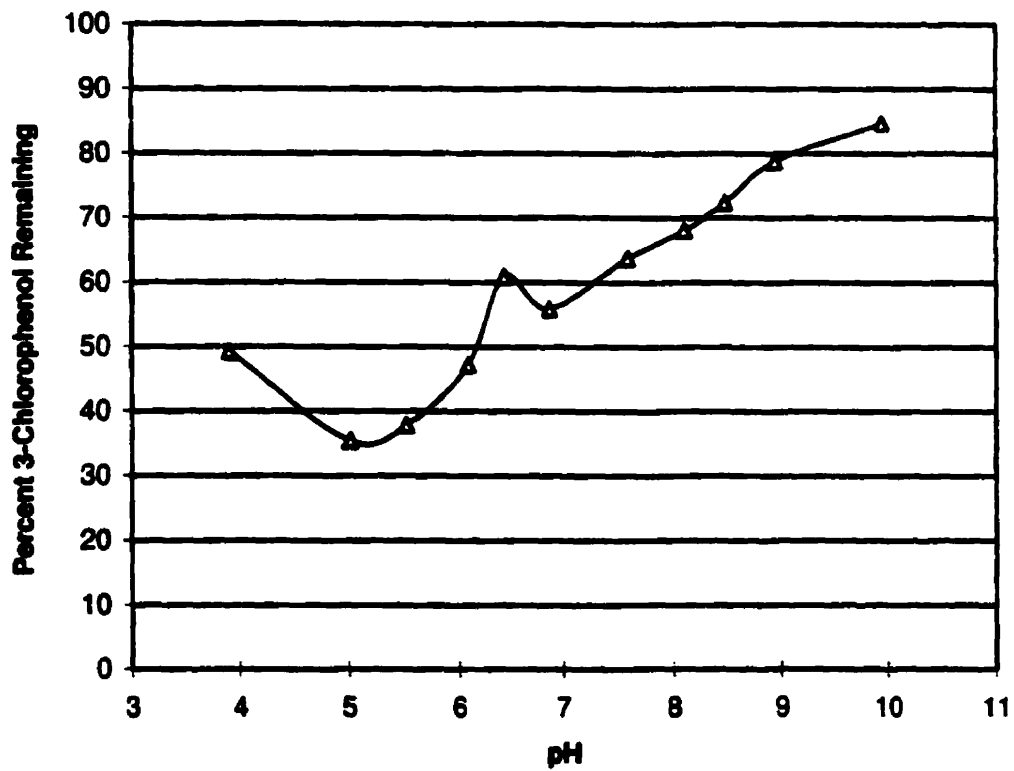


—△— SBP = 0.07 U/mL

**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$   
 PEG = 400 mg/L

Figure 4.1.2: Effect of pH on the Removal of 2-Chlorophenol

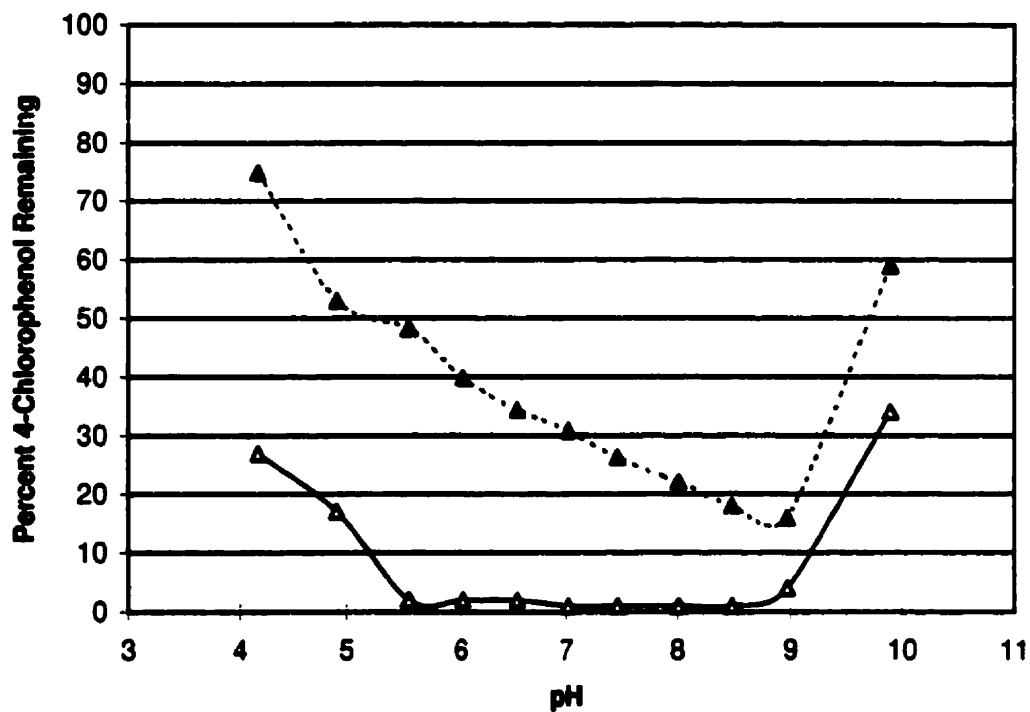




—▲— SBP = 0.10 U/mL

**Reactor Conditions:**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
PEG = 400 mg/L

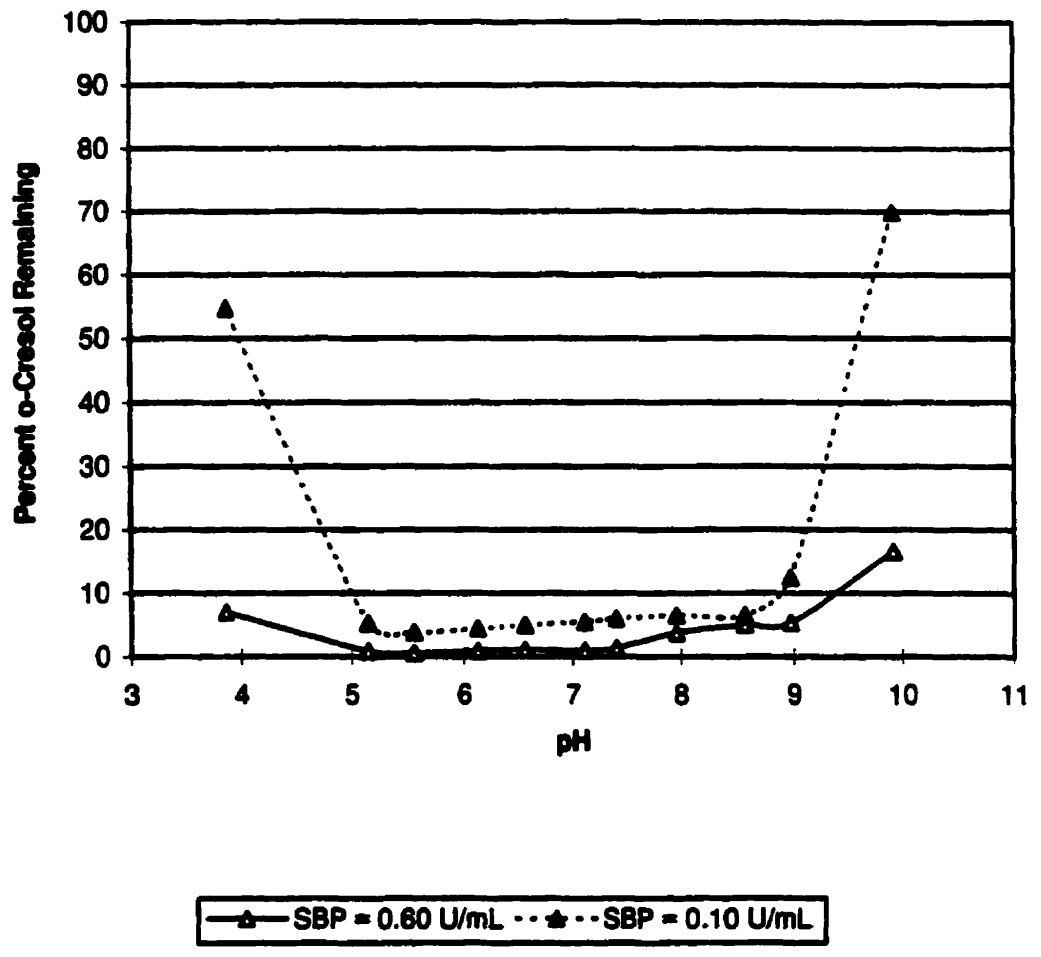
Figure 4.1.3: Effect of pH on the Removal of 3-Chlorophenol



—▲— SBP = 0.2 U/mL    ···▲··· SBP = 0.07 U/mL

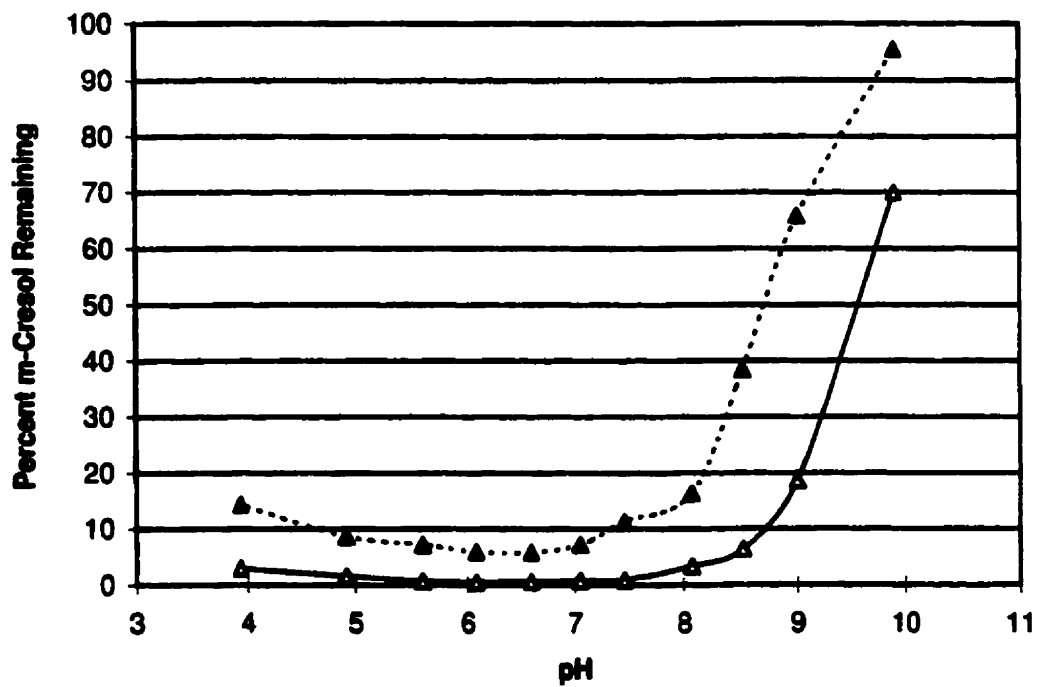
**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$   
 PEG = 400 mg/L

Figure 4.1.4: Effect of pH on the Removal of 4-Chlorophenol



**Reactor Conditions:**  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
 PEG = 400 mg/L

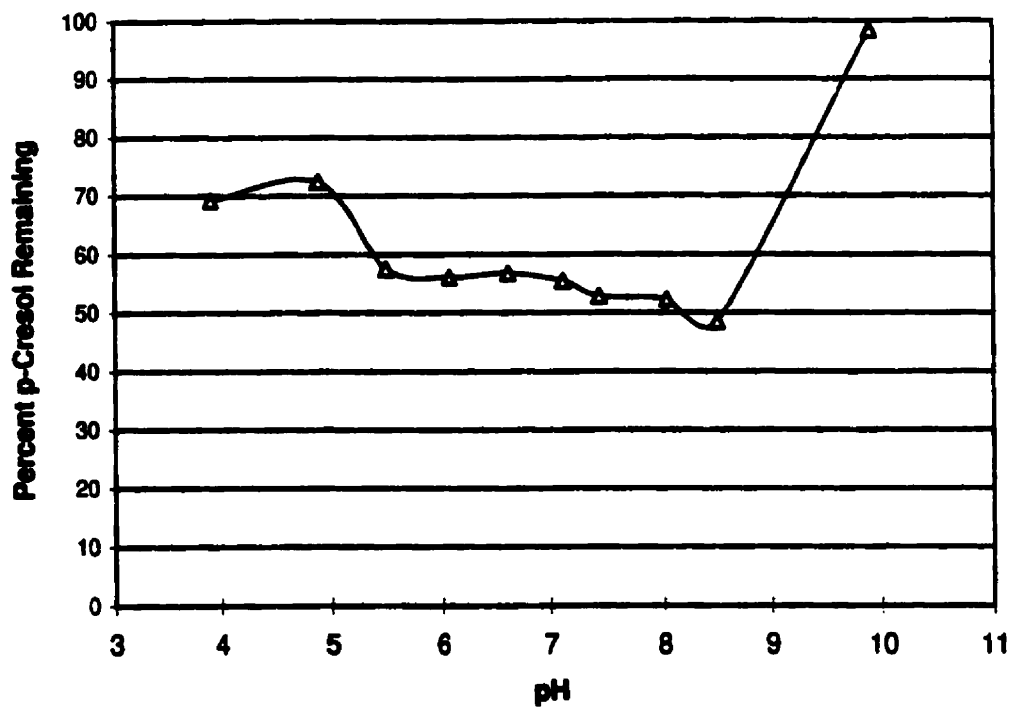
Figure 4.1.5: Effect of pH on the Removal of o-Cresol



—▲— SBP = 0.20 U/mL ···▲··· SBP = 0.05 U/mL

**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$   
 PEG = 400 mg/L

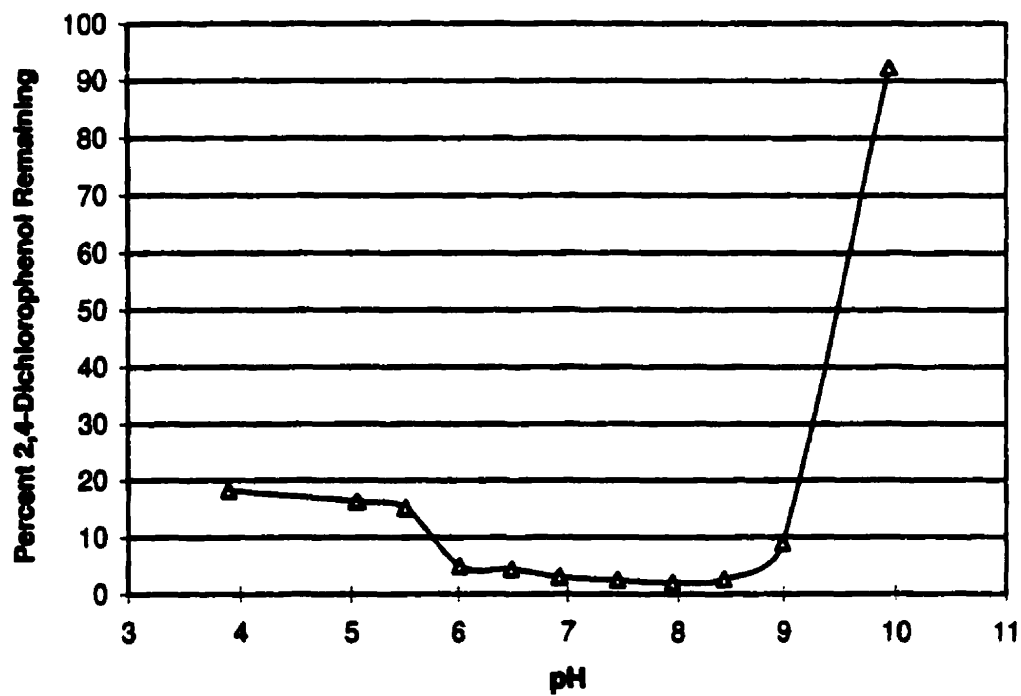
Figure 4.1.6: Effect of pH on the Removal of m-Cresol



—△— SBP = 0.04 U/mL

**Reactor Conditions:**  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
 PEG = 400 mg/L

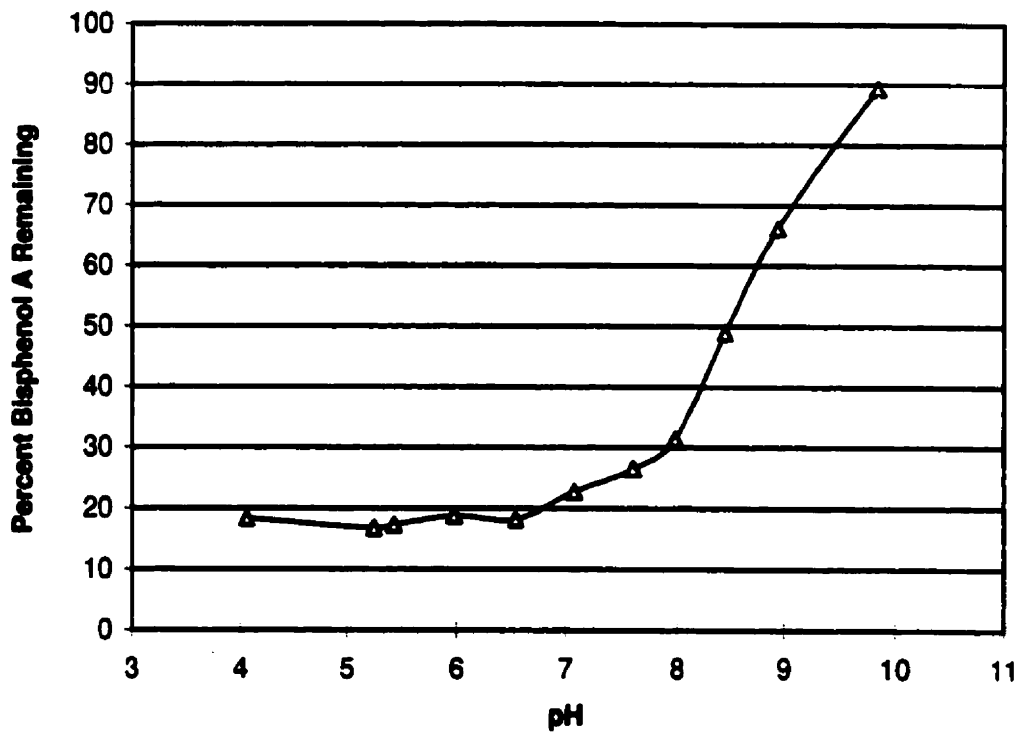
Figure 4.1.7: Effect of pH on the Removal of p-Cresol



—▲— SBP = 0.05 U/mL

**Reactor Conditions:**  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
 PEG = 400 mg/L

Figure 4.1.8: Effect of pH on the Removal of 2,4-Dichlorophenol



—△— SBP = 0.001 U/mL

**Reactor Conditions:**  
Substrate = 0.5mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
PEG = 400 mg/L

Figure 4.1.9: Effect of pH on the Removal of Bisphenol A

The graphs show that most of the compounds studied had a broad optimum pH range. This range generally became wider as the amount of SBP was increased, as can be seen in Figure 4.1.4 for 4-chlorophenol. The results for this compound show that at an enzyme dose of 0.07 U/mL, the optimum pH came to a definite point, whereas for the higher enzyme dose equal to 0.2 U/mL, there was a broad optimum pH between 5.5 and 9. The optimum pH occurred at near neutral conditions except for 3-chlorophenol which had an optimum pH of about 5 and for 4-chlorophenol which had an optimum pH of approximately 8.

It can also be seen from Figure 4.1.1 that a change in the amount of SBP present in solution did not affect the optimum pH. These results are consistent with those found by Wu Y. *et al.* (1997). When the initial amount of SBP was increased, the percent substrate remaining in solution decreased.

**Table 4.1: Optimum pH for Phenolic Compounds**

<b>Aromatic Compound</b>	<b>Optimal pH Range</b>	<b>Optimum pH</b>
Phenol	5.5 - 8.5	6.0
2-Chlorophenol	5.5 - 9.0	7.5
3-Chlorophenol	4.0 - 6.0	5.0
4-Chlorophenol	5.5 - 8.5	8.0
o-Cresol	5.0 - 8.5	7.0
m-Cresol	4.0 - 7.5	7.0
p-Cresol	5.5 - 8.5	7.0
2,4-Dichlorophenol	6.0 - 8.5	7.0
Bisphenol A	4.0 - 6.5	6.5



Experiments were conducted to check the pH after mixing in order to verify that the reactions were carried out at the appropriate pH. The results for 4-chlorophenol are shown in Table 4.2.

**Table 4.2: Change in pH for 4-Chlorophenol**

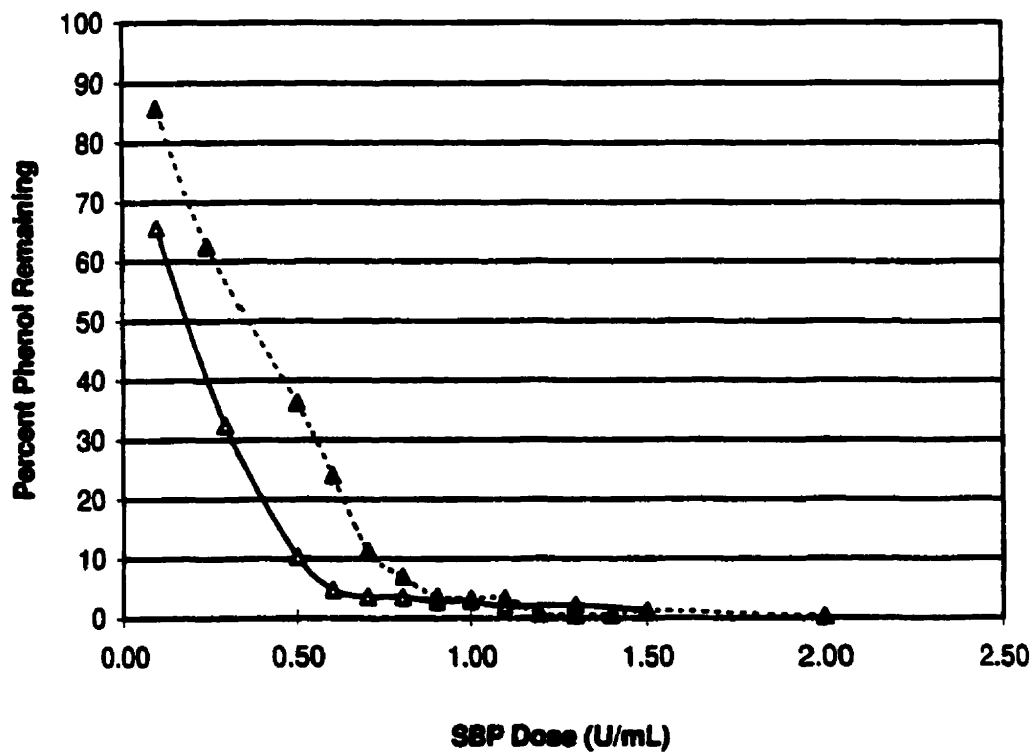
<b>Initial pH (Before Mixing)</b>	<b>Final pH (After Mixing)</b>
4.0	3.5
5.0	3.5
5.5	4.3
6.0	5.8
6.5	6.2
7.0	6.5
7.5	6.8
8.0	6.8
8.5	7.1
9.0	7.5
10.0	9.6

These results, as expected, indicate that there was little change in pH once the reactions were completed. Since the reactions were taking place in unbuffered solutions, the change in pH was determined in order to be sure that the reactions were indeed taking place at the initial pH. The largest change in pH was 1.5 and the smallest was 0.2 and the pH after mixing was always less than the pH before mixing. The results obtained for this compound are consistent with those obtained for the other compounds.

## **4.2 Soybean Peroxidase Dose**

The SBP dose series experiments were conducted at the previously established optimum pH values shown in Table 4.1. The optimum enzyme dose was determined both in the presence (400 mg/L) and in the absence of PEG. The results are presented in Figures 4.2.1 to 4.2.13. The  $[H_2O_2]/[Substrate]$  was kept at 1.2 mM/mM for all compounds, except for one set of experiments for p-cresol where the ratio was 0.9 mM/mM.

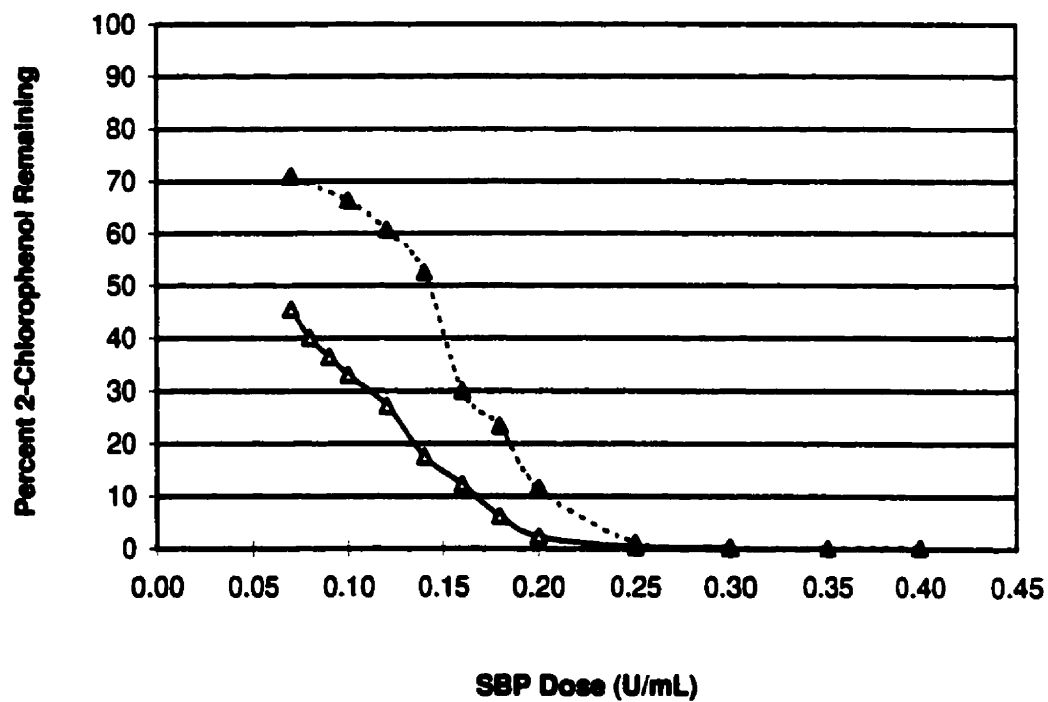
In this section, there are four figures (4.2.7 to 4.2.10) for the compound p-cresol. Figure 4.2.7 shows that 95% removal could not be achieved, even at a high enzyme dose of 0.60 U/mL. It was decided to add salt to the solution and to filter the supernatant, after centrifugation, to see if the removal efficiency would improve. As can be seen from Figure 4.2.8, the addition of salt had little effect, whereas the filtration greatly improved the removal efficiency. The filters used were 25 mm in diameter with a 0.2  $\mu$ m opening. Approximately 5 mL of the supernatant was filtered and discarded, with the sixth milliliter being analyzed. Since the filtration improved the removal of p-cresol, the supernatant for subsequent experiments was filtered. These experiments for p-cresol (Figure 4.2.8) were conducted in the absence of PEG, whereas in Figure 4.2.9, the experiments were done in the presence of 400 mg/L of PEG. The comparison of these two sets of results shows that the removal efficiency in the presence of 400 mg/L of PEG was less than the removal efficiency in the absence of PEG. Therefore, the PEG dose and the  $[H_2O_2]/[Substrate]$  experiments were conducted next and the results are reported later in Sections 4.3 and 4.4. These results indicate that high doses of PEG had a negative effect on the removal of p-cresol which could be due in part to the particular polymerization products of this compound. Therefore, another set of experiments for



—▲— PEG = 400 mg/L    - - ▲ - - NO PEG

**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$

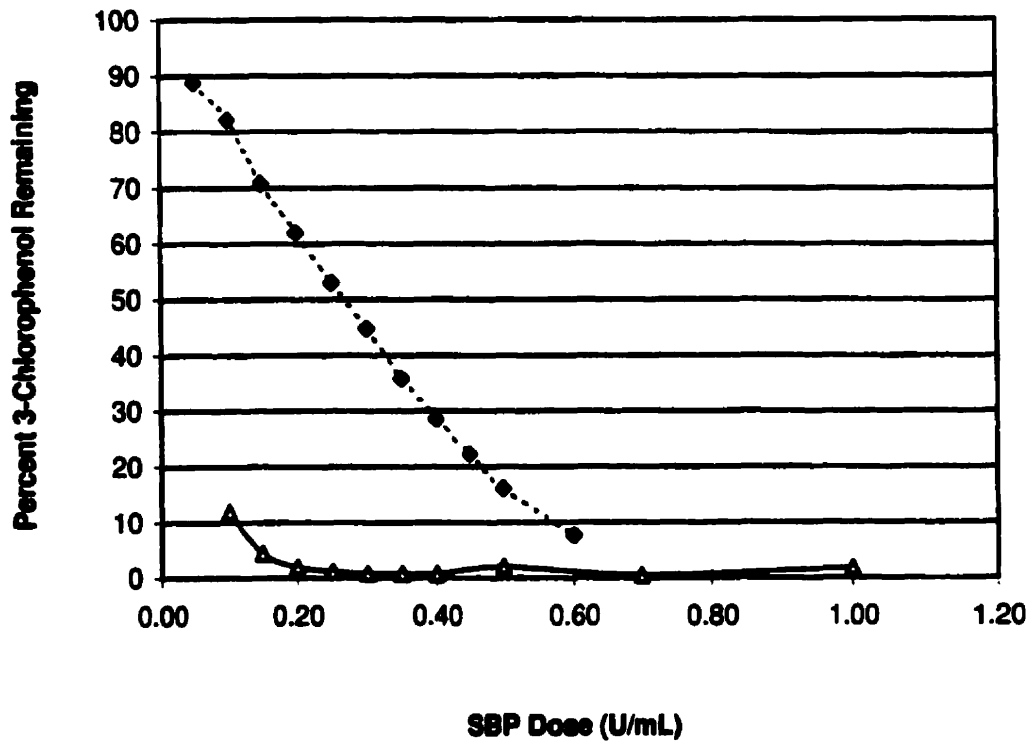
Figure 4.2.1: Effect of SBP Enzyme Dose on the Removal of Phenol



—▲— PEG = 400 mg/L    ···▲··· NO PEG

**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$

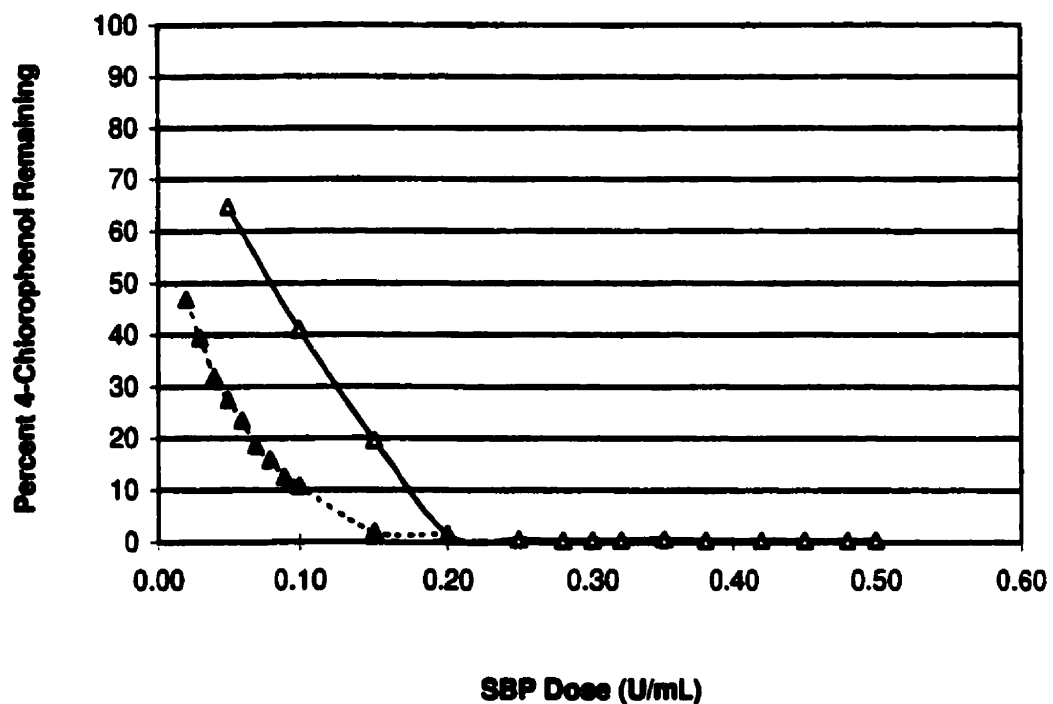
Figure 4.2.2: Effect of SBP Enzyme Dose on the Removal of 2-Chlorophenol



---◆--- No PEG    —▲— PEG = 400 mg/L

Reactor Conditions:  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM

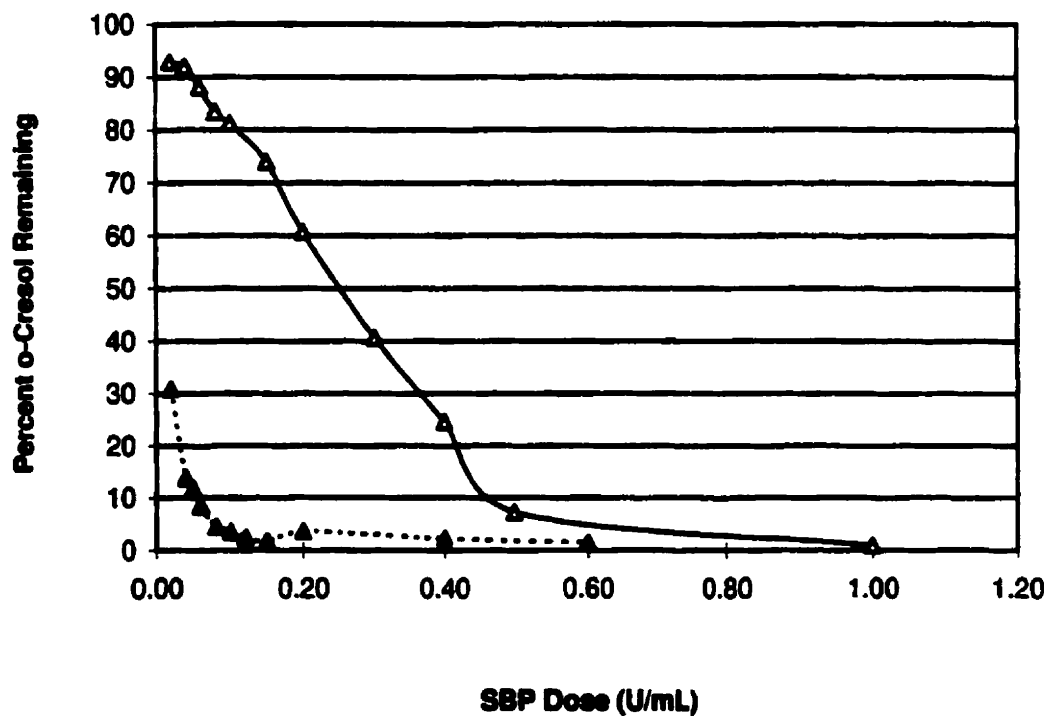
Figure 4.2.3: Effect of SBP Enzyme Dose on the Removal of 3-Chlorophenol



—△— NO PEG    - - ▲ - - PEG = 400 mg/L

**Reactor Conditions:**  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM

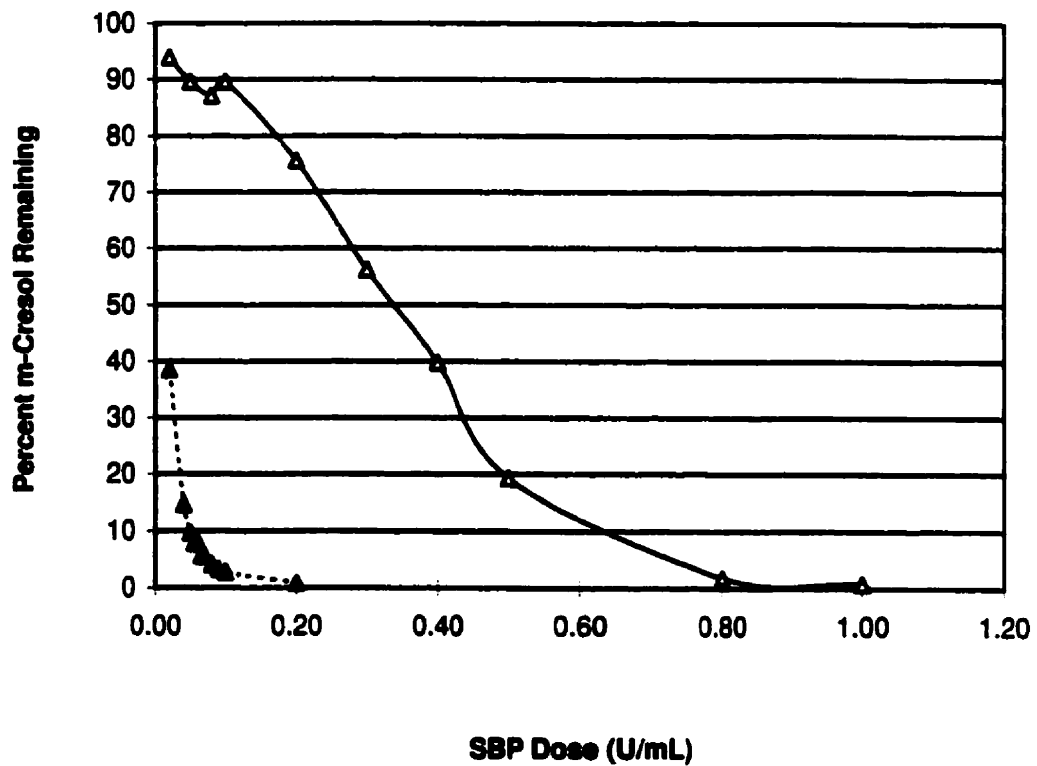
Figure 4.2.4: Effect of SBP Enzyme Dose on the Removal of 4-Chlorophenol



—△— NO PEG ···▲··· PEG = 400 mg/L

**Reactor Conditions:**  
 Substrate = 1 mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$

Figure 4.2.5: Effect of SBP Enzyme Dose on the Removal of o-Cresol

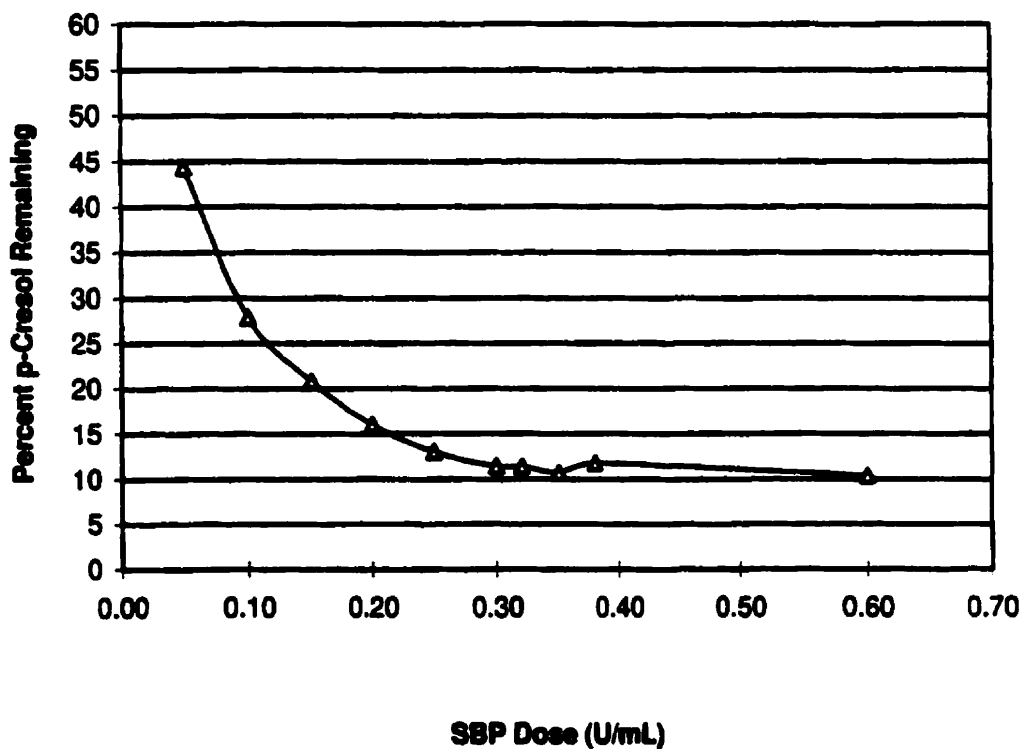


—△— NO PEG    ···▲··· PEG = 400 mg/L

**Reactor Conditions:**  
 Substrate = 1 mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$

Figure 4.2.6: Effect of SBP Enzyme Dose on the Removal of m-Cresol

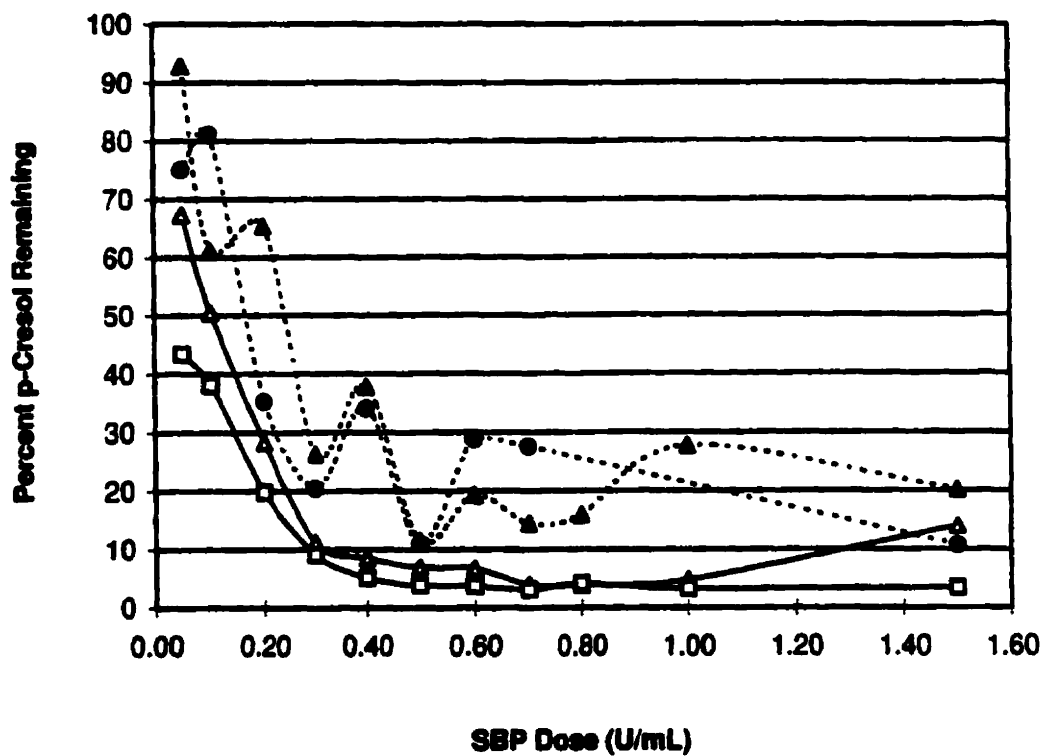




—△— PEG = 400 mg/L

Reactor Conditions:  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM

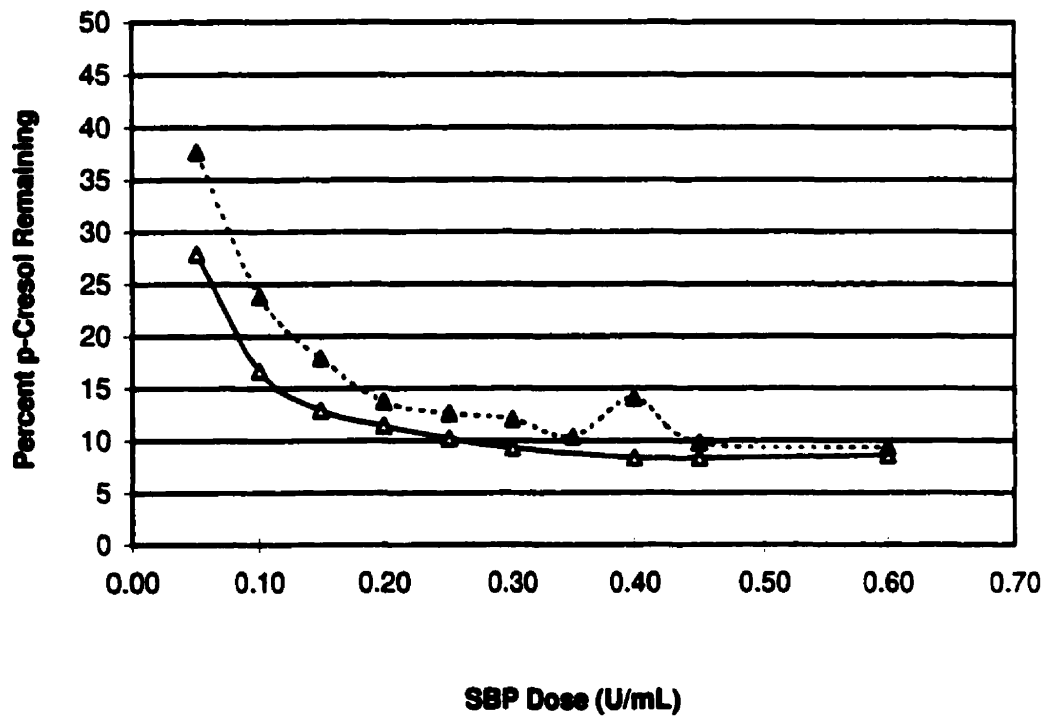
Figure 4.2.7: Effect of SBP Enzyme Dose on the Removal of p-Cresol



—▲— Filtered, No Salt      —□— Filtered, Salt  
 - -▲- - Not Filtered, Salt      - -●- - Not Filtered, No Salt

**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$   
 No PEG

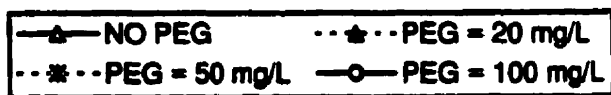
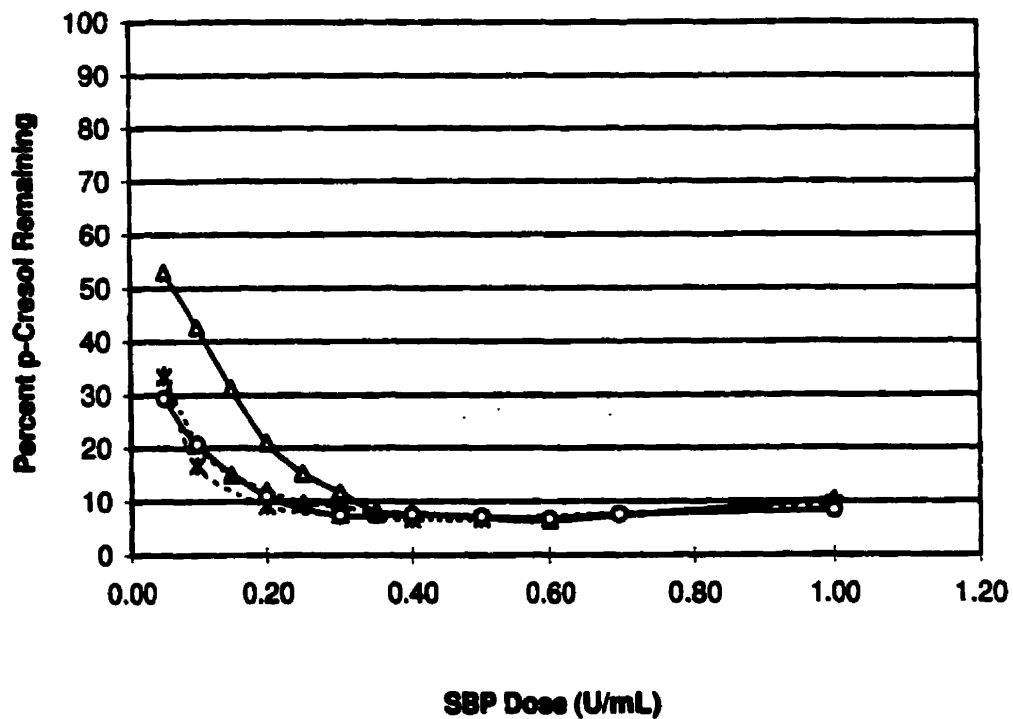
Figure 4.2.8: Effect of Filtration and Addition of Salt on the Removal of p-Cresol in the Absence of PEG



—▲— Filtered    ···▲··· Not Filtered

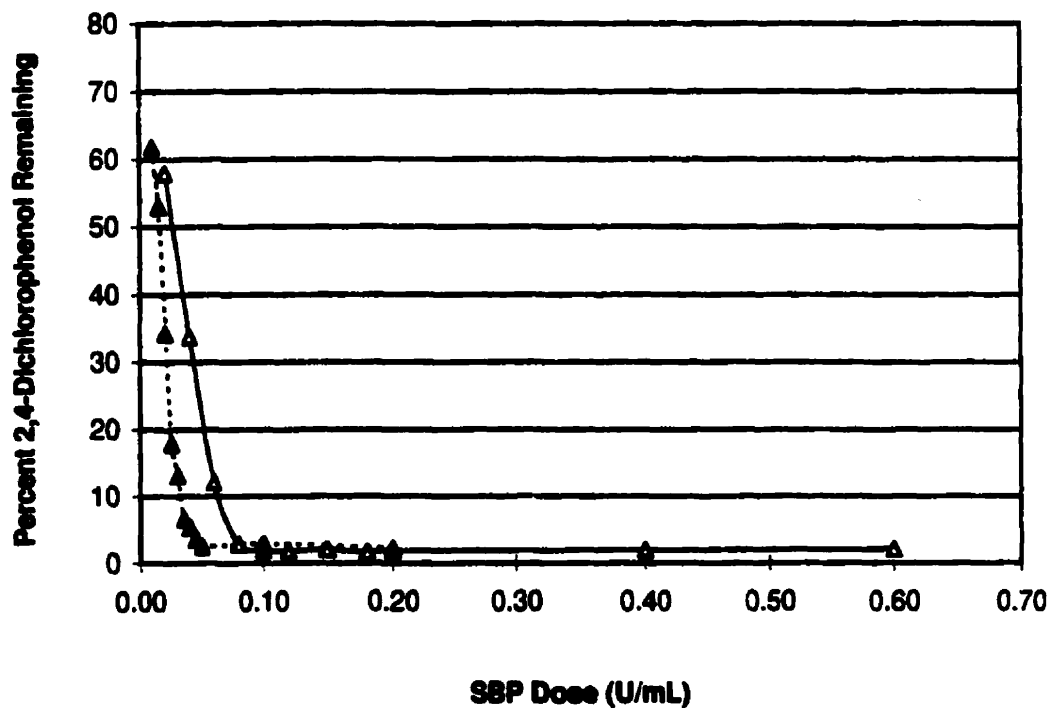
**Reactor Conditions:**  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
 PEG = 400 mg/L

Figure 4.2.9: Effect of Filtration on the Removal of p-Cresol in the Presence of PEG



**Reactor Conditions:**  
 Substrate = 1mM  
 $[H_2O_2]/[Substrate] = 0.9 \text{ mM/mM}$

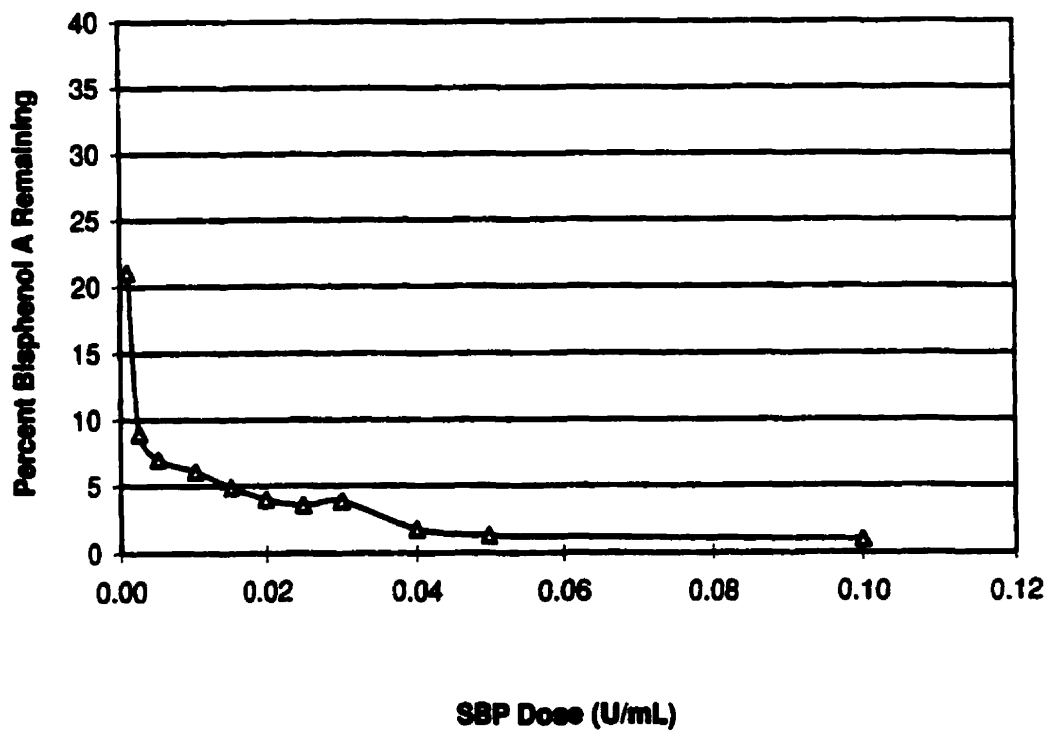
Figure 4.2.10: Effect of SBP Enzyme Dose and PEG Dose on the Removal of p-Cresol



—●— NO PEG    ···▲··· PEG = 400 mg/L

**Reactor Conditions:**  
 Substrate = 1mM  
 [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM

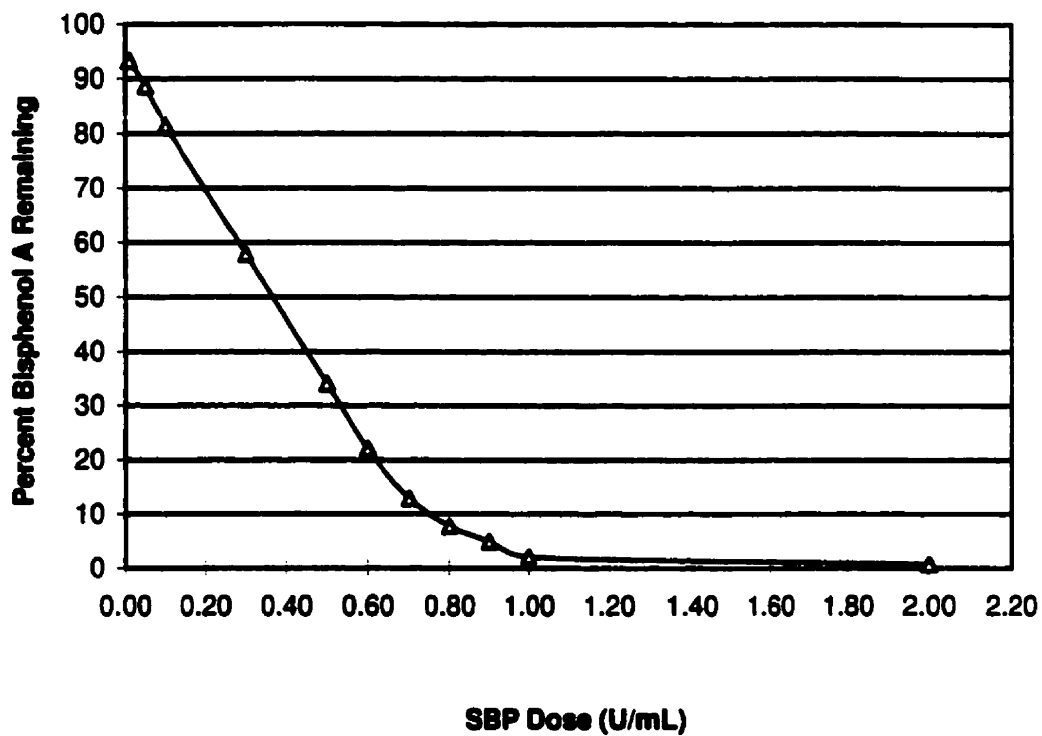
Figure 4.2.11: Effect of SBP Enzyme Dose on the Removal of 2,4-Dichlorophenol



—▲— PEG = 400 mg/L

**Reactor Conditions:**  
Substrate = 0.5 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM

**Figure 4.2.12: Effect of SBP Enzyme Dose on the Removal of Bisphenol A in the Presence of PEG**



—▲— No PEG

**Reactor Conditions:**  
 Substrate = 0.5 mM  
 $[H_2O_2]/[Substrate] = 1.2 \text{ mM/mM}$

**Figure 4.2.13: Effect of SBP Enzyme Dose on the Removal of Bisphenol A in the Absence of PEG**

p-cresol (Figure 4.2.10) was conducted by varying the amount of PEG dose from zero to 100 mg/L and by changing  $[H_2O_2]/[Substrate]$  to the optimum of 0.9 mM/mM.

The results in these figures show that the addition of PEG only slightly reduced the amount of SBP required for 95% removal, which is inconsistent with the results obtained by Wu Y. *et al.* (1997). For example, 2-chlorophenol required 0.19 U/mL of enzyme in the presence of PEG but in the absence of PEG it still only required 0.23 U/mL. This represents an additional requirement of only 1.2 times more SBP enzyme without PEG. The results for p-cresol showed that high doses of PEG actually decreased the removal efficiency instead of improving it. This is the only compound studied that showed these results. The minimum SBP doses with and without PEG are shown in Table 4.3.

**Table 4.3: Optimum SBP Dose in the Presence and Absence of PEG**

<b>Aromatic Compound</b>	<b>Minimum SBP Dose Without PEG (U/mL)</b>	<b>Minimum SBP Dose With PEG (U/mL)</b>	<b>SBP Dose Ratio</b>
Phenol	0.90	0.60	1.5
2-Chlorophenol	0.23	0.19	1.2
3-Chlorophenol	0.65	0.15	4.3
4-Chlorophenol	0.20	0.15	1.3
o-Cresol	0.60	0.08	7.5
m-Cresol	0.75	0.08	9.4
p-Cresol	0.60	0.40	1.5
2,4-Dichlorophenol	0.08	0.04	2.0
Bisphenol A*	0.90	0.015	60.0

Initial substrate concentrations equal to 1.0 mM.

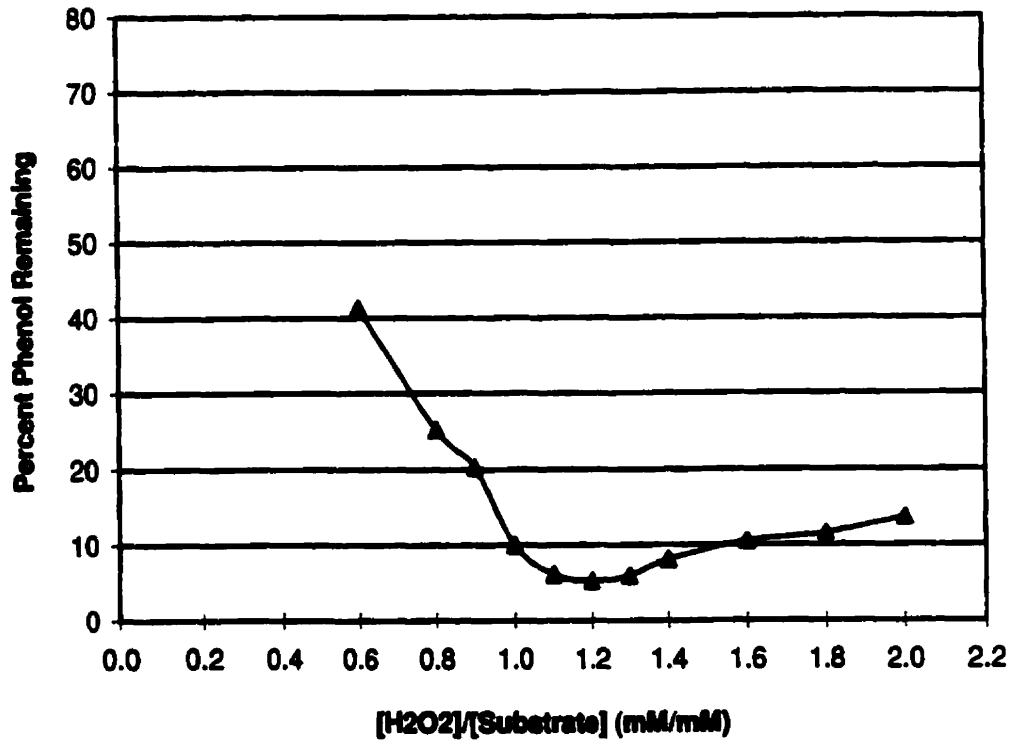
\*Initial bisphenol A concentration equal to 0.5 mM.



Results obtained from previous experiments using SBP (Al-Kassim et al., 1995) indicated higher enzyme requirements for most compounds. Their higher enzyme values could be caused by two factors: (i) a higher H<sub>2</sub>O<sub>2</sub> to substrate ratio dose of 1.5 was used as compared to 1.2; and (ii) a much lower PEG dose of 30 mg/L instead of a dose of 400 mg/L which was used in the SBP dose experiments. The results show that the minimum amount of SBP required for 95% of removal from initial concentration of the aromatic compounds in the absence of PEG was 1.2 to 60 times more than that required with PEG. In the presence of PEG, phenol required the most enzyme for 95% removal, whereas bisphenol A required the least amount of enzyme. This might be explained by the fact that bisphenol is already a dimer and in turn requires less enzyme for its removal from solution. Extra SBP beyond the minimum dose did not result in any significant improvement in removal.

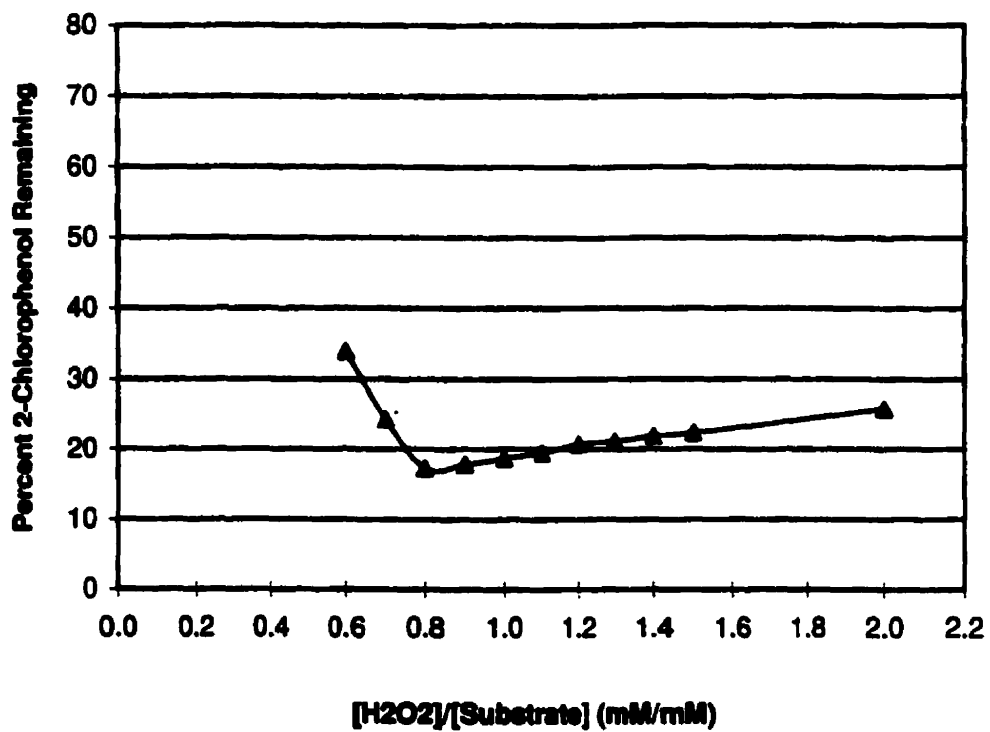
#### **4.3 Hydrogen Peroxide to Substrate Ratio ([H<sub>2</sub>O<sub>2</sub>]/[Substrate])**

The third parameter that was optimized in this study was the molar ratio of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to substrate. These experiments were conducted at the previously determined optimum pH, whereas the initial SBP concentration was less than the optimum dose listed in Table 4.3 in order to see a greater change in removal from point to point. The PEG dose was kept at an excess of 400 mg/L, except for p-cresol, which had a PEG concentration of 20 mg/L. The concentration for all of the phenolic compounds was kept at 1 mM except for bisphenol A which had an initial concentration of 0.5 mM. Results are plotted in Figures 4.3.1 to 4.3.9, and the optimum molar ratios are listed in Table 4.4.



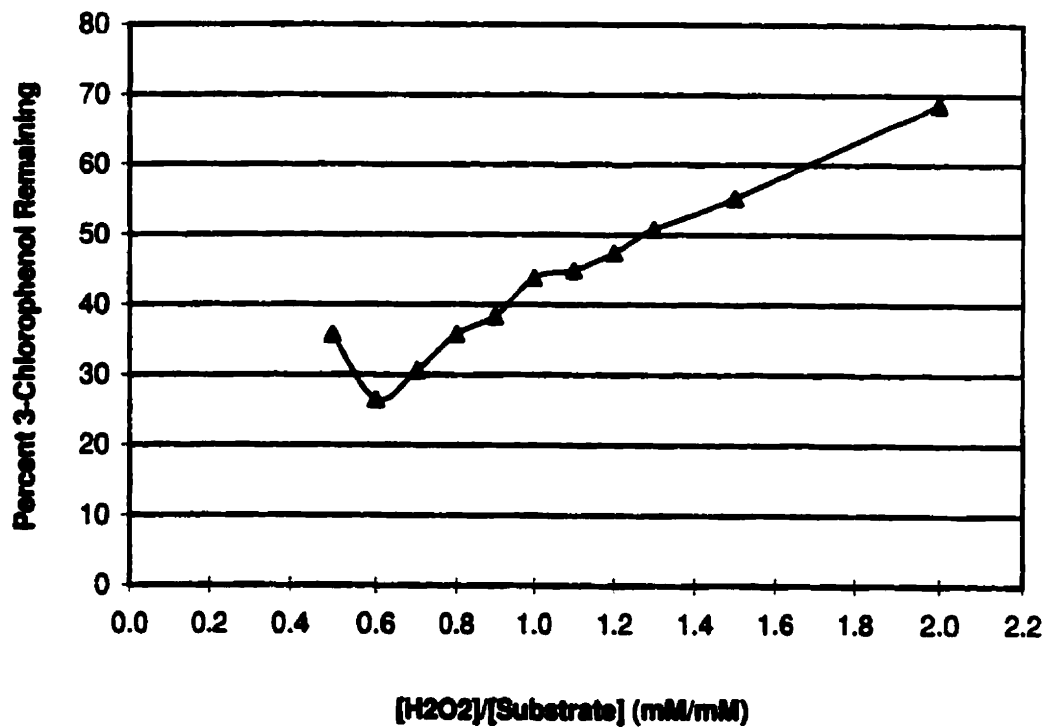
**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.6 U/mL  
PEG = 400 mg/L

Figure 4.3.1: Effect of Hydrogen Peroxide Dose on the Removal of Phenol



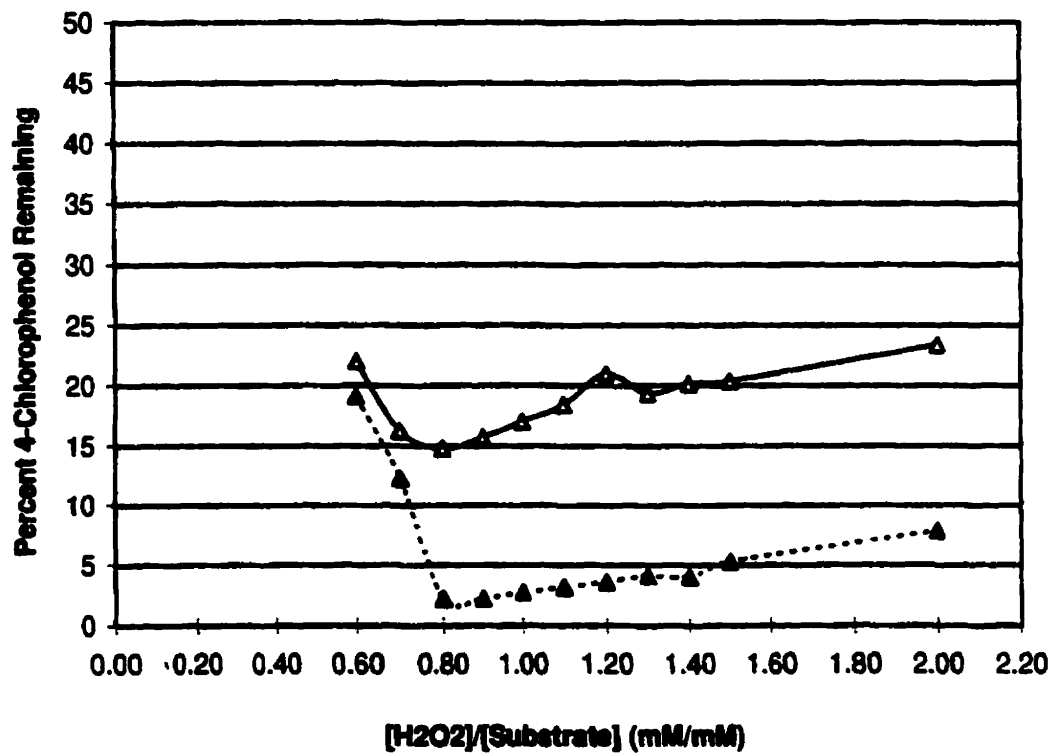
**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.12 U/mL  
PEG = 400 mg/L

Figure 4.3.2: Effect of Hydrogen Peroxide Dose on the Removal of 2-Chlorophenol



**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.05 U/mL  
PEG = 400 mg/L

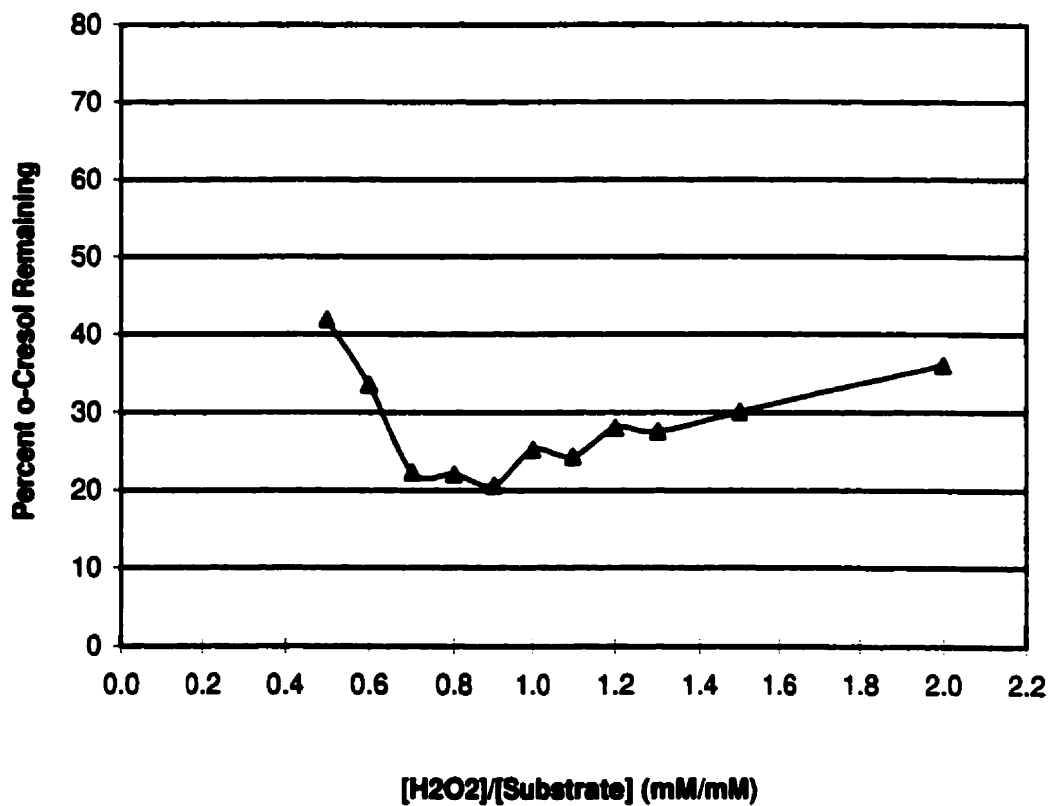
Figure 4.3.3: Effect of Hydrogen Peroxide Dose on the Removal of 3-Chlorophenol



—▲— SBP = 0.07 U/mL ···▲··· SBP = 0.12 U/mL

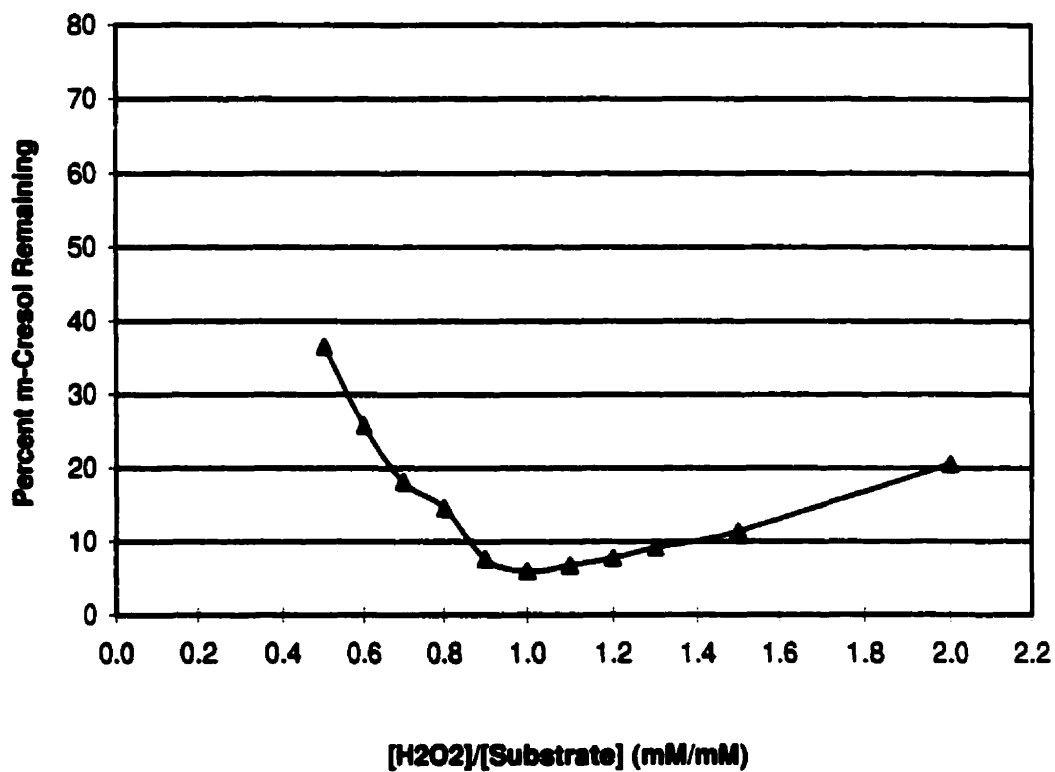
**Reactor Conditions:**  
 Substrate = 1mM  
 PEG = 400 mg/L

Figure 4.3.4: Effect of Hydrogen Peroxide Dose on the Removal of 4-Chlorophenol



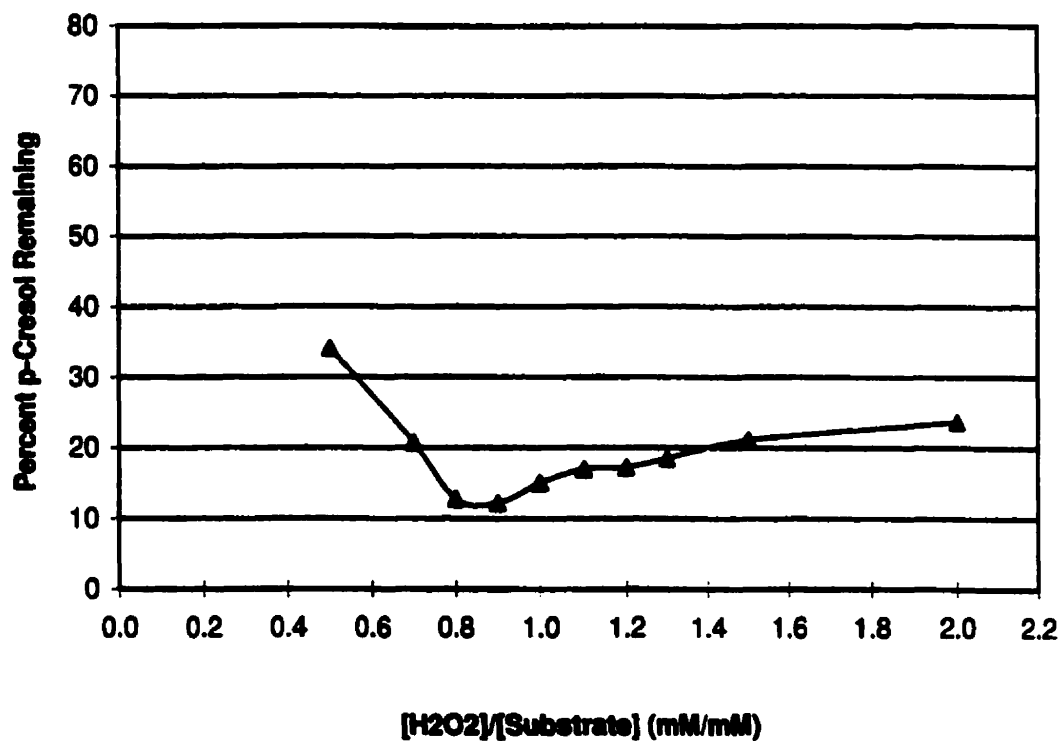
**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.04 U/mL  
PEG = 400 mg/L

Figure 4.3.5: Effect of Hydrogen Peroxide Dose on the Removal of o-Cresol



**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.04 U/mL  
PEG = 400 mg/L

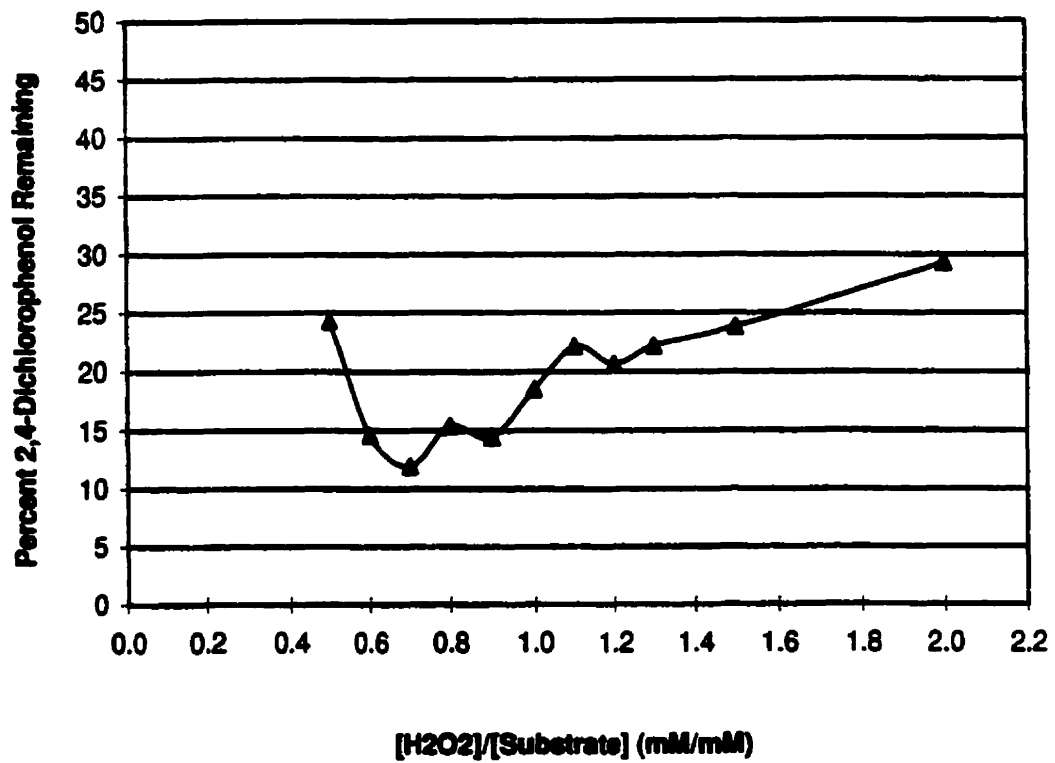
Figure 4.3.6: Effect of Hydrogen Peroxide Dose on the Removal of m-Cresol



**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.15 U/mL  
PEG = 20 mg/L

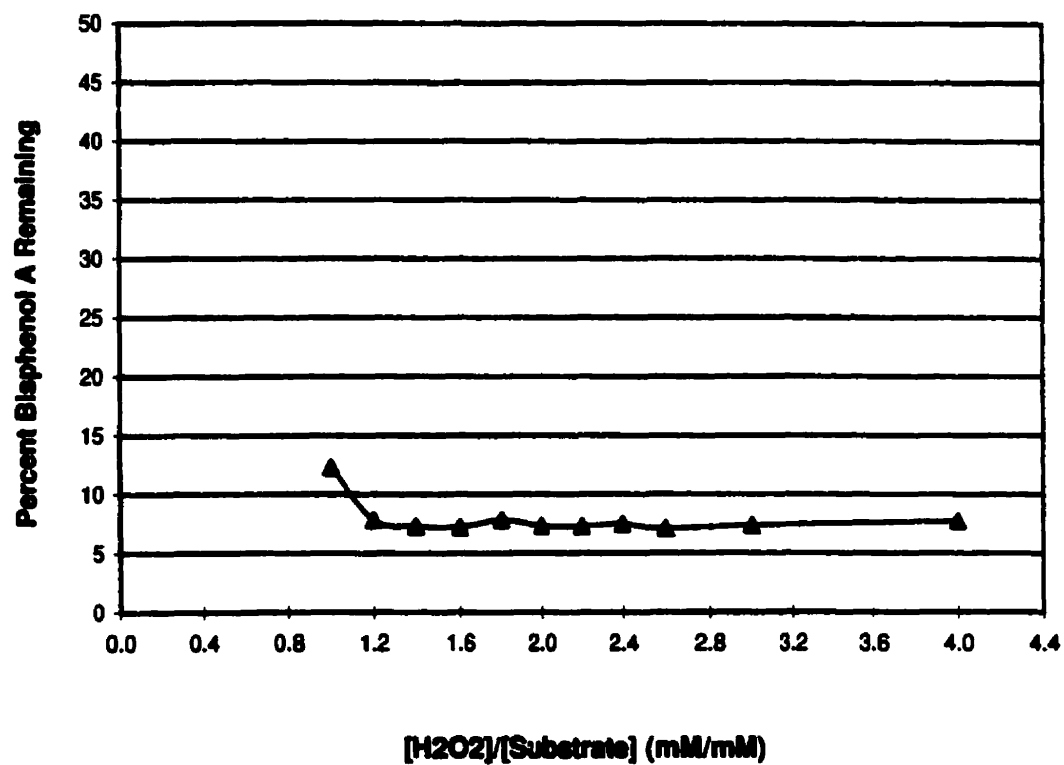
**Figure 4.3.7: Effect of Hydrogen Peroxide Dose on the Removal of p-Cresol**





**Reactor Conditions:**  
Substrate = 1mM  
SBP = 0.02 U/mL  
PEG = 400 mg/L

Figure 4.3.8: Effect of Hydrogen Peroxide Dose on the Removal of 2,4-Dichlorophenol



**Reactor Conditions:**  
Substrate = 0.5mM  
SBP = 0.005 U/mL  
PEG = 400 mg/L

Figure 4.3.9: Effect of Hydrogen Peroxide Dose on the Removal of Bisphenol A

These figures show that as  $[H_2O_2]/[Substrate]$  increased, the removal efficiency also increased until the optimum. These results do not indicate a broad optimum range as was found by Wu Y. *et al* (1997), but instead most compounds show a definite optimum point. Figure 4.3.9 for bisphenol A, on the other hand, is the only compound to have a broad optimum between 1.2 to 4.0 mM/mM. As  $[H_2O_2]/[Substrate]$  increased beyond the optimum, there was a decrease in substrate removal, whereas for bisphenol A, an increase in  $H_2O_2$  did not affect the removal efficiency.

**Table 4.4: Optimum  $H_2O_2$  to Substrate Ratio for Phenolic Compounds**

<b>Aromatic Compound</b>	<b>Optimum <math>H_2O_2</math> to Substrate Ratio (mM/mM)</b>
Phenol	1.2
2-Chlorophenol	0.8
3-Chlorophenol	0.6
4-Chlorophenol	0.8
o-Cresol	0.9
m-Cresol	1.0
p-Cresol	0.9
2,4-Dichlorophenol	0.7
Bisphenol A	1.2

The optimum ratios, as seen in the above table, range from as low as 0.6 for 3-chlorophenol to as high as 1.2 mM/mM for both phenol and bisphenol A. However, it should be noted that since bisphenol A is a biphenol, that a  $[H_2O_2]/[bisphenol\ A]$  of 1.2 is equal to a  $[H_2O_2]/[phenolic\ group]$  of only 0.6. As seen from the results obtained in this

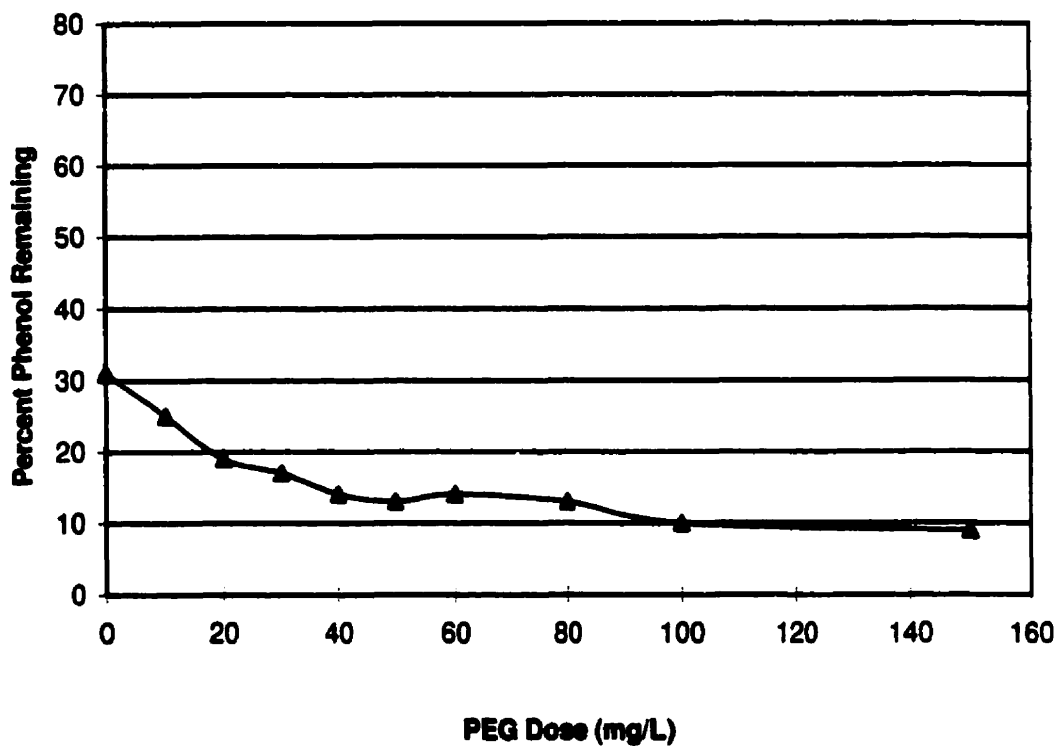
section, an excess of  $\text{H}_2\text{O}_2$  decreased the removal efficiencies. Since the SBP enzyme dose experiments in Section 4.2 were all conducted using a ratio of 1.2, the optimum SBP doses for 3-chlorophenol and 2,4-dichlorophenol (Table 4.3) might be improved at their optimum  $[\text{H}_2\text{O}_2]/[\text{Substrate}]$  of 0.6 and 0.7 respectively.

#### **4.4 Polyethylene Glycol Dose**

Polyethylene glycol dose experiments were conducted at the previously established optimum pH and  $[\text{H}_2\text{O}_2]/[\text{Substrate}]$ . As in the last set of experiments, the SBP concentration was kept at less than the optimum. Results of the removal efficiency as a function of PEG are plotted in Figures 4.4.1 through 4.4.9. The initial concentration of the phenolic compounds was 1 mM for all substrates except bisphenol A which had a concentration of 0.5 mM. The PEG doses in this set of experiments ranged from zero to 600 mg/L.

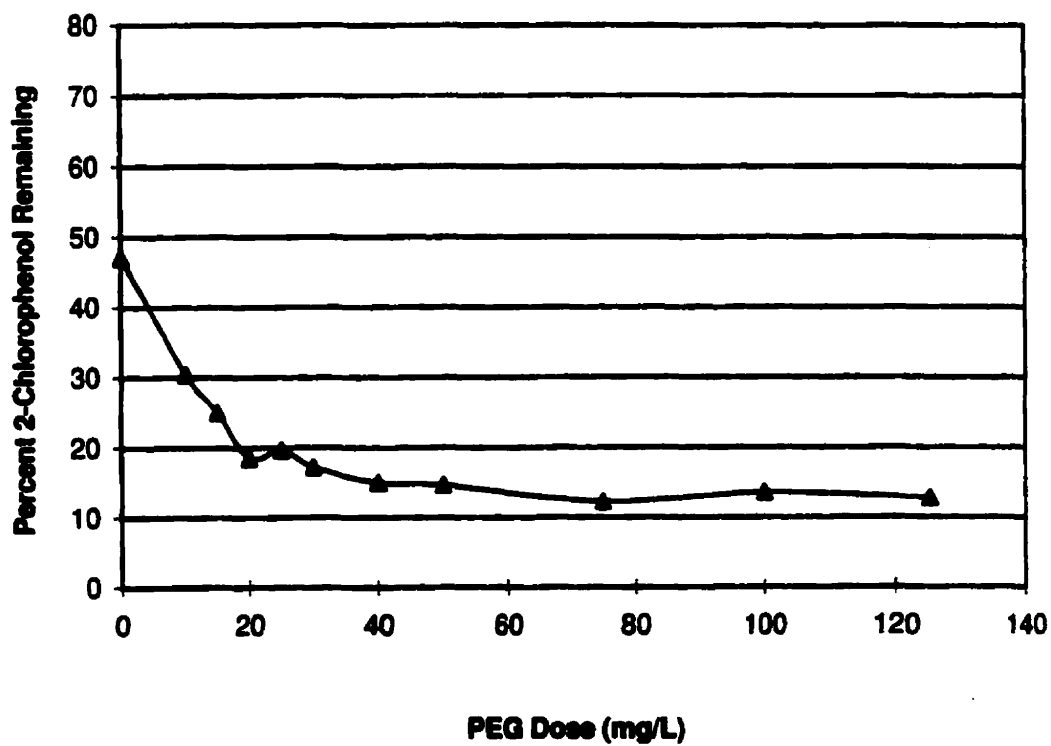
These figures show that the addition of PEG improved the removal efficiency up to an optimum additive dose. Beyond this optimum point, excess PEG neither increased nor decreased the removal efficiency for most compounds studied, as was found by Wu *et al* (1997). However for p-cresol (Figure 4.4.7), excess PEG actually had a negative effect on the amount of substrate that was removed. The minimum effective PEG dose for p-cresol was 20-40 mg/L and had a broad optimum range up to approximately 300 mg/L. With the PEG dose in excess of 300 mg/L, the percent of p-cresol remaining in solution increased drastically. The optimum PEG doses are listed in Table 4.5.

Minimum effective PEG doses varied from 20 to 400 mg/L, depending upon the phenolic compound. These results differ from the ones obtained in previous studies, where the minimum effective PEG doses were lower and less varied. This could either



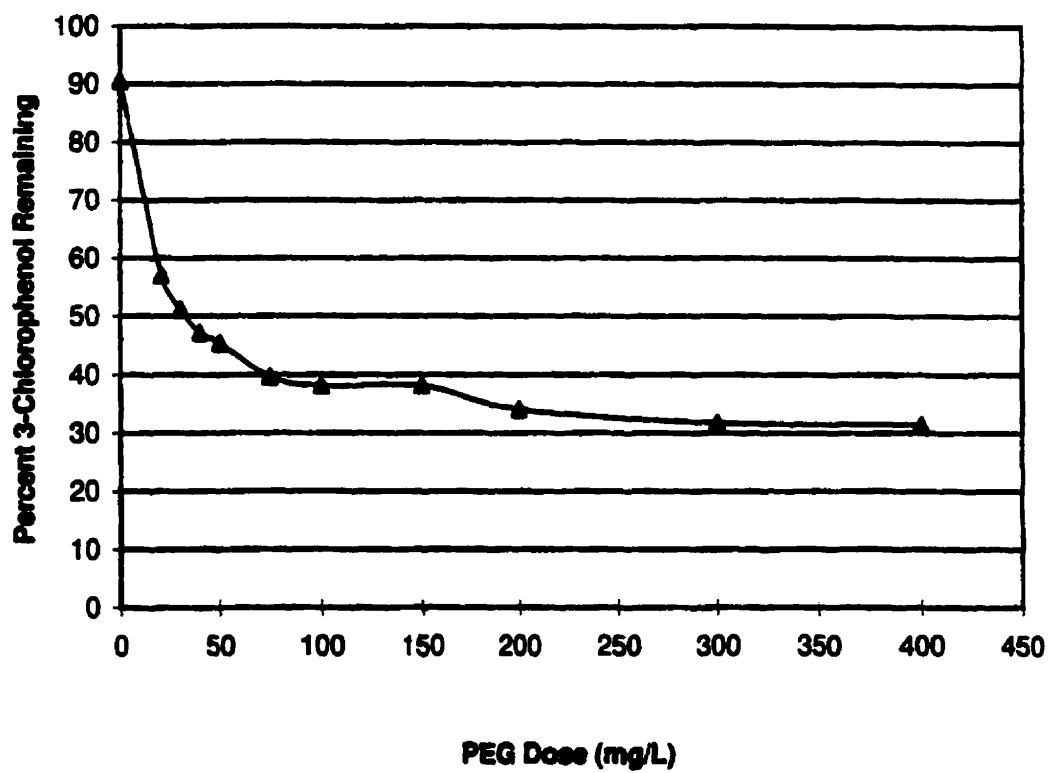
**Reactor Conditions:**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
SBP = 0.6 U/mL

**Figure 4.4.1: Effect of Polyethylene Glycol on the Removal of Phenol**



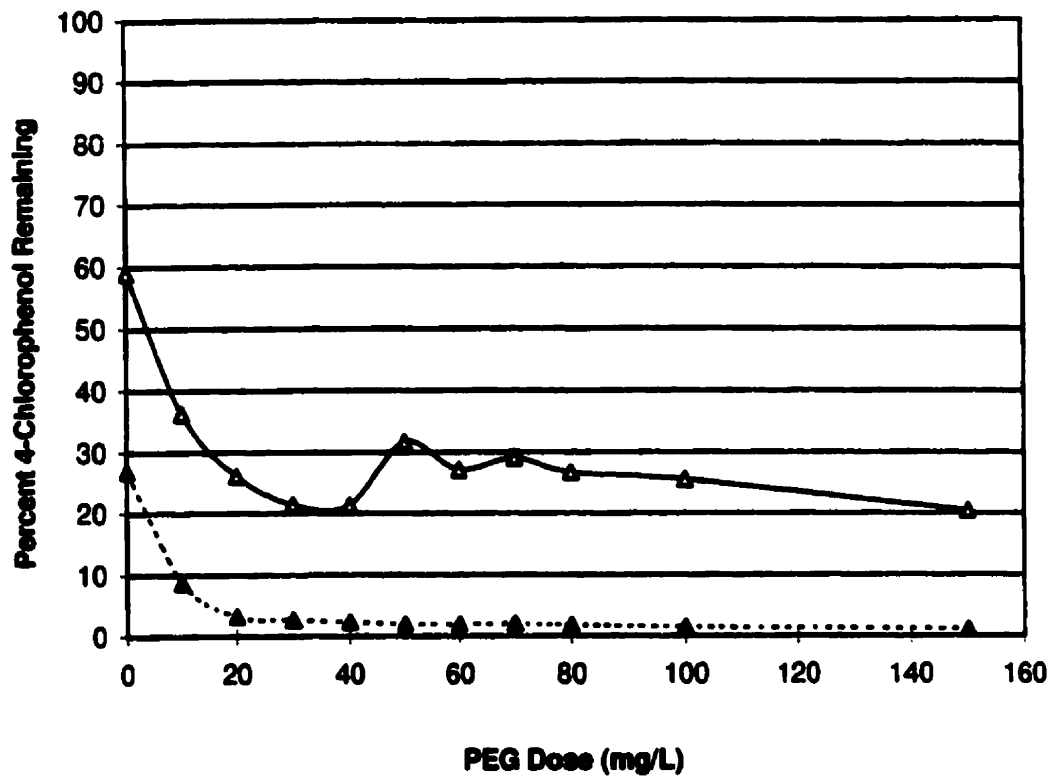
**Reactor Conditions:**  
Substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 0.8 mM/mM  
SBP = 0.12 U/mL

Figure 4.4.2: Effect of Polyethylene Glycol on the Removal of 2-Chlorophenol



**Reactor Conditions:**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 0.6 mM/mM  
SBP = 0.05 U/mL

Figure 4.4.3: Effect of Polyethylene Glycol on the Removal of 3-Chlorophenol

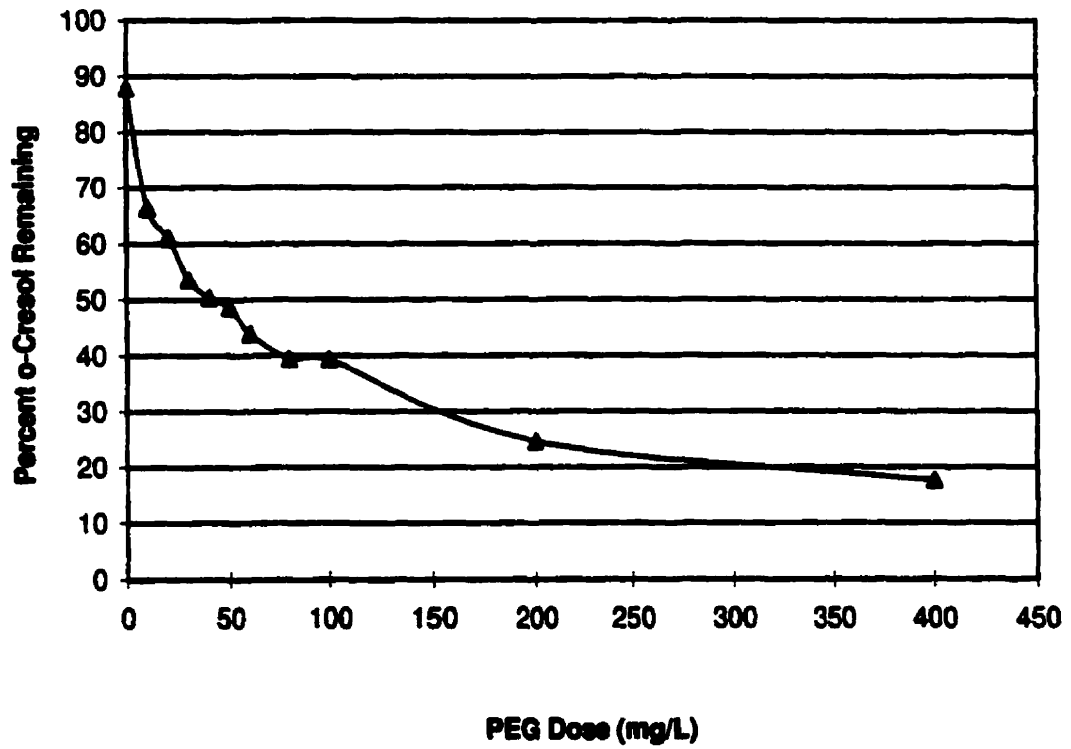


—▲— SBP = 0.07 U/mL    -●- SBP = 0.12 U/mL

**Reactor Conditions:**  
 Substrate = 1 mM  
 $[H_2O_2]/[Substrate] = 0.8 \text{ mM/mM}$

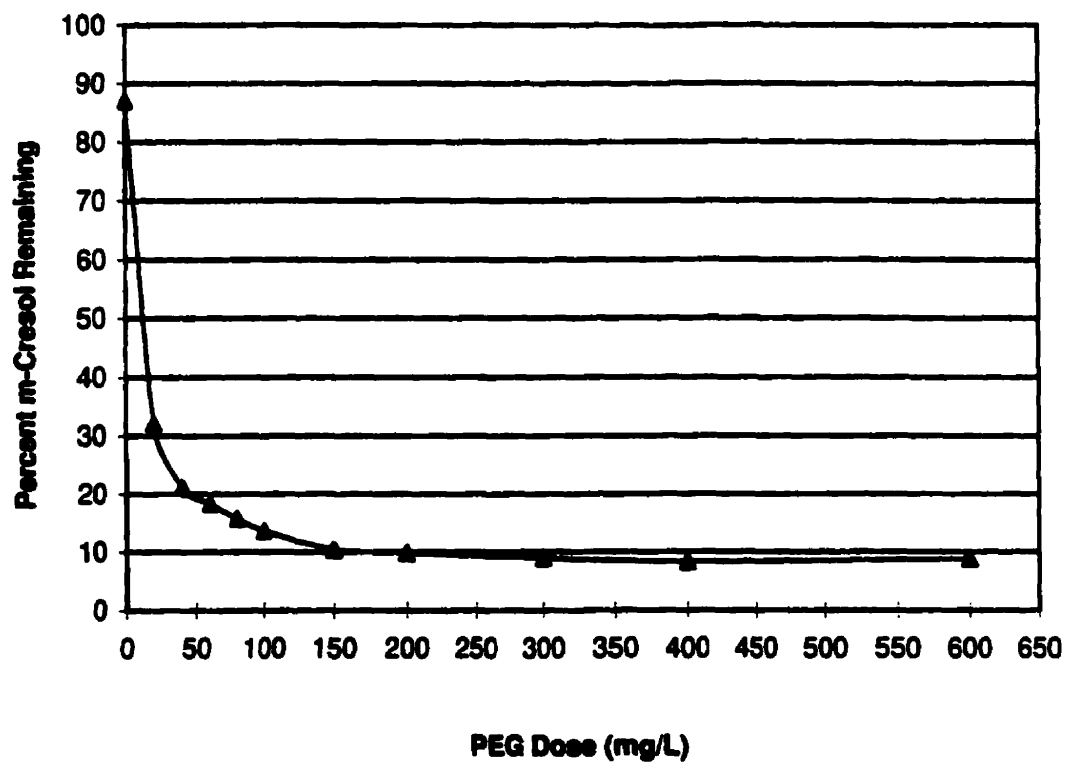
Figure 4.4.4: Effect of Polyethylene Glycol on the Removal of 4-Chlorophenol





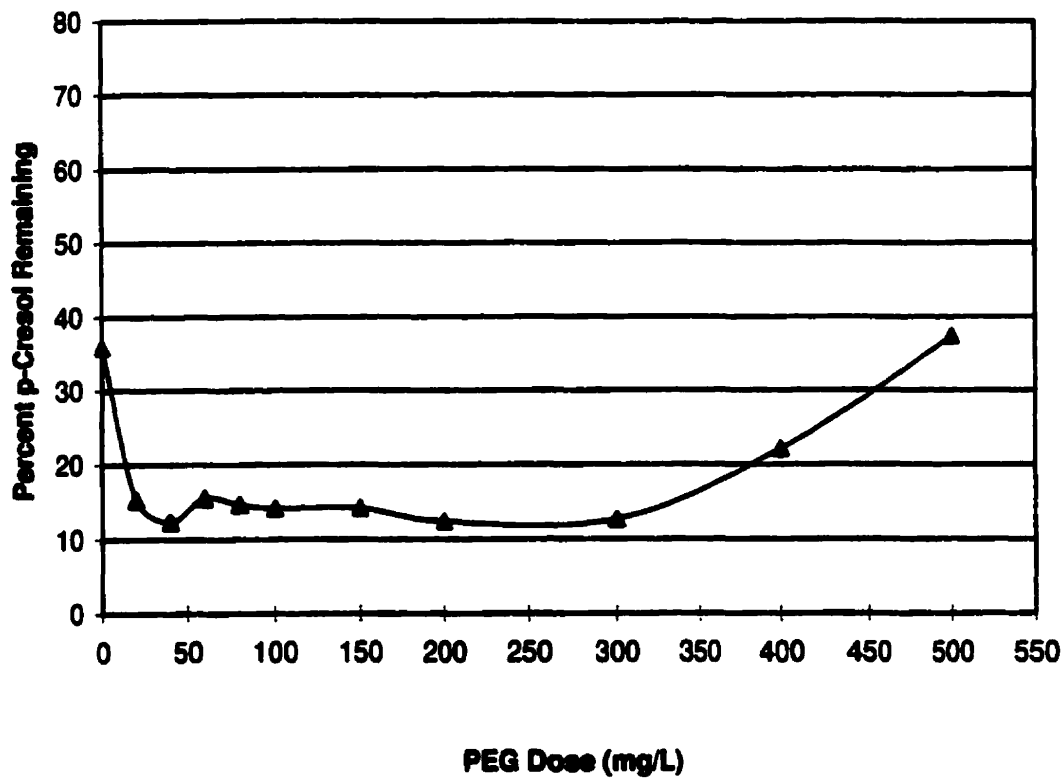
**Reactor Conditions:**  
Substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 0.9 mM/mM  
SBP = 0.04 U/mL

Figure 4.4.5: Effect of Polyethylene Glycol on the Removal of o-Cresol



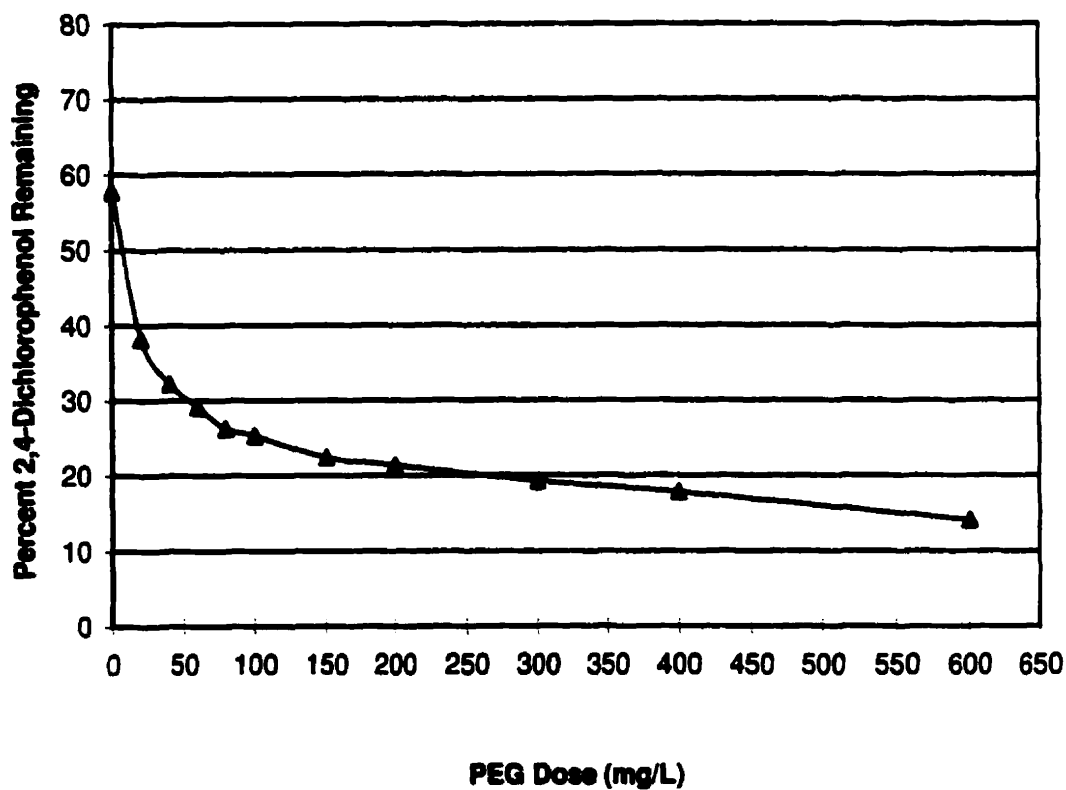
**Reactor Conditions:**  
Substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.0 mM/mM  
SBP = 0.04 U/mL

Figure 4.4.6: Effect of Polyethylene Glycol on the Removal of m-Cresol



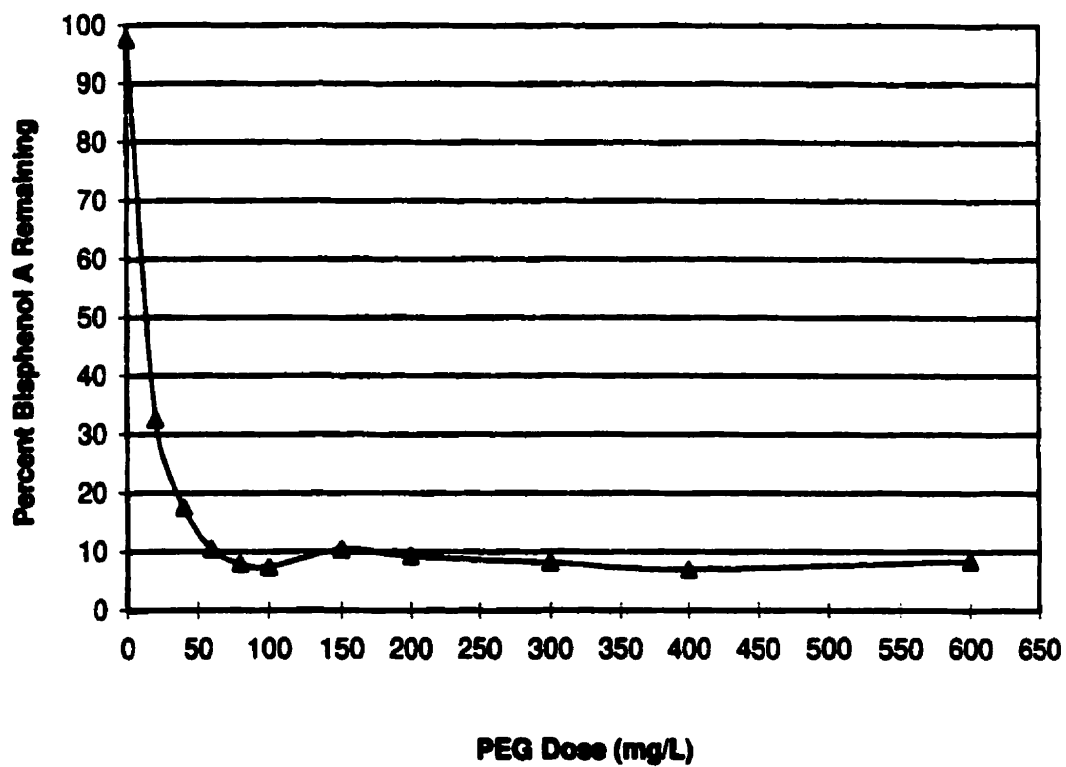
**Reactor Conditions:**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
SBP = 0.15 U/mL

**Figure 4.4.7: Effect of Polyethylene Glycol on the Removal of p-Cresol**



**Reactor Conditions:**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 0.7 mM/mM  
SBP = 0.02 U/mL

Figure 4.4.8: Effect of Polyethylene Glycol on the Removal of 2,4-Dichlorophenol



**Reactor Conditions:**  
 Substrate = 0.5 mM  
 $[H_2O_2]/[Substrate] = 1.6 \text{ mM/mM}$   
 SBP = 0.005 U/mL

Figure 4.4.9: Effect of Polyethylene Glycol on the Removal of Bisphenol A

be due to the fact that the enzyme used was SBP instead of HRP, or to the fact that these experiments were carried out in unbuffered solutions rather than buffered.

**Table 4.5: Minimum Effective PEG Dose for Aromatic Compounds**

<b>Aromatic Compound</b>	<b>Minimum Effective PEG Dose (mg/L)</b>
Phenol	50
2-Chlorophenol	40
3-Chlorophenol	75
4-Chlorophenol	30
o-Cresol	400
m-Cresol	150
p-Cresol	20-40
2,4-Dichlorophenol	150
Bisphenol A	60

#### **4.5 Coprecipitation**

The overall removal efficiency of a phenolic compound depends upon its reactivity toward peroxidase and the solubility of the products. Klibanov *et al.* (1980) observed that easily removed compounds aided in the precipitation of harder to remove compounds.

As seen in Section 4.2, certain compounds required less enzyme than others to reach a removal efficiency of 95% or greater. Phenol, for example, required 0.60 U/mL of SBP enzyme, whereas 2,4-dichlorophenol required only 0.04 U/mL to achieve the

same removal efficiency. Two sets of experiments were conducted to determine the benefit of coprecipitation on the removal of phenol in the presence of more easily removed compounds. The two compounds that were chosen for coprecipitation with phenol were 2,4-dichlorophenol and bisphenol A. The optimum parameters for these three compounds are listed in Table 4.6.

**Table 4.6: Summary of Optimum Parameters for Three Selected Phenolic Compounds**

<b>Aromatic Compound</b>	<b>SBP Dose with PEG (U/mL)</b>	<b>PEG Dose (mg/L)</b>	<b>[H<sub>2</sub>O<sub>2</sub>]/[Substrate] (mM/mM)</b>	<b>pH</b>
Phenol*	0.60	50	1.2	6.0
2,4-Dichlorophenol*	0.04	150	0.7	7.0
Bisphenol A**	0.015	60	1.2	6.5

\*Initial concentration equal to 1 mM.

\*\*Initial concentration equal to 0.5 mM.

In these coprecipitation experiments, the analysis was done using the colorimetric method that was used for all of the other compounds, except p-cresol. The amount of each individual compound was not determined, but instead, only the total percent of substrate remaining was calculated.

The total initial substrate concentration in this set of experiments was 1 mM; and, the concentration of each compound was 0.5 mM. Figure 4.5.1 shows the coprecipitation results for 2,4-dichlorophenol and phenol. The reaction parameters were as follows: pH = 6; PEG dose = 100 mg/L; and [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.0 mM/mM. It can be seen from Table 4.6 that the total enzyme dose required for an individual removal efficiency of 95% at a concentration of 0.5 mM is 0.32 U/mL (0.30 U/mL for phenol plus 0.02 U/mL for

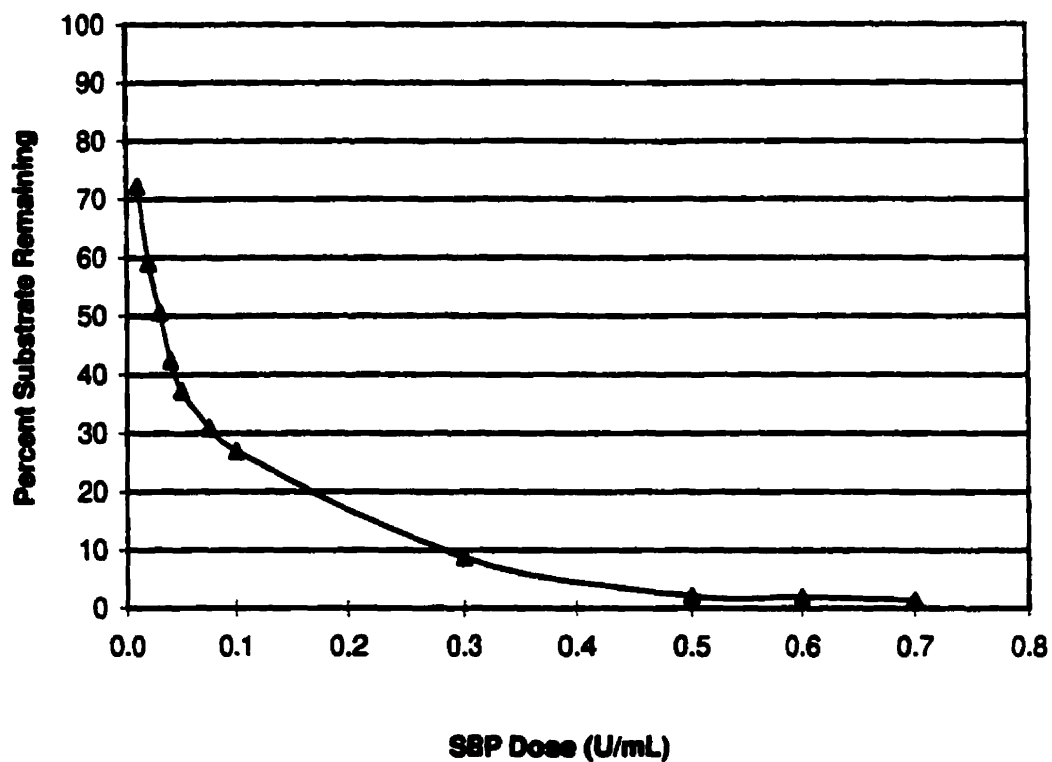
2,4-dichlorophenol). From Figure 4.5.1, the optimum SBP enzyme dose is 0.40 U/mL. Therefore the presence of an easier to remove compound did not aid in the removal of phenol.

The results for the removal of phenol in the presence of bisphenol A are shown in Figure 4.5.2. As in the previous coprecipitation experiment, the total initial substrate concentration was 1 mM, with the individual initial concentrations equal to 0.5 mM each. The experiments were conducted at pH = 6,  $[H_2O_2]/[Substrate] = 1.2$  mM/mM and a PEG dose = 80 mg/L. The total SBP enzyme dose required for 95% removal, calculated from the results in Table 4.6, is 0.315 U/mL (0.30 U/mL for phenol plus 0.015 U/mL for bisphenol A). The optimum SBP dose, from Figure 4.5.2, was found to be 0.25 U/mL, which is slightly less than the calculated requirements. Since there was only a slight improvement in enzyme dose, the presence of bisphenol A also did not significantly aid in the removal of phenol.

#### **4.6 Error Analysis**

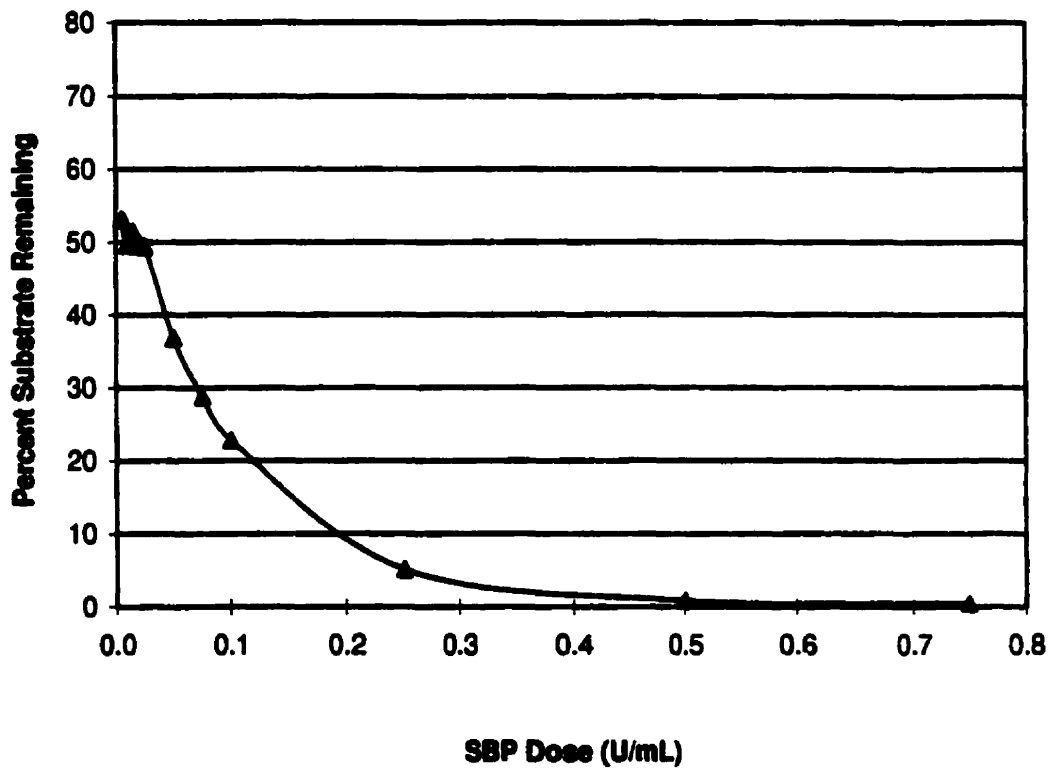
A set of experiments was conducted in order to determine the reliability of the results in this study. The  $H_2O_2$  to substrate ratio series for p-cresol was repeated three times at three different ratios and the percent error was calculated. The solutions were prepared separately to determine the accuracy of analytical techniques. The design parameters were as follows: pH = 7; initial p-cresol concentration = 1.0 mM; PEG dose = 50 mg/L; and SBP enzyme dose = 0.10 U/mL. The results of these experiments are listed in Table 4.7.





**Reactor Conditions:**  
 Total substrate = 1mM  
 $[H_2O_2]/[Substrate] = 1.0mM/mM$   
 PEG = 100 mg/L

**Figure 4.5.1: Effect of Enzyme Dose on the Removal of Two Substrates:  
 2,4-Dichlorophenol & Phenol**



**Reactor Conditions:**  
Total substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
PEG = 80 mg/L

**Figure 4.5.2: Effect of Enzyme Dose on the Removal of Two Substrates: Bisphenol A & Phenol**

**Table 4.7: Error Analysis**

<b>Sample #</b>	<b>[H<sub>2</sub>O<sub>2</sub>]/[Substrate] (mM/mM)</b>	<b>Percent Remaining</b>	<b>Average</b>	<b>Percent Deviation</b>
1	0.5	31.2	30.7	+ 1.63
2	0.5	31.2		+ 1.63
3	0.5	29.8		- 2.93
4	0.8	23.5	23.5	0
5	0.8	23.2		- 1.28
6	0.8	23.9		+ 1.70
7	1.5	28.4	29.1	- 2.41
8	1.5	28.7		- 1.37
9	1.5	30.1		+ 3.44

These results indicate that the deviation was always less than  $\pm 5\%$ , which is within an acceptable range. Therefore, the results obtained in this study are considered to be accurate and reliable.

## **5. CONCLUSIONS AND RECOMMENDATIONS**

### **5.1 Conclusions**

The results of this study have demonstrated the applicability of using soybean peroxidase (SBP) enzyme for treating phenolic wastewater. The reaction parameters, optimized to achieve a removal efficiency of at least 95%, were pH, SBP enzyme dose with and without PEG,  $[H_2O_2]/[Substrate]$ , and PEG dose. Table 5.1 summarizes the optimum doses for all of the reaction parameters.

The optimum pH occurred around pH 7 for most phenolic compounds except for 3-chlorophenol and 4-chlorophenol whose optimum pH was 5.0 and 8.0 respectively. Most of the compounds had a broad optimum pH range that generally became wider as the amount of SBP was increased. An increase in SBP concentration had no effect on the optimum pH.

Excess peroxidase had no significant effect on the removal of phenol; however, limiting the amount of SBP resulted in lower substrate removal efficiencies. The addition of PEG only slightly reduced the amount of enzyme required for 95% removal, except for bisphenol A

The optimum  $H_2O_2$  to substrate molar ratios ranged from 0.6 for 3-chlorophenol to 1.2 for both phenol and bisphenol A. As the molar ratio increased, the removal efficiency also increased until the optimum ratio was reached. Beyond the optimum ratio, there was a significant decrease in removal efficiency. The only compound to have a broad optimum range was bisphenol A. All other compounds had a definite optimum point.

**Table 5.1: Summary of Optimum Reaction Parameters for Various Aromatic Compounds**

<b>Aromatic Compound (1 mM)</b>	<b>Optimum pH</b>	<b>Optimum SBP Dose With PEG (U/mL)</b>	<b>Optimum SBP Dose Without PEG (U/mL)</b>	<b>[H<sub>2</sub>O<sub>2</sub>]/[Substrate] (mM/mM)</b>	<b>Minimum Effective PEG Dose (mg/L)</b>
Phenol	6.0	0.60	0.90	1.2	50
2-Chlorophenol	7.5	0.19	0.23	0.8	40
3-Chlorophenol	5.0	0.15	0.65	0.6	75
4-Chlorophenol	8.0	0.15	0.20	0.8	30
o-Cresol	7.0	0.08	0.60	0.9	400
m-Cresol	7.0	0.08	0.75	1.0	150
p-Cresol	7.0	0.40	0.60	0.9	20-40
2,4-Dichlorophenol	7.0	0.04	0.08	0.7	150
Bisphenol A (0.5 mM)	6.5	0.015	0.90	1.2	60

The addition of PEG had little effect on improving the substrate removal efficiency, except for bisphenol A. As discussed in Section 4.4, this might be due to a lower salt content in the reaction mixture since these experiments were carried out in unbuffered solutions instead of the buffered solutions used in previous studies. There was a wide range of minimum effective doses, that ranged from as low as 20 mg/L to as high as 400 mg/L. An excess of PEG had no measurable effect on the removal efficiency; however, there was one exception to this observation. For p-cresol, excess PEG drastically increased the amount of substrate that remained in solution.

Coprecipitation of phenol with an easier to remove compound did not reduce the SBP requirements for 95% removal. In the presence of 2,4-dichlorophenol, the enzyme requirements for the removal of phenol did not improve. Similarly, the minimum SBP dose for phenol in the presence of bisphenol A was not reduced significantly.

## **5.2 Recommendations**

The results of these experiments have shown that soybean peroxidase is a viable alternative to other enzymes that have previously been studied. In order to implement the enzymatic method of treatment to full scale industrial applications, several other aspects must be considered.

The potential toxicity of the final products must be studied. Once the nature of the by-products is determined, a suitable disposal method can be chosen.

The potential of using the soybean hulls instead of purchasing the enzyme from a chemical manufacturer should be investigated. After the enzyme has been extracted from the hulls, they could then be used as animal feed. This would greatly reduce the amount

of solid waste that is produced and in turn would also reduce costs. Similarly, some researchers have also suggested using crude HRP (Cooper *et al.*, 1996; Dec *et al.*, 1994; Klibanov, 1982) to reduce treatment costs. In contrast to crude SBP, the amount of solid waste produced with the use of crude HRP is much greater. Another advantage of using soybeans is that they are more readily available than horseradishes. Finally, a detailed cost analysis should be done in order to determine the applicability of this process over current treatment methods.

The effect of coprecipitation should continue to be investigated by using different compounds than the ones used in this study. The mixture of other compounds may yield better results.

An investigation on the applicability of using SBP on a real wastewater matrix should be conducted. Other components that are present in an actual wastewater stream may interfere with or improve the removal of the phenolic compounds.

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**APPENDIX A**  
**Enzyme Activity Assay**

## 1. General

The purpose of the enzyme activity assay is to determine the amount of active enzyme that is present in a solution. Under saturating conditions of phenol, AAP and  $\text{H}_2\text{O}_2$ , the initial rate is measured by observing the rate of colour formation in a solution. The reaction between phenol and  $\text{H}_2\text{O}_2$  is catalyzed by the enzyme (SBP) such that the products of the reaction react with AAP to form a red coloured solution which absorbs light at a peak wavelength of 510 nm.

## 2. Reagents

i) Phosphate buffer (0.1 M NaPP, pH 7.4)  
160 mL 0.2 M monobasic sodium phosphate  
840 mL 0.2 M dibasic sodium phosphate  
Distilled water to 2 L

ii) Phenol (0.1 M phenol)  
1882.2 mg phenol  
Phosphate buffer to 200 mL  
Store in refrigerator

iii) 4-Aminoantipyrine (9.6 mM AAP)  
390 mg AAP in flask  
Phosphate buffer to 200 mL  
Store in refrigerator

iv) Hydrogen peroxide (2.0 mM  $\text{H}_2\text{O}_2$ )  
a) 226.7  $\mu\text{L}$  of 30%  $\text{H}_2\text{O}_2$   
Distilled water to 100 mL  
b) 10 mL of  $\text{H}_2\text{O}_2$  solution from a)  
Distilled water to 100 mL

## 3. Procedure

In a semi-micro cuvette, combine in the following order:

50  $\mu\text{L}$  SBP solution  
500  $\mu\text{L}$  NaPP buffer  
100  $\mu\text{L}$  0.1 M phenol  
250  $\mu\text{L}$  9.6 mM AAP  
100  $\mu\text{L}$  2.0 mM  $\text{H}_2\text{O}_2$

The total volume in the cuvette should be 1 mL, and the rate of colour formation must be measured before substrate depletion becomes significant. Immediately after the addition of the sample, shake the cuvette and then monitor the absorbance change with time at 510 nm.

#### 4. Calculation

i) Find the average slope over the linear range of the data in terms of absorbance units per unit time (au/min).

ii) Calculate the activity in the cuvette

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

The activity is in terms of micromoles of hydrogen peroxide converted per minute at 20°C and pH 7.4.

iii) Calculate the activity of the sample

$$\text{Activity in sample (U/mL)} = \text{Activity in cuvette (U/mL)} \times \frac{1000(\mu\text{L})}{\text{sample volume}(\mu\text{L})}$$

## **APPENDIX B**

### **Aromatic Substrate Assay**

## 1. General

This is a colorimetric assay used to measure the concentration of an aromatic substrate in an aqueous sample. It uses ferricyanide and 4-aminoantipyrine as colour generating substrates when in combination with the aromatic sample. The only limiting reagent is the amount of the aromatic compound, and therefore, the degree of colour generated at a peak wavelength of 510 nm is proportional to the amount of aromatic present.

## 2. Reagents

i) Ferricyanide reagent (83.4 mM of  $K_3Fe(CN)_6$  in 0.25 M  $NaHCO_3$ )

2.75 g  $K_3Fe(CN)_6$   
2.1 g  $NaHCO_3$   
Distilled water to 100 mL

ii) 4-Aminoantipyrine reagent (20.8 mM of AAP in 0.25 M  $NaHCO_3$ )

0.423 g AAP  
2.1 g  $NaHCO_3$   
Distilled water to 100 mL

## 3. Procedure

In a semi-micro cuvette combine in the following order:

800  $\mu$ L of aromatic sample (diluted if necessary)  
100  $\mu$ L of AAP reagent  
100  $\mu$ L of ferricyanide reagent

The final assay sample volume should be 1 mL. After a couple minutes, measure the absorbance at 510 nm against a reagent blank.

## 4. Calculation

Using the appropriate calibration curve (Appendix C), convert absorbance readings into desired concentration units.

## **APPENDIX C**

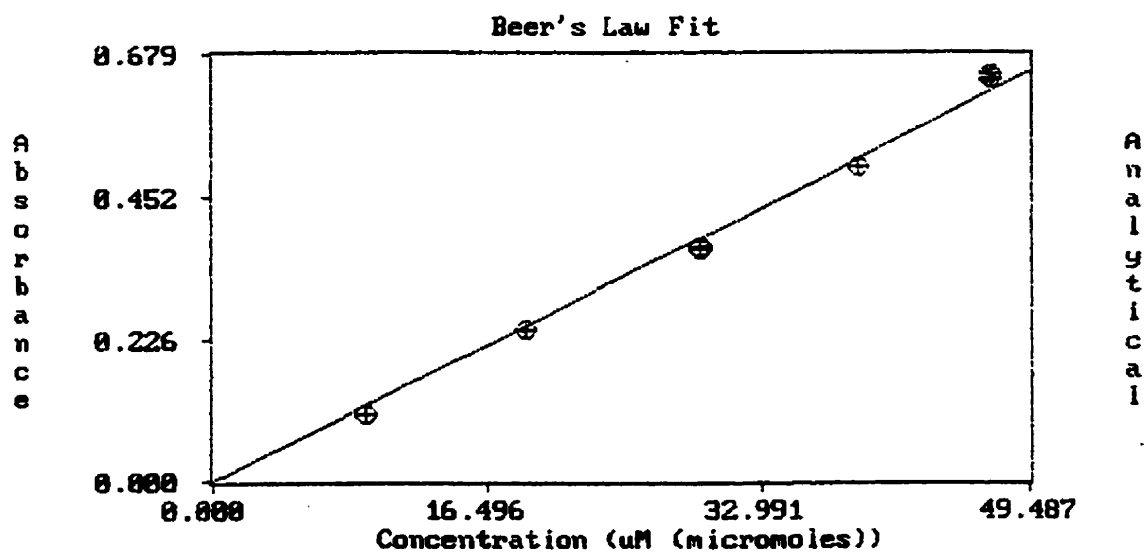
### **Standard Curves for Aromatic Compounds**



# Phenol:

Sample Name : Phenol  
Solvent Name : Deionized water  
Conc Units :  $\mu\text{M}$  (micromoles)

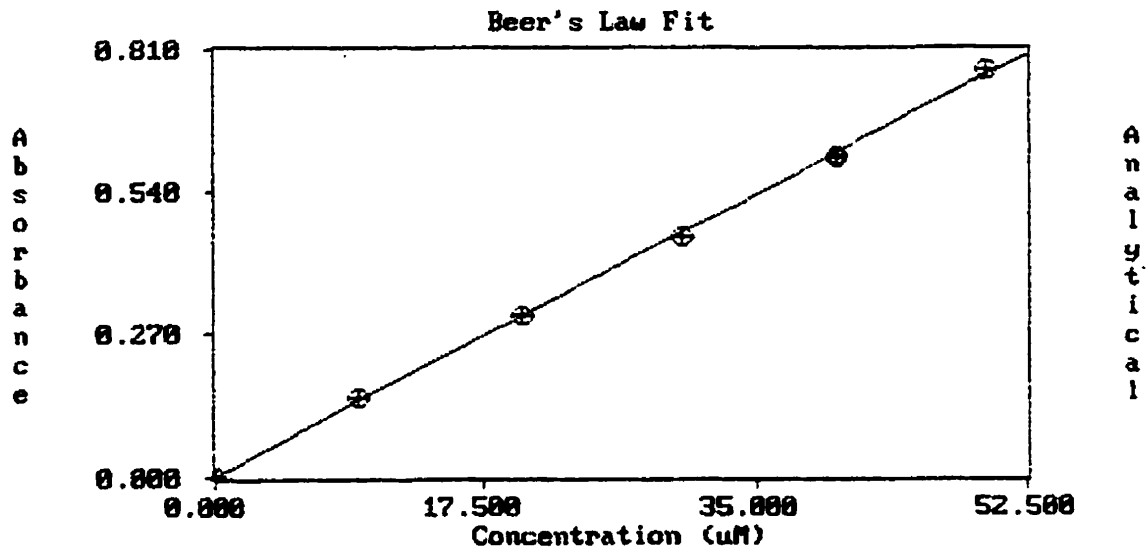
Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



## 2-Chlorophenol:

Sample Name : 2-CHLOROPHENOL  
Solvent Name : DEIONIZED WATER  
Conc Units :  $\mu\text{M}$

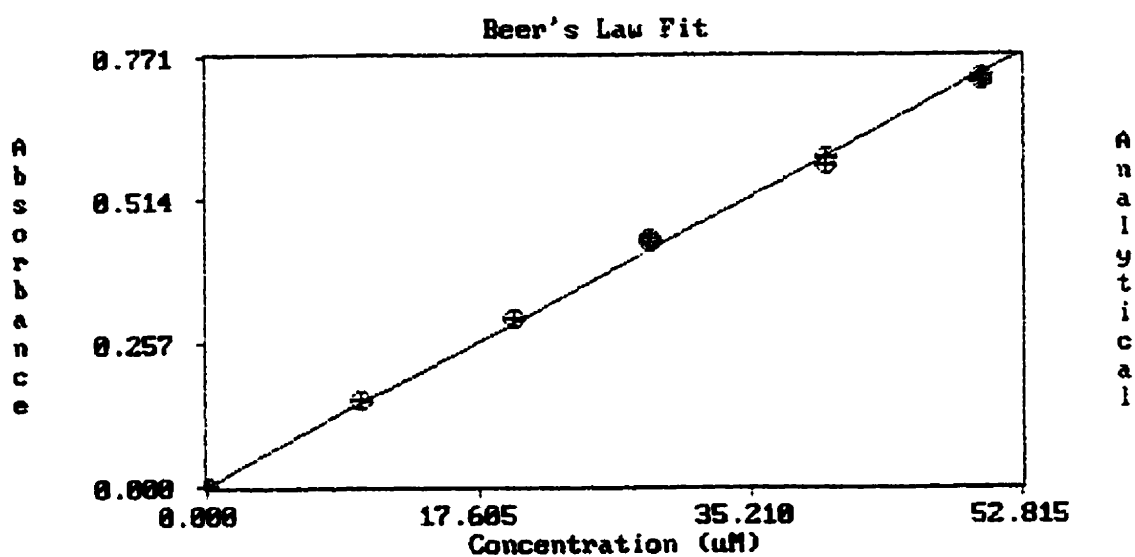
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Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



### 3-Chlorophenol:

Sample Name : 3-chlorophenol  
Solvent Name : deionized water  
Conc Units :  $\mu\text{M}$

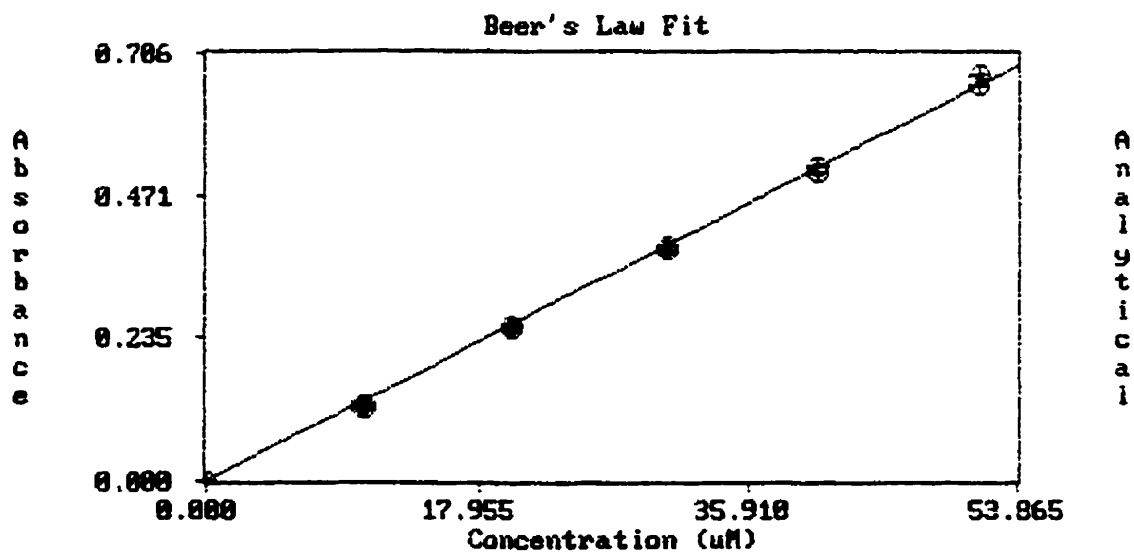
Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



## 4-Chlorophenol:

Sample Name : 4-Chlorophenol  
Solvent Name : Deionized water  
Conc Units :  $\mu\text{M}$

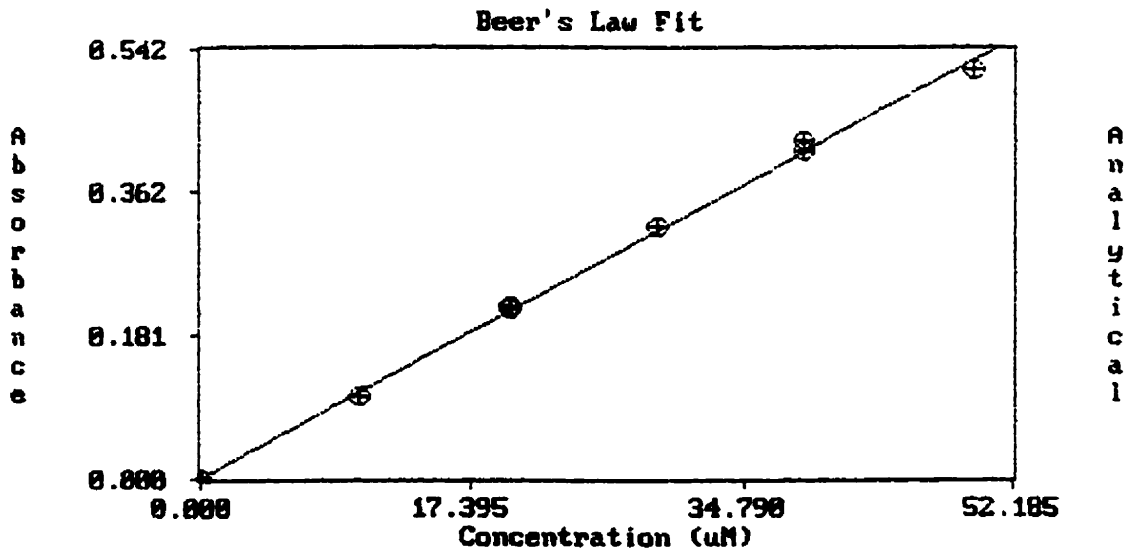
Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



**o-Cresol:**

Sample Name : o-cresol  
Solvent Name : deionized water  
Conc Units :  $\mu\text{M}$

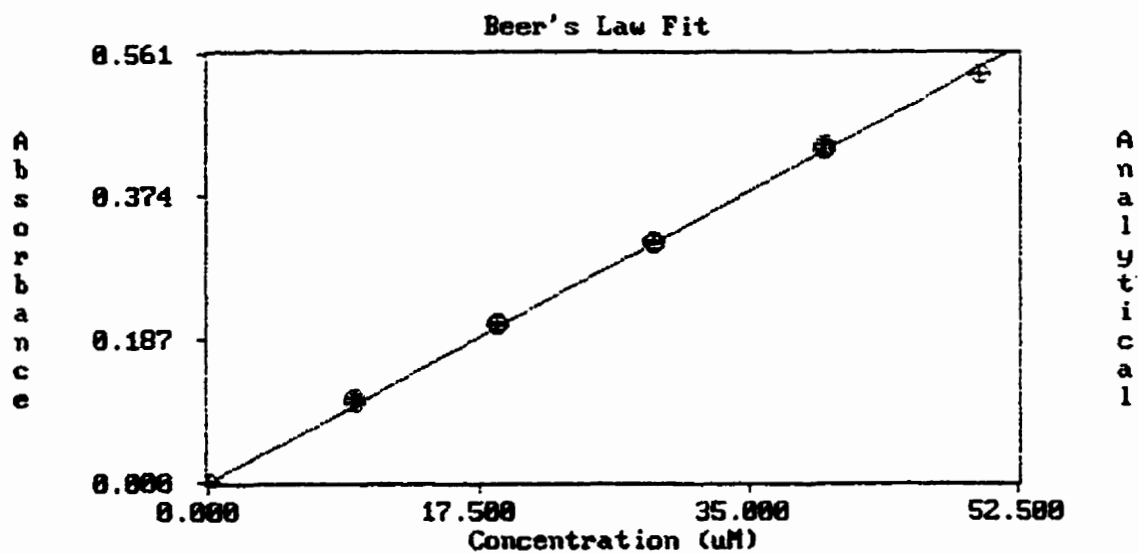
Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



**m-Cresol:**

Sample Name : m-Cresol  
Solvent Name : Deionized Water  
Conc Units :  $\mu\text{M}$

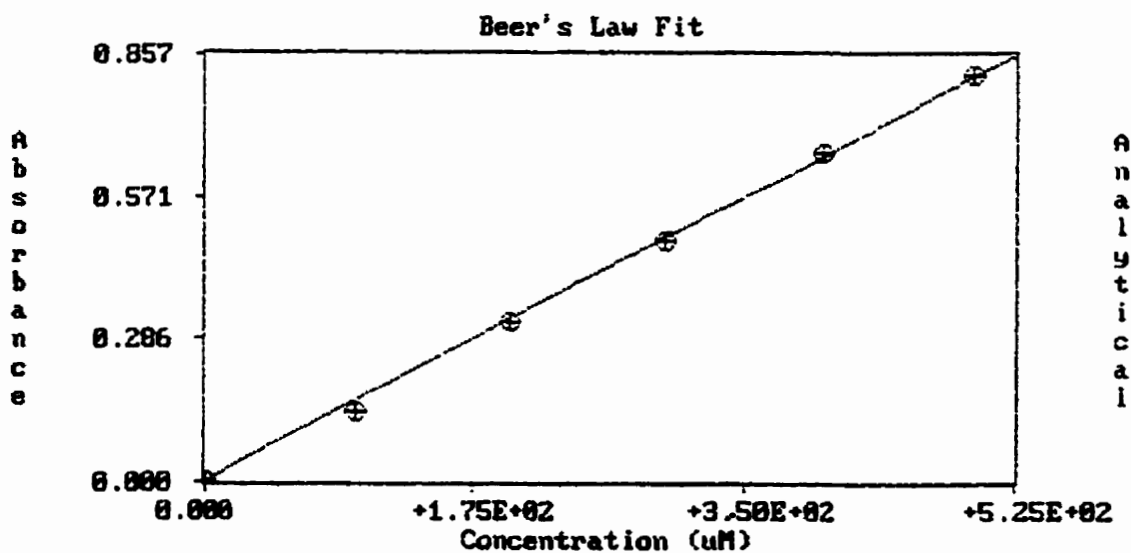
Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



# p-Cresol:

Sample Name : p-Cresol  
Solvent Name : Deionized water  
Conc Units :  $\mu\text{M}$

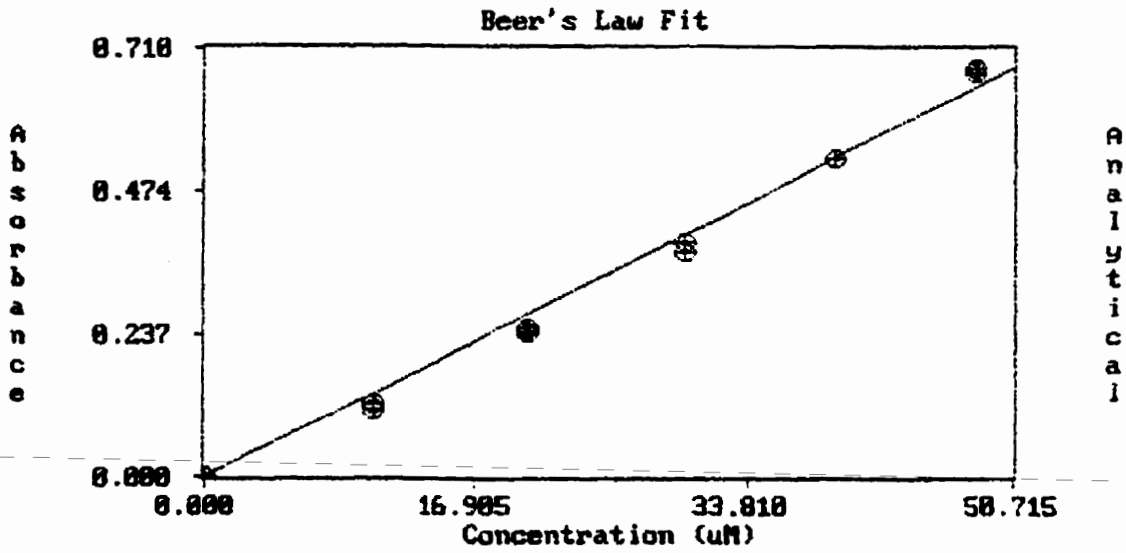
Analytical Wavelength : 278 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



## 2,4-Dichlorophenol:

Sample Name : 24Dichlorophnl  
Solvent Name : Dei. Water  
Conc Units : uM

Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds

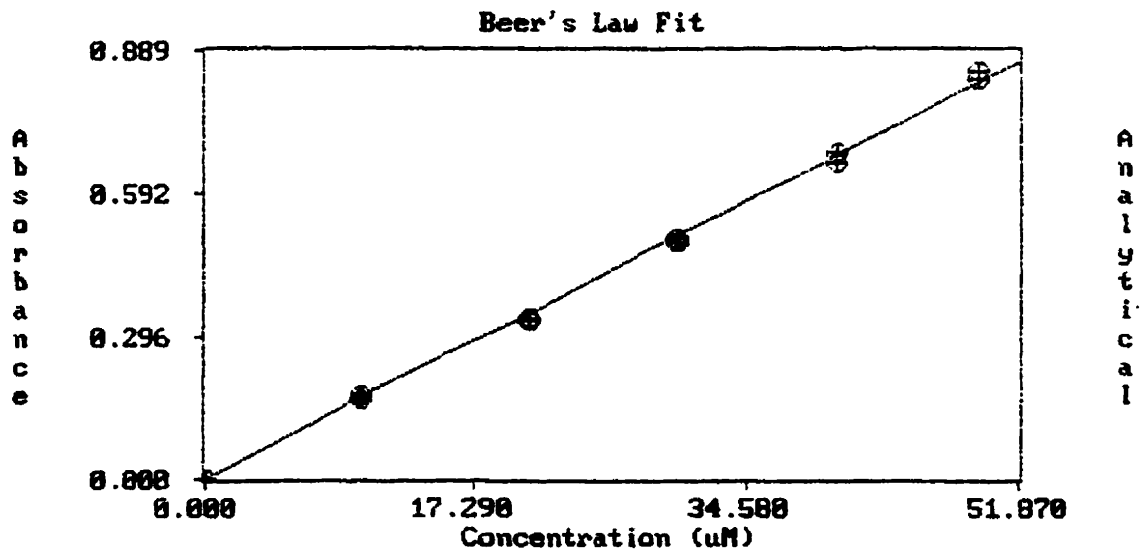




# Bisphenol A:

Sample Name : Bisphenol A  
Solvent Name : Deionized Water  
Conc Units :  $\mu\text{M}$

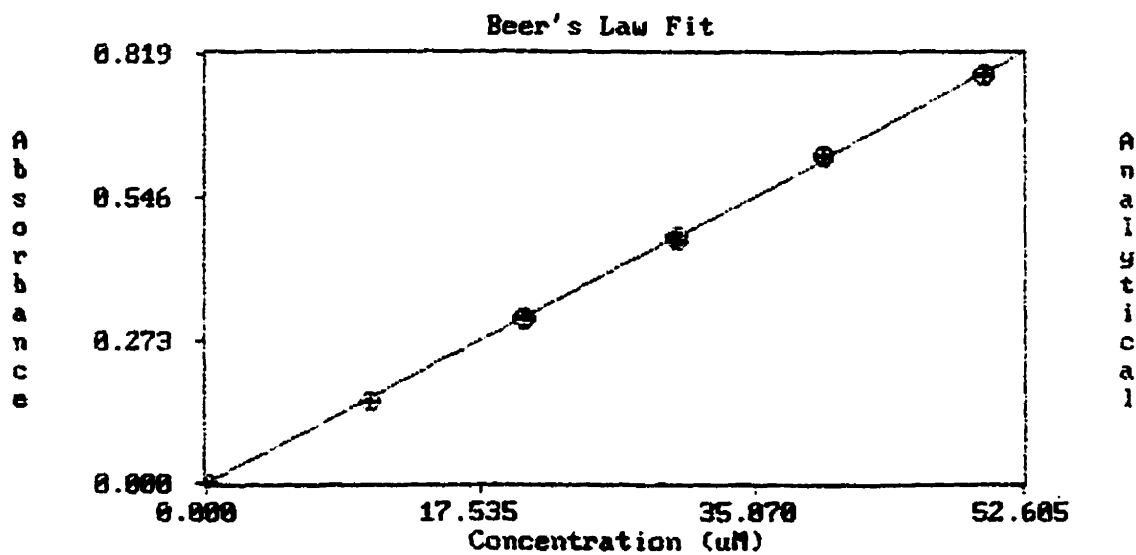
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Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



## 2,4-Dichlorophenol & Phenol:

Sample Name : 24Dichl+Phenol  
Solvent Name : Deionized Water  
Conc Units :  $\mu\text{M}$

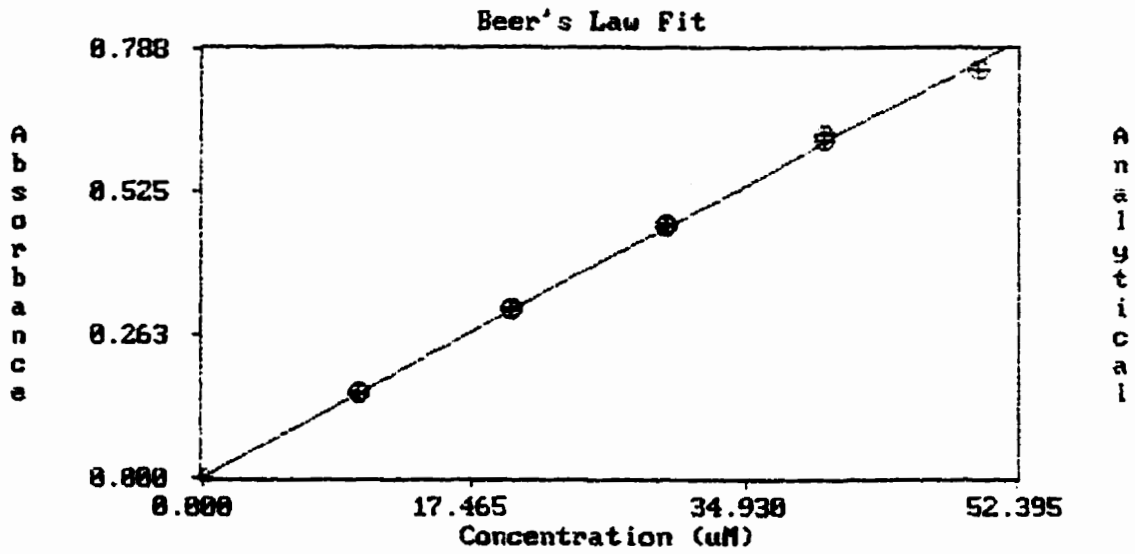
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Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



# Bisphenol A & Phenol:

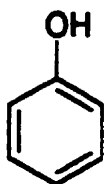
Sample Name : BisphiA+Phni  
Solvent Name : Deionized Water  
Conc Units : uM

Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds

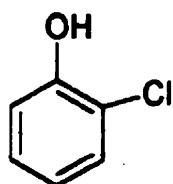


## **APPENDIX D**

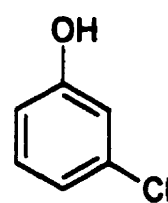
### **Chemical Structures for the Aromatic Compounds**



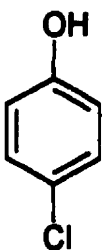
**Phenol**



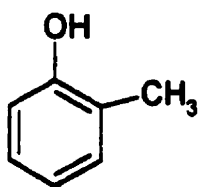
**2-Chlorophenol**



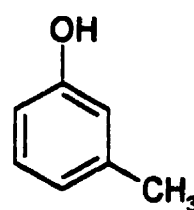
**3-Chlorophenol**



**4-Chlorophenol**



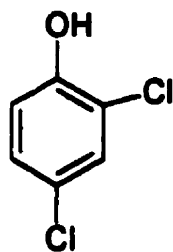
**o-Cresol**



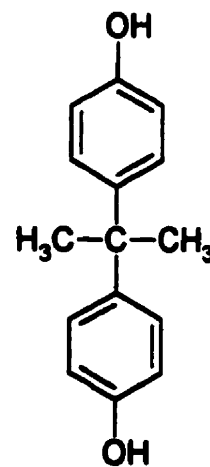
**m-Cresol**



**p-Cresol**



**2,4-Dichlorophenol**



**Bisphenol A**

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