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REMOVAL OF PHENOL DERIVATIVES FROM AQUEOUS SOLUTION BY HORSERADISH PEROXIDASE IN THE PRESENCE OF ADDITIVES

by

Yimin Wu

A thesis submitted to the
Faculty of Graduate Studies and Research
through
the Department of Civil and Environmental Engineering
in partial fulfilment of the requirements for the
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ABSTRACT

It has been observed that horseradish peroxidase (HRP) can be used to remove phenol and its derivatives from wastewater. However, the large amount of HRP needed has limited its practical application. It has been reported recently that additives like polyethylene glycol (PEG) and gelatin can greatly reduce the amount of enzyme required for polymerization of phenolic compounds in high concentrations. Experiments were carried out to investigate the effect of additives on the HRP catalyzed removal of phenol derivatives at lower concentrations. Phenol derivatives studied included chlorinated phenols and methyl phenols. The results showed that there was a wide pH range, usually 6 to 8, for optimal removal of phenol derivatives. The optimum pH is mostly neutral except for 2-chlorophenol which had optimum pH of 5. Polyethylene glycol can reduce the amount of HRP needed for 95 percent or more removal of phenol derivatives from 1/30 to 1/130 and consequently, the turnovers were increased 30 to 130 times. The minimum PEG dose at a phenolic concentration of 1 mM (about 100 mg/L) varied from 30 to 100 mg/L, depending on the nature of phenolic compound . Extra PEG did not improve its effect. The time needed for the completion of polymerization varied from 1 to 3 hours at the minimum HRP and minimum PEG dose condition. For most of the phenol derivatives investigated, the optimum molar ratio of hydrogen peroxide to phenol derivatives was around 1. Coprecipitation can occur after adding additive.

DEDICATION

Dedicated to my wife and our mothers

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CHAPTER 1

INTRODUCTION

Phenolic wastewaters are discharged from a number of industries such as coal conversion, resins and plastics, textiles, dyes and other organic chemicals, timber, soaps and detergents, paving and roofing, iron and steel and petroleum refining. Almost all these phenolic wastes are toxic and many of them have been classified as hazardous pollutants. Regulations concerning phenols are strict. For example, the MISA requires phenols in the wastewater to be treated to as low as 20 μg/L before its release (Environment Ontario, 1988). The provincial water quality objectives of Ontario for surface water requires phenols not exceed 1 μg/L to protect against tainting of edible fish flesh (Ministry of Ontario, 1984). Therefore, it is necessary to remove phenols from industrial wastewater before its discharge to surface waters. Conventional methods for dephenolization of industrial wastewaters, including solvent extraction, microbial degradation, adsorption on activated carbon, and chemical oxidation suffer from such serious drawbacks as high cost, incomplete removal, formation of toxic by-products and applicability to a limited concentration range (Klibanov, 1980).

1.1 ENZYME

The idea of using an enzyme to remove toxic substances in wastewater is not new. However, it is only recently that extensive research has been conducted on enzymatic treatment of wastewater. Fundamentally, biological treatment of wastewater is

based on the microorganisms (mainly bacteria) which produce enzymes to decompose the pollutants. All enzymes are identified as proteins, which are polymers of amino acids combined by peptide bonds. As proteins, enzymes have three-dimensional structure which is essential for catalysis. These structures may be disrupted by pH or temperature changes and also can be made inactive by inhibitors of various types (Trever, 1985).

It is virtually impossible to measure the amount of any enzyme directly. Instead, enzymes are measured by their catalytic activity, i.e. by measuring the rate of reaction that they are catalyzing and comparing it with the rate of the uncatalyzed reaction. The factors affecting enzyme reaction include the enzyme concentration, substrate concentration, pH, temperature, reaction time and inhibitors (Wynn, 1979).

1.2 APPLICATION OF ENZYMES IN WASTEWATER TREATMENT

The usage of enzymes in wastewater treatment was widely investigated in the 1980's in Europe, North America and Japan (Wu, 1992). The wastewater tested included pesticides, aromatic compounds, urea, cyanide and organic compounds, etc. All these investigations showed that use of enzymes to treat the wastewater, which was difficult to treat by biological methods, was practical and more effective.

Most of the research was concentrated on the removal of aromatic compounds using different enzymes, for example, horseradish peroxidase, laccases, tyrosinase and ligninases. Horseradish peroxidase (HRP) proved to be very effective to treat phenols and aromatic amines and applications on actual wastewater were reported. Researchers showed that HRP could be used to transform phenolic or aromatic amine radicals into water-

insoluble polymers which subsequently could be removed easily by filtration or sedimentation (Klibanov, 1980). Although HRP was not effective in removing certain compounds such as polychlorinated biphenyls in water, coprecipitation occurred when treated together with reactive compounds, which improved the removal efficiency.

Some of the advantages include:

(i) enzymes are not affected by many inhibitors of microbial metabolism

(ii) they can be used under a wider range of extreme environmental conditions (pH and temperature, etc) compared to biological treatment

(iii) they have the flexibility to function with a broad range of substrate types and concentrations, and

(iv) usually they need a short reaction time because of their high activity.

The large amount of enzyme needed in this treatment method has limited its practical application to date. However, it has been reported recently that additives such as polyethylene glycol and gelatin can greatly reduce the amount of enzyme required for polymerization of phenolic compounds at high concentrations (Nakamoto, 1992). Studies on low concentrations of phenol solutions showed that additive was still effective in reducing the amount of HRP needed in completing polymerization of phenol (Wu, 1992).

1.3 OBJECTIVE

The objective of this study was to investigate the effect of additives on horseradish peroxidase catalyzed removal of phenol derivatives at low concentration (1 mM, around 100 mg/L) frequently found in industrial wastewaters.

1.4 SCOPE

The scope of this study included:

(i) Phenol derivatives studied were 2-, 3- ,4-chlorophenols, o-, m-, p-cresols and 2,4-dichlorophenol.

(ii) Polyethylene glycol (PEG) and gelatin were used as additives.

(iii) Parameters investigated were: optimum pH range, effect of additive on HRP dose, minimum additive dose, reaction time needed and optimum ratio of hydrogen peroxide dose to phenolic compounds.

(iv) effect of additive on coprecipitation of the mixture of phenolic compounds was also studied.

CHAPTER 2

LITERATURE REVIEW

2.1 HORSERADISH PEROXIDASE

Horseradish peroxidase is among the enzymes which are ubiquitous in the plant and animal kingdoms. Its discovery can be traced back to 1903, and intensive studies have been carried out with it. There are only three important isoenzymes: isoenzyme A (acidic), isoenzyme C (neutral or slightly basic) and a strongly basic HRP. Among them, most commercial preparations are isoenzyme C, and most of the research is based on HRP-C. Horseradish peroxidase consists of 308 amino acid residues. Its molecular weight has been estimated from 33890 to 42100. The purity can be expressed by R.Z. number, a ratio of absorbances at 403 and 280 nm, which is a measure of hemin content using the aromatic amino acid content as reference. Very good preparations of HRP have R.Z. values of 3 to 3.4. However it is not a direct indication of enzymatic activity (Dunford, 1991).

Native HRP is an ideal enzyme. It is stable for long periods of time at room temperature over the pH range 5 - 10. Storage of the enzyme in distilled water at neutral pH and 4 ^{o}C for one month produced no significant loss in activity (Nicell, 1991). Also, storage of the aqueous enzyme, buffered at pH 7.4 at room temperature for 2 days with or without vigorous mixing, produced only 5 % loss in activity. The dry enzyme retains its activity when stored for several years at -15 ^{o}C and for many weeks when stored at 37 ^{o}C . The enzyme activity had a broad optimum over a pH range of 5.7 to 8.5 with

optimal activity occurring at neutral pH. It retained more than 90 % of its activity after incubation for 48 hours at pH's between 6 to 9 and room temperature but was severely reduced outside this range. There is no significant loss in the HRP activity on incubation in the temperature range of 5 ^{0}C and 35 ^{0}C (Nicell, 1991).

2.2 HORSERADISH PEROXIDASE IN WASTEWATER TREATMENT

Early in 1980, Klibanov and his co-workers (Klibanov et al., 1980) reported a new enzymatic method developed for the removal of phenols and anilines from industrial wastewaters. They used horseradish peroxidase and hydrogen peroxide to treat the aqueous solutions containing the pollutants. Over 30 different phenols and aromatic amines were tested using this method. Phenols and aromatic amines were easily removed from water with a high efficiency (exceeding 99%). It was suggested that the corresponding phenolic and aromatic amine radicals were generated during the reaction. These free radicals polymerized to form polyaromatic products, which were practically insoluble in water (see Figure-2.2.1). These insoluble polymers could be easily separated by simple filtration or sedimentation procedures. Optimal pH for phenol derivatives were from 3.5 to 7.0. The minimum H_2O_2 needed for 99.8% removal of 0.8 mM of 2-chlorophenol was 1.0 mM. The peroxidase needed to reach 99.8% removal efficiency could be decreased by increasing the reaction time. The removal efficiency for different concentrations of 2-chlorophenol, in the range 1 mg/L to 150 mg/L was found to be constant within the experimental error. The authors concluded that at least two independent factors affected the overall removal efficiency of a phenol or an aniline from

water: its reactivity toward peroxidase and the solubility in water of the products of peroxidase oxidation. The authors also reported a strategic coprecipitation in which easily removed pollutants aided in the precipitation of other phenols and anilines which had been removed less readily when alone.

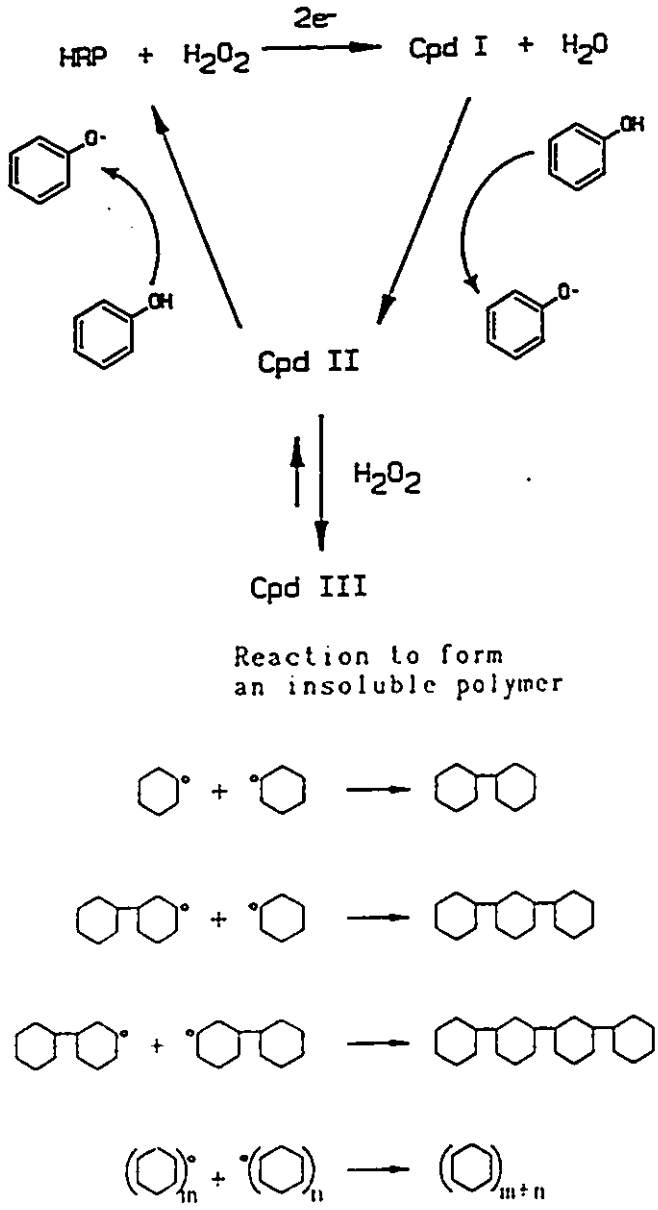


Figure-2.2.1 Catalytic cycle of horseradish peroxidase (Nicell, 1990)

Horseradish peroxidase was applied to dephenolize coal-conversion wastewaters (Klibanov et al., 1983). They investigated a typical coal-gasification wastewater with the following composition: phenol 2000 mg/L, ammonia 5000 mg/L, chloride 19000 mg/L, cyanide 100 mg/L, thiocyanate 1000 mg/L and other constituents that adversely affect bacterial and other methods of dephenolization. The removal efficiency was 97 percent (as compared to 98 percent for a phenol concentration of 2000 mg/L in the absence of other contaminants). When phenol concentration was only 100 mg/L, the removal efficiency was only 10 percent. This implied that components of coal-gasification wastewaters inhibited peroxidase, but this inhibition was insignificant at high concentration of phenol. The reason might be that phenol displaces the inhibitors from the complexes with the enzyme. The authors also found that even pollutants that were not reactive toward peroxidase might be enzymatically precipitated if the waste water also contained other pollutants that were readily removed by the enzyme. The authors proved through experiments that the enzymatic removal of phenol stopped when the peroxidase activity disappeared. Neither phenol nor H_2O_2 alone inactivated the enzyme under the same conditions. They suggested that inactivation of peroxidase took place during the enzymatic reaction, most likely as a result of the interactions of the phenoxy radicals with the enzyme's active center.

A French group studied the effectiveness of this method for removal of low levels of phenolic compounds in drinking water (Maloney et al., 1984, 1986). They observed that at concentrations below 0.01 mg/L of 2-chlorophenol, the conversion efficiencies could be still higher than 95 percent. The problem with the low concentration was that

no precipitation was observed and, therefore, the by-products could not be removed from the drinking water. The potential competition or interference by background organic compounds was also investigated. The results indicated that these background organics, assumed to be humic and fulvic acids, did not interfere with the enzyme reaction. Experiments also showed that by-products yielded two major fractions. One was a highly polar fraction and the other was a high molecular weight non-polar fraction.

Bollag and his co-workers (1988,1990) studied the effectiveness of different enzymes including HRP, laccases and tyrosinase. They found that the removal of phenols through polymerization depended on the chemical structure and concentration of the substrate, pH of the reaction mixture, activity of the enzyme, length of incubation and temperature. All enzymes retained their activity throughout a broad range of pH and temperature. The removal of halogenated phenols decreased with increasing number of chlorines and increasing molecular weight of the substituent. Mass spectra revealed the loss of chlorine atoms during enzymatic polymerization. The release of chloride ions into solution during polymerization amounted up to 20 % of the chlorine initially associated with the 2,4-dichlorophenol molecule. Dechlorination contributes to the overall detoxification effect which results from enzymatic polymerization.

Nicell (1991) studied the effectiveness of HRP in polymerizing phenolic compounds into insoluble precipitates under different pH, HRP concentration, temperature, hydrogen peroxide concentration and contact time. He concluded that the degree of removal that could be achieved by a given dose of enzyme was dependent on the nature of the aromatic substrate. Optimal catalytic lifetime was achieved in the pH range of 7

to 9 for the eight phenolic compounds used in his study, i.e. phenol, chlorinated-phenols, methyl phenols and 2,4-dichlorophenol. Under 35 ^{0}C there is no significant thermal inactivation of HRP. The stoichiometry of the reaction between aromatic substrate and peroxide was found to be unity. Co-precipitation of hard-to-remove compounds with more readily removed compounds was confirmed. Table-2.1 shows the effect of HRP on some often studied substrates (Nannipieri,1991).

The mechanism of inactivation was further studied by Arnao et al. (1990). In the absence of reductant substrates, and with excess H_2O_2, peroxidase showed the kinetic behaviour of a suicide inactivation, H_2O_2 being the suicide substrate. They concluded that a competition was established between two catalytic pathways: the catalase pathway and the compound III-forming pathway, and, the suicide inactivation pathway (formation of inactive enzyme).

Baynton (1992) investigated the inactivation of horseradish peroxidase. Inactivation curves of activity remaining vs. time exhibited a rapid phase (0 - 60 seconds) which was H_2O_2 concentration dependent, and a slow phase characterized by a gradual loss of enzyme activity that was neither time nor H_2O_2 concentration dependent. The process involved both a reversible inactivation pathway leading to Compound III, which likely accounted for the observed rapid inactivation, and a pathway leading to an irreversibly inactivated intermediate, Compound P_{670}, which is predominant at H_2O_2 concentration above 1.0 mM. Phenoxy radicals generated during the oxidation of phenol also inactivate the enzyme in an irreversible, mechanism-based time-dependent inactivation. The inactivation mechanisms can be shown in Figure-2.2.2.

Table-2.1 Enzymatic removal of aromatic amines and phenols from water by Horseradish Peroxidase (Nannipieri and Bollay, 1991)

Compound	Removal (%)	H_2O_2	pH
Benzidine	99.9	1	5.5
3,3`-Dimethozybenzidine	88.9	1	5.5
3,3`-Diaminobenzidine	99.6	1	5.5
3,3`-Dimethylbenzidine	99.6	1	5.5
3,3`-Dichlorobenzidine	99.9	1	5.5
1-Naphthylamine	99.7	5	5.5
3-Naphthylamine	98.3	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-Methyphenol	86.2	1	4.0
3-Methyphenol	95.3	1	4.0
4-Methyphenol	85.0	1	5.5
2-Chlorophenol	99.8	1	7.0
3-Chlorophenol	66.9	1	7.0
4-Chlorophenol	98.7	1	5.5
2,3-Dimethyphenol	99.7	1	4.0
2,6-Dimethyphenol	82.3	1	5.5
4-Phenylphenol	99.9	1	4.0
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
Diphenylamine	80.5	1	7.0
1-Naphthol	99.6	1	4.0
4-Phenylphenol	99.9	1	4.0

2.3 ADDITIVE

A lot of effort has been made to reduce the inactivation during the enzymatic reaction. The most promising breakthrough is the discovery of using additives (Nakamoto et al., 1992). Their experiments showed that apparent inactivation of peroxidase during

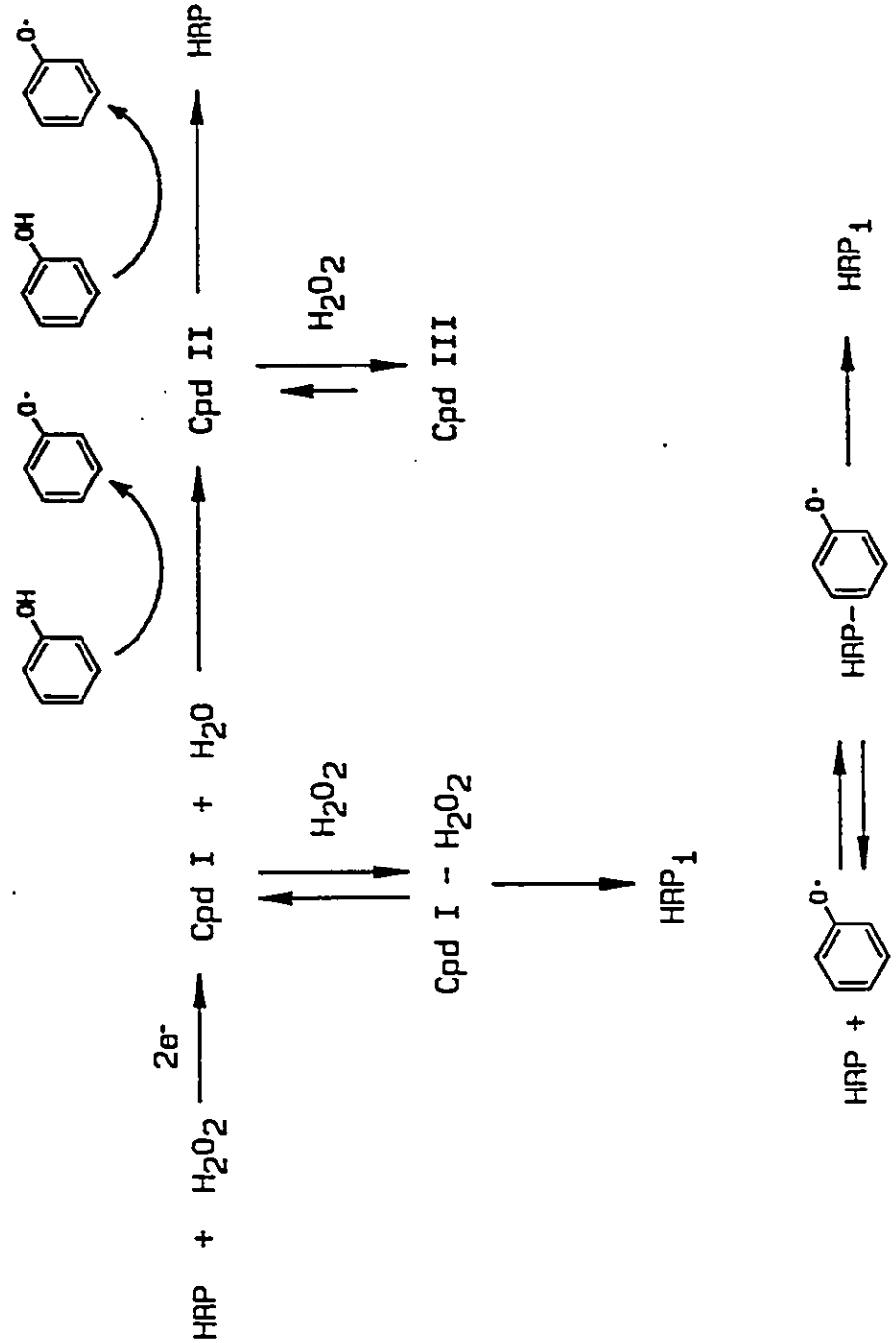


Figure-2.2.2 Inactivation mechanisms of horseradish peroxidase (Nicell, 1990)

the phenol polymerizing reaction was mainly caused by end-product polymer, which adsorbed the enzyme molecules and hindered the access of substrate to the enzyme's active site. By adding proteins or hydrophillic synthetic polymers, the enzyme adsorption was suppressed and the apparent enzyme inactivation was alleviated to drastically reduce the amount of enzyme required. Polyethylene glycol (PEG) and gelatin were found to be the best among the additives. This method was applied to phenol solution containing from 10000 to 30000 mg/L. The reduction in the amount of peroxidase required for phenol removal was up to 200-fold. It was also successfully used to dephenolize actual wastewater containing phenol, 2-propanol and 2-butanone. It was found that PEG's of average molecular weight less than 400 were ineffective in suppressing peroxidase activity loss.

Wu (1993) studied the effects of PEG on the phenol polymerizing reaction at lower concentrations. The results showed that PEG had a significant protective effect on the activity of HRP with the phenol concentrations of 1 to 10 mM (around 100 mg/L to 1000 mg/L). The amount of HRP required was reduced to 40- to 75-fold, respectively, of that required without PEG. The higher the phenol concentration, the more effective was the addition of PEG. The minimum doses of HRP and PEG required for at least 95% removal were 0.05 U/mL and 0.03 mg/L, respectively, for 1 mM phenol solution and 0.4 U/mL and 0.25 mg/L, respectively, for 10 mM phenol solution. PEG did not change the reaction stoichiometry. Under the optimum reaction conditions, the reaction time for at least 95% removal was 3 hours for 10 mM solution and 5 hours for 1 mM solution. An increase in HRP concentration showed a significant reduction in reaction time.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

Horseradish peroxidase (EC 1.11.1.7, type I, RZ=1.0, 78 purpurogallin units/mg solid) was purchased from Sigma Chemical Co., St. Louis, MO. The specific activity of horseradish peroxidase was approximately 120 U/mg dry solid using the assay method specified in Appendix-A, which uses phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide as substrates. One unit of activity is defined as the number of micromoles of phenol converted per minute at pH 7.4 and 25 degrees centigrade. The nominal activity measured by Sigma Chemical Co. was 80 Units/mg dry solid where one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20 degrees centigrade. It was stored as dry powders in a freezer at -15 ^{0}C as required. Aqueous stock solution of peroxidase (1 mg/mL) was stored at -4 ^{0}C. Its activity was checked at least once a month after its preparation.

Catalase (EC 1.11.1.6 12,400 units/mg protein) and polyethylene glycol (average molecular mass = 3,350) were also purchased from Sigma Chemical Co., St. Louis, MO. The nominal activity of the catalase was 9,300 Units/mg dry solid and 12,400 Units/mg protein where one unit will decompose 1.0 micromole of H_2O_2 per minute at pH 7.0 and 25 degrees centigrade while the H_2O_2 concentration falls from 10.3 to 9.2 micromoles per millilitre of reaction mix. Aqueous stock solution of catalase (0.5 mg/mL) was also stored at -4 ^{0}C.

Hydrogen peroxide (30% by weight over volume) was purchased from BDH Inc., Toronto, Ontario. Peroxide solutions for the analytical assays were prepared daily.

Chlorinated phenols and methyl phenols studied were obtained from Aldrich Chemical Co., Milwaukee, WI., and had a purity of 99 % or greater. Stock aromatic aqueous solutions were prepared and stored at -4 ^{0}C.

All other chemicals used were of analytical grade and were supplied by Fisher Scientific Co., Fair Lawn, N.J., J.T. Baker Chemical Co., Phillipsburg, N.J. or Aldrich Chemical Co.

3.2 ANALYTICAL EQUIPMENT

Colour absorbance was measured using a Hewlett Packard Diode Array Spectrophotometer Model 8452A (wavelength range 190 to 820 nm with 2 nm resolution) operated from a HP Vectra ES/12 computer. Quartz spectrophotometer cells (macro and semi-micro with 10 mm path length) were supplied by Hellma (Canada) Ltd. The disposable cuvettes were purchased from Fisher Scientific Co. or Aldrich Chemical Co..

A Sorvall RT6000B Refrigerated Centrifuge, supplied by Du Pont Company, Sorvall Instruments, Wilmington, Delaware, was used to centrifuge samples at 3000 gravities for 30 minutes. The temperature in the centrifuge was kept around 20^{0} C.

Microprocessor Ionanalyzer Model 901, purchased from Orion Research Inc., Cambridge Mass., was used to measure pH of the samples. The pH standards were purchased from BDH Chemicals, Toronto.

3.3 EXPERIMENTAL PROTOCOL

Because there was no significant loss in the HRP activity on incubation in the temperature range of 5 °C to 35 °C, the experiments in this study were conducted at room temperature (Around 20 °C). The effects of additives on pH and HRP dose, minimum PEG dose, molar ratio of hydrogen peroxide to phenol derivatives , time needed for the completion of the reaction, and coprecipitation were investigated.

All experiments were done in batch reactors which consisted of a vial containing 30 milliliters of a mixture of aromatic substrate, hydrogen peroxide, peroxidase enzyme buffer and additive as designed. The components were added in the following order: buffer, additive, HRP enzyme and aromatic substrate. Aliquots for measure of the absorbance of mixture before the reaction were taken before adding hydrogen peroxide to initiate the reaction. The reactors were stirred vigorously using a magnetic stirrer and teflon coated stir bar during the reaction. Catalase (0.5 mg/L) was added to stop the reaction when it was necessary. Alum was then added to the mixture and its pH was adjusted to between 6 to 7 when it was necessary, and mixture was stirred for about twenty minutes to form the floc. The samples for the mixture after the reaction were then taken and centrifuged simultaneously for 30 minutes at 3000 g. The supernatant was analyzed for its absorbance to determine the residual of phenol derivatives. Details about pH and reaction time experiment are described below.

3.3.1 pH

The buffers used in this experiment were prepared according to Methods in Enzymology (Gomori, 1955). The reagents used and the corresponding pH range obtained are listed in Table-3.1.

Components for reaction mixture were added in the batch reactors as described above. Aromatic concentrations were controlled at 1 mM (around 100 mg/L). 13.6 µL of 15% H_2O_2 was added to initiate the reaction after withdrawing 1 mL samples. 250 µL catalase was added in the reactors after 16 hours of reaction time. After 15 minutes, about 100 mg solid alum was added and the reactors were stirred vigorously for several minutes to dissolve the alum. After that, concentrated HCl or NaOH were added to adjust pH between 6 to 7 and contents were gently stirred for 20 minutes for formation of floc. Samples were then collected , centrifuged and analyzed for aromatic compounds remaining by direct determination of UV absorbance or by a colorimetric method based on reaction with AAP (see section 3.4.2).

Table-3.1 Buffers used in the experiments on pH

Buffering Reagents	pH Range
Acetic acid-sodium acetate	4 - 5
Monobasic-dibasic sodium phosphate	6 - 9
Boric acid-acid-borax acid	9
Sodium carbonate-sodium bicarbonate	10

3.3.2 REACTION TIME

Components for the reaction mixtures were added to the batch reactors as described above. In order to stop the reaction at the desired time, 30 μL stock catalase (0.5 mg/L) and 220 μL alum (40 g/L) were added in the centrifuge tube. The tubes were shaken after the 2 mL samples were taken at different time intervals so that samples could be mixed well with catalase and alum. After sampling at different time points, these tubes were taken to undergo centrifugation and analysis.

It should be mentioned that the catalase dose used to halt the reaction in the centrifuge tubes should be controlled because the excessive catalase can remain in the samples and subsequently interfere with the colorimetric method by consuming hydrogen peroxide used in that method.

3.4 ANALYTICAL METHODS

3.4.1 HRP ACTIVITY

A modified assay was employed to measure the HRP enzyme activity using phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide as substrates. The approach in this assay is to provide all components except enzyme near saturation concentration so that the initial rate of reaction becomes directly proportional to the amount of enzyme present. The assay mixture contained 250 μL of 9.6 mM 4-AAP, 100 μL of 100 mM phenol, 100 μL of 2 mM hydrogen peroxide, 500 μL of 100 mM phosphate buffer (pH 7.4) and 50 μL of enzyme solution. The rate of reaction was measured by monitoring the rate of formation of the non-precipitated products which absorbed light at a peak

wavelength of 510 nm upon addition of the enzyme. Thus, one unit of activity used in this study is defined as the number of micromoles of phenol converted per minute at pH 7.4 and 25°C. A detailed explanation of the operation of this assay is described in Appendix-A.

3.4.2 AROMATIC COMPOUNDS

The concentration of aromatic compounds in aqueous solution was determined by direct spectrophotometric or colorimetric methods wherever applicable. The concentrations of phenolic compounds used in this study are expressed in terms of molar quantities for convenience . One millimolar is equal to 128.6 mg/L for chlorinated phenol, 108.2 mg/L for methyl phenol and 163 mg/L for 2,4-dichlorophenol. A colorimetric method was employed to measure the aromatic compounds. This method uses HRP as a catalyst and 4-aminoantipyrine and hydrogen peroxide as colour generation substrates. The colour generated at 510 nm is directly proportional to the concentration of the aromatic substrate. The details about this method are described in Appendix-B. Calibration curves were developed for all phenol derivative (see Appendix-C). The results showed that there was not a linear relationship between absorbance and concentration for 3-chlorophenol and for p-cresol. Hence, all tests on these two chemicals were done using the direct spectrophotometric method.

The direct spectrophotometric method was developed based on the absorbance of ultraviolet light by phenols. Phenol derivatives absorb ultraviolet light with maxima between 270 to 284 nm in a quantity directly proportional to the concentration of phenol

derivatives in the solution. It has been reported that peroxidase, hydrogen peroxide and the buffer solutions used to maintain pH do not interfere in this method (Klibanov, 1980). However, it was observed that the reaction products which probably were different after the addition of additives, might interfere with the accuracy of this method. This phenomenon is clearly demonstrated later in the experiment on PEG effect on HRP dose for p-cresol. The reason for this might at least partially be due to the spectrum change of peak and extinction coefficient after the enzymatic reaction (see table-4.4). A summary of analytical methods used for different aromatic compounds is presented in Table-3.2.

Table-3.2 Summary of the analytical methods used

Aromatic Compounds	Colorimetric Method Works or Not	UV Absorbance Peak (nm)
2-Chlorophenol	yes	274
3-Chlorophenol	no	274
4-Chlorophenol	yes	280
o-Cresol	yes	270
m-Cresol	yes	272
p-Cresol	no	278
2,4-Dichlorophenol	yes	284

3.5 SOURCE OF ERROR

Two kinds of errors can occur in any experiment: systematical error, and random error. The former is due to measuring or analytical techniques and instruments. The latter

is due to the personal or carelessness. For the analytical methods chosen in this study, systematical errors can occur in both direct spectrophotometric method and colorimetric method with very low concentration after the reaction. For the colorimetric method it took 35 to 45 minutes to reach the maximum absorbance while preparing the standard curve using standard solutions. But after the completion of reaction, it took less than 5 minutes to reach the maximum. Maximum readings are supposed to be reported and these depend on individuals. For the direct method, the spectrum of the solution always changed after the completion of the reaction. Sometimes the peaks had formed which, at least partially contributed to the errors. Relatively speaking, the errors from the colorimetric method are more predictable. More discussion can be found in the next Chapter.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 pH

Enzymes are proteins made up of poly-amino-acids. pH can influence their structure and consequently, influence their activity. Many enzymes have highest activity at neutral pH. Enzymes usually lose their activity at extreme pH values because of the change in their structures. Since the activity of HRP at pH below 4 and above 11 is very low, the pH range of 4 to 10 was chosen in this study. Both PEG and gelatin were used to study their effect on the optimum pH range for the HRP catalyzed polymerization of different phenolic compounds under different HRP doses. The concentrations of additive chosen were 5 g/L for PEG and 4 g/L for gelatin based on experiments conducted by Nakamoto et al. (1992) at much higher concentration of phenol.

These experiments were designed so that only the HRP concentration was limiting. Hydrogen peroxide was overdosed (2 mM), which was twice as much as the optimum stoichiometric dose, and additives were also overdosed. The amount of PEG or gelatin used was same as that needed for high concentrations of phenol (Nakamoto et al. 1992). The reaction was stopped after 16 hours , which was considered to be sufficient to complete the reaction. Borax buffer may have had some influence as an additive (Nicell, 1991), but this is masked by overdose of PEG or gelatin. The initial substrate concentration was 1 mM in all cases. Experiments were conducted with different HRP doses and different additives over a range of pH.

The results are shown from Figures 4.1.1 to 4.1.7. All these figures show clearly

that there is a wide range of optimum pH for all of the phenol derivatives studied. Generally, the greater the HRP dose, the wider the optimum pH range. The optimum pH occurred at near neutral condition except for 2-chlorophenol which had an optimum pH of about 5. The optimum pH for each of the compounds studied are shown in Table-4.1.

Table-4.1 Optimum pH for phenol derivatives studied

Aromatic Compounds	Optimal pH Range	Aromatic Compounds	Optimal pH Range
2-Chlorophenol	4.0 - 7.0	o-Cresol	6.0 - 8.5
3-Chlorophenol	5.5 - 8.0	m-Cresol	5.5 - 8.5
4-Chlorophenol	5.0 - 8.0	p-Cresol	5.0 - 8.0
2,4-Dichlorophenol	4.5 - 7.5		

It is also seen from these figures that the optimum pH range is not influenced by the amount of HRP used. Generally, when the initial activity of HRP was increased, the concentration remaining decreased in the entire pH range, as long as pH was in the range of the HRP activity. These observations are consistent with those reported by Nicell (1991) on HRP activity as a function of pH.

When the results with additives in this study are compared to the results from the experiments without additives (Nicell, 1991), it is noticed that optimum pH ranges become wider, and close to neutral in the presence of the additive. Optimum pH range changed towards neutrality in all cases. For example, 2-chlorophenol had an optimum

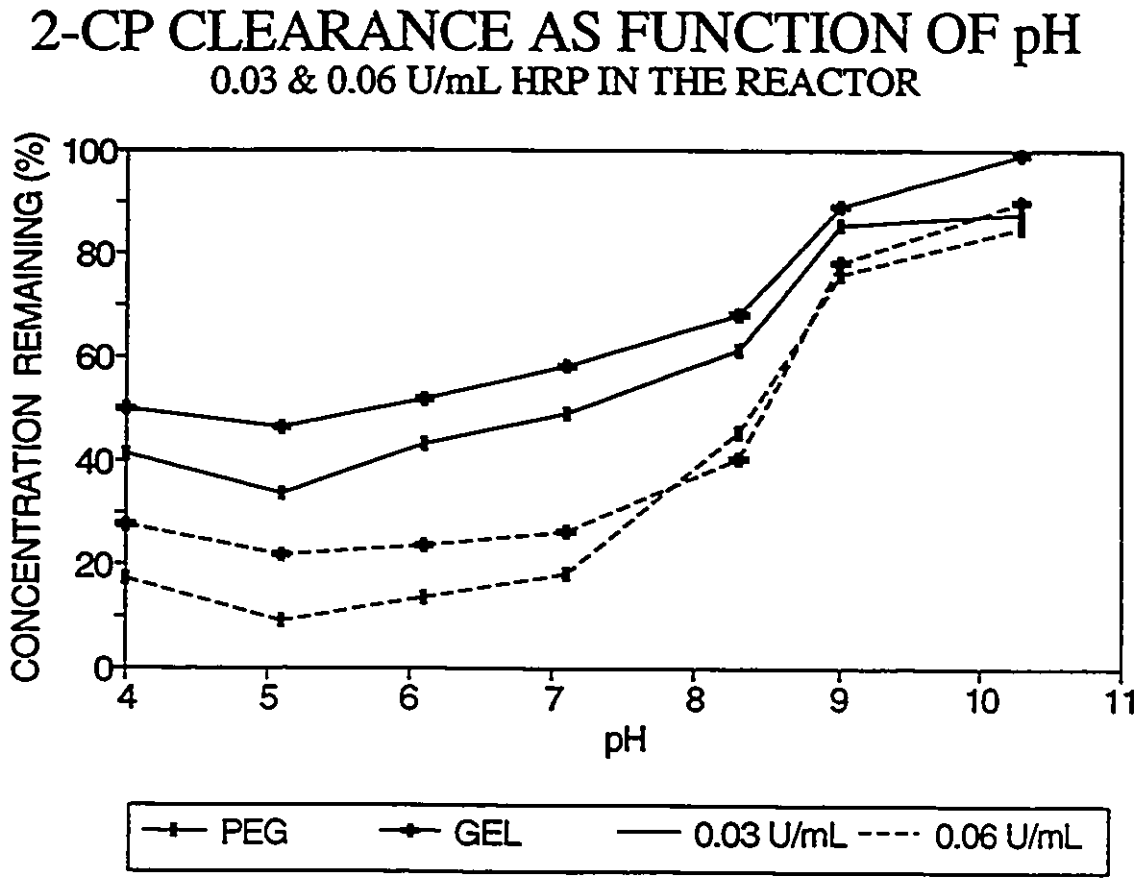


Figure-4.1.1 2-chlorophenol clearance as function of pH

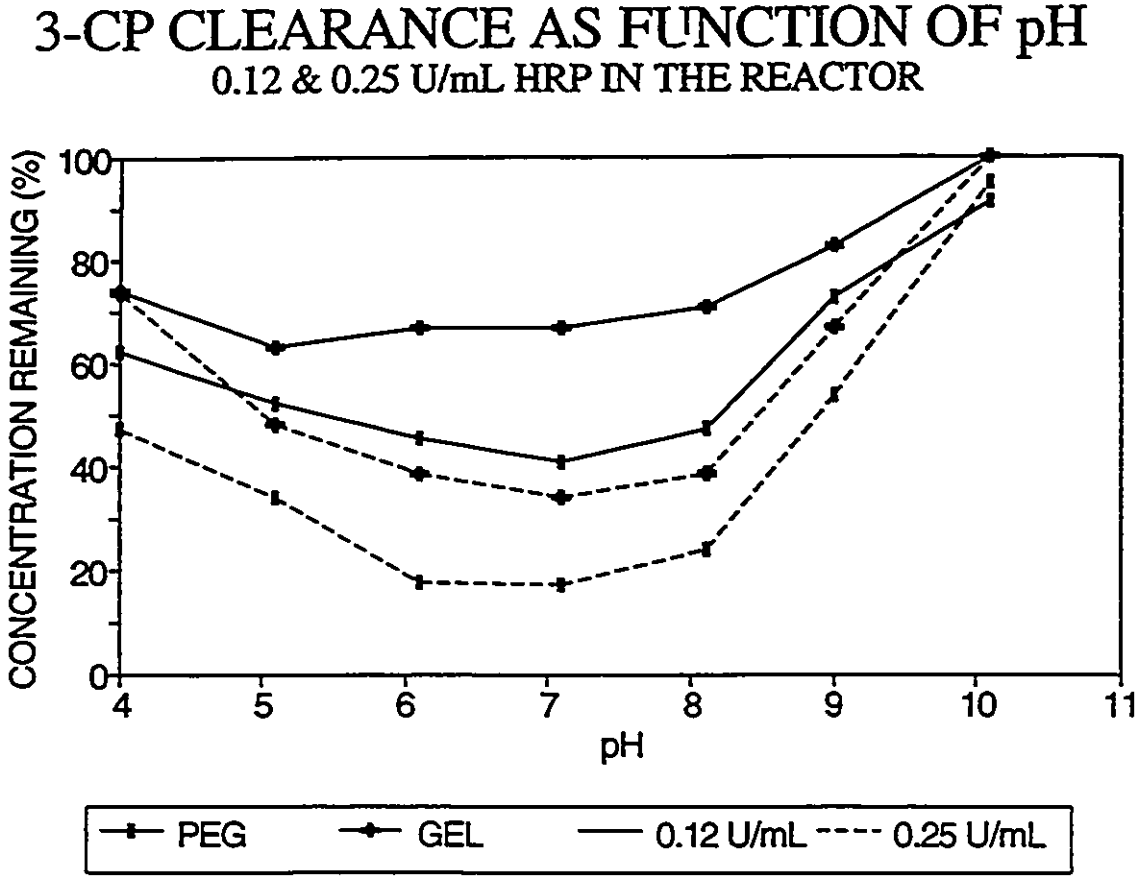


Figure-4.1.2 3-chlorophenol clearance as function of pH

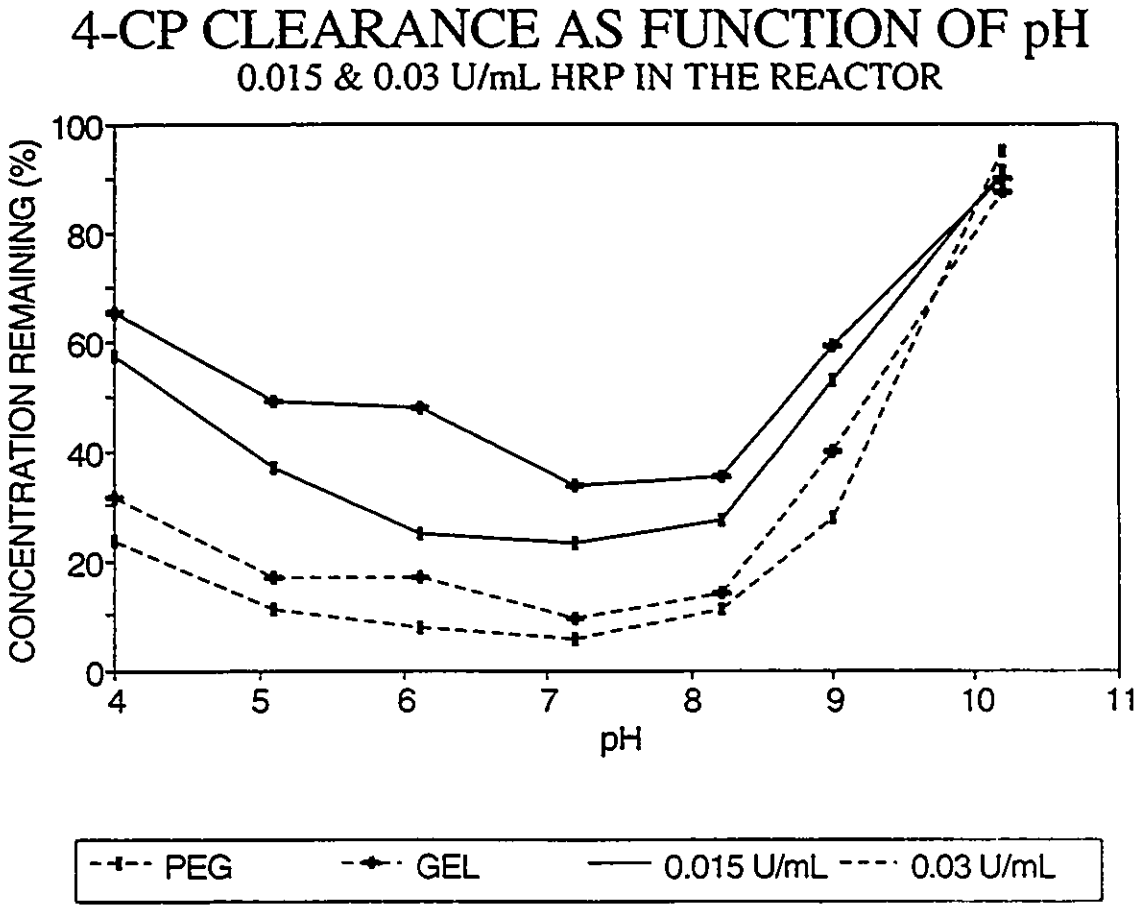


Figure-4.1.3 4-chlorophenol clearance as function of pH

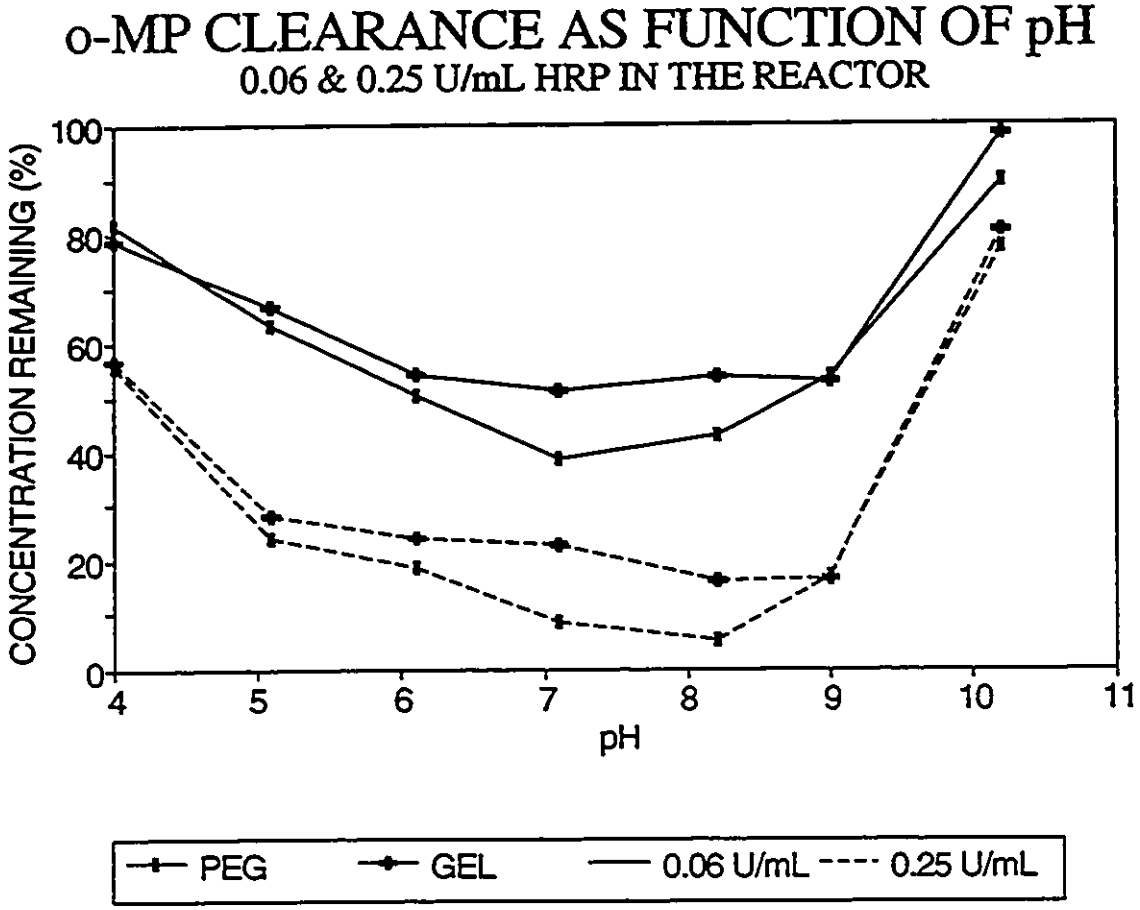


Figure-4.1.4 o-cresol clearance as function of pH

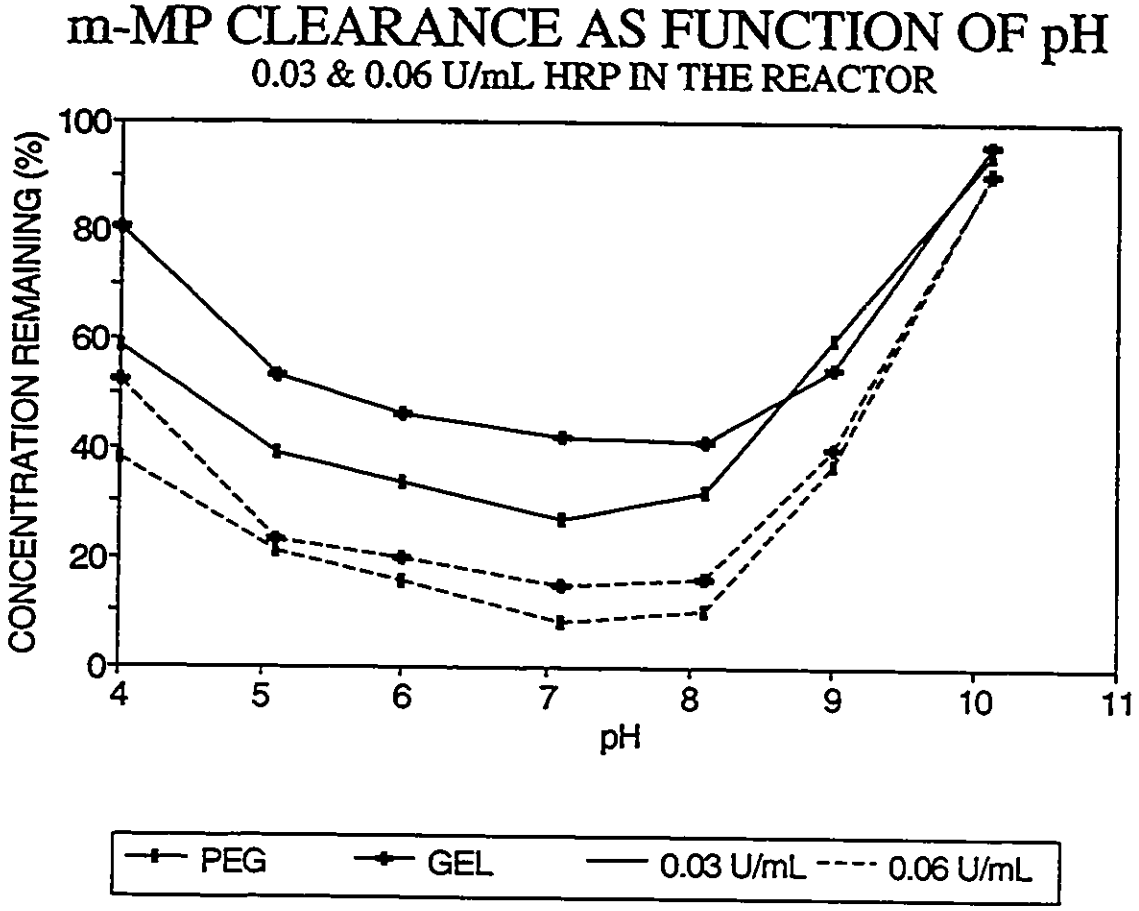


Figure-4.1.5 m-cresol clearance as function of pH

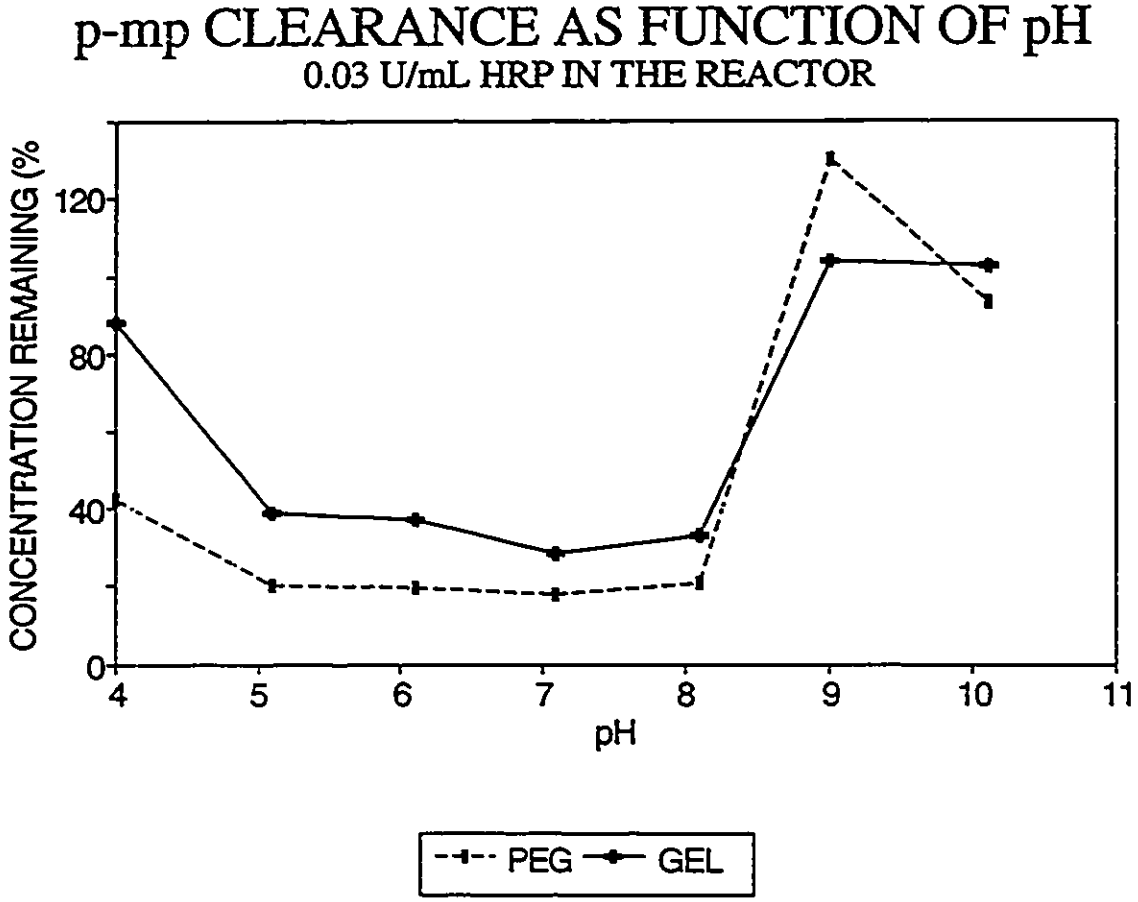


Figure-4.1.6 p-cresol clearance as function of pH

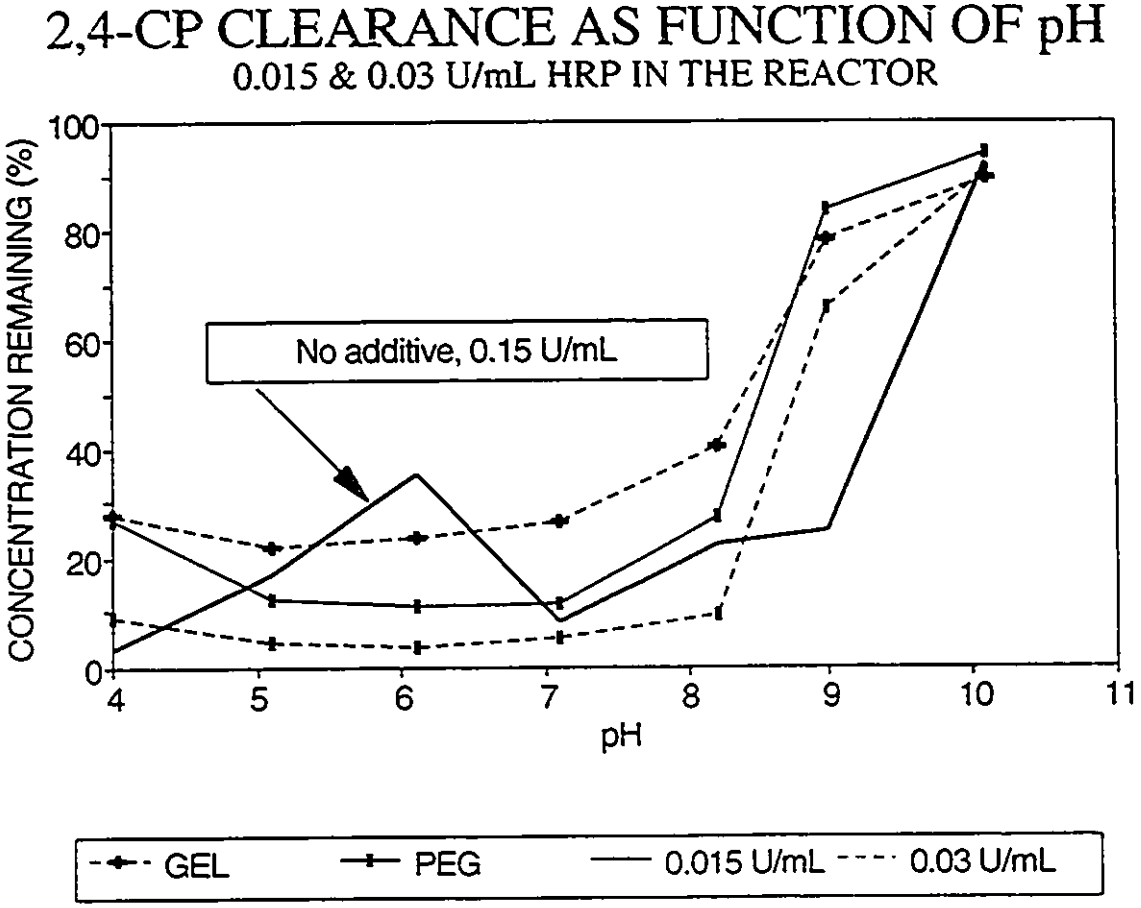


Figure-4.1.7 2,4-dichlorophenol clearance as function of pH

pH range from 8 to 9 without additive, which changed to 4 - 7 with additive. The substrate concentrations remaining were changing smoothly with pH. This influence of additive on widening the pH range is good for field application. This new optimum pH is also good with regard to using alum to settle the polymer formed, because the optimum pH for it is around 6.3 which is almost coincident with that for activity of the HRP enzyme. From the experiment, we can say that additive has influenced the performance of HRP in the polymerization of phenolic compounds.

PEG and gelatin had similar effects on the performance of HRP at different pH values. However, PEG performed better than gelatin not only in terms of removal efficiency but also in terms of the settling behaviour of the final product. Gelatin usually helped to form insoluble polymers with yellow colour in the final supernatant which could not be removed even after adding alum. For example, the supernatant in the reactor of 3-chlorophenol and m-cresol with gelatin had yellow colours but no sediments after adding alum and adjusting pH. However, when PEG was used, white sediments with no colour in the supernatant were observed. When the UV method was used to measure the substrate concentration remaining for p-cresol, some of the remaining concentration values were higher than the initial ones. This might be due to the influence of final products. From the spectrum, a new absorbance peak was observed at 290 nm instead of 278 nm at higher pH (pH 8 - 10). A similar observation was also made when using the UV method to check the result from the colorimetric method for 2,4-dichlorophenol where the peak changed from 286 to 306 nm, and this might contribute to the higher residual absorbance.

4.2 HRP DOSE

Since the PEG was found to be better than gelatin, PEG was chosen for the subsequent experiments conducted to study the effect of additive on the HRP dose required to achieve complete , i.e. , greater than 95 percent substrate removal. The concentrations of all aromatic compounds were kept at 1 mM and 2 mM of hydrogen peroxide was used. The duration of the reaction was 6 hours. The additive (PEG) was still overdosed at 5 g/L. All experiments were conducted at the optimum pH values shown in Table-4.2.

Table-4.2 pH chosen for each compounds in HRP dose experiments

Aromatic Compounds	pH Chosen	Aromatic Compound	pH Chosen
2-Chlorophenol	5.0	o-Cresol	7.0
3-Chlorophenol	7.0	m-Cresol	7.0
4-Chlorophenol	7.0	p-Cresol	7.0
2,4-Dichlorophenol	7.0		

The results are shown in Figures 4.2.1 to 4.2.7. From these figures, it can be seen that the addition of PEG greatly reduced the amount of HRP needed for the completing polymerization (95 percent or more removal). For example, 4-chlorophenol needed only 0.015 U/mL of enzyme to complete the reaction when PEG was added as compared to 2 U/mL required without PEG. It meant that there was a 132 fold improvement in

peroxidase utilization. The minimum HRP doses required for different organic compounds are listed in Table-4.3.

Table-4.3 Minimum HRP dose for completion of polymerization

Aromatic Compounds	Minimum HRP Dose U/mL Without PEG	Minimum HRP Dose U/mL Without PEG	HRP Dose Ratio
2-Chlorophenol	3.0	0.03	100
3-Chlorophenol	5.0	0.50	10
4-Chlorophenol	2.0	0.015	132
o-Cresol	3.0	0.10	30
m-Cresol	2.8	0.03	93
p-Cresol	1.0	0.03	33
2,4-Dichlorophenol	0.5	0.015	33

This table shows that the amount of HRP required for complete polymerization in the presence of PEG was 10- to 133-fold less than that required without PEG. Therefore, the turnovers (moles of substrate removed/mole of HRP inactivated) had increased by 10 to 132 times.

These observations confirm that PEG can also protect HRP activity at low concentrations of phenolic compounds. Without additives, HRP activity was lost during the reaction. However, extra HRP beyond the minimum needed did not help in getting any additional removal. The removal of compounds was almost directly proportional to the amount of HRP used when less than the minimum was added.

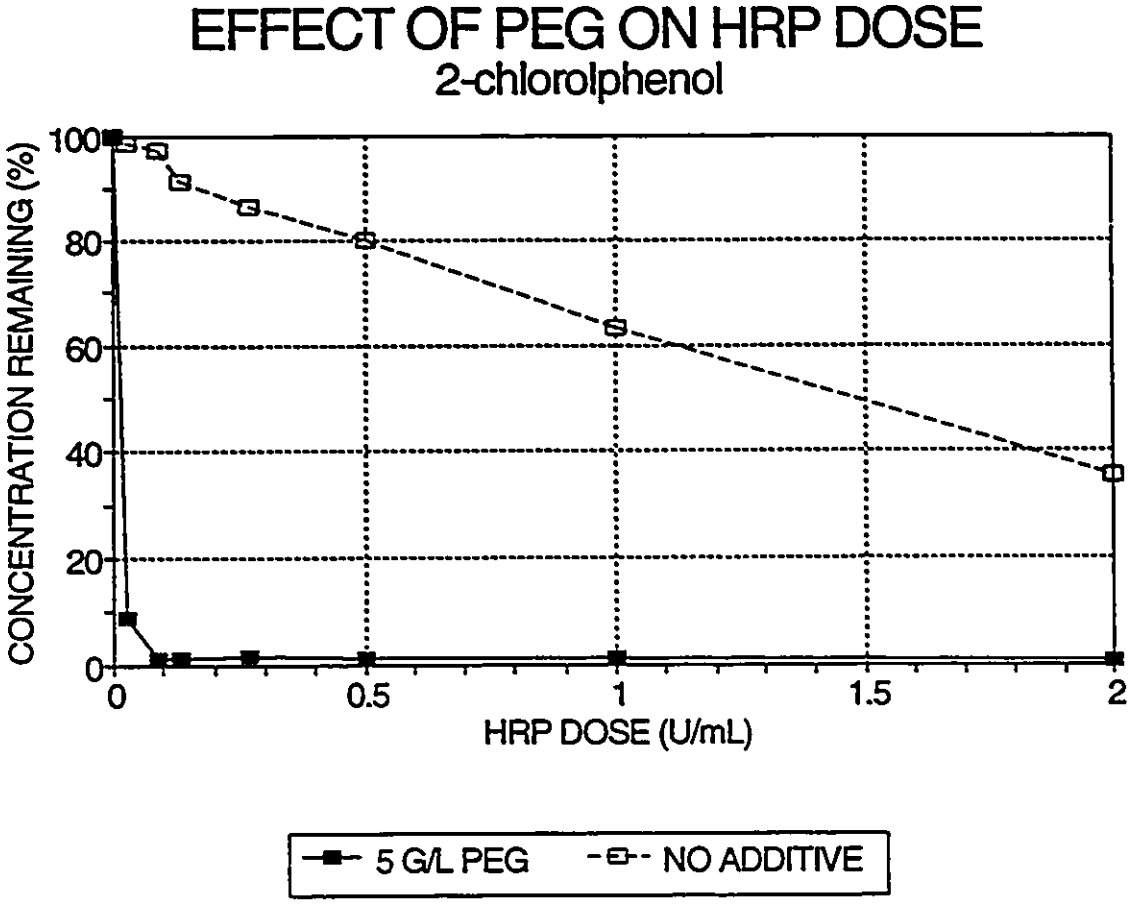


Figure-4.2.1 PEG effect on HRP dose for 2-chlorophenol

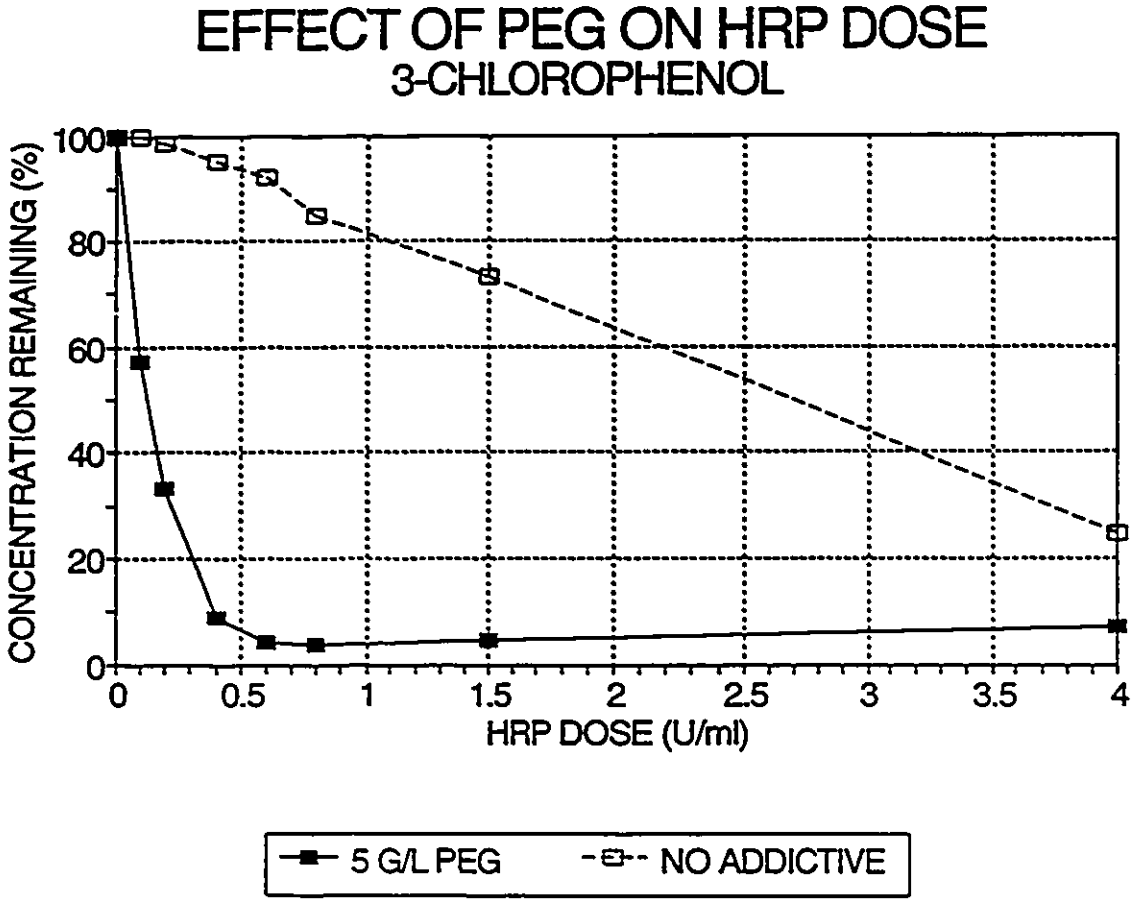


Figure-4.2.2 PEG effect on HRP dose for 3-chlorophenol

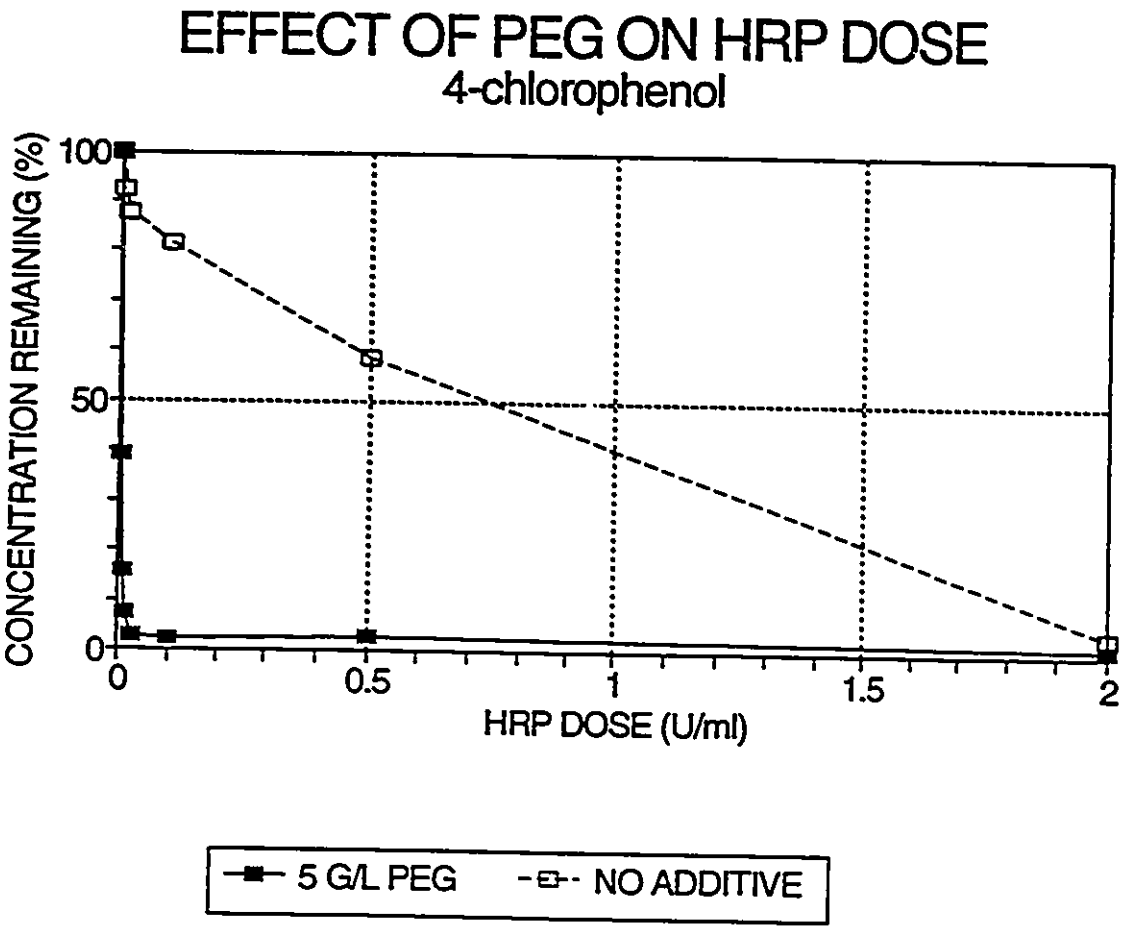


Figure-4.2.3 PEG effect on HRP dose for 4-chlorophenol

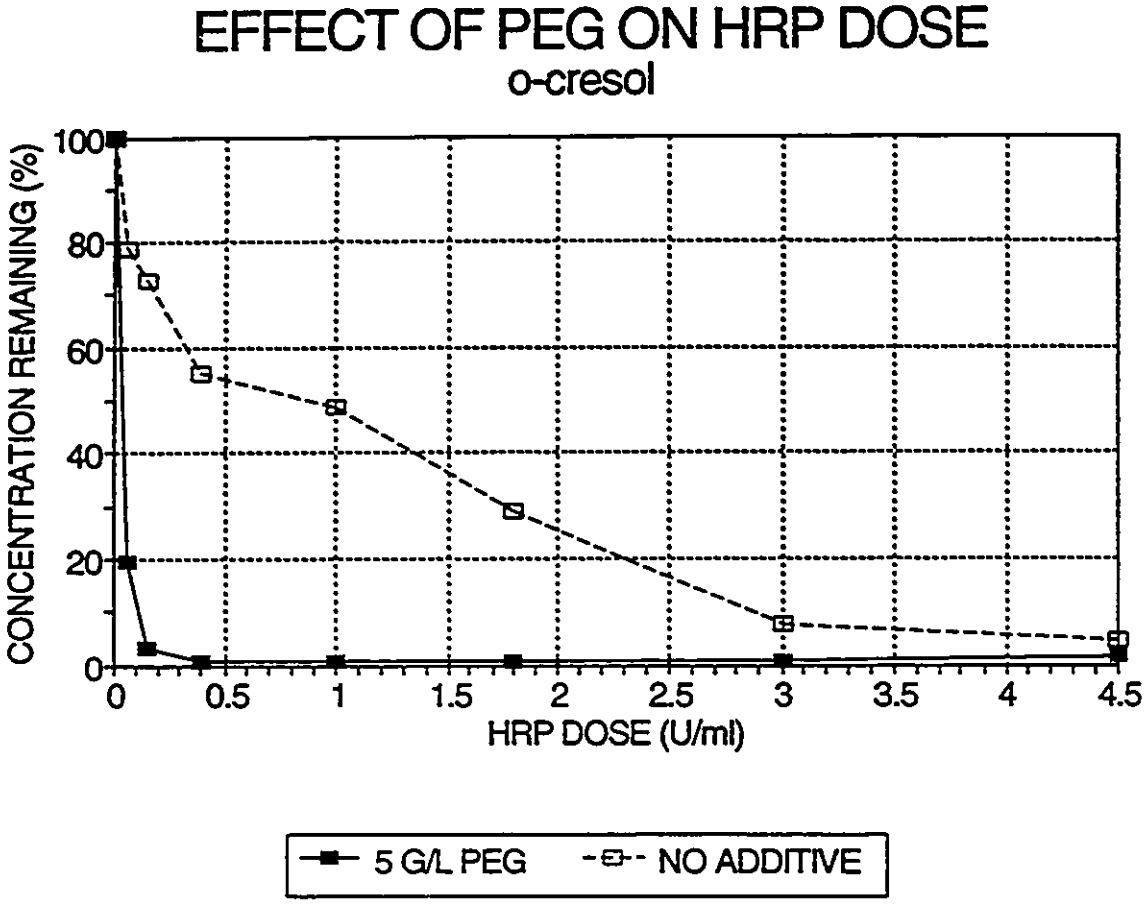


Figure-4.2.4 PEG effect on HRP dose for o-cresol

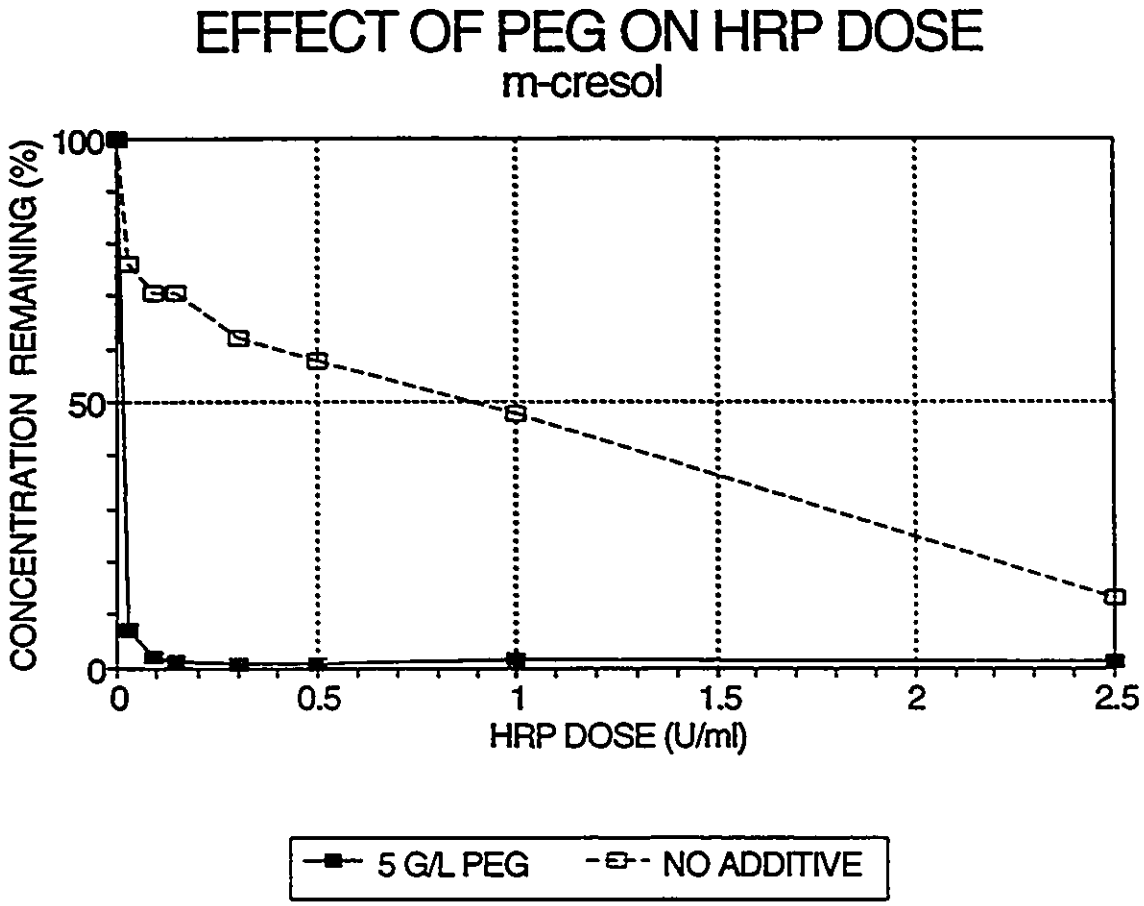


Figure-4.2.5 PEG effect on HRP dose for m-cresol

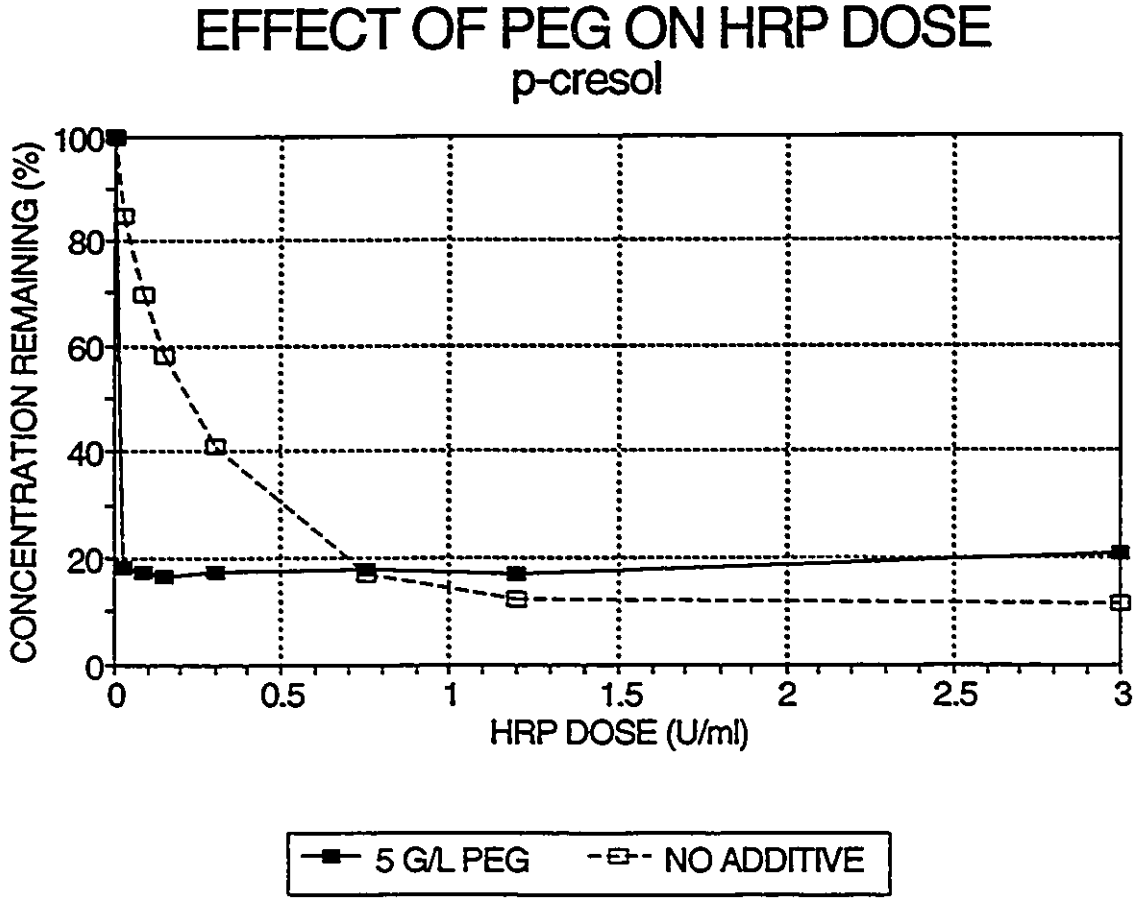


Figure-4.2.6 PEG effect on HRP dose for p-cresol

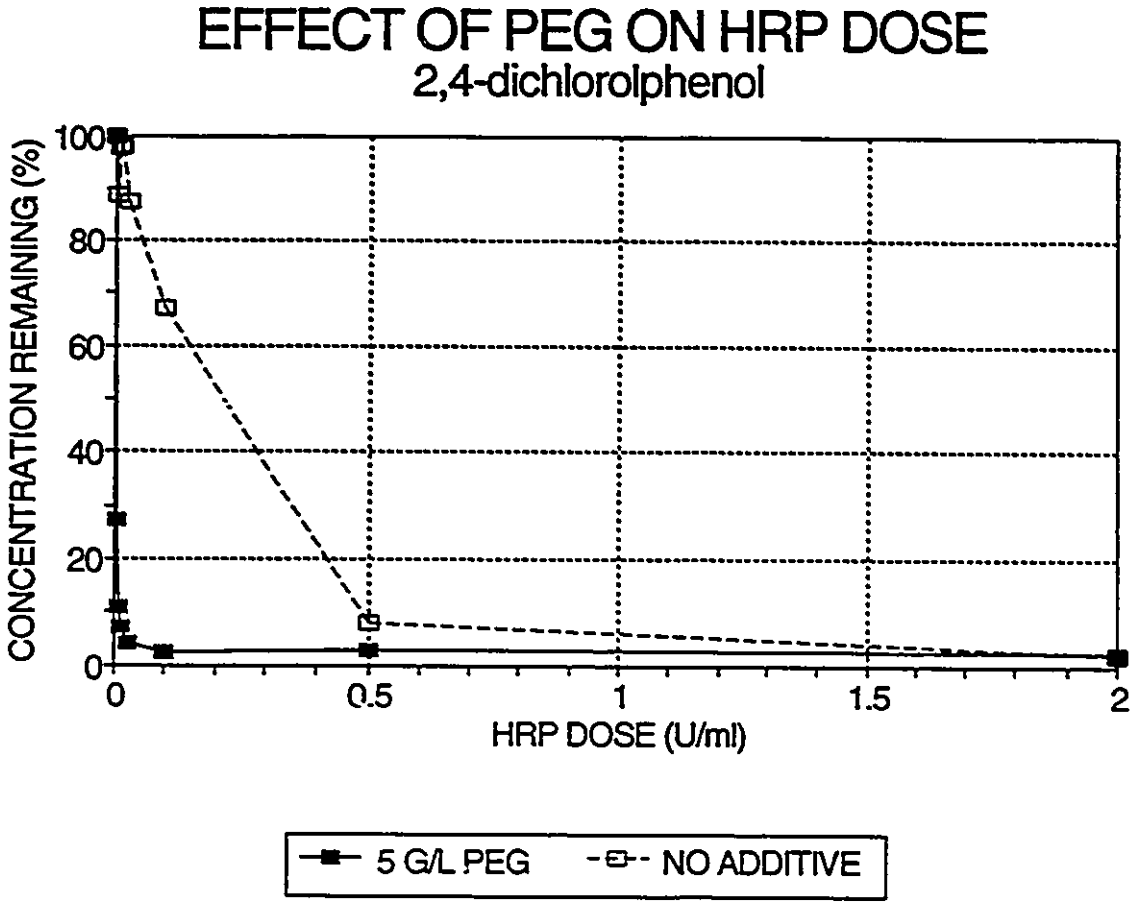


Figure-4.2.7 PEG effect on HRP dose for 2,4-dichlorophenol

It was mentioned earlier that the final product after adding PEG might be different from those without PEG. Again, it was observed that the reactors with PEG had more precipitates than those without PEG, and the particles formed in the reactors with PEG were finer than those in the reactors without PEG. For instance, after adding PEG, the final precipitates in the low HRP dose reactors of 2-chlorophenol were more than those in reactors without additive. Furthermore, the former had a dark brown colour, while the latter had a light brown colour.

It is suggested that HRP dose might have influenced the nature of final product when it is below the minimum HRP dose. When using the UV method for p-cresol, it was observed that the absorbance peak stayed at 278 nm without enough HRP, and it had shifted to 294 nm with excess HRP after the completion of the reaction. When the HRP dose was somewhere between, the absorbance peak was also somewhere between. Table-4.4 shows this behaviour for p-cresol.

Table-4.4 Absorbance peak change with HRP dose for p-cresol

HRP Dose (U/mL)	0.03	0.09	0.15	0.30	0.75	1.20	3.00
Abs. Peak (nm)	278	278	278	278	284	296	296
Abs. Remaining (%)	85	70	58	42	18	12	12

* No PEG added

It implied that the final product had an absorbance peak at 296 nm which was mentioned in section 4.1. The Figure-4.2.6 for p-cresol also suggests that PEG might have interfered with the UV method, because the final absorbances remaining with PEG addition were higher than those without PEG addition, which is apparently wrong. These inconsistencies occurred with other compounds also.

4.3 PEG DOSE

Experiments were conducted using the minimum HRP dose for different phenol derivatives at their optimum pH's to determine the minimum PEG amount needed for its protective effect on the activity of HRP. The concentrations of phenolic compounds were kept at 1 mM and the hydrogen peroxide concentration was 2 mM. The maximum PEG dose used was 5 g/L, which was used in all of the pH and HRP dose experiments. The results are shown in Figure 4.3.1 to Figure 4.3.7. Since the concentration remaining of the 5 g/L reactors was the same as that of 1 g/L reactors, all figures were plotted to 1 g/L.

From these figures, one can see that minimum PEG dose varies from 10 to 100 mg/L, depending on the phenolic compounds. For example, 2-chlorophenol and 3-chlorophenol needed 100 mg/L, while the others needed less than 30 mg/L. Any extra PEG (up to 5 g/L in the experiments) added to the reactors neither improved its effect on protecting enzymes, nor did it worsen its effect. Different compounds had a different minimum PEG dose, therefore, the minimum PEG needed for protection of enzyme activity was related to the structure of the specific phenolic compound, just as has been reported for the minimum HRP dose. The minimum PEG doses required for different

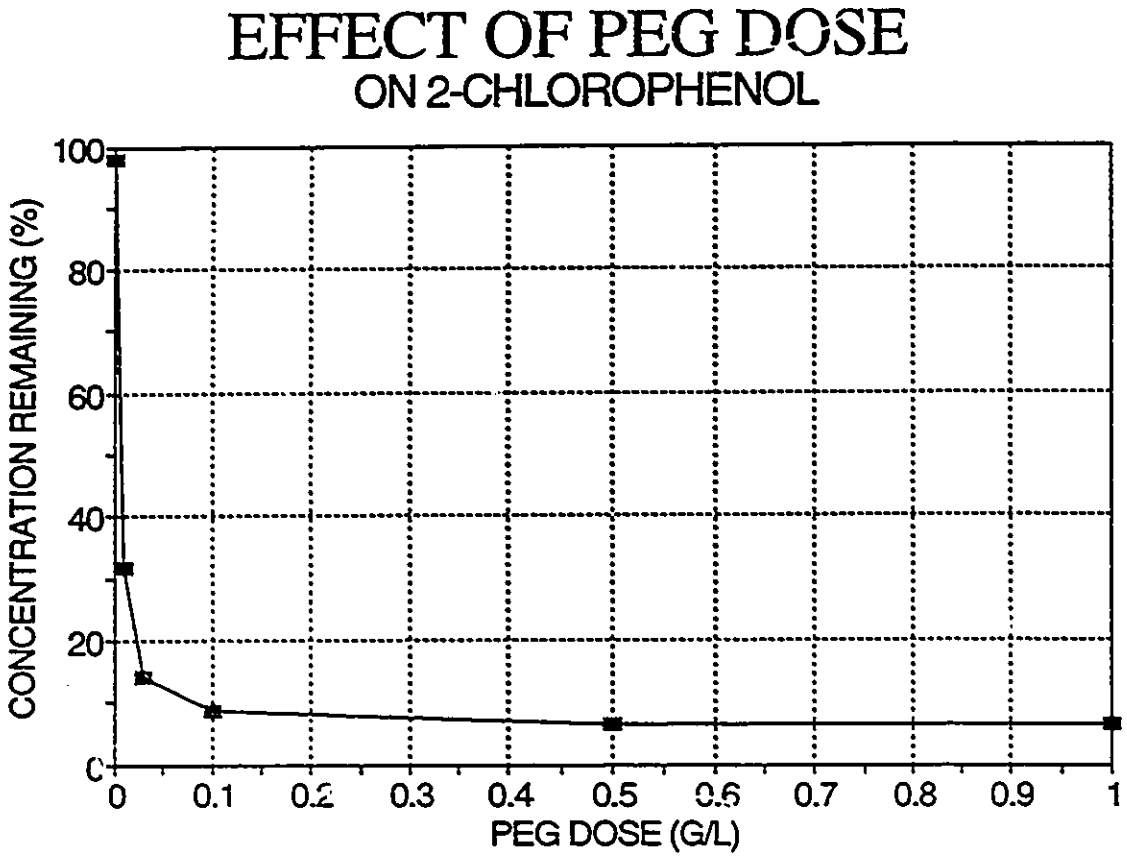


Figure-4.3.1 PEG dose effect on concentration remaining for 2-chlorophenol

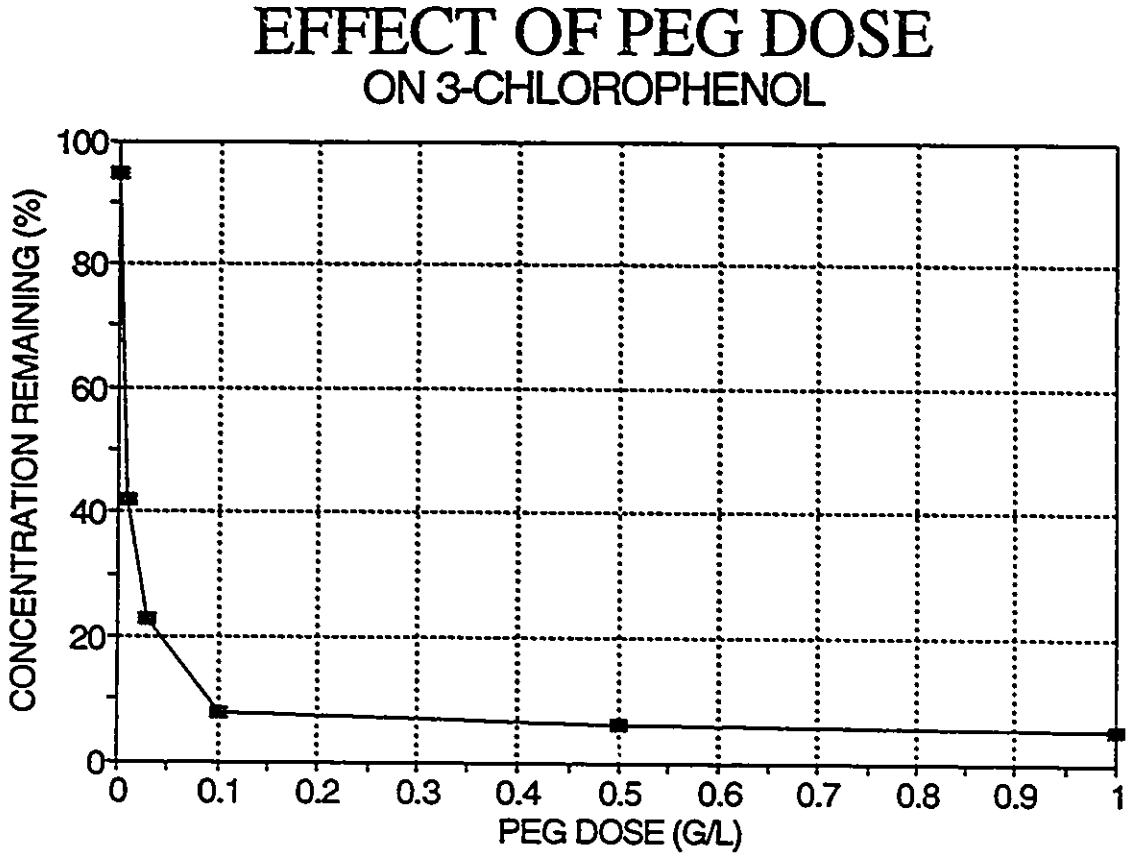


Figure-4.3.2 PEG dose effect on concentration remaining for 3-chlorophenol

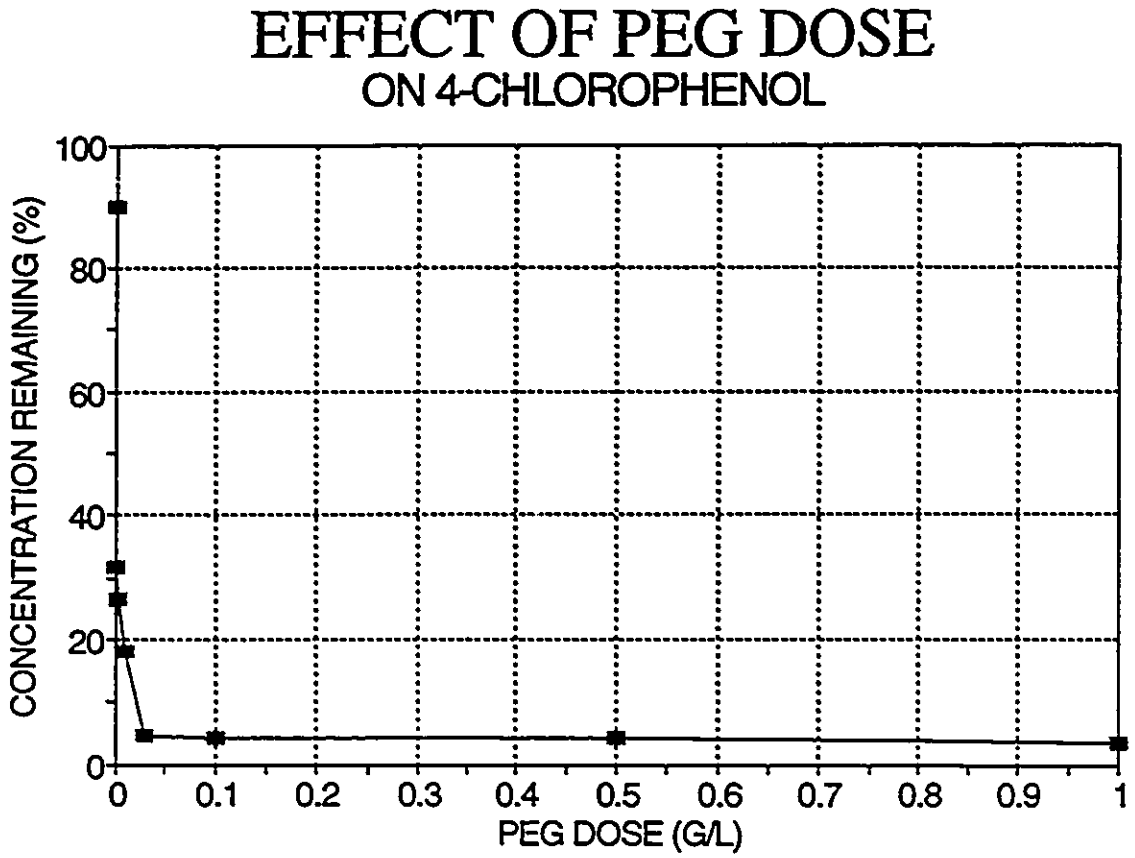


Figure-4.3.3 PEG dose effect on concentration remaining for 4-chlorophenol

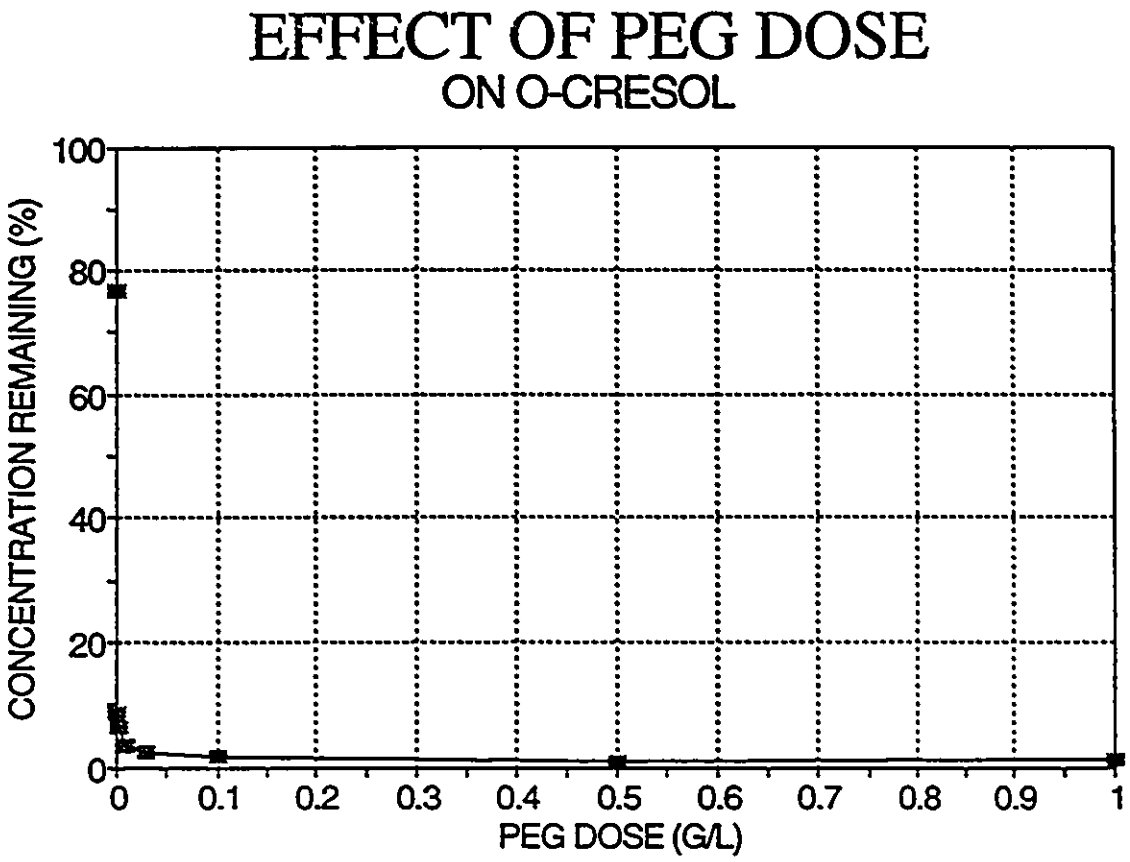


Figure-4.3.4 PEG dose effect on concentration remaining for o-cresol

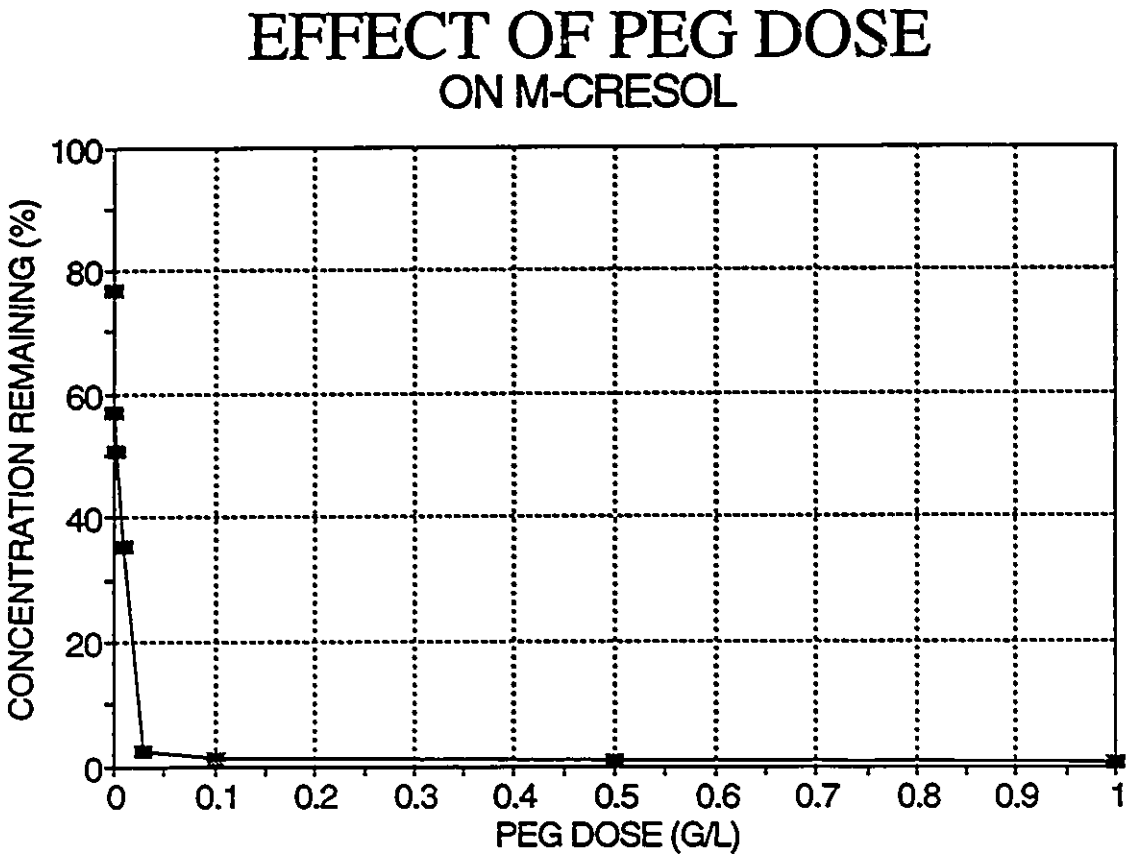


Figure-4.3.5 PEG dose effect on concentration remaining for m-cresol

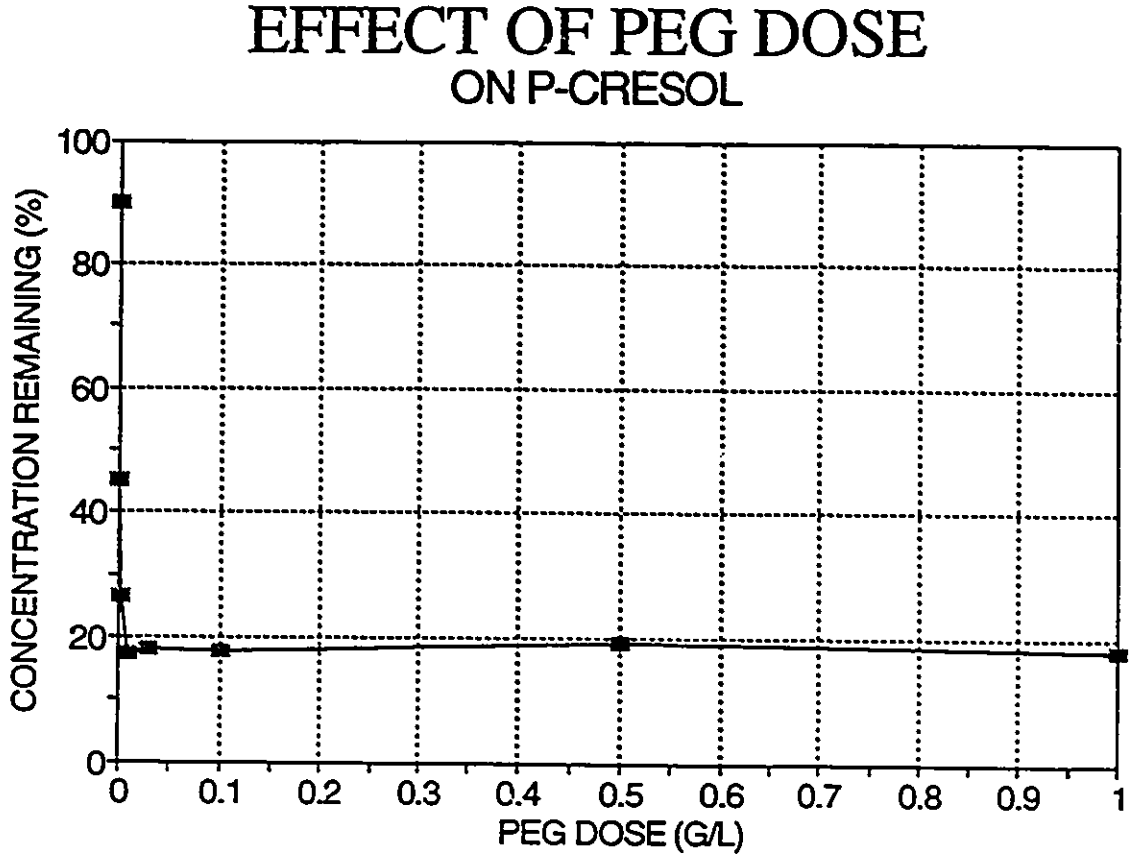


Figure-4.3.6 PEG dose effect on concentration remaining for p-cresol

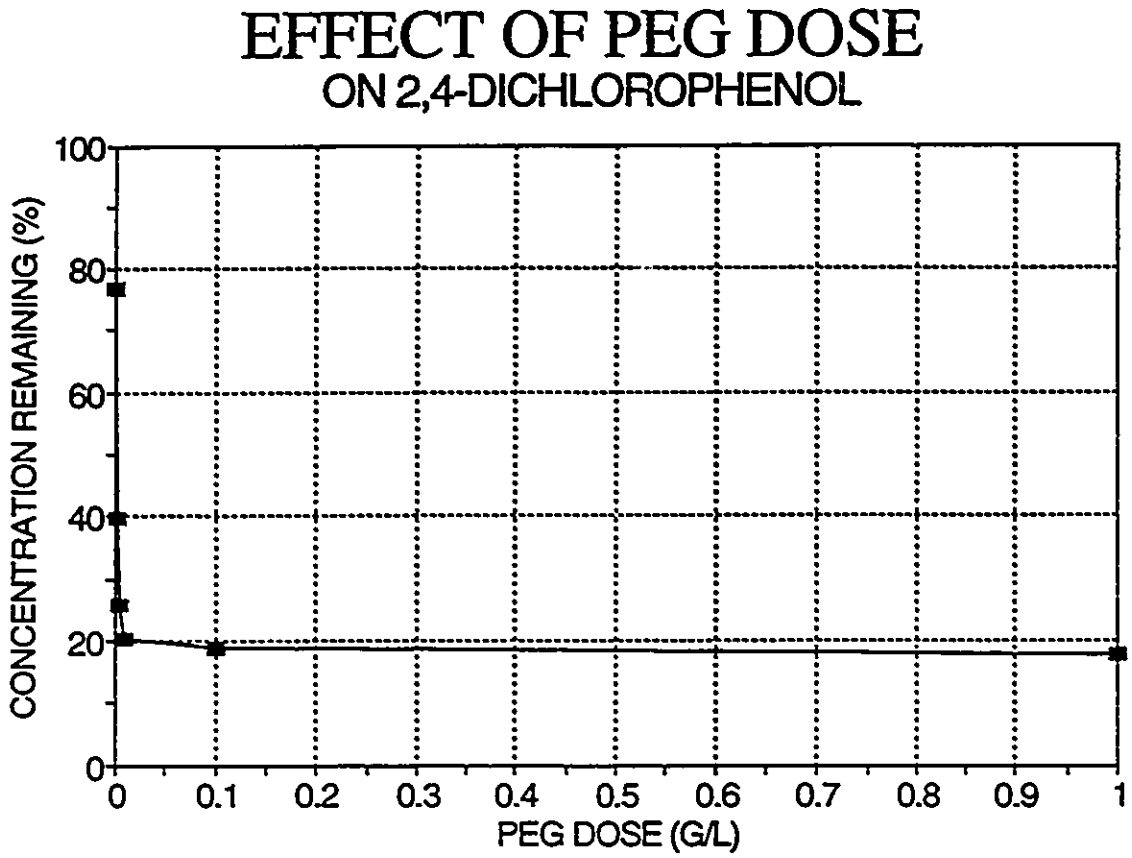


Figure-4.3.7 PEG dose effect on concentration remaining for 2,4-dichlorophenol

compounds are listed in Table-4.5. No relationship was observed between minimum PEG dose and minimum HRP dose.

While using the UV method to check the spectrum, it was found that the absorbance peak of the final product did not change with the change in PEG dose. Overdose of PEG did not have any reverse influence on its effect. 5 g/L PEG had the same effect on concentration removal and final spectrum as minimum PEG dose did (less than 100 mg/mL). This observation confirmed the results for pH and HRP dose experiments.

Table-4.5 Minimum PEG dose for phenol derivatives

Aromatic Compounds	Minimum PEG Dose (mg/L)	Aromatic Compound	Minimum PEG Dose (mg/L)
2-Chlorophenol	100	o-Cresol	10
3-Chlorophenol	100	m-Cresol	30
4-Chlorophenol	30	p-Cresol	30
2,4-Dichlorophenol	10		

4.4 REACTION TIME

The time needed to complete the polymerization reaction was studied by using minimum PEG and HRP doses for different phenol derivatives at 1 mM in the presence of 2 mM of hydrogen peroxide. Samples were taken from the reactors at predetermined

time intervals until 24 hours. After 7 hours, the substrate concentration remaining did not change significantly. So, the change of substrate concentration remaining with time was shown only for the first seven hours in Figures 4.4.1 to 4.4.7. Those plots showed that the minimum time needed for complete polymerization varied from 1 to 3 hours with the minimum HRP and PEG doses. Between 80 to 90 percent of the organics were removed in the first hour, while the remaining organic concentration decreased very slowly after the first hour. The slow reaction rate after 1 hour could be due to the low concentration of phenolic compound remaining (about 10 percent), and the corresponding low concentrations of horseradish peroxidase and hydrogen peroxide. Listed in Table-4.6 are the reaction times required for complete polymerization.

The time required for the enzymatic reaction is related to the amount of enzyme used, with and without the additives. The higher the initial enzyme activity, the less time required for completion of polymerization (Nicell, 1991; Wu, 1993). In all these experiments, the HRP concentration was very low at the end of the reaction. Because the minimum HRP dose was used in these experiments, the times listed here were maximum time required to complete the polymerization. These reaction times can be shortened by adding more enzyme. Since adding PEG greatly reduced the amount of HRP required for the reaction, the time for reaction had increased (Wu, 1993). Obviously, adding PEG has negative influence on its practical application because the cost of reactors volume will increase.

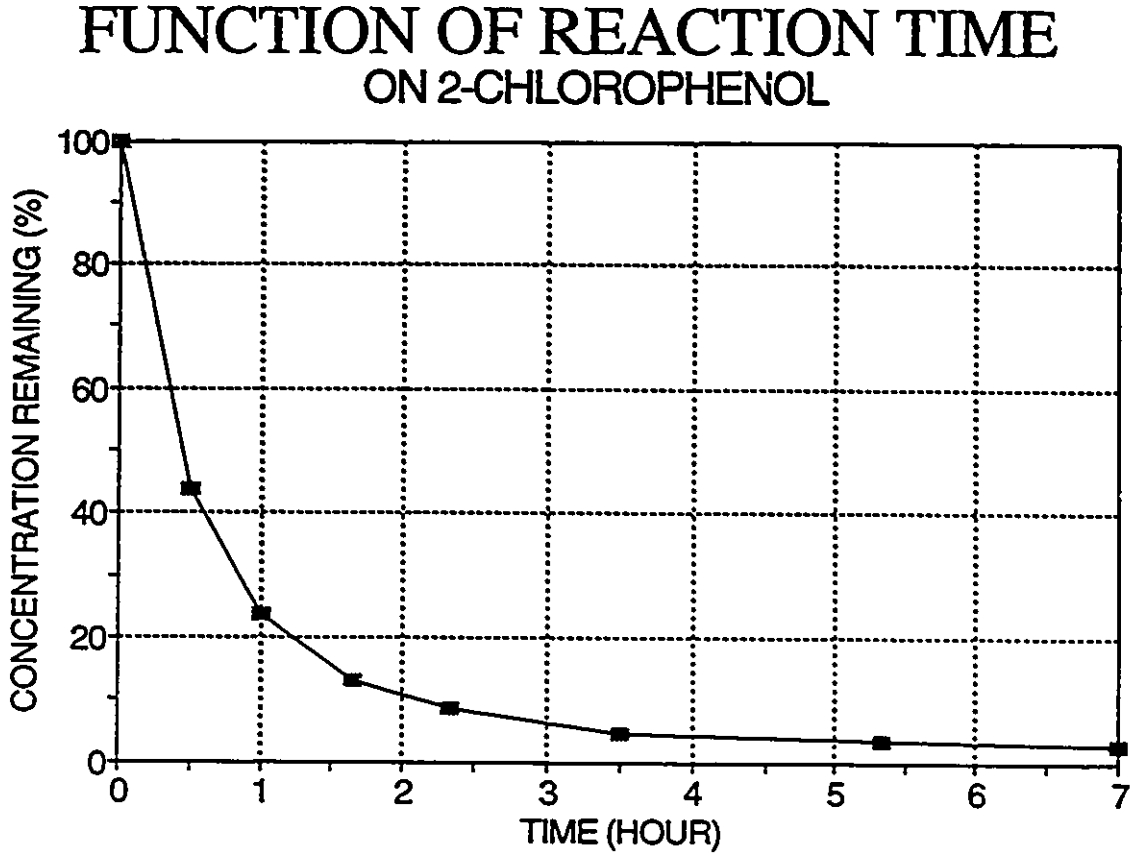


Figure-4.4.1 Effect of reaction time on concentration remaining for 2-chlorophenol

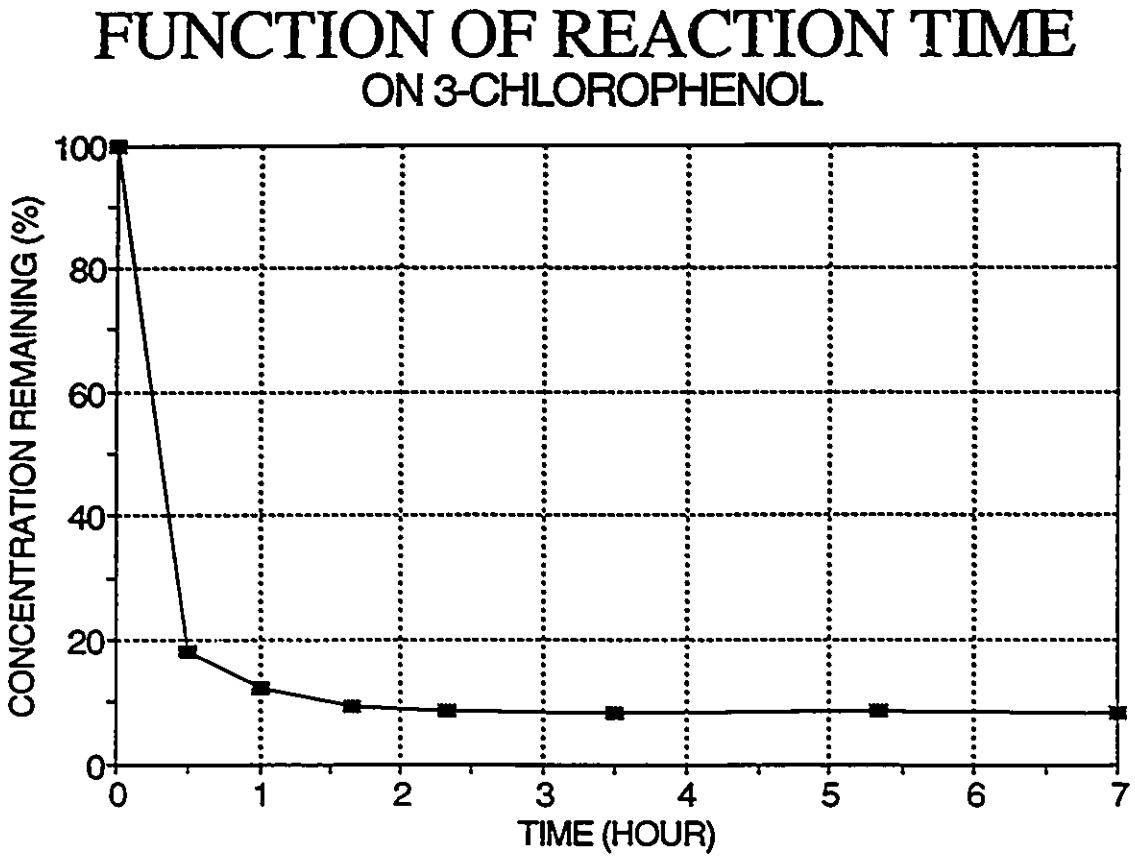


Figure-4.4.2 Effect of reaction time on concentration remaining for 3-chlorophenol

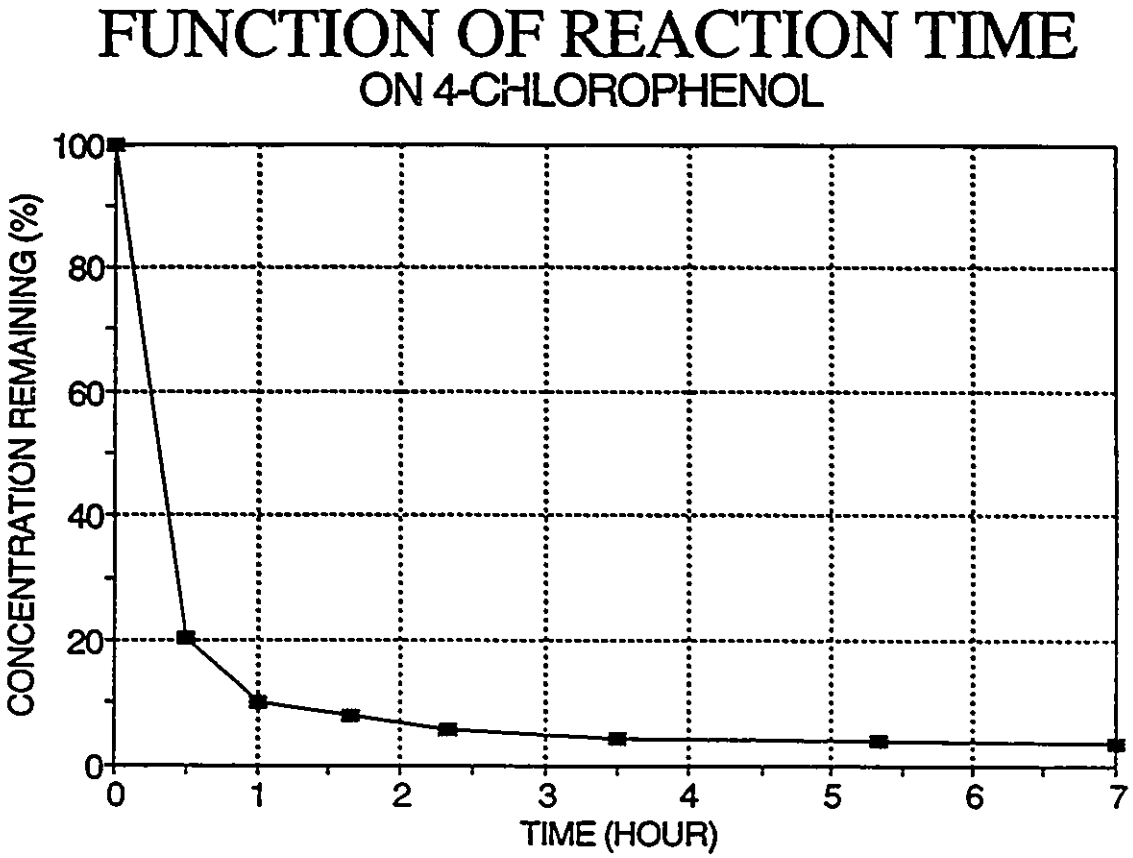


Figure-4.4.3 Effect of reaction time on concentration remaining for 4-chlorophenol

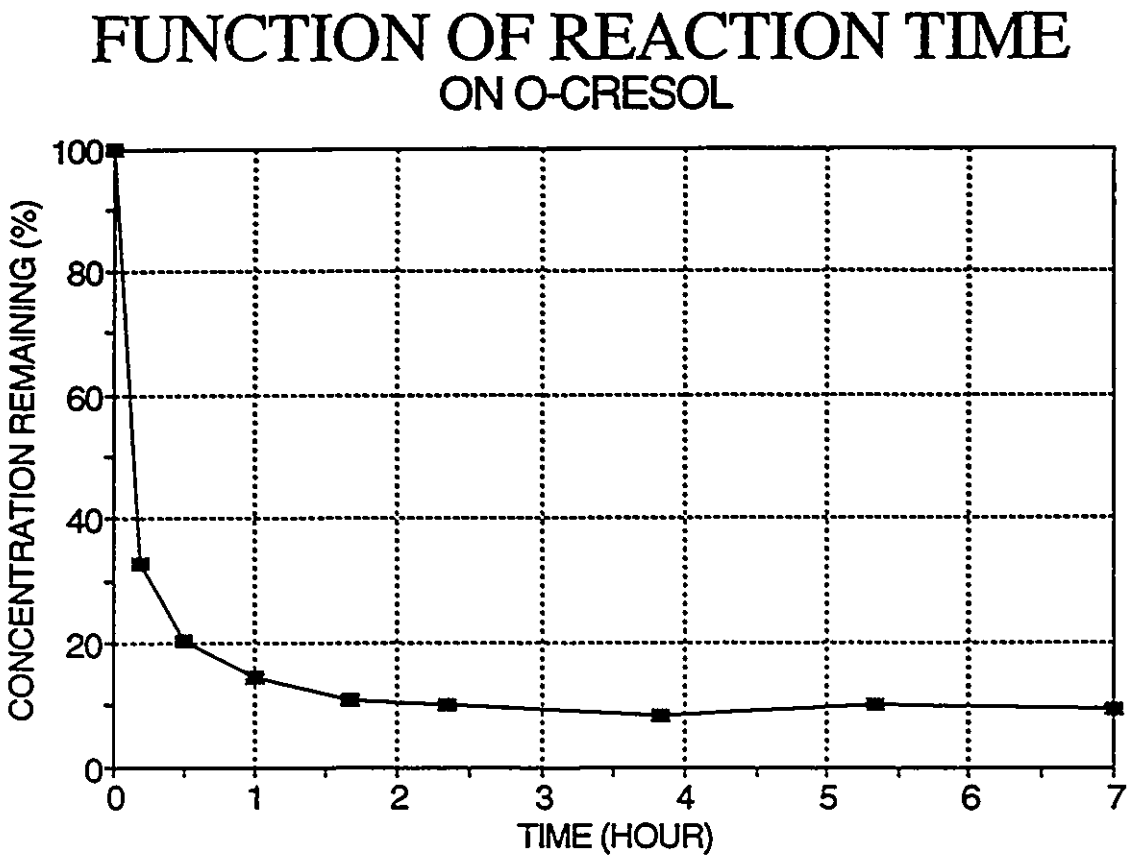


Figure-4.4.4 Effect of reaction time on concentration remaining for o-cresol

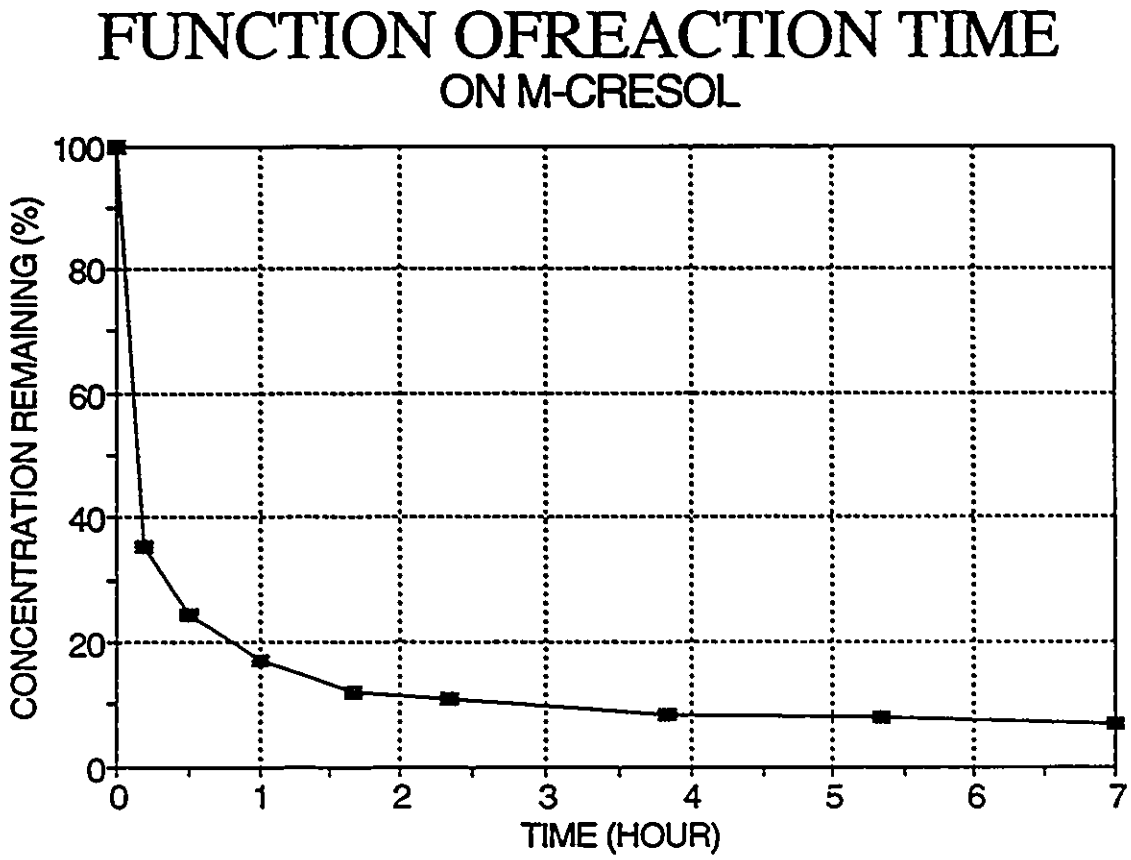


Figure-4.4.5 Effect of reaction time on concentration remaining for m-cresol

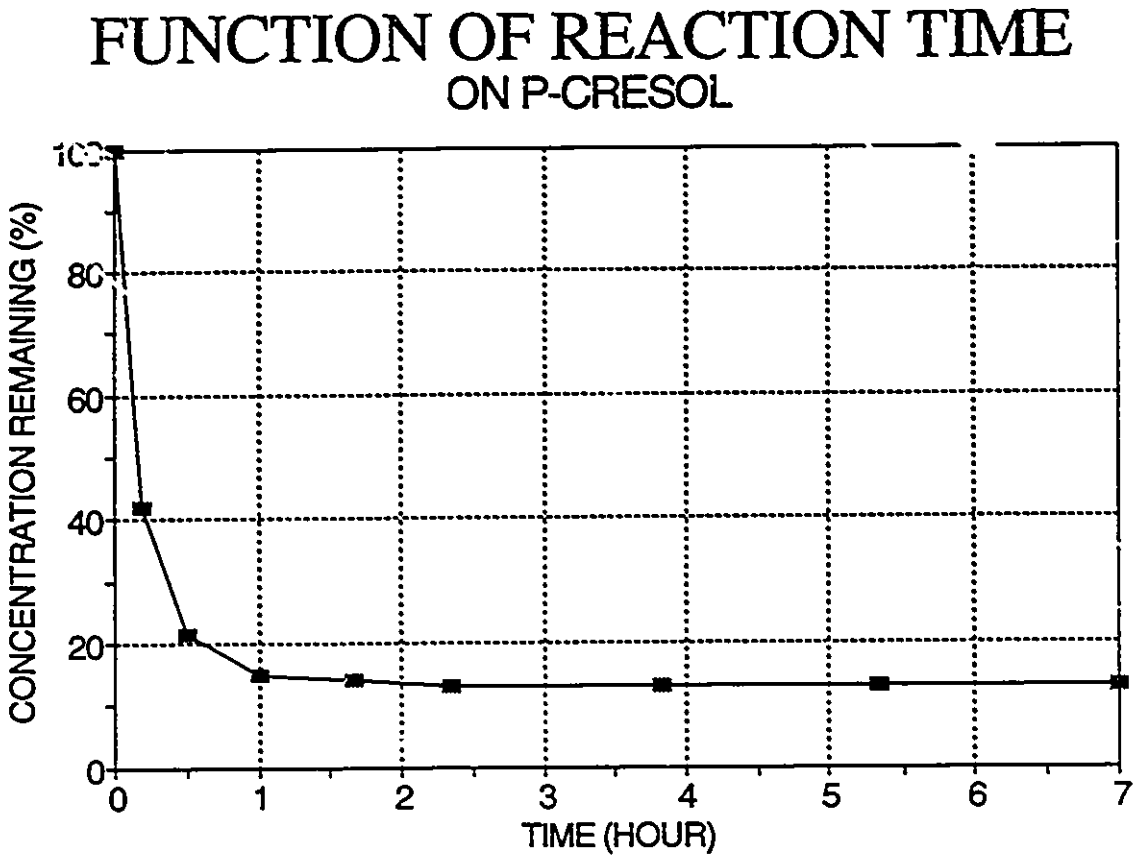


Figure-4.4.6 Effect of reaction time on concentration remaining for p-cresol

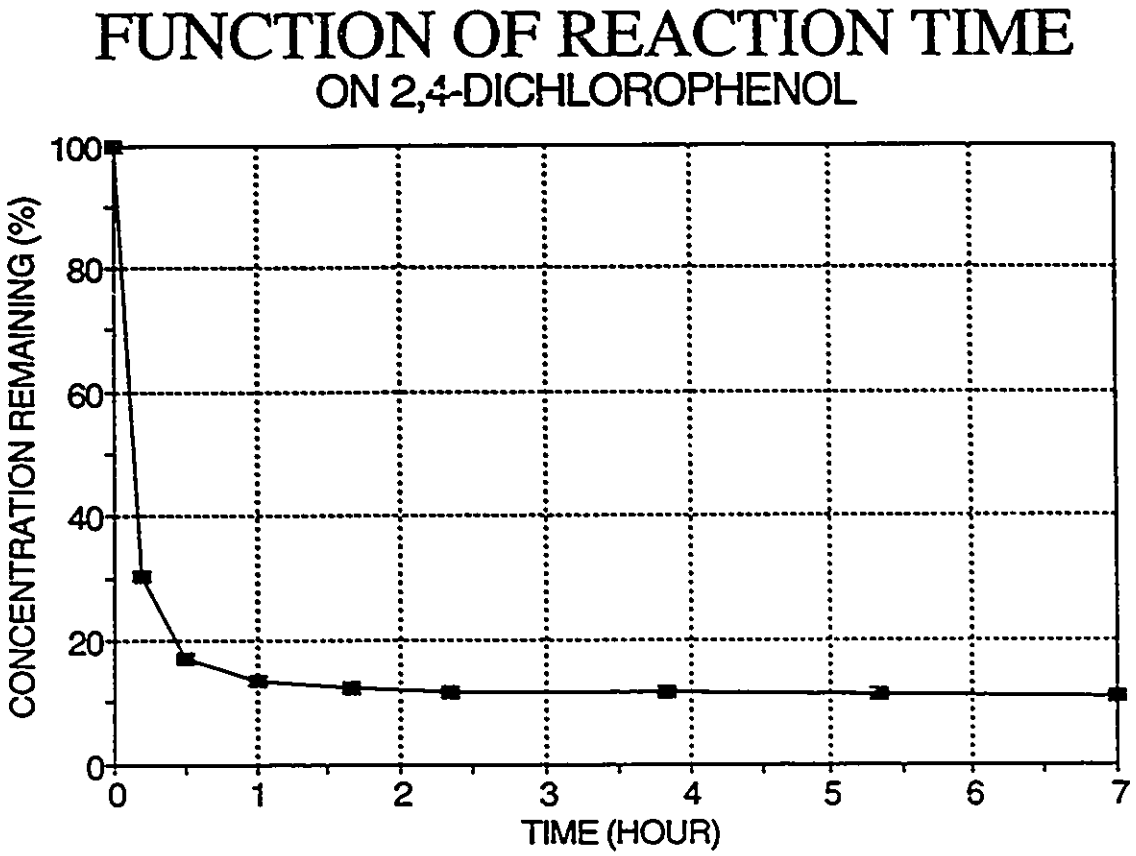


Figure-4.4.7 Effect of reaction time on concentration remaining for 2,4-dichlorophenol

Table-4.6 Reaction time needed for polymerization of different phenolic compounds

Aromatic Compounds	Time Required For Completion	Removal of Absorbance in the First Hour (%)
2-Chlorophenol	3.0	80
3-Chlorophenol	2.0	88
4-Chlorophenol	2.5	90
o-Cresol	2.0	85
m-Cresol	2.5	82
p-Cresol	1.5	85
2,4-Dichlorophenol	1.5	88

4.5 $[H_2O_2]$ TO SUBSTRATE RATIO

The effect of PEG on the $[H_2O_2]$ to substrate ratio for the HRP catalyzed polymerization of phenolic compounds was studied by varying H_2O_2 concentration. Minimum HRP dose and PEG dose were used in these experiments. Phenolic compound concentrations were kept at 1 mM while hydrogen peroxide concentration was varied from 0.45 to 2 mM. Phenol derivatives studied were 2-, 4-chlorophenol, o- and m-cresol. The results are plotted in Figures 4.5.1 to 4.5.4. The optimum range and the recommended optimal point of H_2O_2 concentration are listed in Table-4.7.

These plots show that concentration remaining decreases sharply with an increase in $[H_2O_2]$/[Phenol] before the optimal point (lowest concentration). It shows that the hydrogen peroxide is limiting in this range. After the optimum point, the remaining concentration increases with an increases in $[H_2O_2]$/[Phenol]. It can be explained that an

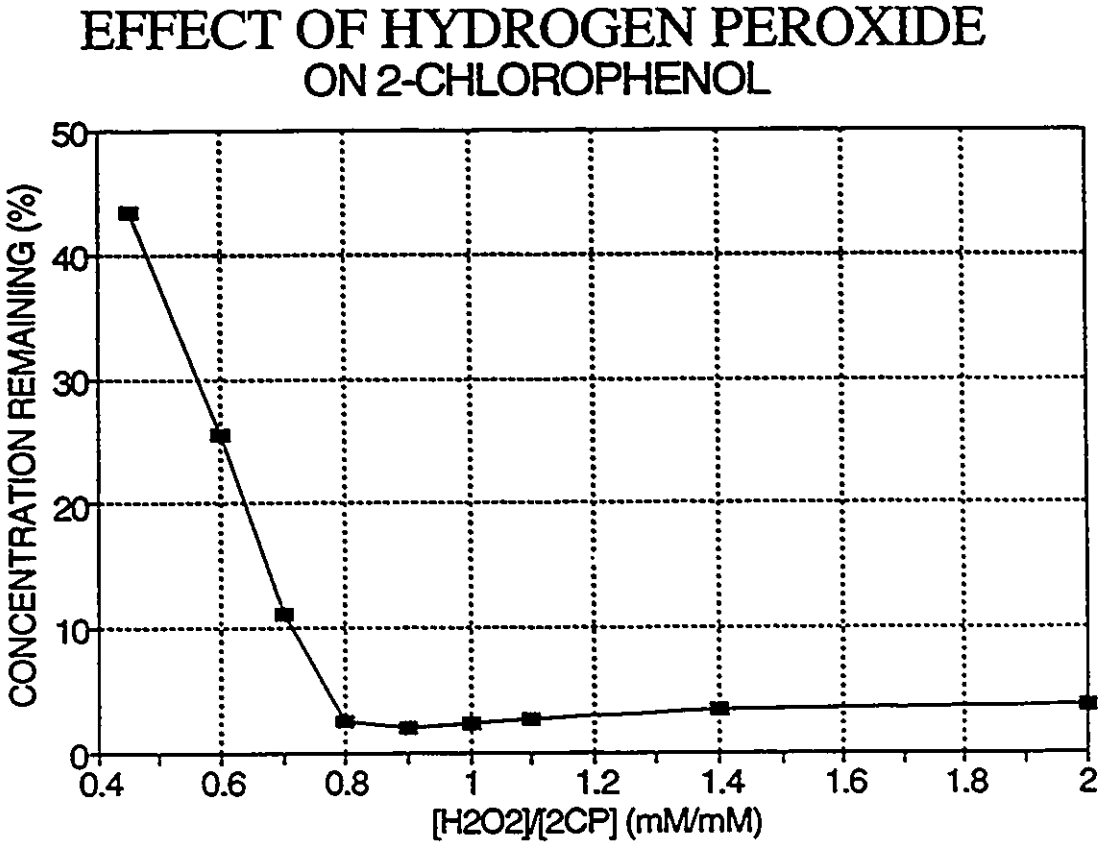


Figure-4.5.1 Effect of peroxide on concentration remaining for 2-chlorophenol

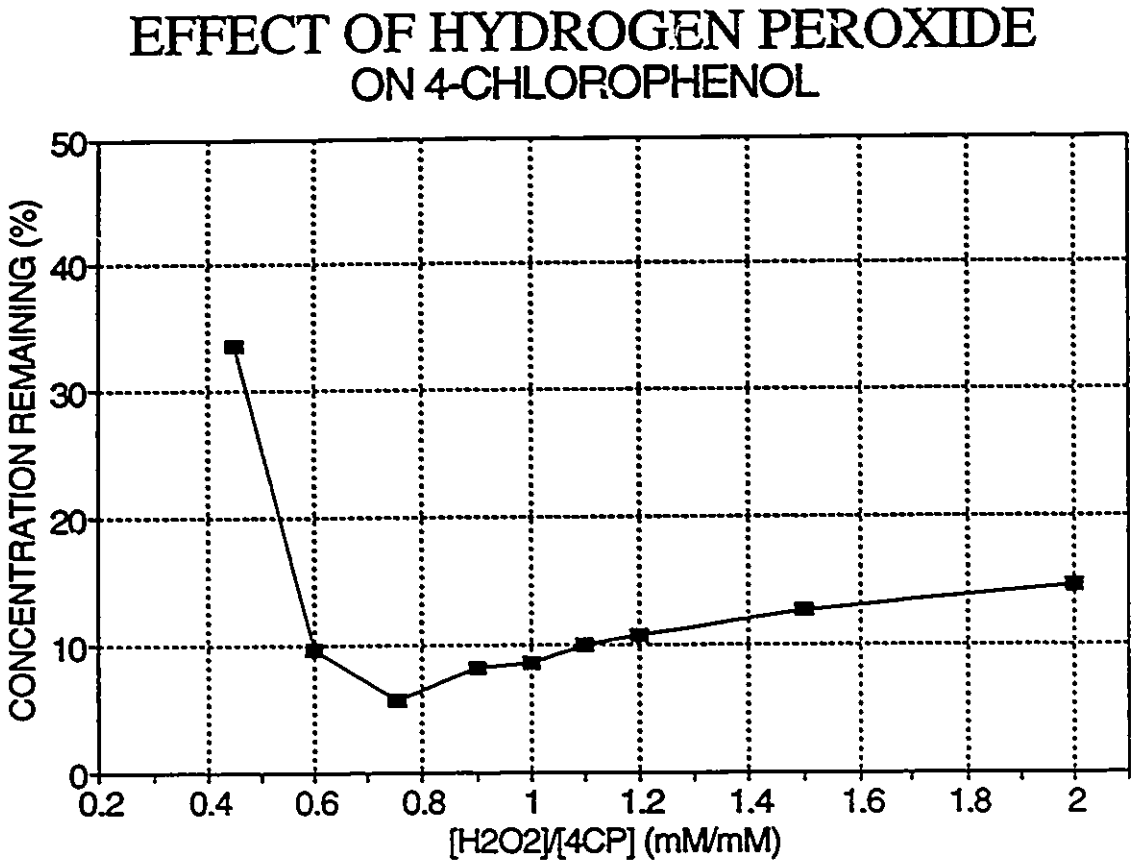


Figure-4.5.2 Effect of peroxide on concentration remaining for 4-chlorophenol

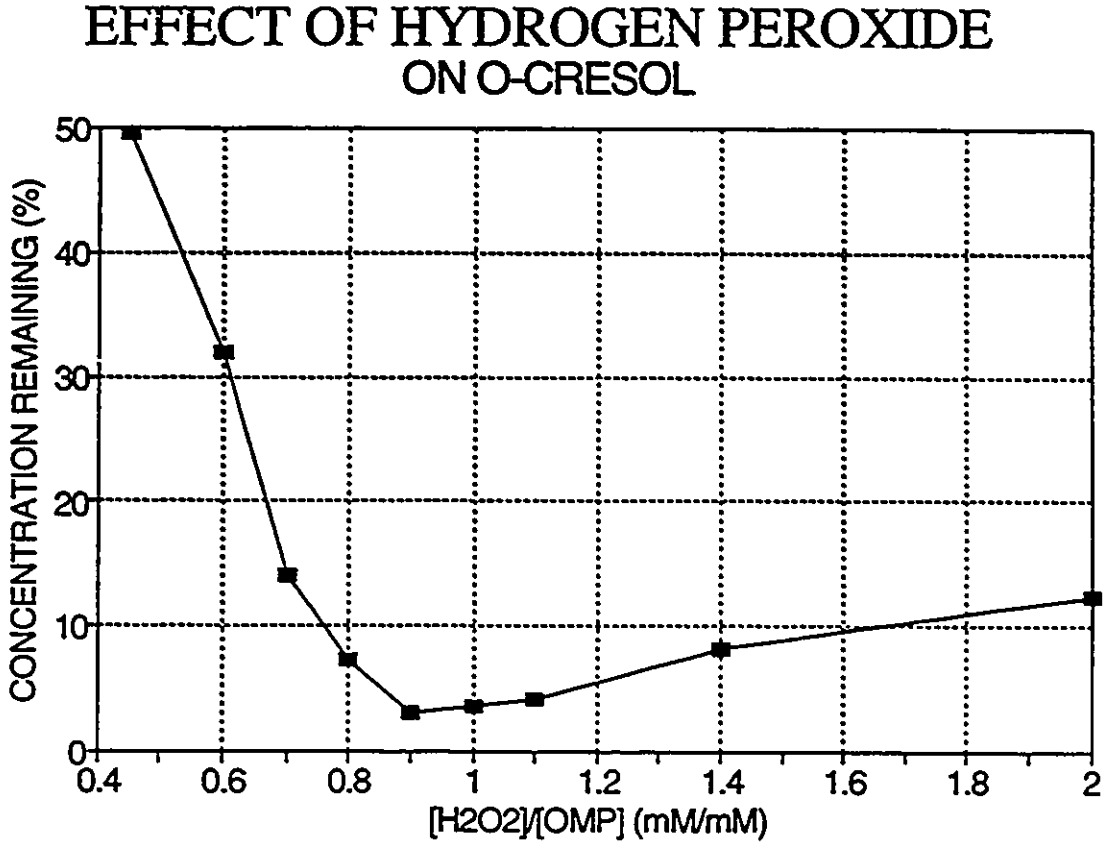


Figure-4.5.3 Effect of peroxide on concentration remaining for o-cresol

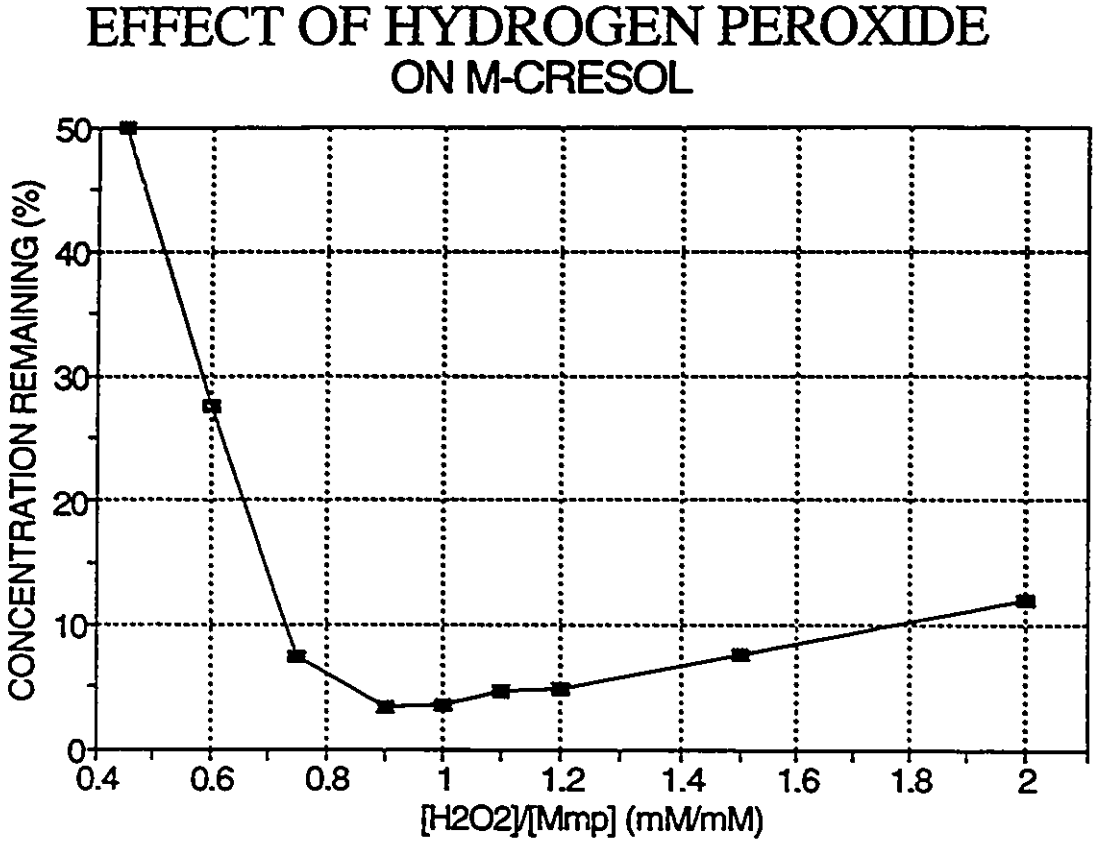


Figure-4.5.4 Effect of peroxide on concentration remaining for m-cresol

excess of hydrogen peroxide would produce more intermediate products which may inhibit the activity of the enzyme, or more lower molecular weight products were formed, which were soluble.

Table-4.7 Optimum H_2O_2 concentration for phenolic compounds

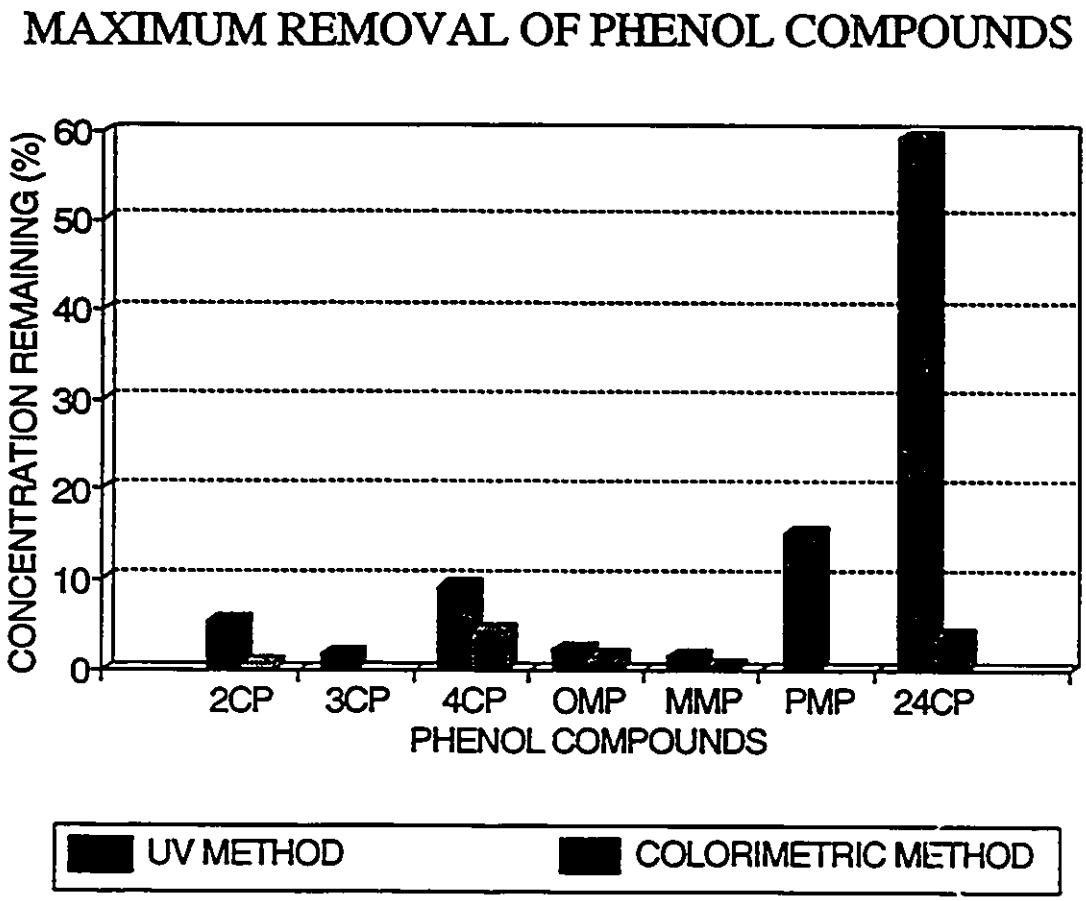
Aromatic Compounds	$[H_2O_2]/[Phenol]$ Optimal Point	$[H_2O_2]/[Phenol]$ Optimum Range
2-Chlorophenol	0.90	0.80 - 1.2
4-Chlorophenol	0.75	0.60 - 1.1
o-Cresol	0.90	0.85 - 1.2
m-Cresol	0.90	1.85 - 1.2

From these plots and the Table, it is obvious that optimum range of $[H_2O_2]/[Substrate]$ for various compounds are quite wide. This wide range for $[H_2O_2]/[Substrate]$ ratio, mostly from 0.85 to 1.2, is good for the change of phenol concentration in field application on actual wastewaters. The optimal point remained between 0.9 to 1, with the exception for 4-chlorophenol which has an optimal point at 0.75. Nicell (1991) has reported this value to be 1 without additives. The $[H_2O_2]/[Substrate]$ ratio did not change after adding the additive and remained around 1.

4.6 COPRECIPITATION

The degree to which a compound may be removed from solution by enzyme catalyzed polymerization is dependent on its reactivity toward peroxidase and the solubility of the products of the peroxidase oxidation. One or both of these factors could adversely affect the precipitation of aromatics from water, which would appear thereby limiting the application only to those compounds which can be efficiently removed from solution. Coprecipitation (Klibanov, 1980) was observed in which easily removed compounds aid in the precipitation of other compounds which cannot be removed to the same extent.

The maximum removal of different phenol derivatives was checked using excess HRP and PEG, 1 U/mL and 100 mg/L, respectively. The $[H_2O_2]$/[Substrate] ratio for these experiments was 1, and the initial total concentration of phenolic compounds was kept at 1 mM. Neutral pH was chosen and the reactions were carried out for 12 hours. The results are shown in Figure-4.6.1. Whenever possible, both the direct method and the colorimetric method for estimation of phenol were used to compare the results. Generally, the colorimetric method indicated a lower concentration remaining than the direct method. The difficulty in removing certain compounds did not change after adding the PEG. For instance, 2,4-chlorophenol and 4-chlorophenol showed a high residual which could not be further reduced according to the colorimetric method. All compounds showed less than 5 % concentration remaining. P-cresol showed the highest residual which could not be removed according to the UV method. It is believed that PEG had some interference in direct method for 2,4-chlorophenol, which showed an absorbance remaining of around



2,3,4CP: 2-,3-,4-Chlorophenol; O,M,PMP: O-,M-,P-Cresol; 24cp:2,4-Dichlorophenol

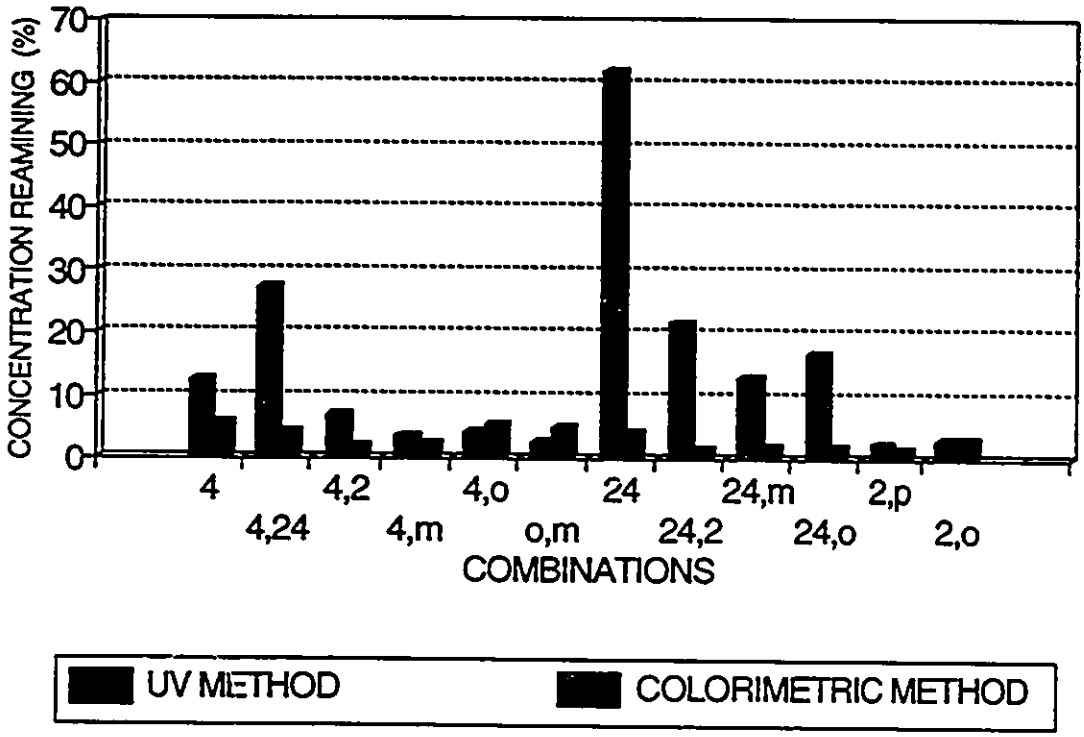
Figure-4.6.1 Maximum removal of phenol compounds studied

60 %, and possibly for some other compounds also.

Experiments were conducted to check the coprecipitation of 2,4-chlorophenol and 4-chlorophenol with other compounds. Other compounds were also mixed together to see if there was any enhancement in removal. All the experiment conditions were the same as the maximum removal experiment above except that 0.5 mM of each phenolic compound was used so that total concentration was still maintained at 1 mM. The results can be seen in Figure-4.6.2. This Figure shows that the total concentrations remaining for 2,4-chlorophenol and 4-chlorophenol are lower when they are mixed with other phenolic compounds. It suggests that coprecipitation occurs when they are mixed with other phenolic compounds. However when other phenolic compounds are mixed together, there is no improvement. For example, when o-cresol and m-cresol are mixed together, the final concentration remaining is slightly higher than that for individual chemicals. This suggests that enhancement due to coprecipitation does not always occur when two or more phenol derivatives are treated together.

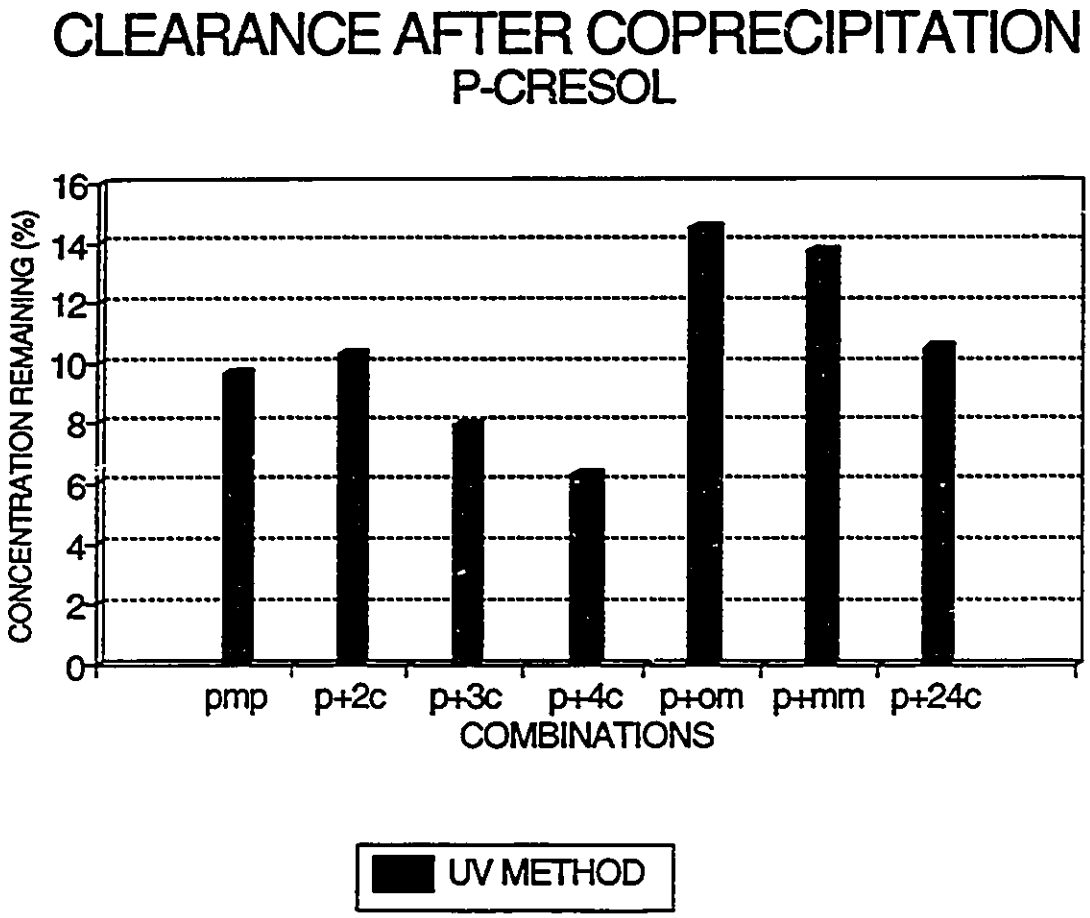
Similar experiments were conducted on p-cresol using UV method. The results are shown in Figure-4.6.3. Although the addition of 3-chlorophenol and 4-chlorophenol showed a slight improvement in coprecipitation, the addition of the other compounds did not cause coprecipitation.

CLEARANCE AFTER COPRECIPITATION
2,4-DICHLOROPHENOL and 4-CHLOROPHENOL



2, 4: 2-,4-Chlorophenol; O, M: O-,M-Cresol; 24: 2,4-Dichlorophenol; p: Phenol

Figure-4.6.2 Concentration remaining in coprecipitation of 2,4-dichlorophenol and 4-chlorophenol



pmp, p: p-Cresol; 2c,3c,4c: 2-,3-,4-chlorophenol;
om,mm: o-,m-Cresol; 24c: 2,4-Dichlorophenol

Figure-4.6.3 Concentration remaining in coprecipitation of p-cresol coprecipitation.

Visual observations should be mentioned here. When p-cresol and 2,4-dichlorophenol were treated separately, the final products of the polymerization did not settle even after adding alum. However, when p-cresol was mixed with other compounds, precipitates settled without adding alum. Also, when p-cresol was mixed with o-cresol, the absorbance peak at 408 for o-cresol had disappeared after the polymerization. These two observations suggest that interactions between the free radicals from different phenolic compounds did occur to form the mixed polymers which have the hydrophobic characteristics of the polymers formed by the more efficiently removed compound (Nicell, 1990).

It is not proper to overemphasize the effect of coprecipitation in the practical application of the enzymatic method. There are three reasons for that. First of all, the high concentration removal efficiency of the easy-to-remove compounds have some mathematical influence on the final lowered concentration removal efficiency of the mixed solution. Secondly, the difference between the concentration remainings before and after the mixing is not of significance in terms of analytical errors. Thirdly, it is possible that the final concentration remaining of the mixture of two phenolic compounds is higher than that when they were treated separately. Real industrial wastewaters are a mixture of different phenolic compounds, so, the negative effect might alleviate the effect of coprecipitation.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSIONS

Based on the previous discussion, the following conclusions are drawn for the low concentrations (1 mM, around 100 mg/L) of phenol derivatives (2-, 3-, 4-chlorophenol, o-, m-, p-cresol and 2,4-dichlorophenol):

1. PEG can greatly reduce the minimum amount of HRP needed for the completion of the polymerization of phenolic compounds. The amount of enzyme can be 30- to 130-fold less than that required without additive. The exception is 3-chlorophenol, which provides only 10 times improvement.

2. After adding PEG or gelatin, the optimum pH range becomes wider and optimal pH is close to neutral except for 2-chlorophenol. The influence of pH is not dependent on the amount of enzyme used.

3. PEG and gelatin have similar behaviour as additive, but PEG is better than gelatin in terms of removal efficiency and final products.

4. Addition of extra enzyme beyond the minimum dose does not improve the removal. The minimum amount of HRP needed for the completion of reaction depends on the specific compound.

5. The minimum PEG dose varies from 10 - 100 mg/L, depending on the specific phenolic compound. There is no general relationship between the minimum PEG dose and minimum HRP dose for all phenolic compounds.

6. With minimum HRP and PEG doses, the time required for completing polymerization is usually less than 3 hours. Most of the phenolic compound (eighty to ninety percent) is removed in the first one hour.

7. Addition of PEG does not change the reaction stoichiometry. The optimum $[H_2O_2]$/[Phenol] ratio is around 1. Lack of hydrogen peroxide reduces the removal efficiency.

8. Coprecipitation can occur after adding additive.

9. Final product seemed to be related to the enzyme dose, reaction time and additives. Mixing different phenolic compounds also affect the final products.

5.2 RECOMMENDATIONS

1. The mechanism of the effect of additive in the HRP catalyzed polymerization of phenol derivatives should be studied in order to explore other additives for practical use.

2. Potential toxicity of the final products should be studied for the safe application of this method.

3. New analytical methods for phenolic compounds should be employed in order to avoid interferences at the low concentration of phenolic compounds after the reaction.

4. Experiments should be conducted on the actual wastewater to check the effect of additive. Possible interference from the components existing in the actual wastewater should also be studied.

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APPENDIX A

HRP Activity Assay

1. General:

The purpose is to determine the amount of active enzyme that is contained in a stock solution. Under saturating condition of substrates (phenol, AAP and H_2O_2), the initial rate is measured by observing the rate of colour formation in a solution in which a reaction between phenol and H_2O_2 is catalyzed by HRP 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:

a. Phosphate **buffer** (0.1 M NaPP, pH 7.4)

160 mL 0.2M monobasic sodium phosphate +

840 mL 0.2M dibasic sodium phosphate +

distilled water to 2L

b. Phenol **(0.1 M Phenol)**

1882.2 mg Phenol in flask (200mL) +

phosphate buffer to 200mL

store in refrigerator

c. 4-aminoantipyrine **(9.6 mM AAP)**

390 mg AAP in flask (200 mL) +

phosphate buffer to 200 mL +

store in refrigerator

d. Hydrogen peroxide (**2.0 mM H_2O_2**)

226.7 µL of 30% H_2O_2 in flask (100mL) .

then remove 10mL of which in a same flask . dilute it to 100 mL. remake it daily.

3. Procedure: in a semi-micro cuvette place:

100 µL 0.1 mM Phenol +

250 µL 9.6 mM AAP +

100 µL 1.0 mM H_2O_2 +

500 uL NaPP buffer +

50 uL Sample (diluted)

The sample volume must 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 510nm.

4. Calculations

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

b. Calculation

$$Activity(U/mL) = \frac{slope(au/min)}{6,000 auL/mol} \times 10^6 \frac{\mu mol}{mol} \times \frac{1L}{1000mL}$$

The activity is in terms of micromoles of Phenol converted per minute at 20°C, pH 7.4 with 0.1 mM Phenol, 2.4mM AAP and 0.2 mM H_2O_2.

$$SampleActivity(U/mL) = Activity(U/mL) \times \frac{1000\mu L}{samplevol.(\mu L)}$$

5. Interferences

Aromatics which are substrates of HRP interfere with this reaction because of excess colour formation that is dependant on the type and quantity of the aromatic in the sample. This test should only be used on samples containing no aromatic -even in trace quantities.

APPENDIX-B

Aromatic Substrate Assay

1. General:

This is a colorimetric assay used to measure the concentration of aromatic substrate in a sample. It uses Horseradish Peroxidase as catalyst and 4-aminoantipyrine as a colour generating cosubstrate in combination with the aromatic in the sample. In this assay, the amount of aromatic introduced into the assay sample is the only limiting reactant and therefore the degree of colour developed in the reaction at the peak wavelength is proportional to the aromatic concentration at a peak wavelength of 510 nm.

2. Reagents:

a. Phosphate **buffer** (0.1 M NaPP, pH 7.4)

160 mL 0.2M monobasic sodium phosphate +

840 mL 0.2M dibasic sodium phosphate +

distilled water to **2L**

b. Horseradish peroxidase stock **(1 mg/mL HRP)**

200 mg HRP in flask (200mL) +

phosphate buffer to 200mL

store in refrigerator

c. 4-aminoantipyrine **(9.6 mM AAP)**

390 mg AAP in flask (200 mL) +

phosphate buffer to 200 mL +

store in refrigerator

d. Hydrogen peroxide (**1.0 mM H_2O_2**)

113.3 uL of 30% H_2O_2 in flask (100mL) ,

then remove 10mL of which in a same flask , dilute it to 100 mL. remake it daily

3. Calibration Procedure

Make up a stock solution of aromatic substrate with a concentration of 1 mM. From the stock solution create 25 mL solutions of aromatic substrate ranging in concentration from 0 to 0.25 mM. In a semi-micro cuvette place in the following order:

200 uL Aromatic Sample +

200 uL NaPP Buffer +

100 uL HRP Stock +

250 uL 9.6 mM AAP +

250 uL 1.0 mM H_2O_2

The sample volume must be 1 mL. Keep the aromatic concentration in the cuvette below 50 μM. Immediately after the addition of the H_2O_2 , shake the cuvette and then wait for the colour is fully developed (mostly after 35 minutes). Read the maximum amount of absorbance at the peak wavelength at 510nm. Make a plot of absorbance versus concentration of aromatic in the cuvette.

4. Measurement of Aromatic Substrate

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

50-200 uL Aromatic Sample +

200-350 uL NaPP Buffer +

100 uL HRP Stock +

250 uL 9.6 mM AAP +

250 uL 1.0 mM H_2O_2

The sample volume must be 1 mL. Keep the aromatic concentration in the cuvette below 50 μM. Immediately after the addition of the H_2O_2 , shake the cuvette and then wait for the colour is fully developed. Read the maximum amount of absorbance at the peak wavelength at 510 nm. Notice that for the samples of low concentration after the HRP catalyzed reaction, the maximum colour might be formed in less than 5 minutes.

5. Calculations

Calculate the sample aromatic concentration from:

$$[Aromatic]_{sample} = [Aromatic]_{cuvette} \times \frac{1000\,(uL)}{samplevol.\,(uL)}$$

where $[Aromatic]_{cuvette}$ is determined according to the calibration curve.

APPENDIX-C Standard Curves for Phenol derivatives

2-Chlorophenol:

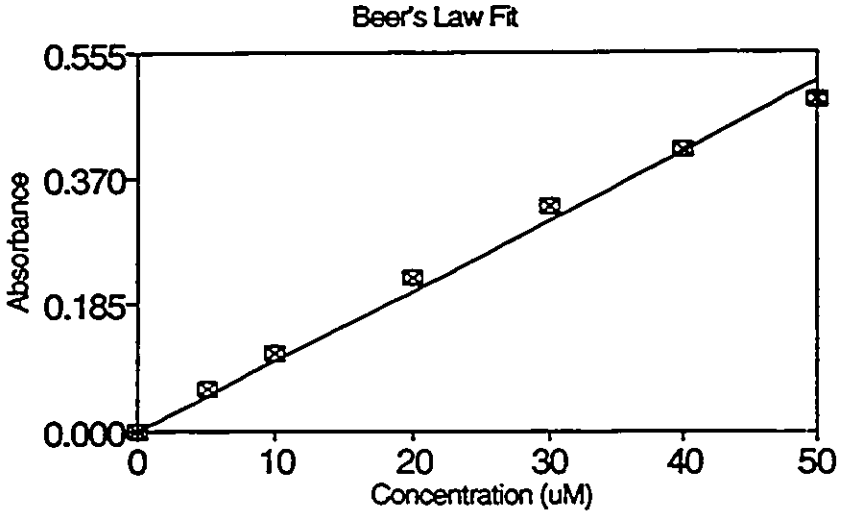
---> Standard Calibration Report <---

Date : 09-04-1992
Time : 16:29:57
Operator : Not Entered

File Name : C:\UV\DATA\2cp45min.510

Sample Name :
Client Name :
User Units :

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



3-Chlorophenol:

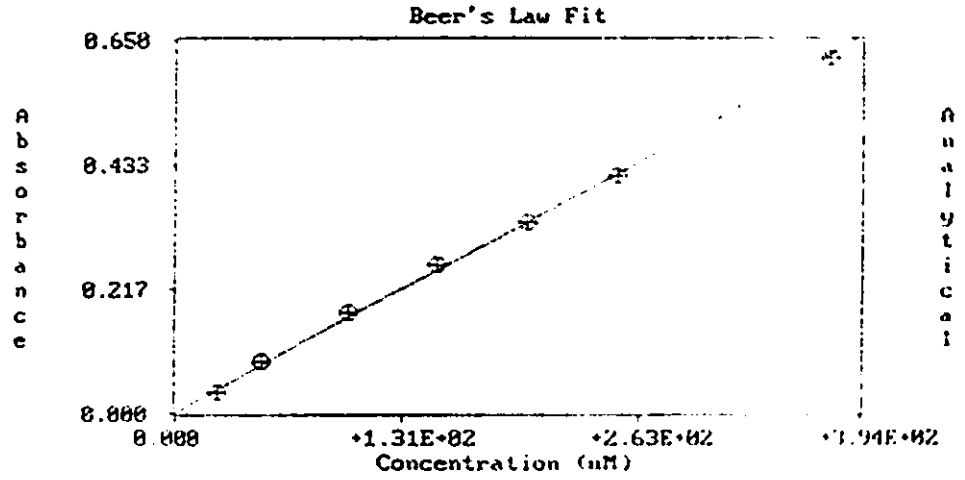
---> Standard Calibration Report <---

Date : 09 03 1992
Time : 15:10:44
Operat : Not Entered

File Name : C: \UV\DATA\3cp20min.STD

Sample Name : 3cp
Solvent Name : pH=7.5
Conc Units : uM

Analytical Wavelength : 274 nm
Reference Wavelength : None Selected
Confirmation Wavelength : None Selected
Integration Time : 1 seconds



4-Chlorophenol:

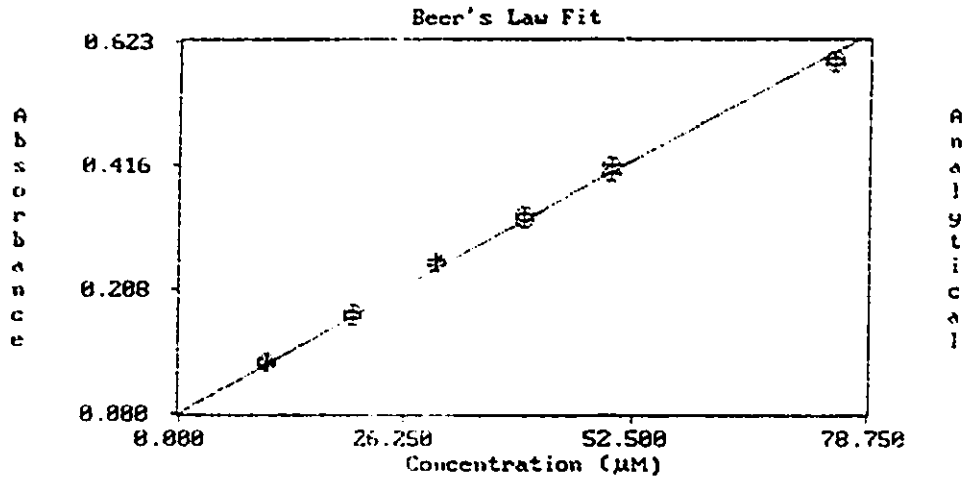
-- > Standard Calibration Report <---

Date : 09-04-1992
Time : 16:29:57
Operator : Not Entered

File Name : C:\UV\DATA\4cp45min.510

Sample Name :
Solvent Name :
Conc Units :

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



o-Cresol:

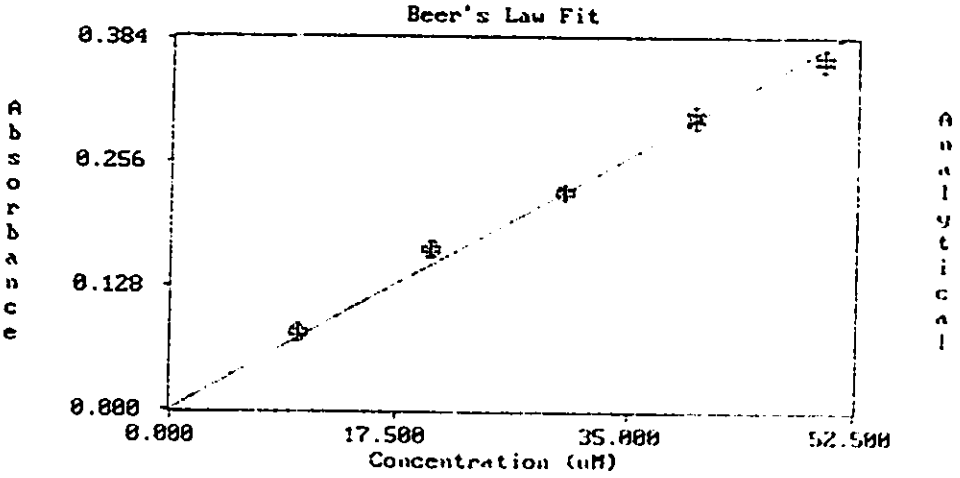
---> Standard Calibration Report <---

Date : 09 01 1992
Time : 15:24:3
Operator : Not Entered

File Name : a:omparone.STD

Sample Name : o-cresol
Solvent Name : water
Conc Units : uM

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



m-Cresol:

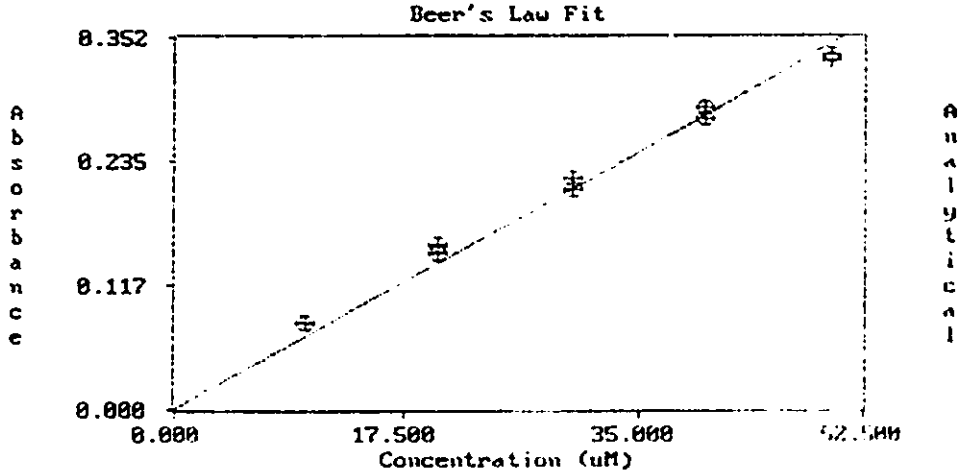
---> Standard Calibration Report <---

Date : 09-01-1992
Time : 16:27:10
Operator : Not Entered

File Name : a:mmparone.STD

Sample Name : m-cresol
Solvent Name : water
Conc Units : uM

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



p-Cresol:

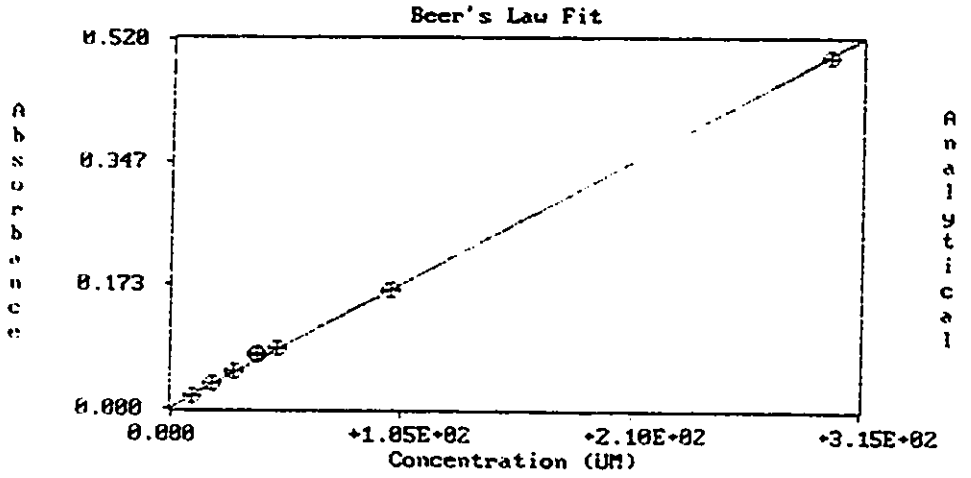
---> Standard Calibration Report <---

Date : 09-08-1992
Time : 11:15:03
Operator : Not Entered

File Name : a:pmp278.STD

Sample Name : PMP
Solvent Name : DW
Conc Units : UM

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



2,4-Dichlorophenol:

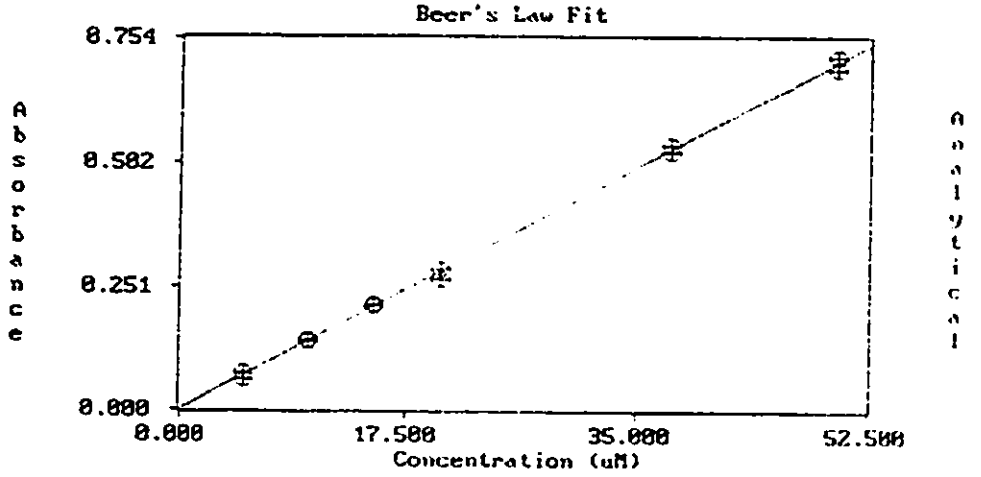
---> Standard Calibration Report <---

Date : 09-08-1992
Time : 15:41:58
Operator : Not Entered

File Name : Data not stored yet

Sample Name : 24cp
Solvent Name : dw
Conc Units : uM

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



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