Application of additives in horseradish peroxidase-catalyzed removal of phenol derivatives from aqueous solution.

Yimin. Wu
University of Windsor

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UMI
APPLICATION OF ADDITIVES IN HORSERADISH PEROXIDASE
CATALYZED REMOVAL OF PHENOL DERIVATIVES
FROM AQUEOUS SOLUTION

by

Yimin Wu

A Dissertation

Submitted to the Faculty of Graduate Studies and Research
through the Department of Civil and Environmental Engineering
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
1996

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ABSTRACT

Additives can be used in the horseradish peroxidase (HRP) catalyzed polymerization of phenolic compounds to increase the enzyme turnovers by more than 100-fold. In addition to polyethylene glycol (PEG) and gelatin, some polyelectrolytes may also prove effective as additives. The HRP saving is contingent on the nature of the additives and phenolic compounds. PEG appears to be the best choice from all perspectives. In the presence of additives, the optimum pH range becomes wider and the optimal pH is closer to neutral. Enzyme inactivation is mainly caused by the polymer products. Most of the additives are incorporated covalently with the polymer product during the reaction. HRP and additive interact with the polymer product according to their ratio of partition affinities for the polymer product. Enzyme inactivation is alleviated because of the small ratio.

A computer model has been developed which includes a second order Michaelis-Menten equation with respect to the concentrations of phenol and hydrogen peroxide, and two equations based on inactivations caused by both polymer product and hydrogen peroxide. Experimental data prove that the model output can predict experimental behavior realistically under a variety of reaction conditions. The model has been verified by predicting some independent experimental results. Although the model is obtained from batch reactors, it can also be applied to plug flow reactors and continuous stirred tank reactors as well.

Experiments prove that there is no need to stir the reaction mixture during the entire reaction period. Batch reactors and plug flow reactors have an identical reaction curve. Because of the addition of additives, continuous stirred tank reactors need very long times to complete the reaction at the minimum HRP dose. A plug flow reactor system is recommended for the reaction in the presence of additives. Such a system consists of a small mixing tank followed by a tank in which the reaction and the settling occur simultaneously.
DEDICATION

Dedicated to each of those, especially my family, who made this work possible
ACKNOWLEDGMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Unit</th>
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<tbody>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
<td>mM</td>
</tr>
<tr>
<td>HRP&lt;sub&gt;ia&lt;/sub&gt;</td>
<td>Inactivated Horseradish Peroxidase</td>
<td>mM</td>
</tr>
<tr>
<td>ARP</td>
<td>Peroxidase from <em>Arthromyces Ramosus</em></td>
<td>mM</td>
</tr>
<tr>
<td>SBP</td>
<td>Peroxidase from soybean</td>
<td>mM</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
<td>mg/L</td>
</tr>
<tr>
<td>PE</td>
<td>Polyelectrolyte</td>
<td>mg/L</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen Peroxide</td>
<td>mM</td>
</tr>
<tr>
<td>AH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Aromatic Compound, e.g., Phenol</td>
<td>mM</td>
</tr>
<tr>
<td>[ ]</td>
<td>Molar Concentration</td>
<td>mM</td>
</tr>
<tr>
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<td>Michaelis constant of one-substrate Michaelis-Menten equation</td>
<td>mM</td>
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<tr>
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<td>mM</td>
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<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
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<tr>
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<td>Reaction stoichiometry between PEG and phenol</td>
<td>mg/L $\cdot$ mM</td>
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<td>mM/mM</td>
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<tr>
<td>PADR</td>
<td>Practical Additive Dose Range</td>
<td>mg/L</td>
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CHAPTER 1  INTRODUCTION

1.1 PHENOLIC WASTEWATER AND ITS TREATMENT

Phenolic compounds are present in the wastewaters of a number of industries such as coal conversion, resins and plastics, textiles, dyes and other organic chemicals, timber, soaps and detergents, iron and steel and petroleum refining. The concentrations of phenolic compounds in these wastewaters may range from very low to as high as tens of grams per liter. Almost all these phenolic wastes are toxic and many of them have been classified as hazardous pollutants because of their potential harmfulness to human health. Phenols can enter the human body through water and food ingested, air breathed and skin contact (USPHS, 1989). Repeated exposure to low levels of phenol in drinking water has been linked with diarrhea and mouth sores in humans. The phenolic compounds in water accumulate in fish through the food chain, causing an odor when the fish is served as food. Chlorophenols are more toxic than phenol to all life forms and the toxicity increases with the degree of chlorination (Apajalahti, 1987).

Regulations concerning phenols are strict. For example, the MISA (Municipal and Industrial Strategical Abatement) requires phenols in wastewater to be treated to as low as 20 μg/L before its release (Environment Ontario, 1988). The provincial water quality objective of Ontario for surface water requires that phenols do not exceed 1 μg/L to protect against tainting of edible fish flesh (Ministry of Ontario, 1984). Therefore, proper treatment is necessary to remove phenols from industrial wastewaters before their discharge to surface waters.

Available conventional methods for removal of phenol from industrial wastewater include solvent extraction, microbial degradation, adsorption on activated carbon and chemical oxidation. However, they may suffer from such serious drawbacks as high cost, incomplete removal, formation of toxic by-products and applicability to a limited concentration range (Klibanov et al., 1980). For example, biological treatment processes
are sensitive to shock loads and need a hydraulic retention time of several days. Furthermore, phenolic compounds may be evaporated into air during the long period of aeration if an activated sludge process is used, thus causing secondary contamination. Activated carbon can not handle economically flows with high concentrations of phenol, while solvent extraction can handle only high concentration flows and needs further treatment. It is beneficial to find an innovative method that is versatile for a wide range of reaction conditions. The enzymatic method serves that purpose.

1.2 APPLICATION OF ENZYMES IN WASTEWATER TREATMENT

Fundamentally, biological treatment of wastewater is based on the microorganisms (mainly bacteria) that produce enzymes to decompose the pollutants. All enzymes are identified as proteins, which are polymers of amino acids combined by peptide bonds. Most enzymes can be separated from living bodies and function as well as in the living bodies. The two most distinctive features of enzymes are their high activity and specificity. As proteins, enzymes have three-dimensional structure that is essential for catalysis. The factors affecting enzyme reaction usually include the enzyme concentration, substrate concentration, pH, temperature, reaction time and inhibitors (Wynn, 1979).

The idea of using enzymes to remove toxic substances in wastewater is not new. The application of enzymes to wastewater treatment was widely investigated in the 1980's in Europe, North America and Japan (Wu, 1992). The wastewaters tested included those containing pesticides, aromatic compounds, urea, cyanide and organic compounds. All these investigations showed that the use of enzymes to treat wastewater, which was difficult to treat by biological methods, was practical and more effective.

The phenomenon of oxidative coupling of phenols by enzymes was well recognized in the early of 60's (Brown, 1967), but its application to wastewater treatment occurred about fourteen years later (Klibanov et al., 1981). Most of the research
concentrated on the removal of aromatic compounds using different enzymes, for example, peroxidases, laccases, tyrosinases and ligninases (Al-Kassim et al., 1993a, 1993b, 1994). Peroxidases from different sources (horseradish (HRP), soybean (SBP), Coprinus macrorhizus (CMP) and Arthromyces Ramosus (ARP)) proved to be effective in treating phenols and aromatic amines. Applications on actual wastewater were reported at the same time. Researchers showed that HRP could be used to transform phenolic or aromatic amine radicals into water-insoluble polymers that subsequently could be removed by filtration or sedimentation (Klibanov et al., 1980). While it is true that HRP was not effective in removing certain compounds such as polychlorinated biphenyls from water, the removal efficiency could be improved when they were treated together with reactive compounds. This phenomenon was called coprecipitation.

Since enzymes act independently as catalysts, they possess some remarkable advantages over other methods:

- enzymes can be used under a wider range of extreme environmental conditions (pH and temperature) compared to biological treatment;
- they are not affected by many inhibitors of microbial metabolism;
- they have the flexibility to function with a broad range of substrate concentrations and types including those toxic or biorefractory to microbes;
- enzymatic operation is in a catalytic manner, so the process control is simpler. For instance, there is no shock loading effect or delay associated with shutdown/startup;
- usually, the reaction time needed is shorter because of the high activity of the enzyme.

A potential obstacle in the application of this method is associated with the large amount of enzyme needed in this treatment. The corresponding high cost has limited its practical application to date. However, it was reported recently that additives, such as polyethylene glycol (PEG) and gelatin, could greatly reduce the amount of enzyme required for polymerization of phenolic compounds at high concentrations (Nakamoto and
Machida, 1992). Studies on low concentrations of phenol also showed that PEG was effective in reducing the amount of HRP needed in completing polymerization of phenol (Wu J. et al., 1993).

Several kinetic models (Nicell, 1991; Siddique, 1992; Yu et al., 1994) have been developed to simulate the HRP catalyzed removal of phenolic compounds. However, each of these models has certain deficiencies. Some of these models do not show the effect of hydrogen peroxide on the reaction, while others do not predict the enzyme inactivation accurately. Consequently, these models cannot be used to aid reactor design, especially in the presence of additives. Research on the reactor design has focused mainly on the effect of the semibatch addition of hydrogen peroxide or HRP on the removal efficiency (Nicell, 1991; Wu, J. et al., 1993). Thus, there is still a need for satisfactory understanding of how to select and design a reactor under different reaction conditions with the aid of a well performing computer model.

1.3 OBJECTIVES

Based on the above discussion, the following objectives were set for this study:

- 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.
- Examine the effects of additives on the apparent activity of HRP and its inactivation under various conditions.
- Determine the fate of additives after the completion of polymerization to see if they possess any negative effect on the effluent.
- Propose a possible mechanism of additive's protection of enzymes for application of new additives.
- Establish a kinetic model which can be used to select and design reactors.
• Compare different reactor configurations and recommend a reaction process for real wastewater treatment in the presence of additives.

• Recommend a general design procedure for the enzymatic removal of phenolic compounds from the wastewater.

1.4 SCOPE

The scope of this study is described below:

• Enzyme used in this study was exclusively horseradish peroxidase.

• Phenol derivatives, 2-, 3-, 4-chlorophenols, o-, m-, p-cresols and 2,4-dichlorophenol, were investigated along with phenol.

• Polyethylene glycol (PEG), gelatin and certain polyelectrolytes were tested as additives.

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

• Inactivations caused by temperature, hydrogen peroxide, intermediate products during reaction and the final polymer products were studied.

• Computer model included four variables: phenol concentration and hydrogen peroxide concentration, PEG concentration and apparent activity of HRP. It reflected the effects of HRP, hydrogen peroxide and PEG concentration on the removal of phenol at low concentrations (1 - 3 mM).

• Reactors compared were batch, semi-batch, plug flow and continuous stirred tank. Semi-batch operation of plug flow reactor was also studied.
CHAPTER 2 LITERATURE REVIEW

2.1 HORSERADISH PEROXIDASE

2.1.1 Properties

Horseradish peroxidase is among the enzymes that are ubiquitous in the plant and animal world. Its discovery can be traced back to 1903 and extensive 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 (Dunford, 1991). Among them, most commercial preparations are isoenzyme C and most of the research is based on HRP-C. The horseradish peroxidase molecule consists of 308 amino acid residues. The molecular mass has been estimated to range from 33,890 to 42,100. Its purity can be expressed by the R.Z. number, a ratio of its absorbances at 403 and 280 nm, which is a measure of hemin content using the aromatic amino acid content as a reference. A good preparation of HRP has a R.Z. value of 3.0 to 3.4. However, it is not a direct indication of enzymatic activity.

Native HRP is an ideal enzyme. Over the pH range of 5 to 10, it is stable for a long period of time at room temperature (Dunford, 1991; Nicell, 1991; Bollag et al., 1990, 1992). Storage of the enzyme in distilled water at neutral pH and 4 °C for one month produced no significant loss in activity. 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 its activity (Nicell, 1991). The dry enzyme can retain its activity for several years when stored at -15 °C and for many weeks at 37 °C. The enzyme activity has a broad optimum pH range of 5.7 to 8.5 with optimal activity occurring at neutral pH. It retains more than 90% of its activity after incubation for 48 hours at pH values between 6 to 9 and room temperature, but the activity is severely reduced outside this range. There is no significant loss in the HRP activity on incubation in the temperature range of 5 °C to 35 °C (Nicell, 1991).
2.1.2 Reaction Scheme

In simplified terms, the normal peroxidase cycle may be represented by the following scheme:

![Reaction Scheme Diagram]

Figure 2-1 Reaction Cycle of HRP Catalyzed Oxidation of Aromatic Compound

This can be considered as a modified type of ping-pong kinetics, referred to as peroxidase ping-pong by Dunford (1991). Native HRP is oxidized by hydrogen peroxide and converted to Compound I (HRP-I), an active intermediate form of HRP. Compound I can oxidize an aromatic compound (AH₂) into a free radical (*AH), while Compound I itself becomes reduced to another active form of HRP, Compound II (HRP-II). Similarly, Compound II can oxidize another aromatic compound into a free radical, and itself be reduced to the native form of HRP. The free radicals formed in this cycle can react with each other, in a non-enzymatic process, to form oligomers that are subject to further oxidation by HRP to constitute higher polymers.
2.1.3 Inactivation of Horseradish Peroxidase

In the absence of reductant substrates and with excess hydrogen peroxide, peroxidase showed a kinetic behavior of suicide inactivation, hydrogen peroxide being the suicide substrate (Arnao et al., 1990a, 1990b; Baynton, 1992, 1994). Inactivation curves of activity remaining versus time exhibited a rapid phase (0 - 60 seconds) which was hydrogen peroxide concentration dependent, and a slow phase characterized by a gradual loss of enzyme activity that was neither time nor hydrogen peroxide concentration dependent. The process involved a reversible inactivation pathway leading to Compound III (HRP-III in Figure 2-2), which likely accounted for the observed rapid inactivation, and a pathway leading to an irreversibly inactivated intermediate, Compound P670, which was predominant at hydrogen peroxide concentration above 1.0 mM. They concluded that a competition was established between two catalytic pathways: the catalase Compound III-forming pathway, and the suicide inactivation pathway (formation of inactive enzyme).

![Figure 2-2 Pathway for Inactivation of HRP by Hydrogen Peroxide](image)
In the presence of reductant substrates, horseradish peroxidase showed a different inactivation mechanism which was irreversible, mechanism-based and time-dependent. It was suggested that this inactivation was most likely the result of the interactions of phenoxy radicals with the enzyme's active center during the oxidation of phenol (Klibanov et al., 1983; Dunford, 1991; Baynton et al., 1994). On the contrary, Nakamoto and Machida (1992) argued that it was due to the adsorption of enzyme onto the polymers formed during the reaction (see Section 2.3).

2.2 HORSE RADISH PEROXIDASE IN WASTEWATER TREATMENT

Applications of this method to wastewater were first reported around 1980 (Klibanov and Morris, 1981). Over 30 different phenols and aromatic amines were tested using this method (Nannipieri et al., 1991). Phenols and aromatic amines were easily removed from water with a high efficiency (exceeding 99%). The products of the polymerization were practically insoluble in water. Instead of purified HRP, the crude form of HRP from horseradish root was also tested and was found to be as effective as the pure one. Moreover, the crude form of the enzyme could be reused up to 30 times. Its effectiveness was similar to that of the pure enzyme (Dec and Bollag, 1994). HRP immobilized on different support surfaces was also successfully applied to remove 4-chlorophenol (Siddique, 1992).

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 (Bollag et al., 1990, 1992). Optimal reaction parameters (pH, HRP concentration, temperature, hydrogen peroxide concentration and contact time) were also investigated (Klibanov et al., 1980, 1981; Nicell, 1991). The range of pH for phenol derivatives was from 3.5 to 7.0. The peroxidase needed to reach 99.8% removal efficiency could be decreased by increasing the reaction
time. The minimum hydrogen peroxide needed for 99.8% removal of 0.8 mM of 2-chlorophenol was 1.0 mM. Extra hydrogen peroxide reduced the removal efficiency. The stoichiometry of the reaction between aromatic substrate and peroxide was found to be unity. The removal efficiency for different concentrations of 2-chlorophenol in the range of 1 mg/L to 150 mg/L was found to be constant within the experimental error. At least two independent factors affected the overall removal efficiency of a phenol or an aniline derivative from water, i.e., its reactivity toward peroxidase and the solubility of the products of peroxidase oxidation in water (Klibanov et al., 1980).

Table 2-1 Enzymatic Removal of Aromatic Amines and Phenols from Water by Horseradish Peroxidase (Nannipieri and Bollag, 1991)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Removal</th>
<th>$\text{H}_2\text{O}_2$ (mM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzidine</td>
<td>99.9</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>3,4'-Dimethoxybenzidine</td>
<td>99.9</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>3,3'-Diaminobenzidine</td>
<td>99.6</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>3,3'-Dimethylbenzidine</td>
<td>99.6</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>3,3'-Dichlorobenzidine</td>
<td>99.6</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>Phenyl-1-naphthol</td>
<td>99.7</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>1-Naphthylamine</td>
<td>98.3</td>
<td>5</td>
<td>5.5</td>
</tr>
<tr>
<td>2-Naphthylamine</td>
<td>98.3</td>
<td>5</td>
<td>5.5</td>
</tr>
<tr>
<td>5-Nitro-1-Naphthylamine</td>
<td>99.6</td>
<td>5</td>
<td>5.5</td>
</tr>
<tr>
<td>N,N-Dimethylaniline</td>
<td>99.2</td>
<td>3</td>
<td>5.5</td>
</tr>
<tr>
<td>Phenol</td>
<td>85.3</td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td>2-Methoxyphenol</td>
<td>98.0</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>3-Methoxyphenol</td>
<td>98.6</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>4-Methoxyphenol</td>
<td>89.1</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>2-Methylenol</td>
<td>86.2</td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td>3-Methylenol</td>
<td>85.3</td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td>4-Methylenol</td>
<td>85.0</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>99.8</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>66.9</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>98.7</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>2,3-Dimethylenol</td>
<td>99.7</td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td>2,6-Dimethylenol</td>
<td>82.3</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>Aniline</td>
<td>72.9</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>4-Chloroaniline</td>
<td>62.5</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>4-Bromoaniline</td>
<td>84.5</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>Phenylamine</td>
<td>86.7</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>1,3-Diaminophenol</td>
<td>98.6</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>Diphenylamine</td>
<td>80.5</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>99.6</td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td>2-Nitro-1-naphthol</td>
<td>98.9</td>
<td>1</td>
<td>4.0</td>
</tr>
</tbody>
</table>
A French group studied the effectiveness of this method for removal of phenolic compounds in drinking water at low concentrations (Maloney et al., 1984, 1986). They observed that the conversion efficiencies were still higher than 95 percent at initial concentrations below 0.01 mg/L of 2-chlorophenol. 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.

A phenomenon of coprecipitation was originally reported by Klibanov (1980) and later confirmed by independent research by Nicell (1991) and Wu Y. et al. (1993). It was observed that certain pollutants which were unreactive or less reactive toward peroxidase were enzymatically precipitated if the wastewater also contained other pollutants that were readily removed by the enzyme.

Horseradish peroxidase treatment proved effective when it was applied to real wastewaters from coal-conversion (Klibanov et al., 1983), petroleum and iron casting (Dantus, 1995). A typical coal-gasification wastewater had the following composition: 2,000 mg/L phenol, 5,000 mg/L ammonia, 19,000 mg/L chloride, 100 mg/L cyanide, 1,000 mg/L thiocyanate and several other constituents that adversely affect biological method 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 (Klibanov et al., 1983). 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 higher concentrations of phenol. The reason might be that phenol displaced the inhibitors from the complexes with the enzyme. An industrial wastewater, contaminated with 2,4-
dichlorophenol (up to 840 mg/L), was successfully treated by crude HRP with removal efficiency higher than 99% (Dec and Bollag, 1994).

Various dimers and trimers were determined or suggested as the intermediate products of the coupling of free radicals (Yu et al., 1994). These oligomers could again act as substrates of HRP. Yu suggested that the predominant polymerization bonds in the products might be the oxygen-para connection. Experiments at low concentration of 4-chlorophenol showed that by-products yielded two major fractions: one was a highly polar fraction and the other was a high molecular mass non-polar fraction (Maloney et al., 1984, 1986). The removal of halogenated phenols decreased with the increasing number of chlorines and the increasing molecular mass of the substituent (Bollag, 1990; Bollag et al., 1992). 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 contributed to the overall detoxification effect that resulted from enzymatic polymerization. The toxicity of the final product depended on the enzyme used and the pH (Aitken et al., 1994). The HRP could detoxify most of the phenols.

2.3 ADDITIVE

Additives considered in this study were some high molecular mass materials, such as polyethylene glycol and gelatin. Polyethylene glycol has been widely used to purify protein, modify or activate enzymes in biochemistry and biological research (Harris, 1992). It can interact with or affect the structure of proteins/enzymes, consequently affecting their function. Gelatin is also considered to be a high mass polymer, which is heterogeneous with respect to molecular mass and shape (Veis, 1964). It is unique among proteins owing to the absence of appreciable internal order and is used in the food industry and other scientific research. Polyelectrolytes are widely used as coagulants or coagulant
aides in water and wastewater treatment. They may possess a negative charge, positive charge or an overall neutral charge (Dentel, 1991).

Efforts have been made to reduce the inactivation during the enzymatic reaction. The most promising breakthrough was the discovery of using additives (Nakamoto and Machida, 1992). Experiments showed that apparent inactivation of peroxidase during the phenol polymerizing reaction was mainly caused by the end-product polymers, which adsorbed the enzyme molecules and hindered the access of substrate to the enzyme's active site. They argued that it would be impossible to reduce the amount of the enzyme if the enzyme was inactivated by phenoxy radicals. However, 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 solutions containing from 10,000 to 30,000 mg/L of phenol. The reduction in the amount of peroxidase required for phenol removal was up to 200 times. It was found that PEGs having average molecular mass less than 400 were ineffective in suppressing peroxidase activity loss.

Wu J. et al. (1993) studied the effects of PEG on the phenol polymerizing reaction at lower concentrations. The results showed that PEG still had a significant protective effect on the activity of HRP in the phenol concentrations of 1 to 10 mM (around 100 mg/L to 1000 mg/L). The amount of HRP required was reduced to 1/40 to 1/75, respectively, of that required without PEG. The effectiveness of PEG increased with an increase in phenol concentration. The minimum doses of HRP and PEG required for at least 95% removal were 0.05 U/mL and 0.03 g/L, respectively, for 1 mM phenol solution and 0.4 U/mL and 0.25 g/L, respectively, for 10 mM phenol solution. The addition of 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.

2.4 KINETICS OF REACTIONS INVOLVING HRP

During HRP catalyzed polymerization of aromatic compounds, both the concentration of aromatic compounds and the apparent activity of HRP decline with the time. Inactivation of HRP by hydrogen peroxide can happen either in the absence of or in the presence of aromatic compounds as discussed in Section 2.1.3.

2.4.1 Inactivation of HRP

It is reported in the literature that HRP can be inactivated by both hydrogen peroxide and products formed during the reaction.

2.4.1.1 Hydrogen Peroxide

In the absence of reducing substrates and with excess hydrogen peroxide, HRP showed a kinetic behavior of a suicide inactivation (Arnao et al., 1990a, 1990b; Baynton, 1992). It was assumed that HRP acted as a catalase while hydrogen peroxide served as an oxidant and as a reductant. A pathway of kinetic model was proposed in which the suicide inactivation was attributed to the production of Ei (P-670) from the complex of Compound I\( \cdot \)H\(_2\)O\(_2\). A first order inactivation constant, \( k_i \), of \( 3.92 \pm 0.06 \times 10^{-3} \text{s}^{-1} \) was obtained through a series of mathematical calculations based on a double-exponential assumption (Arnao et al., 1990a, 1990b). In contrast, the inactivation was also reported to appear to be second-order with respect to hydrogen peroxide and enzyme concentration (Baynton, 1992). The rate constant was estimated around 0.023 M\(^{-1}\)s\(^{-1}\) through a series of experiments in a wide range of hydrogen peroxide concentrations. The overall
inactivation was obviously related to the concentration of hydrogen peroxide, which made the second order assumption more amenable.

2.4.1.2 Products Formed During the Reaction

In the presence of reducing substrates, inactivation of HRP can happen either in the presence or in the absence of additives. Most of the early researchers attributed the inactivation to the attack of phenoxy radicals formed during the reaction to the active center of enzyme. It was applied to estimate the inactivation rate using a second order expression with respect to enzyme concentration and phenoxy radical concentration (Yu et al., 1994). However, the intermediate and final forms of enzyme involved in this particular inactivation mechanism were unknown. Following a series of experiments within a wide range of HRP and phenol concentrations, a second order rate constant with respect to the concentrations of enzyme and phenol was determined to be 0.0193 M⁻¹s⁻¹ (Baynton, 1992). Generally, the decay rate of enzymes showed a first order depletion model with respect to enzyme concentration (Cornish-Bowden and Wharton, 1979). This model was successfully employed to predict the enzyme inactivation (Siddique, 1992) together with one-substrate Michaelis-Menten equation. Nicell (1991) assumed that the inactivated enzyme was directly proportional to the aromatic compounds removed with the factor of 1/(Turnover Number, i.e., $k_{cat}$). Nevertheless, the turnover number was not a constant because of its being related to the concentrations of phenol and enzyme. Nakamoto and Machida (1992) argued that this inactivation was due to the adsorption of the enzyme onto the final products, but neither the inactivation kinetics nor the constants were available from their work.
2.4.2 Kinetics of Aromatic Compound Conversion

Dunford (1991) proposed a modified type of ping-pong kinetics that was based on the peroxidase cycle as described in Figure 2-1. The reaction rate, \( v \), if defined as \(-d[AH_2]/dt\), was derived from that scheme in the following form:

\[
\frac{2[HRP]_0}{v} = \frac{(k_2 + k_3)}{k_2k_3} \frac{1}{[AH_2]} + \frac{1}{k_1[H_2O_2]} \tag{2-1}
\]

This expression was applied in kinetic modeling of the reactions both in the presence (Yu et al., 1994) and in the absence (Nicell, 1992) of PEG together with different assumptions on enzyme inactivation. Nicell assumed that the effect of hydrogen peroxide on the inactivation was not significant; therefore, it was not incorporated into the model. In addition to that, his model included a Compound III that could be converted back to native form of HRP by hydrogen peroxide (Figure 2-1). As a result of these assumptions, the outputs from that model often overestimated active enzyme present in the reaction mixture, which consequently predicted a faster reaction rate than experimental data showed. The effect of hydrogen peroxide on the model output was especially unsatisfactory. Yu et al. (1994) also adopted the above expression as the reaction rate, but, he fixed the value for \([AH_2]/[H_2O_2]\) and thus eliminated the effect of hydrogen peroxide in his model. In that case, a one-substrate Michaelis-Menten equation could also model the reaction well (Siddique, 1992).

2.5 REACTOR DESIGN

Most of the research discussed earlier was carried out with batch reactors. It was found that enzyme turnover (Phenol removed/Enzyme consumed, mM/Unit) with respect to HRP could be increased through semibatch addition of HRP when additives were not used, whereas semibatch addition of hydrogen peroxide had little effect (Nicell, 1992). On the basis of these observations from the experiments, a CSTR was chosen and proved to
be better than a batch reactor because of lowered concentrations of the reactants and the enzyme. Furthermore, it was found that CSTRs in series performed better than a single CSTR with the same total detention time.

In contrast, enzyme turnover could only be improved through semibatch addition of hydrogen peroxide when additives were applied (Wu J. et al., 1994). Under that operation, the concentration ratio of maximum hydrogen peroxide to initial HRP was found to control the reaction rate. The optimum range was determined to be between 10 and 25 μmol/U. This effect was obvious only when hydrogen peroxide and phenol concentration were higher than 3 mM at 1:1 stoichiometry of hydrogen peroxide and phenol.

The differences in the behaviors of semi-batch addition of HRP and hydrogen peroxide between the presence and the absence of additive may not be due to the additive itself, but the absolute concentration of enzyme and the relative value of hydrogen peroxide to enzyme concentration. In both cases of semi-batch operation, the improvement in enzyme turnover was not as large as obtained by the addition of additives.
CHAPTER 3 MATERIALS AND METHODS

3.1 MATERIALS

Crystalline horseradish peroxidase (EC 1.11.1.7, type I, R.Z. = 1.0, 78 purpurogallin units/mg solid) was purchased from Sigma Chemical Co., St. Louis, MO. The nominal activity measured by Sigma Chemical Co. was 78 Units/mg dry solid where one unit forms 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20 °C. The specific activity of horseradish peroxidase was approximately 120 Units/mg dry solid using the assay 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 °C. The enzyme was stored as a dry powder in a freezer at -15 °C until required. An aqueous stock solution of peroxidase (1 mg/mL) was made by dissolving HRP into 100 mM phosphate buffer (pH = 7.4), which was stored at 4 °C. Its activity was measured at least once a month after its preparation to monitor the change in activity.

Catalase (EC 1.11.1.6; 12,400 units/mg protein), gelatin (Type B: from bovine skin, approximate 75 Bloom) and polyethylene glycol (average molecular mass = 300 and 3,350) were 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. One unit decomposes 1.0 micromole of hydrogen peroxide per minute at pH 7.0 and 25 °C, while the hydrogen peroxide concentration falls from 10.3 to 9.2 micromoles/millilitre of reaction mix. Aqueous stock solution of catalase (0.5 mg/mL) was stored at 4 °C.

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

Phenolic compounds were obtained from Aldrich Chemical Co., Milwaukee, WI., and had a purity of 99% or greater. Stock aromatic aqueous solutions were prepared with buffer solution (pH = 7.4) and stored at 4 °C.
Three polyelectrolytes, PERCOL LT24 (cationic as PE₁), PERCOL LT20 (non-ionic as PE₂), Aquafloc 6465 (anionic as PE₃), were acquired from Windsor Water Treatment Plant, Windsor, Ontario, and another two (cationic as PE₄, anionic as PE₅) from West Windsor Pollution Control Plant, Windsor, Ontario. All of them were being used in the actual operation of the plant.

All other chemicals used in this study 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., Milwaukee, WI.

3.2 ANALYTICAL AND EXPERIMENTAL EQUIPMENT

Color absorbance was measured with a Hewlett Packard Diode Array Spectrophotometer, Model 8452A (wavelength range 190 to 820 nm with 2 nm resolution), operated through 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, DE, was used to centrifuge samples at 3000 rpm (gravity = 2200) for 30 minutes. The temperature in the centrifuge was kept around 20 °C by the refrigerator installed inside.

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

A DC-180 automated TOC Analyzer, purchased from Dohrmann Division, Rosemount Analytical, California, was used to measure the TOC content in reaction mixtures. The method used in the machine was ultraviolet light promoted potassium
persulfate oxidation. The CO₂ detector was a linearized non-dispersive infrared detector (NDIR). Syringe mode was used in all tests. The machine had a precision of ± 2% measured value or ± 5 μg/L, whichever was greater.

A peristaltic pump (Model 89052B), purchased from Hewlett Packard, was used to deliver solutions into the system of continuous flow reactors. A Harvard Syringe Pump 22 (Model 2400 multi), purchased from Harvard Apparatus, South Natick, MA., was utilized to convey reaction solutions to the reactors. Its accuracy was within ± 1 %, and its reproducibility was within ± 0.1 %. The Becton Dickinson plastic syringes, supplied by CanLab Scientific Ltd., Ontario, were applied together with the syringe pump. Food grade silicon and Tygon tubing were used for flexible joints and peristaltic pump tubing.

Temperatures in water bath were maintained using a Haake D1 Temperature Controller, produced by Haake of Germany.

3.3 EXPERIMENTAL PROTOCOL

The experiments in this study were conducted at room temperature (around 20 °C), because there was no significant loss in the HRP activity on incubation in the temperature range of 5 °C to 35 °C (Nicell, 1991). All experiments were carried out at pH controlled with buffer solutions.

3.3.1 Buffer Preparation

The buffers used in this experiment were prepared according to Methods in Enzymology (Gomori, 1955). The reagents used and the corresponding pH ranges obtained are listed in Table 3-1. The buffer concentration prepared with this method was 0.1 M. The pH value of the reaction mixture was determined with pH meter and was used in plotting experimental results.
Table 3-1 Buffer Components Used in the Experiments

<table>
<thead>
<tr>
<th>Buffering Reagents</th>
<th>pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid-sodium acetate</td>
<td>4 - 5</td>
</tr>
<tr>
<td>Monobasic-dibasic sodium phosphate</td>
<td>6 - 9</td>
</tr>
<tr>
<td>Boric acid-acid-borax acid</td>
<td>9</td>
</tr>
<tr>
<td>Sodium carbonate-sodium bicarbonate</td>
<td>10</td>
</tr>
</tbody>
</table>

3.3.2 Batch and Semi-Batch Operation

Batch reactors consisted of vials containing 30 milliliters buffered mixture of aromatic substrate, hydrogen peroxide, additive and HRP (Figure 3-1). The components were added in the following order: buffer, additive, HRP enzyme and aromatic substrate. Aliquots were taken to measure the absorbances of the mixture before the reaction. Then, hydrogen peroxide was added to initiate the reaction. The reactors were stirred vigorously during the reaction using magnetic stirrers and teflon coated stir bars. Stock catalase solution (0.5 mg/mL) was pipetted into centrifuge tubes for the purpose of stopping the reaction when required. It was enough for 100 µL catalase to consume 1 mM hydrogen peroxide in 1 mL in a short time. Aliquots were taken using Eppendorf pipettes into centrifuge tubes at different time intervals and were shaken for a while so that samples could be mixed well with catalase. Immediately after all specimens were collected, the sample mixtures were centrifuged simultaneously for 30 minutes at 3000 rpm. The supernatant was analyzed for its absorbance to determine the residual of phenol derivatives by direct determination of UV absorbance or by one of the colorimetric methods described in Section 3.4.2.

In the experiments concerning the effect of PEG on the reaction parameters in Chapter 4, solid alum was added into the reactors to help settling the polymers. Reactors were stirred vigorously for several minutes to dissolve the alum. After that, HCl or NaOH
was added to adjust pH within 6 to 7, and the reaction contents were gently stirred for 20 minutes for formation of flocs. Nevertheless, experimental results showed that adding alum and adjusting pH were not essential to the separation of precipitate from solution because of the high speed used in the centrifugation. So, this step was omitted in the subsequent experiments.

![Diagram](image)

**Figure 3-1 Batch Reactor System**

Semi-batch operation was basically the same as that for the batch reactor except that hydrogen peroxide or HRP was added in more than one spike. Hydrogen peroxide or HRP was added discontinuously in two or three spikes at different time intervals. The total amount of hydrogen peroxide or HRP was controlled to be the same as that used in batch reactors when the purpose of the experiments was to compare the semi-batch addition to the batch operation.
3.3.3 Plug Flow Operation and Semi-Batch Operation of Plug Flow System

The plug flow reactor system used in this study is shown in Figure 3-2. The system primarily consisted of three glass reaction tubes. The details of the plug flow reactor are illustrated in Figure 3-3. Each tube had a volume of 70 mL and served as a reactor. A storage tank and a syringe tube were connected to the first reactor through a peristaltic pump and a syringe pump. The semi-batch operation for the plug flow system was established, when one or two more syringe tubes were connected to the subsequent reactors by the same pump.

Figure 3-2 Plug Flow Reactors System
The buffered reaction mixture of HRP, PEG and phenol in the storage tank was pumped into the system using the peristaltic pump. Hydrogen peroxide, buffered at the same pH value, was introduced into the system using the syringe pump. The two flow streams were connected with and mixed in a Y-Shaped tubing connector (purchased from Canadawide Scientific) just before their entering a reactor. Upflow systems were used in almost all of the experiments.

The experiments were carried out in the following steps:

- Prepare stock solution in the storage tank according to the experimental design.
- Measure the actual concentration of phenol and activity of HRP.
- Set the flowrate for peristaltic pump according to its calibration curve. Measure the actual flowrate at the effluent end and adjust it as close as possible to the design value.
• Set the flowrate of the syringe pump according to the designed flowrate ratio value (Flowrate of syringe pump over flowrate of peristaltic pump).

• Let the system run for one quarter or one third of a reactor detention time. Check the total flowrate at the same time.

• Add catalase into centrifuge tubes. The catalase dose must be large enough to halt the reaction in a very short time. This dose must be determined through experiments.

• Finally, start the timer, take aliquots of samples from different sampling points at the desired time and measure activities at the same time. The precipitates accumulated in the reactors can be controlled within the sludge zone by removing the precipitates, if any, with a small syringe.

Semi-batch addition of hydrogen peroxide was also studied using the set-up in Figure 3-2. The whole procedure was identical to the plug flow operation except that one or two more syringes were installed on the same syringe pump. The hydrogen peroxide streams were introduced into the second and the third reactor, respectively. The flowrates from different syringes were identical because the same size syringes were used in all the experiments. The concentration of hydrogen peroxide in each syringe was determined through calculation according to the experiment design.

3.3.4 Continuous Stirred Tank Reactor Operation

The continuous Stirred Tank Reactor (CSTR) system used in this study is demonstrated in Figure 3-4. Two syringe tubes of the same size were installed on the syringe pump. One of them was filled with the solution of hydrogen peroxide buffered at a certain pH value. The other one was filled with the mixture of HRP, PEG and phenol, buffered at the same pH. The reaction solutions were introduced into a reaction container by the syringe pump, through a Y-shaped connector, at exactly the same flowrate. The concentrations of the reaction components were predetermined according to the design.
The reaction container was a sealed glass or plastic bottle with an inlet at the bottom and an outlet at the top. The reactor was stirred vigorously using a magnetic stirrer and teflon coated stir bar during the entire reaction. The reactor volume varied from 20 mL to 230 mL depending on the desired detention time. Aliquots were taken at different time intervals as desired. All other steps were similar to those for the plug flow reactor operation.

![Diagram of Continuous Stirred Tank Reactor System](image)

Figure 3-4 Continuous Stirred Tank Reactor System

### 3.4 ANALYTICAL METHODS

#### 3.4.1 HRP Activity

The enzyme structures may be disrupted by pH or temperature changes and also can be made inactive by inhibitors of various types (Palmer, 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.

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
was to provide all components except enzyme near saturation concentration so that the initial rate of reaction became 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, 450 to 500 µL of 100 mM phosphate buffer (pH 7.4) and 50 to 100 µL of enzyme solution. The rate of reaction was measured by monitoring the rate of formation of the 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 hydrogen peroxide converted per minute at pH 7.4 and 25°C. A detailed explanation of this assay is given in Appendix A-1.

3.4.2 Aromatic Compounds

Because it takes long time (more than 15 minutes) for chromatographic methods to detect the phenol concentration in a solution, the concentrations of aromatic compounds in aqueous solution were 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 94.1 mg/L for phenol, 128.6 mg/L for chlorinated phenol, 108.2 mg/L for methyl phenol (cresol) and 163 mg/L for 2,4-dichlorophenol, respectively.

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 et al., 1980). However, it was observed that the reaction products, which probably were different after the addition of additives, interfered with the accuracy of this method. This phenomenon was clearly demonstrated in the experiment on PEG effect on HRP dose for p-cresol. The reason for this might be at least partially due to the change in the maximum
wavelength and extinction coefficient after the enzymatic reaction. A summary of analytical methods used for different aromatic compounds is presented in Table 3-2.

<table>
<thead>
<tr>
<th>Aromatic Compounds</th>
<th>Colorimetric Method Applicable or not</th>
<th>UV Absorbance Peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>yes</td>
<td>272</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>yes</td>
<td>274</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>no</td>
<td>274</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>yes</td>
<td>280</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>yes</td>
<td>270</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>yes</td>
<td>272</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>no</td>
<td>278</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>yes</td>
<td>284</td>
</tr>
</tbody>
</table>

Two colorimetric methods were used in this study. The first method used HRP as a catalyst and 4-aminoantipyrine and hydrogen peroxide as color generating substrates. The color generated at 510 nm was directly proportional to the concentration of aromatic substrates. The details about this method are described in Appendix A-2. Calibration curves were developed for all phenol derivatives investigated in this study (see Appendix B-1). The results showed no 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.

Because the accuracy of this method might be affected by the components of the reaction, another method was developed, in which no enzyme reaction component was involved. The assay contained two reagents: one was Ferricyanide reagent (83.4 mM of \( \text{K}_3\text{Fe(CN)}_6 \) in 0.25 M \( \text{NaHCO}_3 \)), and the other was AAP reagent (20.8 mM of AAP in 0.25 M \( \text{NaHCO}_3 \)). The absorbance was measured at 510 nm versus reagent blank. Further details are provided in Appendix A-3. Standard curves for the phenolic compounds studied were established as shown in Appendix B-1. The results showed that there was a
linear relationship between the absorbance and the concentration for all phenolic compounds studied except p-cresol. Again, this method was not useful for p-cresol, therefore, results with regard to it were obtained from the direct UV method.

3.4.3 Hydrogen Peroxide

A modified assay was employed to measure the hydrogen peroxide using phenol and 4-aminoantipyrine (AAP) as the substrates of HRP. The approach in this assay was to provide all components except hydrogen peroxide near saturation concentration. The color generated at 510 nm was directly proportional to the amount of hydrogen peroxide present. The assay mixture contained 210 μL of 9.6 mM 4-AAP, 100 μL of 100 mM phenol, 460 to 510 μL of 100 mM phosphate buffer (pH 7.4), 50 to 100 μL HRP solution (1 mg/mL) and 50 μL of hydrogen peroxide sample. A detailed explanation on the preparation of the assay reagents can be found in Appendix A-4.

3.4.4 Total Organic Carbon

The remaining amount of additive in the solution was determined by TOC test. A Dohrmann DC-180 Total Carbon Analyzer was used for this test. Potassium persulfate reagent (20 grams of reagent grade K₂S₂O₈ dissolved in 1 liter of reagent water with 2 mL of concentrated phosphoric acid) was used as the oxidizing agent while ultraviolet light acted as the catalyst. The carbon dioxide generated was detected by a linearized non-dispersive infrared detector (NDIR). Each day before the measurement, the instrument was calibrated according to the procedures given in the Operation Manual published by the company. Potassium hydrogen phthalate (2000 mg/L) was used as the standard solution (425 micrograms of reagent grade C₈H₅O₄K, dried to a constant mass, was dissolved in 100 mL deionized water and 0.1 mL reagent grade concentrated phosphoric acid was added before filling it to the mark of 100 mL). The organic carbon
determinations were made by injection mode with 250 μL injection volume each. Buffer solution was used as the blank.

Separated standard curves were developed for each additive and the phenolic compound involved in the reaction. The TOC contributed by phenolic compound was calculated using the standard curve. The difference between the TOC amount measured and the TOC caused by phenolic compound was used to estimate the TOC caused by additive remaining in the samples.

3.5 SOURCE OF ERRORS

Two kinds of errors can occur in any experiment: systematical errors and random errors. The former are due to the measuring or analytical techniques and the instruments. The latter are due to the personal factors, e.g. carelessness. For instance, systematical errors can occur in both direct spectrophotometric method and colorimetric method when very low concentrations are present after the reaction. The first colorimetric method took 35 to 45 minutes to reach the maximum absorbance while preparing the standard curve using standard solutions. However, after the completion of reaction, it took less than 5 minutes to reach the maximum. Maximum readings were supposed to be reported and these depended on the individuals. For the direct UV method, the spectrum of the solution always changed after the completion of the reaction. Sometimes new peaks had been formed which, at least partially contributed to the errors. Relatively speaking, the errors from the colorimetric method are more predictable.

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

- Experiments were designed and run in such a way that everything was exactly the same except the parameter being investigated. For example, the same volume of PEG solution with different concentrations was added to the same volume of reaction
mixture, instead of adding different volumes of PEG with the same concentration to different volumes of reaction mixture.

- The protocol of the experimental design and analysis procedures was followed strictly. The order of the steps was not changed. No step was omitted without any reason.
- Unnecessary treatments on the samples were minimized.
- Those assays that did not contain any reaction component were selected if possible.
- Same volume of sample in assay was used when relative values were desired.
CHAPTER 4 RESULTS AND DISCUSSION

4.1 SELECTION OF EFFECTIVE ADDITIVES

A series of experiments was conducted to test the effectiveness of a variety of compounds as additive in the HRP catalyzed polymerization. These compounds included polyethylene glycol with average molecular mass 3550 (PEG3550) and 300 (PEG300), gelatin from bovine skin, five polyelectrolytes as described in Section 3.1 and polyacrylamide (PAA). The experimental results, plotted in Figure 4-1, confirmed that PEG3550 and gelatin were effective in protecting HRP, while PEG300 was not effective (Nakamoto and Machida, 1992). Two kinds of cationic polymers (PE1 and PE4) and one non-ionic polymer (PE2) were also effective while two kinds of anionic polymers (PE3, PE5) could not function as effective additives. It seemed that electrical charges of the polyelectrolytes influenced their effectiveness as additives. However, polyacrylamide, which belonged to nonionic polyelectrolytes, also proved ineffective. Thus, the structure of polyelectrolytes, as well as their electrical charges, may influence their function as additives.

![Graph showing fraction remaining for different additives](image_url)

**Figure 4-1**: 4-Chlorophenol Clearance as Function of Additive

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4.2 EFFECT OF ADDITIVES ON THE REACTION PARAMETERS

4.2.1 pH

Enzymes are proteins made up of poly-aminoacids. The value of pH can influence their structure and consequently influence their activity. Many enzymes have highest activity at neutral pH and usually lose their activity at extreme pH values. Since the activity of HRP at pH below 4 and above 11 is very low, a pH range of 4 to 10 was chosen in this study. PEG\textsubscript{3550}, gelatin, PE\textsubscript{1} and PE\textsubscript{2} were selected to study their effect on the optimum pH range for the HRP catalyzed polymerization of different phenolic compounds.

These experiments were designed so that only the HRP concentration was limiting. The concentration of additive was kept at 5 g/L for PEG and 4 g/L for gelatin based on experiments conducted at a much higher concentration of phenol by Nakamoto and Machida (1992). Hydrogen peroxide was overdosed at 2 mM, which was twice the optimum stoichiometric dose. The reaction was stopped after 16 hours, which was considered to be sufficient time to complete the reaction. Borax buffer may have had some influence as an additive (Nicell, 1991), but this was masked by overdose of PEG or gelatin. The initial substrate concentration was 1 mM in all cases.

The results for 4-chlorophenol and m-cresol are shown in Figures 4-2 and 4-3. The optimum pH values for all compounds studied are listed in Table 4-1. The figures and the table show clearly that there was a wide range of optimum pH for all of these phenol derivatives. Generally, the greater the HRP dose, the wider the optimum pH range. The optimum pH occurred near neutral condition except for 2-chlorophenol which had an optimum pH of about 5.
Figure 4-2: 4-Chlorophenol Clearance as Function of pH

Figure 4-3: m-Cresol Clearance as Function of pH
<table>
<thead>
<tr>
<th>Aromatic Compound</th>
<th>Optimal pH Range</th>
<th>Optimum pH</th>
<th>HRP Range (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Chlorophenol</td>
<td>4.0 - 7.0</td>
<td>5</td>
<td>0.05 - 0.05</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>5.5 - 8.0</td>
<td>7</td>
<td>0.12 - 0.25</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>5.0 - 8.0</td>
<td>7</td>
<td>0.015 - 0.03</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>4.5 - 7.5</td>
<td>6</td>
<td>0.015 - 0.03</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>6.0 - 8.5</td>
<td>8</td>
<td>0.06 - 0.25</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>5.5 - 8.5</td>
<td>7</td>
<td>0.03 - 0.06</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>5.0 - 8.0</td>
<td>7</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Note: PEG dose = 5.0 g/L; Gelatin dose = 4.0 g/L; [H₂O₂]/[AH₂] = 2 : 1

It is also seen from Figures 4.2 and 4.3 and Table 4-1 that the optimum pH was 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 were consistent with those reported by Nicell (1991) on HRP activity as a function of pH without additive.

When the results with additives in this study were compared to the results from the experiments without additives (Nicell, 1991), it was noticed that optimum pH ranges became wider, and closer to neutral in the presence of the additive. Optimum pH shifted towards neutrality in all cases. For example, the optimum pH range for 3-chlorophenol changed from 8 - 9 without additive to 5.5 - 8 with additive and the optimum pH from 8.5 to 7. The substrate concentrations remaining changed more smoothly with pH. This influence of additive in widening the pH range is beneficial for field applications. The new optimum pH is also favorable with regard to using alum to settle the polymers formed during the reaction, because the optimum pH for it is around 6.3 which is coincident with that for HRP activity. It can be concluded from these experiments that additive had improved 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 behavior of the final product. Gelatin usually
helped to form soluble polymer with a dark color remaining in the final supernatant. This color could not be removed either by adding alum or by centrifugation. For example, the supernatant in the reactor of 3-chlorophenol and m-cresol with gelatin had a yellow color, and no sediment could be separated after adding alum and adjusting pH. However, when PEG was used, white sediments with no color in the supernatant were observed, which could be separated by gravity. Later, this phenomenon proved to be the effect of an overdose of gelatin (Section 4-3).

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 the final products. In the spectrum, a new absorbance peak was observed at 290 nm instead of 278 nm at higher pH (8 - 10). A similar observation was also made when comparing the UV results with the colorimetric results for 2,4-dichlorophenol, where the peak switched from 286 to 306 nm. This might contribute to the higher readings in the residual absorbances.

Experiments with the polyelectrolytes demonstrated a similar result to that from PEG and gelatin as shown in Figure 4-4. The effect of PE on the formation of color and particles was similar to that of gelatin. PE₁ and PE₂ had similar behaviors.

4.2.2 HRP Dose

4.2.2.1 PEG

PEG₃₅₅₀ was first evaluated for its effect as an additive on the minimum HRP dose required to achieve more than 95 percent substrate removal. The concentrations of all aromatic compounds were kept at 1 mM, and hydrogen peroxide concentration was set at 2 mM. The duration of the reaction was 6 hours. PEG concentration was overdosed at 5 g/L. All experiments were conducted at the optimum pH values, i.e. pH = 7, for all aromatic compounds except for 2-chlorophenol where pH of 5 was used.
Figure 4-4 PE effect on Clearance as Function of pH
Note: Phenol or 4-cp = 1 mM, HRP= 0.03 U/mL, PE = 10 mg/L.

The results for 4-chlorophenol are shown in Figure 4-5. The figure demonstrates that the addition of PEG_{3550} can greatly reduce the minimum amount of HRP needed for completing the polymerization. It needed only 0.015 U/mL of enzyme to complete the reaction in the presence of PEG_{3550} as compared to 2 U/mL required without it. This represents a 132-fold improvement in peroxidase utilization. The minimum HRP doses required for different organic compounds are tabulated in Table 4-2.

<table>
<thead>
<tr>
<th>Aromatic Compound</th>
<th>HRP Dose (U/mL)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without PEG *</td>
<td>With PEG *</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>5.0</td>
<td>0.50</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>2.0</td>
<td>0.015</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>3.0</td>
<td>0.10</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>3.8</td>
<td>0.03</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>1.0</td>
<td>0.03</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>0.5</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* These values were obtained with 2 mM hydrogen peroxide and 1 mM of aromatic compounds.
Figure 4-5 PEG Effect on HRP Dose for 4-Chlorophenol

Table 4-2 indicates that the minimum HRP doses required to complete the polymerization in the presence of PEG were reduced to 1/10 to 1/132 of those required without PEG. Therefore, the enzyme turnovers (moles of substrate removed/mole of HRP consumed) had increased by 10 to 132 times. These observations confirmed that PEG could protect HRP activity at low concentrations for a variety of phenolic compounds. Without additives, HRP activity was lost during the reaction. Excess HRP beyond the minimum value did not help in achieving additional removal. The removal of substrates was almost directly proportional to the amount of HRP added when it was less than the minimum dose.

It was mentioned in the previous section that the final products after adding additives might be different from those obtained without them. It was observed that the particles formed in the reactors with PEG were finer than those in the reactors without PEG. For example, after adding PEG, the final precipitates in the reactors of 2-chlorophenol took more time to settle than those in the reactors without additive.
addition, the color of the particles was different. The former had a light brown color, while the latter had a very dark brown color.

4.2.2.2 Gelatin

Further experiments were conducted to examine the gelatin effect. However, instead of overdosing gelatin and hydrogen peroxide this time, the minimum gelatin dose (from Section 4.2.3.2) and the optimum hydrogen peroxide dose (Section 4.2.4.2) were applied. The results for 2,4-dichlorophenol and m-cresol are plotted in Figure 4-6, which shows that these substrates needed less HRP in the presence of gelatin than in the presence of PEG. The reason is that the optimized hydrogen peroxide dose of 1:1 was used here. The optimized dose produced a small reduction in the minimum HRP dose and consequently resulted in more HRP savings in Table 4-3 than in Table 4-2, because the minimum HRP doses in Table 4-3 were still obtained with a 2:1 ratio of hydrogen peroxide to phenols. If 1:1 ratio was used, they would be smaller correspondingly. So, the actual saving achieved with gelatin would not necessarily be better than that achieved with PEG. Actually, the comparison between PEG, gelatin and the PEs under optimum reaction conditions showed that PEG functioned a little better than gelatin in terms of removal efficiency. Because the ferricyanide method used in the test did not work for p-cresol, experimental data of p-cresol are not available.

<table>
<thead>
<tr>
<th>Aromatic Compound</th>
<th>HRP Dose (U/mL)</th>
<th>HRP Dose Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Gelatin *</td>
<td>With Gelatin **</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>5.0</td>
<td>0.03</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>5.0</td>
<td>1.50</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>2.0</td>
<td>0.01</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>3.0</td>
<td>0.06</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>2.8</td>
<td>0.02</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>0.5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* These values were obtained with 2 mM H₂O₂, ** obtained with 1 mM H₂O₂
Figure 4-6 Gelatin Effect on HRP Dose for m-Cresol and 2,4-Dichlorophenol
Note: Minimum HRP doses without gelatin are listed in Table 4-3

4.2.2.3 PEs

Experiments were conducted with polyelectrolytes as additives for 4-chlorophenol removal with a 1:1 reaction stoichiometry. The results, illustrated in Figure 4-7, reveal that all three polyelectrolytes with different electrical charges had a beneficial effect to a certain extent. The anionic polyelectrolyte (PE₃) improved the removal efficiency at low HRP activities but the effect was very limited. The nonionic polyelectrolyte (PE₂) also reduced the minimum HRP dose, but the improvement was only 20-fold. The cationic polyelectrolyte (PE₁) produced the highest HRP saving (65-fold improvement) among the three polyelectrolytes. When PE₃ was combined with PE₁ or PE₂, it had no influence on the function of PE₁ or PE₂. This again indicated that the electrical charge was not important for the function of polyelectrolytes as additive as PAA had showed.
4.2.3 Minimum Additive Dose

4.2.3.1 PEG

Experiments were conducted to determine the minimum PEG dose required for its complete protective effect on the activity of HRP, while using the minimum HRP doses (Table 4-2) for different phenol derivatives at their optimum pH values. The concentrations of phenolic compounds were kept at 1 mM, and hydrogen peroxide concentration was 2 mM. The maximum PEG dose was 5 g/L, which was used in all of the pH and HRP dose experiments. The results for some of the compounds are illustrated in Figure 4-8. Since the phenol concentration remaining in the 5 g/L PEG reactors was the same as that in the 0.3 g/L PEG reactors, the PEG dose was plotted up to 0.3 g/L. The figure demonstrates that minimum PEG dose varied from 10 to 100 mg/L, depending on
the phenolic compounds. For example, 3-chlorophenol needed 100 mg/L PEG while o-cresol needed only 10 mg/L. Any extra PEG (up to 5 g/L in this experiment) added to the reactors neither improved its effect on protecting enzymes, nor worsened its effect. Different compounds had different minimum PEG doses; therefore, the minimum PEG needed for protection of enzyme activity was related to the structure of a specific phenolic compound, just as had been reported for the minimum HRP dose. The minimum PEG doses required for different compounds are listed in Table 4-4. No relationship existed between the minimum PEG doses and the minimum HRP doses.

While using the UV method to examine the spectrum of the supernatant after centrifugation, it was found that the absorbance peak of the final product did not change with the change in PEG dose. The spectrum for different PEG doses showed the same
pattern. An overdose of PEG had no overdose influence on its effectiveness. A PEG dose of 5 g/L had the same effect on removal efficiency and the final spectrum as the minimum PEG dose of less than 100 mg/L. This observation confirmed the results obtained previously during pH and HRP dose experiments.

<table>
<thead>
<tr>
<th>Table 4-4 Minimum Additive Doses for Phenol Derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic Compounds</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Phenol</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
</tr>
<tr>
<td>α-Cresol</td>
</tr>
<tr>
<td>m-Cresol</td>
</tr>
<tr>
<td>p-Cresol</td>
</tr>
</tbody>
</table>

* Phenol concentration = 3 mM, ** Phenol concentration = 5 mM

4.2.3.2 Gelatin

Minimum gelatin dose was investigated under the minimum HRP dose for each phenolic compound (Table 4-3) at its optimum pH value. The concentrations of the compounds were 1 mM, and a 1:1 ratio was used for [H2O2]/[Phenol] in all these tests. Unlike PEG, gelatin had a minimum dose requirement of 100 mg/L for most of the compounds as presented in Figure 4-9 and Table 4-4. The exception was α-cresol which required a minimum gelatin dose of only 30 mg/L.

In contrast to PEG, an excess amount of gelatin in the reaction solution had a negative impact on the removal of phenolic compounds in terms of both removal efficiency and final product. With excess gelatin, removal efficiency was usually lower. More significantly, excess gelatin produced a dark color in the final reaction mixture but
no particles were formed. Different phenolic compounds produced different colors such as brown, purple or white. This meant that the phenolic compounds were actually converted into other soluble compounds, possibly higher oligomers of phenol, which could not be removed from the solution. The UV spectrum showed new absorbance peaks for these compounds after reaction. The behavior of excess gelatin in the reaction suggested clearly that the addition of gelatin affected the final product. The new product of polymerization did not inactivate or mildly inactivated HRP. In the case of PEG, the polymerization continued to form particles under any PEG doses. However, the addition of excess gelatin might stop the polymerization at an early stage and result in no particle formation. It is concluded that the removal efficiency for gelatin in the optimum pH study (Figures 4-2 and 4-3) should have a better percentage value similar to that for PEG if optimum gelatin doses were used.
4.2.3.3 PEs

The minimum doses for polyelectrolytes were investigated under minimum HRP dose and optimum reaction stoichiometry. The experimental results for phenol are plotted in Figure 4-10, which shows a minimum PE dose requirement of 10 mg/L for both PE₁ and PE₂. Higher phenol concentrations needed higher PE doses. Different phenolic compounds needed different PE doses, as shown in Table 4-4.

![Graph showing PE Dose Effect on Phenol Removal]

Figure 4-10 PE Dose Effect on Phenol Removal

Similarly to gelatin, excess PE prevented the formation of insoluble product of the polymerization. No particles were formed and only color was produced. Removal efficiency was also decreased. Both gelatin and PE have electrical charges, and this can be considered as the reason why the additive effect deteriorated with excess amount. If the additive dose, at which particles are formed and can be separated from solution, is defined
as the practical additive dose range (PADR), then it was 10 to 20 mg/L for PE₁, and 5 to 20 mg/L for PE₂. When PE dose was less than the minimum, no particles were formed. This suggests that either the extent of polymerization had decreased, or the polymerization chain was shorter. This again implies that the final product was different after adding additive.

4.2.4 Hydrogen Peroxide To Substrate Ratio

Experimental results in Figure 4-11 show that hydrogen peroxide consumption increased as its dose was increased. However, excess hydrogen peroxide was not necessarily used in the conversion of phenol. Hydrogen peroxide itself was not stable and it could have decomposed over time (Figure 4-12). Somehow, this depletion was protected in the presence of phenol even when phenol was not removed. Hydrogen peroxide was also consumed while it inactivated HRP.

![Graph showing the fate of H₂O₂ after completion of reaction](image)

Figure 4-11 H₂O₂ Fate after Completion of Reaction
Reaction Condition: HRP = 1.5 U/mL, 4-cp = 1 mM, pH = 7, Td = 6 hr., no additive
4.2.4.1 PEG

The effect of PEG on the hydrogen peroxide to substrate ratio for different phenolic compounds was studied by varying hydrogen peroxide concentration. Minimum HRP and PEG doses were used in these experiments. Phenolic compound concentrations were kept at 1 mM while hydrogen peroxide concentrations varied from 0.45 to 2 mM. Phenol derivatives studied were 2-, 4-chlorophenol, o- and m-cresol. The results for 2-cp and o-mp are plotted in Figure 4-13. The optimum range and the recommended optimal value of hydrogen peroxide concentration are tabulated in Table 4-5.

It can be seen from Figure 4-13 that the concentration remaining decreased sharply with an increase in [H$_2$O$_2$]/[Substrate] before reaching an optimal point (the one with the lowest concentration remaining). This indicates that hydrogen peroxide was the limiting component in this range. Beyond the optimum point, the remaining concentration
increased with an increase in \([\text{H}_2\text{O}_2]/[\text{Substrate}]\). In this case, an excessive amount of hydrogen peroxide was not effective in phenol conversion, but mainly intensified the inactivation of HRP by hydrogen peroxide.

Figure 4-13 and Table 4-5 show that these compounds had a wide optimum range of hydrogen peroxide to substrate ratio. This wide range, mostly from 0.85 to 1.2, is beneficial to accommodate the fluctuating phenol concentrations in field application on actual wastewaters. Nicell (1991) had reported this value to be 1.0 without additives. The optimal point shifted a little to the lower value of about 0.9. It had even a much smaller optimal value of 0.75 for 4-chlorophenol. This suggests that the final oligomers had a shorter chain, or the extent of the polymerization was lower. Thus, the final product might be different after adding PEG.
Table 4-5 Optimum Stoichiometry for Phenols with Additives

<table>
<thead>
<tr>
<th>Aromatic Compound</th>
<th>[H₂O₂]/[Substrate] Optimum Range</th>
<th>[H₂O₂]/[Substrate] Optimal Point Recommended</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG</td>
<td>Gelatin</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>0.80 - 1.2</td>
<td>0.9 - 1.4</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>0.60 - 1.1</td>
<td>0.7 - 1.2</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>0.85 - 1.2</td>
<td>0.9 - 1.4</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>0.85 - 1.2</td>
<td>0.9 - 1.4</td>
</tr>
</tbody>
</table>

4.2.4.2 Gelatin

Reaction stoichiometry in the presence of gelatin was investigated. Minimum HRP and gelatin doses for 1 mM phenol were used in these experiments. The results for 4-cp and m-mp are plotted in Figure 4-14 and are summarized in Table 4-5.

Gelatin differed from PEG₃₅₅₀ in two aspects. First, more hydrogen peroxide was needed to complete the polymerization. The optimum point remained at 1.0 (except for 4-cp) compared to 0.9 for PEG. The optimum range also shifted to a higher domain in the range of 0.9 to 1.4 (0.7 to 1.2 for 4-cp). Second, excess hydrogen peroxide led to color formation, instead of particles, as the final product. This was similar to what happened in gelatin dose study in the previous section; however, this time it was caused by an excess amount of hydrogen peroxide and not gelatin. It is concluded that, in the presence of gelatin, the final product constitution was affected not only by gelatin but also by hydrogen peroxide.

4.2.4.3 PE

Tests were conducted using PE₁ with 4-cp and m-mp under the optimized reaction conditions. Again, 4-Chlorophenol needed less hydrogen peroxide than m-mp (Figure 4-15). In the presence of PE₁, the optimal points were the same as those with PEG (Table 4-5). Excess PE₁ resulted in no particle formation. Instead, color was formed, which was similar to the color produced in the presence of gelatin.
Figure 4-14 Effect of Peroxide on the Clearance of Phenols with Gelatin

Figure 4-15 Effect of Peroxide on the Clearance of Phenols with PE1
4.2.5 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 concentration in the presence of 2 mM of hydrogen peroxide. Samples were taken from the reactors at predetermined time intervals up to 24 hours. After 7 hours, the residual substrate concentration did not change significantly. Therefore, only the results of the first seven hours are plotted in Figure 4-16. Listed in Table 4-6 are the reaction times required for 95% polymerization of different compounds. Figure 4-16 and Table 4-6 show that the minimum time needed varied from 1 to 3 hours with the minimum HRP and PEG doses. About 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 was due to the low concentration of phenolic compound remaining (less than 10 percent), and the corresponding low concentrations of horseradish peroxidase.

Figure 4-16 Effect of PEG on the Reaction Time with PEG
The time required for the enzymatic reaction is related to the amount of enzyme used, with and without additive. The higher the initial enzyme activity, the lower would be the time required for completion of polymerization (Nicoll, 1991; Wu J. et al., 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 time listed in Table 4-6 was the maximum time required to complete the polymerization. These reaction times can be shortened by adding extra enzyme. Since adding PEG greatly reduced the amount of HRP required for the reaction, the time needed for the reaction had increased (Wu J. et al., 1993). Thus, the addition of PEG will have some negative influence on the practical application in terms of the increased reactor volume. A complete kinetic behavior in the presence of PEG is presented later in Chapter 5: Kinetic Modeling.

<table>
<thead>
<tr>
<th>Table 4-6 Reaction Time Needed in the Presence of PEG</th>
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<tbody>
<tr>
<td>Aromatic Compound</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
</tr>
<tr>
<td>o-Cresol</td>
</tr>
<tr>
<td>m-Cresol</td>
</tr>
<tr>
<td>p-Cresol</td>
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</tbody>
</table>

4.2.6 Coprecipitation

The extent of the removal of a compound from a solution by enzyme-catalyzed polymerization is dependent on its reactivity toward peroxidase and the solubility of the products of peroxidase oxidation. One or both of these factors can adversely affect the precipitation of aromatics from wastewaters, thereby limiting the application only to those compounds which can be efficiently removed from the solution. Klibanov et al. (1980) observed the coprecipitation phenomenon in which easily removed compounds aided in
the precipitation of other compounds which could not, by themselves, be removed to the same extent.

Experiments were conducted to investigate the coprecipitation of p-cresol with other compounds and the UV method was used for analysis. Excess HRP (1 U/mL) and PEG (100 mg/L) were used in these reactions. The hydrogen peroxide concentration remained at 1 mM. It was observed earlier that p-cresol had a very high residual which could not be further reduced. One half mM of each phenolic compound was combined with p-cresol so that the total concentration was maintained at 1 mM. The results in Figure 4-17 show that the addition of 3-chlorophenol and 4-chlorophenol made a slight improvement in removal of total concentration while the addition of other compounds caused no improvement in treatment efficiency.

![Graph showing total phenolic concentration remaining in coprecipitation with p-Cresol.](image)

**Figure 4-17** Total Phenolic Concentration Remaining in Coprecipitation with p-Cresol.

*Note: p: p-Cresol; 2c, 3c, 4c: 2-, 3-, 4-chlorophenol; om, mm: o-, m-Cresol; 24c: 2,4-Dichlorophenol*
Certain visual observations were noted in these experiments. When p-cresol was treated separately, the final product of the polymerization did not settle even after adding alum. However, when p-cresol was mixed with other compounds, precipitate 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 had the hydrophobic characteristics of the polymers formed by the more efficiently removed compound (Nicell, 1991).

The coprecipitation effect should not be overemphasized for four reasons. Firstly, the high concentration removal efficiency of the easy-to-remove compounds may have some mathematical influence on the final lowered removal efficiency of the mixed solution. Secondly, the difference between the concentration remaining before and after combining different compounds is not of significance in terms of analytical errors. Thirdly, it is possible that final concentration remaining of the mixture of different phenolic compounds is higher than that when they are treated separately. Real industrial wastewaters are usually a mixture of a variety of phenolic compounds; consequently, the negative effect might alleviate the effect of coprecipitation. Finally, it is not a good practice to add some toxic chemicals in order to remove others.

4.3 EFFECT OF ADDITIVES ON THE FUNCTION OF HRP

Additives can save HRP during the reaction, possibly by having influence on its activity and its inactivation. The study included the effect of additive on the activity of the same amount of HRP enzyme in the presence or absence of the reaction components. The inactivation under different conditions was also investigated. This may help to reveal the mechanism of the protective effect of additives and to establish the kinetic model of the reaction.
4.3.1 HRP Activity

4.3.1.1 Activity Increase without Reaction

HRP was incubated separately in the presence of various additives (50 - 100 mg/L). Activity tests were repeated twice after 20 minutes and the average activities were expressed as percentage increases over control (no additives) in Figure 4-18. The results paralleled the effectiveness of additives in Figure 4-1. For example, PEG\textsubscript{300} and PAA were not effective additives in Figure 4-1 and they did not show an increase in activities in Figure 4-18. Other additives were effective and they showed increases in activities. This implies a correlation between the increase in activity and the effectiveness of additives. After measuring the activity as a function of incubation time, it was found that this effect was instantaneous. The maximum increase was achieved in less than 30 seconds. The magnitude of the increase in activity was found to be related to the additive dose. As seen in Figure 4-19, this increase had reached its maximum value when PEG\textsubscript{3550} concentration was 1 g/L.

![Activity Increase Graph]

Figure 4-18 Activity Increase Caused by Additives
Note: Incubation conditions: pH = 7, PEG, Gelatin = 100 mg/L, PEs = 50 mg/L
HRP\textsubscript{control} = 0.08 U/mL; sample volume in activity assay = 100 μL
The Activity Assay in Appendix-A was also found to be influenced by additives. A HRP solution of 0.5 U/mL was prepared and its activity was measured using the assay. Activity was measured again, but buffered with PEG$_{3550}$ solution instead of buffer only as specified in the assay. Different PEG concentrations in cuvette were used to observe its dose effect. Each activity was measured twice. It was found (Figure 4-20) that the average activity in the cuvette with PEG$_{3550}$ increased about 7 - 8 %. This increase was irrespective of PEG dose, which is different from the activity increase in reactors shown in Figure 4-18. Thus, PEG$_{3550}$ did influence the assay, but it was not dose-dependent. The increase was almost the same over the wide range of concentrations of PEG$_{3550}$ used in the cuvette.
4.3.1.2 Activity Increase in Reaction

In addition to the increase in activity in the presence of additives without hydrogen peroxide, initial activity increased significantly when hydrogen peroxide was added to start the reaction. An activity of 0.2 U/mL, soon after hydrogen peroxide was added, was measured for a reaction mixture with an initial activity of 0.1 U/mL, which is discussed in Section 4.3.2.3. The increase was related to initial HRP activity and the concentrations of initial phenol and hydrogen peroxide. Further detailed discussion can be found in the activity sub-model in Section 5.6.

4.3.2 HRP Inactivation

Several factors influence HRP activity, such as temperature, pH, additives. The additives improved the enzyme turnover and therefore may have an impact on the HRP inactivation during the reaction. Three kinds of inactivation were studied: inactivation
caused by (a) temperature, (b) hydrogen peroxide and (c) the polymerization product. PEG\textsubscript{3550} was chosen as a representative additive in these experiments.

4.3.2.1 Temperature Inactivation

The effect of additive on the inactivation caused by temperature was examined by measuring the change in HRP activity over time. Three culture tubes were filled with 5 mL buffer (pH 7). Two of them contained PEG\textsubscript{3550} and PEG\textsubscript{300}, respectively, at the final concentration of 500 mg/L. They were preheated in a water bath of 45°C for more than 15 minutes. At the same time, a HRP solution of 55 U/mL was prepared. Then, 50 μL of the HRP solution was distributed to the two culture tubes with PEG already preheated to 45°C. The final initial HRP activity was about 0.5 U/mL. The activity change was measured over time. The relative activity change is plotted in Figure 4-21. The 100 % activity represents that at 45°C without PEG.

![Figure 4-21 Effect of Additive on Thermal Inactivation](image)

*Figure 4-21 Effect of Additive on Thermal Inactivation*

*Note: 100 % HRP = 0.5 U/mL, pH = 7, temperature = 45 °C*
Figure 4-21 demonstrates that initial activity had increased by about 20% in the presence of PEG₃₅₀₀ even when the temperature was significantly higher than room temperature. Again, PEG₃₀₀ showed no increase in initial activity. Without additive, the activity had decreased by about 10 to 12% in a 10 minute period. It is almost identical to the reduction of 12% with PEG₃₅₀₀, although the absolute activity decrease was bigger (0.05 U/mL compared to 0.04 U/mL). This suggests that the inactivation rate was the same with or without the presence of PEG₃₅₀₀. In other words, PEG₃₅₀₀ did not protect against the inactivation caused by temperature.

4.3.2.2 Hydrogen Peroxide Inactivation

Hydrogen peroxide inactivation was studied by using 0.1 U/mL of HRP solution incubated with 2 mM Hydrogen peroxide. A 100 mg/L of PEG was used in these experiments. The activity changes over time are plotted in Figure 4-22. There was almost no difference between the inactivation due to PEG₃₅₀₀ or PEG₃₀₀ and without additive. Thus, additive did not protect HRP inactivation caused by hydrogen peroxide.

![Graph](image)

Figure 4-22 Effect of Additive on Inactivation by H₂O₂
4.3.2.3 Hydrogen Peroxide and Phenol Inactivation

HRP inactivation during polymerization was studied first without additive. Two vials of 30 mL containing 1 U/mL of HRP solution were prepared. One of them was used to measure total activity; and the other one for soluble activity (using 0.2 µm filter). The difference between the two values provided the activity on solids. The results shown in Figures 4-23 and 4-24 confirm that active HRP was adsorbed during the reaction in absence of PEG$_{3550}$. The activity adsorbed on solid reached a peak value sometime during the reaction and gradually reduced thereafter. The maximum HRP activity adsorbed on solid was as high as 30 % and occurred in a short period. Also at that time, the activity on the solid was more than that in solution. Thus, the polymer product formed during the reaction without PEG had a strong ability to adsorb active HRP enzyme. However, this did not necessarily support the hypothesis that inactivation of HRP was caused by adsorption (Nakamoto and Machida, 1992).

![Graph of activity vs time]

Figure 4-23 Inactivation during Polymerization without Additive (0.5 mM phenol)

Note: HRP = 1 U/mL, pH = 8, phenol = 0.5 mM, H$_2$O$_2$ = 1 mM
Total activity was lost in a short time period. At the end of the reaction, almost no active HRP remained in solution. It was also observed that the higher the phenol concentration, the faster was the inactivation. For example, it took 5 minutes to lose almost all activity for 1 mM phenol reaction (Figure 4-24), while 10 minutes were required for 0.5 mM phenol (Figure 4-23).

In the presence of PEG\textsubscript{3550}, the inactivation of HRP was much slower as shown in Figure 4-25. The precipitate in this reaction also adsorbed HRP. However, the adsorption was no more than 10%, as compared with 30% in the absence of PEG\textsubscript{3550}. It was observed that, in the presence of PEG\textsubscript{3550}, the initial activity increased after adding hydrogen peroxide to start the reaction. In this experiment, the initial activity had increased almost to twice the value before reaction (0.1 U/mL). At the end of the reaction, the enzyme remaining adsorbed on solids as well as in solution was negligible. This phenomenon was similar to that observed in the absence of PEG\textsubscript{3550}; however, time scale
was significantly different. Because 0.1 U/mL was twice the amount of minimum HRP
dose, it indicates that excess HRP was also inactivated even in the presence of PEG$_{3550}$. This is demonstrated further in Figure 4-43 later. It suggests that the inactivation
mechanism was the same both with or without PEG$_{3550}$, and the difference was in the
inactivation rate. The inactivation rate with PEG was significantly slower.

![Graph](image)

**Figure 4-25** Inactivation during Polymerization with Additive (1 mM phenol)

Note: HRP = 0.1 U/mL, pH = 8, phenol = 1 mM, H$_2$O$_2$ = 1 mM

### 4.3.3 Polymerization Product on the HRP Inactivation

Additive had no effect on temperature and hydrogen peroxide inactivation. This
suggests that the reason for HRP inactivation was related to the polymerization process in
which phenoxy radicals and higher oligomers were involved. Because phenoxy radicals
were extremely unstable, only the precipitate or the polymer product could be separated
from the reaction mixture. Therefore, precipitate was made from the enzyme-catalyzed
reaction and used to incubate with fresh HRP solution to examine its effect on HRP
inactivation.
To make precipitate from the reaction, a certain volume of 2 mM phenol solution was mixed with HRP and was allowed to undergo reaction for a period of time with 2 mM hydrogen peroxide in the presence or absence of PEG\textsubscript{3550}. Activity and phenol concentration before and after reaction were measured to ensure phenol removal. Then, the reaction mixture was put under centrifugation. The supernatant was removed and the solid was resuspended in water to remove the remaining HRP and other reactants from solution. The washing procedure was repeated until no activity remained in the resuspended solution.

Different precipitates were collected by changing HRP dose and by adding or not adding PEG. The washed precipitates collected in this way were added into the fresh HRP solution for a period of time to incubate with the enzyme. Then, both total activity and the activity in solution (by using 0.2 μm filter) were measured over time to evaluate the effect of different precipitates on fresh HRP.

4.3.3.1 HRP Inactivation Caused by Polymerization Product

Three vials with 30 mL of 5 U/mL HRP were used to make precipitates in this study. 500 μL catalase was used to stop the reaction in two vials at 1 minute and 3 minute, respectively. The precipitates were collected after 3 hours together with another vial in which catalase was not used. The precipitates prepared from these vials were subsequently used to incubate 30 mL HRP solution of 2.5 U/mL in another three vials without the presence of additive and phenol. The changes in activity during these experiments are illustrated in Figures 4-26 and 4-27. These figures demonstrate that the polymerization product itself caused the inactivation of HRP. It was observed that the more mature precipitates caused more HRP inactivation. For example, the precipitate collected at 3 h inactivated 0.9 U/mL HRP while only 0.3 U/mL inactivation resulted due to the precipitate made at 1 min reaction. As soon as the precipitates were mixed with HRP, part of the HRP (about 0.3 U/mL) was adsorbed on the precipitates almost
Figure 4-26 Inactivation of HRP by Polymer Product (Total)
Note: Precipitate was from a reaction of 5 U/mL HRP w/o PEG. Phenol = 2 mM

Figure 4-27 Inactivation of HRP by Polymer Product (in Solution)
Note: Precipitate was from a reaction of 5 U/mL w/o PEG. Phenol = 2 mM
instantly. The precipitate of 3 h kept this proportion all the time, suggesting that its adsorption ability was almost saturated. In contrast, activity adsorbed on the solids from 1 min reached its peak after about 15 minutes and gradually receded thereafter. Also, the more mature were the precipitates, the longer was the inactivation effect. The precipitate from 3 h was still inactivating HRP after 1 hour. Therefore, the main inactivation of HRP during a turnover reaction in the absence of additive is caused the products of polymerization. Phenoxy radicals formed during a turnover reaction might have inactivated HRP but did not account for such magnitude.

The catalase added in the precipitate-making process did not interfere with the activity assay because catalase was not used in the collection of the precipitate at 3 h. In addition, excess catalase was washed out of solution and might have stayed on solids only. The catalase adsorbed on solids, similar to the HRP adsorbed on solids, may be in an inactivated form and, therefore, posed no problem in the activity assay.

4.3.3.2 Additive Protection on Such Inactivation

The effect of additive on the inactivation caused by polymer products, formed in the absence of PEG_{3550}, was further studied. Again, experimental results proved that additives could prevent such inactivation from happening. Figures 4-28 and 4-29 showed that the precipitate collected from a reaction of 4 U/mL without PEG_{3550} had inactivated HRP as discussed in the previous section. However, if the HRP solution was mixed with PEG_{3550}, the same precipitate stopped inactivating HRP. In the presence of PEG_{3550}, less HRP activity stayed on solids. The addition of PEG_{3550} again caused an increase in the initial activity.

At the same time, precipitate was collected from the reaction in the presence of PEG_{3550} and used to do similar experiments. As shown in Figures 4-30 and 4-31, this precipitate did not inactivate HRP. The addition of PEG_{3550} also increased the activity of HRP. However, unlike the instant increase that PEG had produced in HRP activity
Figure 4-28 Protection of PEG on Polymer Product Inactivation (1)
Note: Precipitate was from a reaction of 4 U/mL HRP w/o PEG at 1 min

Figure 4-29 Protection of PEG on Polymer Product Inactivation (2)
Note: Precipitate was from a reaction of 4 U/mL HRP w/o PEG at 3 h
Figure 4-30 Effect of Precipitate from Reaction with PEG on HRP Activity (1)
Note: Precipitate was from a reaction of 0.05 U/mL HRP w 100 mg/L PEG at 3 h

Figure 4-31 Effect of Precipitate from Reaction with PEG on HRP Activity (2)
Note: Precipitate was from a reaction of 2 U/mL HRP w 100 mg/L PEG at 3 h
without the precipitate (Section 4.3.1.1), this increase was a slow process and could last for as long as an hour (Figure 4-30). The results show clearly that this type of precipitate also adsorbed HRP on it, but such adsorption did not result in any loss of activity.

4.3.3.3 Adsorptive Power of Product Polymer

In order to quantify the adsorption of HRP on polymer products, precipitates were collected after reactions both in the presence and absence of PEG$_{3550}$. After the precipitate was collected according to the procedure in Section 4.3.3, it was evenly divided into eight centrifuge tubes containing different additives. The concentration of additives was 100 mg/L in each tube. The additives were mixed thoroughly with the precipitates by shaking the tubes vigorously. After centrifugation, the supernatant was collected to undergo centrifugation again. Then, TOC test was done on the final supernatant. Phosphate buffer served as a blank. A hundred percent TOC represented the amount of additive (mg/L) added in the tubes.

The results in Figure 4-32 show again that both precipitates can adsorb HRP, but the precipitate formed with PEG adsorb only half of the amount of HRP compared to that formed without PEG$_{3550}$. The precipitate formed with PEG combined with much less PEG$_{3550}$, probably due to the PEG$_{3550}$ already in it. Both types of precipitates did not interact with PEG$_{300}$, which again confirmed previous results. Both types of precipitates could incorporate gelatin and PEs, except PE$_2$. This may explain why they had similar behavior as additives.

The precipitates were then resuspended in deionized water and the supernatant after centrifugation underwent a TOC test. The results demonstrated that the additives (including HRP) adsorbed on precipitates formed from the reaction without PEG could not be dissociated from the precipitates into water, while they could be partially desorbed from precipitates formed in reactions with PEG. The TOC test also showed that the greater the amount of additive added, the more of them incorporated into the precipitate.
Activity measurement was used to do an HRP activity balance in order to study the effect of such absorption on HRP activity. Precipitate was made from 250 mL of 2 mM phenol treated by 5 U/mL HRP at 1:1 hydrogen peroxide for 1 hour. Half of the precipitate suspended in 7 mL buffer was used to incubate 2.5 mL HRP stock with 25 mg PEG_{3550}. Another half was used to treat the HRP solution without PEG. The corresponding HRP balance is tabulated in Table 4-7.

Approximately 50 out of 375 units of activity were lost during the mixing with the precipitate without the addition of PEG. PEG_{3550} again prevented such inactivation and induced an activity increase. PEG_{3550} caused less adsorption of fresh HRP (11 %) on solids, which was similar to the results obtained for the adsorption during reaction in
Figure 4-25. After resuspending the solids into buffer, more activity stayed on the precipitate with PEG although they adsorbed less HRP.

<table>
<thead>
<tr>
<th>Table 4-7 Activity (U/mL) Balance for HRP Adsorption by Precipitate</th>
</tr>
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<tbody>
<tr>
<td><strong>Experiment Steps</strong></td>
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<td>5</td>
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</tbody>
</table>

Note: Precipitate was from a reaction of 2 mM of Phenol with 1:1 hydrogen peroxide

4.4 FATE OF ADDITIVES AFTER REACTION

It is important to know the fate of additives in the enzyme polymerization of phenolic compounds. If additives stay in solution after reaction, it may be difficult to discharge the treated wastewater. Also, knowledge of the fate of an additive will help in understanding the mechanism of the additive's protection of HRP. Experiments were conducted in the presence of additives and TOC measurements were used to estimate the additive remaining in solution. TOC values were converted to equivalent amount of additive according to the calibration curves in Appendix-B. Commercially available protein assays were attempted to determine the remaining amount of gelatin and HRP but these assays failed because of the interference from phenol.
4.4.1 PEG

PEG fate was investigated first with 1 mM phenol and m-cresol. Then, a similar test was conducted with 5 mM phenol to increase the accuracy of TOC test and to confirm the results obtained from 1 mM experiments.

The experiments followed these steps. First, a reaction solution was prepared according to the experimental design. Before the reaction, samples were taken for the measurement of initial activity and phenol concentration. Then, the solution was distributed into vials containing different amounts of PEG_{3550}, thus reaction mixtures with different PEG doses were prepared. A 1 mL aliquot was taken from each vial for the measurement of initial TOC. The reaction stoichiometry between hydrogen peroxide and phenol was 1:1. After 6 hours, 5 mL reaction mixture was taken from each vial to undergo centrifugation for the measurement of remaining TOC and phenol concentration. Finally, the supernatant was transferred into culture tubes, and the TOC and phenol content in each tube were measured. The experimental results are plotted in Figures 4-33 to 4-35. The calculations of material balance for 1 mM phenol are tabulated in Table 4-8.

<table>
<thead>
<tr>
<th>Table 4-8 Material Balance before and after Reaction (1 mM Phenol)</th>
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<tr>
<td>PEG dose (mg/L)</td>
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</tr>
<tr>
<td>Total TOC Remaining</td>
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<tr>
<td>Phenol Remaining</td>
</tr>
<tr>
<td>TOC of Phenol</td>
</tr>
<tr>
<td>TOC of PEG</td>
</tr>
<tr>
<td>PEG Remaining</td>
</tr>
<tr>
<td>PEG Used</td>
</tr>
</tbody>
</table>

Note: Units are mg/L, TOC of Buffer = 1.3 mg/L, TOC remaining of PEG = Total TOC - TOC remaining of phenol - TOC of buffer
Figure 4-33 PEG₃₅₅₀ Fate after Completion of Reaction (1 mM Phenol)

Figure 4-34 PEG₃₅₅₀ Fate after Completion of Reaction (1 mM m-Cresol)
These figures clearly demonstrate that, at the minimum PEG dose for phenol reactions, PEG was almost completely removed from solution with the precipitate. For 1 mM phenol, an increase in PEG addition resulted in an increase in PEG removal from solution with precipitate. For 5 mM phenol, the amount of PEG consumed increased with PEG addition and approached a limit of 150 mg/L. For m-mp, only a certain amount of PEG (around 30 mg/L) was used up and the excess PEG remained in solution. At the minimum PEG dose, all of the PEG added had combined with the precipitate and separated from the solution together with the phenol removed as precipitate.

In order to examine if the PEG removed with the precipitate might go into solution again, the precipitates from the vials of the minimum PEG doses were resuspended into water. The reason for choosing the vials of minimum PEG doses was that the water present in the precipitate could not be completely separated from the precipitate by centrifugation. In case of excess PEG, any water incorporated with the precipitate, would
contribute TOC in the solution of resuspension and would make it very difficult to differentiate between TOC contributed by PEG in water in the precipitate and PEG in the precipitate itself.

Therefore, 30 mL sample was taken from the vials with the minimum PEG dose to undergo centrifugation. The supernatant was removed from the centrifuge tubes and the solids were resuspended into 10 mL deionized water. The resuspension mixture was put under centrifugation again and the supernatant was used as the sample for TOC test. The resuspension and the TOC analysis were repeated twice. The expected PEG was the computed concentration of PEG based on the assumption that all PEG in precipitate had gone into water. The results, plotted in Figures 4-36 and 4-37, clearly reveal that no PEG was redissolved into solution in all three resuspensions. Thus, the PEG was incorporated onto the precipitate and could not be separated from the precipitate anymore. It is possible that PEG had covalently combined with the precipitate, which is why recycling of the precipitate could not protect HRP in reactions in the absence of additives.

![Graph](image)

**Figure 4-36 Desorption of PEG_{3550} from Precipitate (1 mM Phenol)**

Note: the precipitate was from a reaction of removal of 1 mM phenol
4.4.2 Gelatin

Attempts were made to use commercially available protein assays (Bio-Rad, STD, Lowry STD, etc.) from Sigma Co. to measure gelatin as protein. Although those assays could measure gelatin with an excellent linear correlation in the absence of phenol, there was serious interference caused by phenol remaining in the solution. Therefore, the TOC test had to be used as before. Experiments were conducted with 1 mM and 3 mM phenol solutions and HRP dose was 0.1 and 0.25 U/mL, respectively, which could remove all phenol present. Gelatin dose varied from 60 to 500 mg/L. However, both excess and inadequate gelatin doses resulted in the formation of color only and no precipitate was formed, as discussed in Section 4.2.3.2. Thus, although phenols were converted, they still remained in solution and could not be separated from it. Because these phenolic products could contribute to TOC, only those vials with the formation of precipitate were tested for...
TOC. Therefore, the results are available for a relatively narrow range. Experimental procedure and the calculations were similar to that described in the previous section.

As plotted in Figures 4-38 and 4-39, a profile different from PEG was found for gelatin. Although most of the gelatin added was removed from the solution together with the precipitate, a substantial amount of gelatin remained in solution after reaction (12 to 32 mg/L for 1 mM and 24 to 100 mg/L for 3 mM phenol, respectively). This constituted 15 to 30 percent and 12 to 34 percent of gelatin added, respectively. Even with the minimum dose of 80 mg/L for 1 mM phenol and 200 mg/L for 3 mM phenol, a significant amount of gelatin still remained in solution. When gelatin dose was higher than the minimum, the gelatin remaining in solution was almost directly proportional to the gelatin added. This remaining gelatin can contribute to BOD and organic nitrogen in the solution. When it was less than the minimum, all the gelatin added was removed through incorporation in the precipitate.

Figure 4-38 Gelatin Fate after Completion of Reaction (1 mM Phenol)
Figure 4-39 Gelatin Fate after Completion of Reaction (3 mM Phenol)

4.4.3 PE

Phenol and 4-cp were used as substrates to investigate PE$_1$ remaining after reaction. HRP activity used was 0.25 or 0.3 U/mL for 1 mM of phenolic concentration. Hydrogen peroxide to phenol ratio was 1:1. The removal efficiency was more than 95% after 6 hours. TOC remaining for phenol reaction, as shown in Figure 4-40, was less than 3 mg/L, i.e. 7.5 mg/L of PE$_1$. Most of the PE$_1$ (more than 50%) added was removed together with the precipitate. Although the computed value for TOC remaining for phenol was negative at 15 mg/L, it was within the range of the accuracy of the test. The negative value indicated a nearly complete removal of PE$_1$ added. For 4-cp reaction, the result was illustrated in Figure 4-41. As the PE$_1$ dose was increased, the amount of PE$_1$ combining with the precipitate had increased. Test on 1 mM m-mp showed a pattern similar to phenol.
Figure 4-40 Fate of PE₁ after Completion of Reaction (1 mM Phenol)
Reaction condition: 1 mM Phenol, Cationic PE, 0.3 U/mL HRP, 1:1 H₂O₂, pH = 8

Figure 4-41 Fate of PE₁ after Completion of Reaction (1 mM 4-cp)
Reaction condition: 1 mM 4-cp, Cationic PE, 0.25 U/mL HRP, 1:1 H₂O₂, pH = 8
4.4.4 HRP

The fate of HRP after reaction was also investigated to determine the possible mechanism of additive's protection on HRP enzymes. A phenol concentration of 10 mM was used as the substrate for HRP both in the presence and absence of PEG. The experimental and the calculation procedures were similar to that in Section 4.4.1. Activities both before and after reaction were measured to estimate the HRP amount. Again, protein assay could not be used because of the interference from phenol. The TOC test was used to determine the HRP concentration in the solution after reaction and the results are plotted in Figures 4-42 (reaction without PEG) and 4-43 (reaction with PEG).

In the absence of PEG, more than 95% of phenol was removed at 25 U/mL of HRP. Any activity beyond that was excessive. This excessive amount of HRP (up to 20 U/mL at 45 U/mL) was almost completely inactivated. If the HRP remaining was based simply on the activity remaining, only a very small amount of HRP (less than 2%) remained in solution and the rest was adsorbed by the solids formed during reaction. This seemed to support the mechanism proposed by Nakamoto and Machida (1992) that HRP was adsorbed on precipitate. However, the measured activity represented only the active form of HRP. It was just like that HRP still remained in solution when HRP was inactivated under extreme pH conditions. In Figure 4-42, TOC test showed that 27 to 35 percent of HRP added remained in solution after the reaction. The rest was separated from the solution together with precipitate. The active enzyme was a small part (less than 10%) of HRP remaining in solution. Most of the active HRP stayed on solids. It should be mentioned that the TOC test was fairly accurate because of the relatively large value. The experiment with 5 mM phenol showed exactly the same pattern for HRP remaining and thus supports the conclusion. The ratio of HRP-TOC remaining in solution to HRP-TOC remaining on solid was close to that of the HRP activity remaining in solution to HRP activity remaining on solids. This means that both active and inactive HRP were evenly
distributed between the solids and the solution. Most of the HRP remaining in solution after reaction became inactive.

![Graph showing HRP fate after completion of reaction (10 mM Phenol without PEG)](image)

*Figure 4-42 HRP Fate after Completion of Reaction (10 mM Phenol without PEG)*

In the presence of 333 mg/L of PEG<sub>3550</sub>, 0.2 U/mL of HRP could achieve 97.1% removal of 10 mM phenol. Only 0.02 U/mL of HRP remained active after reaction. However, additional 1 U/mL HRP improved the removal efficiency only 0.1 percent and the extra amount of HRP was almost completely inactivated. This means that even in the presence of PEG, HRP could still be inactivated severely and excessive HRP could not be protected by PEG. Only a very small percentage of HRP (less than 6%) added remained in solution. The TOC tests showed negative values for the remaining HRP, which suggests that the estimations from activity test were reliable. The reason for the negative value was the difficulty in estimating the PEG remaining when its value was small. It could be
concluded that most of the HRP was also removed from solution with precipitate. HRP
could also be incorporated with the polymer product in the presence of PEG$_{3550}$. The
inactivation mechanism might be the same in both conditions, i.e. the polymer product
inactivated the HRP.

![Graph showing HRP Fate after Completion of Reaction](image)

In summary, in the absence of PEG, HRP was inactivated by the polymers formed
during the reaction and inactivated HRP stayed both in the solution and on the precipitate.
In the presence of PEG, most of the inactivated HRP stayed on the precipitate, therefore,
HRP was also inactivated by the polymers. Any excess HRP under both conditions was
inactivated whether or not there was PEG present in the solution. The inactivated HRP
stayed either on precipitate or remained in solution.
4.5 DISCUSSION

4.5.1 Comparison Among the Additives

Experiments were conducted to compare the effect of additives under the optimized conditions obtained in Section 4.2. The experimental results showed that, in terms of removal efficiency, PEG was the best additive that provided most savings in HRP. Gelatin and PE₁ showed similar effectiveness and were better than PE₂. All reactors showed the formation of settleable particles during reaction. Similar results were obtained for both 1 and 3 mM phenol concentrations.

In terms of practical application, PEG₃₅₅₀ has high solubility and it is easy to make 40 g/L PEG₃₅₅₀ solution. In contrast, a lot of effort was needed to make 1 g/L gelatin and PE solutions. It took hours to dissolve them even after heating and stirring.

In terms of practical additive dose range (PADR), PEG was also the best among the additives studied. Any excess PEG posed no negative effect on the polymerization, while excess gelatin and PE reduced the treatment removal efficiency. The PADR were fairly narrow for gelatin and PEs. For instance, PADR for gelatin was 80 to 100 mg/L, and PE₁ and PE₂ showed 10 to 20 mg/L at 1 mM of phenol. Moreover, excess hydrogen peroxide adversely affected the treatment removal efficiency when gelatin or PEs was used. With excess gelatin or PE dose, not only removal efficiency had been reduced but also there was no formation of particles and the polymerization products of phenolic compounds could not be separated from the solution. The color produced in reaction may be subject to further treatment requirements.

In terms of the effluent quality, with minimum PEG dose, there would be little PEG left in solution after completion of reaction. Most of the PEG added would combine with the particles and be separated from solution as precipitate. In addition, PEG is a non-toxic chemical which is approved for internal consumption by FDA (Harris, 1992). Therefore, it is not a problem even if some PEG is discharged into water. However, there was still a considerable amount of gelatin remaining in solution even at the minimum
gelatin dose. This would contribute to the BOD, COD and organic nitrogen in the final treated solution. This can pose a potential problem for the treatment process. Also, gelatin would produce more precipitate than PEG.

In terms of cost, PEG is more competitive than the others. In conclusion, PEG is the best choice as an additive based on this study.

4.5.2 Protection Mechanism

Additives were able to increase the activity with the same amount of HRP, but this increase (around 20%) could not explain how additives protected the HRP and improved the enzyme turnover up to 200-fold. Twenty percent increase in activity produced only a small increase in removal efficiency in the absence of additive (Figure 4-5). Also, if this was the reason for such protection, it should have been effective with all aromatic compounds, which was not true based on the experimental results.

There was evidence that additive effectiveness was related to both aromatic compound structure and the additive itself. For example, with PEG$_{3550}$, HRP turnover improvement was less than 10-fold for 3-cp, while it was more than 133-fold for 4-cp. Different additives had different effectiveness as reported in Section 4.4.2. This suggests that the enzyme protection mechanism lies in the interrelation between the aromatic compound and the additive.

Experimental observations suggest that the final product in the presence of additives was different from that in their absence. Five observations from experiments supported this point. Firstly, the particles in the presence of additive looked finer and their formation was slower. Color was formed before particles, and this color varied with the type of phenolic compounds and additives. In contrast, the particles formed without additives were heavier and their formation was faster. Secondly, the color of the particles was different. For example, the particles formed during phenol removal had a dark brown color without additives. However, the particle formed with PEG and PE showed whitish-
brown color. It was not a coincidence that gelatin led to yellowish precipitate at the same time. This became more obvious when all the water was evaporated from the precipitate. For instance, white crystals appeared in brown solids from the reaction with PEG. Thirdly, extra gelatin or PE dose led to the formation of color and no formation of particles, but phenolic compounds were still converted and HRP was also protected in that situation. The new polymerization product, probably represented by the color in the solution, was different from the settling polymer product in the absence of additive. Fourthly, a more direct evidence was that particles separated from reaction without additive were able to inactivate HRP while those from reactions with additives did not. In addition, the precipitate from the reaction without additive did not inactivate HRP when it was treated with PEG. This affirmed explicitly that the polymer products in the reaction itself, in the absence of additives, caused the inactivation of HRP. Finally, TOC tests provided direct evidence that most of the additives were removed from solution together with precipitate. No PEG could be dissociated from the solids by resuspension, and thus recycling of the precipitate would not act as additive. It showed clearly that it was the final product that inactivated HRP instead of the phenoxy radicals proposed by Klibanov et al. (1980). The addition of PEG or other additives could prevent such inactivation. All these observations lead to a conclusion that additives are incorporated into polymer product, which makes the final product have different characteristics.

It should be emphasized that this proposed mechanism is different from that proposed by Nakamoto and Machida (1992). Firstly, it is the final products and not necessarily the polymer particles that inactivate HRP because the product can take both solid and soluble forms. Secondly, it was an inactivation process that was not simply caused by physical adsorption but by chemical partition between the HRP and the polymer product. This argument is supported by the following three observations from experiments.
( i ), although a high percentage of conversion was achieved in the reaction and no particle formed with o-mp in the absence of additive, HRP enzyme was inactivated during the reaction. The final product of o-mp in the reactor was a yellowish color. The presence of additive prevented the inactivation. This indicated that the soluble final product, probably represented by the color in the solution, inactivated HRP. This inactivation could not be explained by adsorption. The addition of PEG could prevent such inactivation and improve the enzyme utilization by up to 30-fold. Therefore, the inactivation caused by soluble product could also be prevented by additives.

( ii ), in the absence of additive, the activity remaining in solution did not correspond to the HRP remaining in solution as shown in Section 4.4.4. It represented only a very small percentage of HRP present in the solution. Most of the HRP inactivated still remained in solution but not on the solid products.

( iii ), the precipitate obtained from the reaction with PEG could adsorb HRP, but it did not inactivate it. Therefore, the adsorption of HRP did not necessarily mean the inactivation of HRP.

Based on the above discussion, the mechanism for the additive protection of HRP is proposed:

The formation of polymer products is a sequential process in which phenolic compounds are first converted to dimers, then trimers and/or tetramers (Yu et al., 1994). These lower oligomers are substrates for HRP, and do not inactivate HRP. These lower oligomers are further converted to insoluble higher oligomers which lead to the removal of phenolic compounds from the solution. In the absence of additive, the higher oligomers inactivate HRP enzyme during their formation. Because high oligomers have sites similar to the functional group of phenol to active site of HRP, active HRP can interact with them. This process can not lead to the formation of further higher oligomers, while HRP begins to be inactivated during the process. Although HRP can dissociate from the high oligomers as shown in Figure 2-1, more HRP stays with the high oligomers (solid form or
soluble form) and is inactivated at the same time. The interaction between HRP and the polymer product can be described as a partition affinity. If additives are present in this reaction, additives, instead of HRP, will interact with these active sites because of their higher partition affinity with these sites than HRP. The partition affinity order is: PEG\(_{3550}\) > Gelatin > PE\(_1\) > PE\(_2\) > PE\(_3\) > HRP > PEG\(_{300}\). Because PEG\(_{300}\) is a short chain polymer (tetramer), it can not cover all active sites of higher oligomers and, therefore, it is not effective as an additive. Also, different polymer products from different phenolic compounds have different affinities to HRP and additives. That is why different phenolic compounds require different minimum HRP dose in the absence of additives. During the interaction, additive is permanently incorporated with the oligomers. This coupled product of oligomers and additive has no or few sites for interaction with HRP. Therefore, HRP is protected in the further reaction. However, a small percentage of HRP can still interact with a few of these active sites. In the presence of additive, HRP and additive interact with polymer product at the same time according to their ratio of affinities to the polymer product ([value of HRP partition affinity for polymer]/[value of additive partition affinity for polymer]). Less HRP will interact with polymer product if this ratio is small, which means less HRP is inactivated, and vice versa. Because this ratio is different, saving in HRP is different for different phenolic compounds. That is why some additives work better than others. Thus, the polymer product incorporated with additives inactivated enzyme at a much slower rate in the presence of additive. This can be further supported by the results from Wu J. et al. (1993). Without the presence of additive, HRP dose of at least 2 and 30 U/mL was needed to remove 1 and 10 mM phenol, respectively. This represents 16.7 and 250 mg/L HRP in the solution, respectively. Not coincidentally, about 30 and 250 mg/L PEG\(_{3550}\) permitted the reduction of the HRP concentration to 0.05 and 0.4 U/mL for the removal of 1 and 10 mM phenol, respectively. In another independent study, the minimum PEG dose for 1 mM phenol was reported almost exactly as 16.7 mg/L (Saadi, 1993).
In conclusion, the effect of additive protection on HRP enzyme can be summarized as in Figure 4-44. In the absence of additive, active HRP is inactivated by the polymer product formed during the reaction (process A in the figure) because of the formation of polymer-HRP conjugates. In the presence of additive, HRP is first modified and activated by additive and the activity of the HRP becomes higher (process B in the figure). Additive can covalently combine with the polymers during the reaction (process C in the figure). Because the additive has a higher partition affinity with polymer than HRP, most of the polymers are coupled with additive so that lesser HRP interacts with the polymer. HRP still combines with polymer product and becomes inactivated but at a much slower rate. Thus, HRP is protected by the additive.

![Figure 4-44 Proposed Additive Protection Mechanism on HRP Enzyme](image)

Figure 4-44 Proposed Additive Protection Mechanism on HRP Enzyme
CHAPTER 5 KINETIC MODELING

Kinetic modeling of the peroxidase catalyzed reaction is important in developing its reactor system. It can prove to be a helpful tool in selection and design of reactors. A well-designed model can be used to predict the behavior of the reaction under different conditions and help reduce the cost of design and operation of reactors. A good kinetic model can also help to reveal the possible reaction mechanism.

A good model requires that its output should fit the data collected from experiments. The output under the extreme reaction conditions must also be consistent with the reality. The model must satisfy the following reaction phenomena observed in HRP catalyzed reactions:

- When there is not enough HRP or hydrogen peroxide present in the reaction mixture, the conversion of phenol will not possibly be completed.
- When there is extra hydrogen peroxide, the phenol removal efficiency will be lower.
- When there is extra PEG, there will be no improvement in the phenol removal efficiency.

Failure to satisfy these requirements will demand further search for a new model.

With the above considerations in mind, kinetic data were collected from experiments and the outputs from the existing models were compared with these data using curve-fitting techniques. The drawbacks of these models were identified and modifications were made to help establish a new model. Other possible models were also tested to explore the best fitting model.

The model developed in this study is an unsteady state model that consists of a set of differential equations governing the entire reaction. Although it is possible to solve these equations analytically, it is too complicated to do so in practice. Therefore, numerical integration was used to solve those equations. This model satisfies the requirements for a good model described previously.
5.1 MODEL DEVELOPMENT

5.1.1 Evaluation of the Existing Models

Before proposing any new model, it is essential to evaluate the existing models so that both their merits and weaknesses can assist in establishing a better model. Several models were used to predict the behavior of the reactions in the presence or absence of additives.

Model 1: The HRP catalyzed polymerization of phenol in the presence of PEG was shown to be approximately a pseudo-first order reaction with respect to phenol concentration (Yu et al., 1994). This means:

\[- \frac{d[AH_2]}{dt} = k[AH_2] \quad 5-1\]

where AH₂ represents phenol or phenolic compound. \([ \quad ]\) represents molar concentration and \(k\) is the first-order rate constant. The constant in turn was related to the initial concentrations of phenol, hydrogen peroxide and HRP. This simple model needed the final removal efficiency to be given when the concentration of hydrogen peroxide or HRP present in the solution is insufficient to complete the reaction.

Model 2: Another simple model was a combination of a one-substrate Michaelis-Menten equation and a first order depletion of enzyme (Siddique, 1992). This gave:

\[- \frac{d[AH_2]}{dt} = \frac{k_{cat}[HRP]_a[AH_2]}{K_m + [AH_2]} \quad 5-2\]

\[- \frac{d[HRP]_a}{dt} = k_a[HRP]_a \quad 5-3\]

where \([HRP]_a\) represents the concentration of active form of HRP. \(K_m\) is the Michaelis constant for phenol, \(k_{cat}\) is the turnover number, and \(k_a\) is the inactivation rate constant.
This model could predict the reaction quite well when hydrogen peroxide concentration was fixed at the optimum stoichiometry. The problem with this model was that the constants were functions of hydrogen peroxide concentration. In addition, the PEG effect was not included. Consequently, it does not satisfy the requirements of this study.

**Model 3:** The model, proposed by Yu et al. (1994), employed the phenol conversion kinetic expression (Equation 2-1) of Dunford (1991), and an enzyme depletion equation from an assumption that enzyme was inactivated by phenoxy radicals. The final expressions were as follows:

\[
\frac{\mathrm{d}[AH_2]}{\mathrm{dt}} = \frac{2[HRP]_a}{(k_2 + k_3) \frac{1}{k_2 k_3 [AH_2]} + \frac{1}{k_1[H_2O_2]}}
\]

\[
-\frac{\mathrm{d}[HRP]_a}{\mathrm{dt}} = k_a \sqrt{k_{ph}[HRP]_a^3 [AH]_2}
\]

where \(k_{ph}\) is the overall second order constant of phenol conversion with respect to phenol concentration and HRP activity. \(k_1, k_2\) and \(k_3\) are the constants in Figure 2-1.

The value of \([AH_2]/[H_2O_2]\) in this model was fixed. This treatment leads to the same situation as in Model 2. In addition, the inactivation equation was based on an assumption that was not verified by experiments in the presence of PEG. Furthermore, the PEG effect was modeled into its effect on \(k_a\), which was derived from curve fitting as:

\[
k_a = \frac{3.255}{\left(\frac{[PEG]}{[HRP]_0}\right)^{0.429}}
\]

where \([PEG], [HRP]_0\) are the initial PEG and HRP concentration, respectively.
It was concluded that the protection on the HRP enzyme by PEG asymptotically approached a maximum value with the increase in \([\text{PEG}]/[\text{HRP}]\). The estimation for \(k_a\) was obviously not reasonable when PEG concentration approached zero, because \(k_a\) could never be infinity under that condition. This estimation was also contradictory to the fact that PEG concentration had no further effect on the initial removal of phenol as long as it was more than the minimum dose (Wu Y., 1993).

**Model 4:** A more complicated model was derived from the reaction schemes described in Figure 2-1 and Figure 2-2 (Nicell, 1991). This model was defined by the following differential equations:

\[
- \frac{d[\text{AH}_2]}{dt} = \frac{[\text{HRP}]_a}{(k_2 + k_3) \frac{1}{k_2 k_3} \frac{1}{[\text{AH}_2]} + \frac{1}{k_1 [\text{H}_2\text{O}_2]}} \tag{5-7}
\]

\[
- \frac{d[\text{HRP}]_{\text{iii}}}{dt} = \frac{k_{\text{app}} [\text{HRP}]_a}{(k_2 + k_3) \frac{1}{k_2} \frac{1}{[\text{AH}_2]} + \frac{k_3}{k_1 [\text{H}_2\text{O}_2]}} - k_7 [\text{HRP}]_{\text{iii}} \tag{5-8}
\]

\[
[\text{HRP}]_{\text{inact}} = \frac{1}{K_s} ([\text{AH}_2]_0 - [\text{AH}_2]) \tag{5-9}
\]

\[
[\text{HRP}]_a = [\text{HRP}]_0 - [\text{HRP}]_{\text{iii}} - [\text{HRP}]_{\text{inact}} \tag{5-10}
\]

where \([\text{HRP}]_{\text{iii}}\) is the concentration of Compound III and \([\text{HRP}]_{\text{inact}}\) is the inactivated HRP concentration. \(K_s\) is the enzyme turnover. \(k_{\text{app}}\) and \(k_7\) control the following reactions:

\[
\text{HRP}_{\text{ii}} + \text{H}_2\text{O}_2 \xrightarrow{k_{\text{app}}} \text{HRP}_{\text{iii}} + \text{H}_2\text{O}
\]

\[
\text{HRP}_{\text{iii}} \xrightarrow{k_7} \text{HRP} + \text{O}_2^{-}.
\]

The following assumptions were made in this model:
• The amount of enzyme inactivated was directly proportional to the amount of aromatic substrate removed from solution, i.e. $K_S$ was a constant.

• The inactivation of enzyme by hydrogen peroxide and the corresponding consumption of hydrogen peroxide were not significant. Therefore, they were neglected in the model.

• Aromatic substrate and hydrogen peroxide were consumed with a one-to-one stoichiometry.

It should be mentioned that this model was developed without the presence of PEG. Although this model included several more parameters than the others, it still had some critical deficiencies. The major deficiency was the overprediction of active enzyme at the initial stage. This might be partially owing to the reactivation of HRP$_{iii}$ to native form of HRP as defined by $k_7$. That led to an estimation of higher reaction rate. The output from this model usually showed a stiffness, i.e. a fast removal of phenol followed by a fast approach to a flat stage. The reason for that was a combination of the underestimation of $E_{inact}$, the omission of inactivation caused by hydrogen peroxide and the regeneration of active enzyme from E$_{iii}$. Experiments had shown that inactivation by hydrogen peroxide was not negligible in the presence of PEG (Wu Y., 1993). This makes it imperative to include hydrogen peroxide in the new model.

Another major deficiency was the assumption that $K_S$ was a constant. Experiments demonstrated that it was related to HRP and phenol concentrations. Thus, a relationship between $K_S$ and [Phenol]/[HRP] must be established to use the model to predict the reaction under different reaction conditions. This relation was not available in the model. Furthermore, the reaction stoichiometry was shown to vary with initial phenol concentration in the presence of PEG (Wu Y., 1993). The optimal ratio for 1 mM phenol was 1:1:1, while the ratio for 10 mM was 1.0:1. This observation also had an impact on the model, especially when hydrogen peroxide concentration was lower than the reaction stoichiometry in solution.
When the Modified Ping-Pong model (or the Peroxidase Ping-Pong model) was used as the phenol conversion rate expression, as used in Models 3 and Model 4, the major difficulty was that, theoretically, it could not possibly be a pseudo-first order expression with respect to phenol concentration. This was interpreted through the following explanation.

The original Modified Ping-Pong model can be expressed as in Equation 2-1:

\[
\frac{2[\text{HRP}]_0}{v} = \frac{(k_2 + k_3)}{k_2k_3} \frac{1}{[\text{AH}_2]} + \frac{1}{k_1[\text{H}_2\text{O}_2]}
\]

where \( v = -\frac{d[\text{AH}_2]}{dt} \). Therefore,

\[
v = -\frac{d[\text{AH}_2]}{dt} = \frac{2[\text{HRP}]_0[\text{H}_2\text{O}_2][\text{AH}_2]}{(k_2 + k_3)[\text{H}_2\text{O}_2] + \frac{1}{k_1[\text{AH}_2]}}
\]

Let \( K_1 = \frac{k_{\text{cat}}}{k_1} \) and \( K_2 = \frac{k_{\text{cat}}(k_2 + k_3)}{k_2k_3} \).

Then,

\[
-\frac{d[\text{AH}_2]}{dt} = \frac{2k_{\text{cat}}[\text{HRP}]_0[\text{H}_2\text{O}_2][\text{AH}_2]}{K_1[\text{AH}_2] + K_2[\text{H}_2\text{O}_2]}
\]

or

\[
-\frac{d[\text{AH}_2]}{dt} = \frac{2k_{\text{cat}}[\text{HRP}]_0[\text{AH}_2]}{K_1[\text{AH}_2] + K_2[\text{H}_2\text{O}_2]}
\]

where the first order constant, \( \frac{2k_{\text{cat}}[\text{HRP}]_0}{K_1[\text{AH}_2] + K_2[\text{H}_2\text{O}_2]} \), keeps on decreasing because \([\text{HRP}] \) is continuously decreasing due to the inactivation and the value of \([\text{AH}_2]/[\text{H}_2\text{O}_2]\) is almost a constant (about 1 when initial ratio is 1 (Yu et al., 1994)). Thus, the Peroxidase Ping-
Pong model can not be a first order expression with respect to phenol concentration. This analysis suggested that modifications of the phenol conversion expression were necessary.

The following table summarizes the models discussed earlier.

<table>
<thead>
<tr>
<th>Parameters in Model</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
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<td>Phenol, E, PEG, H₂O₂</td>
<td>[Phenol]</td>
<td>[Phenol], [E]</td>
<td>[Phenol], [E], [H₂O₂]</td>
<td>[Phenol], [E], [H₂O₂]</td>
</tr>
<tr>
<td>Inactivation Caused by</td>
<td>N.A.</td>
<td>N.A.</td>
<td>Radicals only</td>
<td>Phenol only</td>
</tr>
</tbody>
</table>

5.1.2 Testing of the Existing Models

**Model 1:** Experimental data could be modeled by a pseudo-first order reaction with respect to phenol concentration quite well so long as the concentrations of enzyme or hydrogen peroxide were equal to or greater than the optimal dose. The curve diverted from the first order course at the final stage when these chemicals were used up. Figure 5-1 shows an example of the model application.

When enzyme or hydrogen peroxide concentration was less than the minimum, then the kinetic expression of the final removal efficiency took the following form, which can be solved analytically:

\[
\ln \frac{[AH₂]_f - [AH₂]_0}{[AH₂]_f - [AH₂]} = kt
\]

where \(k\) is the first order constant and \([AH₂]_f\) and \([AH₂]_0\) are the final and initial concentrations of phenol, respectively.
Model 2: The concentration of hydrogen peroxide was not included in this model, but it had effect on the reaction constants (turnover number, $k_{\text{cat}}$, and inactivation constant, $k_{i}$). When initial HRP concentration changed, the model could predict the reaction very well. An example of comparison between the model output and the actual data is plotted in Figure 5-2. Different lines represent different initial HRP activities (0.065, 0.1 and 0.3 U/mL).
Figure 5-2 Example of Model 2 Fit to the Experimental Data

**Model 3:** A lot of effort was made to establish a relationship between $k_a$ and the concentration of PEG. Among the possible relations tested were the following two expressions:
\[
\frac{dk_s}{dC_{\text{PEG}}} = -kC_{\text{PEG}}, \text{ and}
\]

\[
k_s = f\left(\frac{C_{\text{PEG},0}}{C_{\text{PEG, min}}}, [\text{HRP}]_0\right)
\]

However, all these attempts proved unsuccessful. The detailed results are discussed in Section 5.4.4.2.

**Model 4:** Model 4 was also tested, but constants could not be adjusted to fit the experimental data from this study. The reason might be that this model was developed in the absence of PEG. The model always overpredicted the phenol conversion rate, thus resulting in an overall stiffness of the output curves.

### 5.1.3 Proposed Modifications

In the above analysis, it is shown that the combination of one-substrate Michaelis-Menten equation and an enzyme depletion equation could be used to model the reaction well. Thus, the proposed model could be based on this concept, i.e. one Michaelis-Menten equation plus one enzyme depletion equation as the basic equations. In order to take the concentration of hydrogen peroxide into account, a two-substrate Michaelis-Menten equation might be employed. When the Michaelis-Menten equation is used in the modeling of biological treatment of wastewater, it is a one-substrate model. The substrate inhibition on that reaction can be modeled by a modified Michaelis-Menten equation, as proposed by Andrews (1993). This suggested that the effect of hydrogen peroxide on the enzymatic model might be achieved by adding variable containing hydrogen peroxide concentration into the one-substrate Michaelis-Menten equation as shown below:

\[
-\frac{d[AH_2]}{dt} = \frac{k_{\text{cat}}[\text{HRP}]_0[AH_2]}{K_1[AH_2] + K_2}
\]

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Modifying the above equation by an item \( \frac{[AH_2]^2}{K'} \) into the equation, as in Andrews' equation, gives

\[
\frac{d[AH_2]}{dt} = \frac{k_{cat}[HRP]_0[AH_2]}{K_1[AH_2] + K_2 + \frac{[AH_2]^2}{K'}}
\]

When another parameter containing \( H_2O_2 \) is added, the equation becomes:

\[
\frac{d[AH_2]}{dt} = \frac{k_{cat}[HRP]_0[AH_2]}{K_1[AH_2] + K_2 + K'[AH_2][H_2O_2]}
\]

or

\[
\frac{d[AH_2]}{dt} = \frac{k_{cat}[HRP]_0[AH_2][H_2O_2]}{K_1[AH_2][H_2O_2] + K_2[H_2O_2] + K'[AH_2]}
\]

This is the form of a two-substrate Michaelis-Menten equation. It can be transformed into a one-substrate Michaelis-Menten equation as below:

\[
\frac{d[AH_2]}{dt} = \frac{k_{cat}[HRP]_0[AH_2]}{K_1[AH_2] + (K_2 + K' \frac{[AH_2]}{[H_2O_2]})}
\]

Let \( K_m = K_2 + K' \frac{[AH_2]}{[H_2O_2]} \)

When initial ratio is 1:1, then

\[
\frac{d[AH_2]}{dt} = \frac{k'_{cat}[HRP]_0[AH_2]}{K'_m + [AH_2]}
\]

where \( k'_{cat} = \frac{k_{cat}}{K_1}, K'_m = \frac{K_m}{K_1} \).
It was decided that the two-substrate Michaelis-Menten equation (Eq. 5-18) should be used in the new model.

For HRP inactivation, an apparent inactivation method instead of detailed kinetic pathways was used for three reasons. Firstly, the detailed pathways might not include all possible inactivation pathways. Exclusion of any essential one could be detrimental to the final model. Secondly, all inactivation pathway schemes were obtained without the presence of additives. The effects of additives on the pathways were still not known. So, it was decided to consider the reactions as a whole and estimate total inactivation by linearly summing two second-order inactivations discussed in Section 2-4. Finally, the detailed pathways involved many kinetic constants that were hard to manipulate.

5.2 MODEL DESCRIPTION

5.2.1 Assumptions

Based on the discussion in Section 5.1, the conclusions in Chapter 4 and results from another study (Wu J. et al., 1993), a new model is proposed with the following assumptions:

- With the catalysis of HRP, phenol and hydrogen peroxide are converted to polyphenolic compounds (polymers) and water, respectively. The conversion of phenol obeys the Ping-Pong Bi-Bi mechanism.

- In the presence of PEG, HRP is inactivated by polymers formed during the reaction but at a much lower rate. The inactivation is an apparently second order reaction with respect to the concentrations of HRP and phenol (Baynton et al., 1994).

- Inactivation by hydrogen peroxide takes place simultaneously with that by polymers, which is also an apparently second order reaction with respect to the concentrations of HRP and hydrogen peroxide (Baynton et al., 1994).

- Total inactivation can be estimated by simply adding the two inactivations.
- PEG is bound onto polymers with chemical bonds during reaction, while HRP inactivated by polymers is also bound on polymers (Chapter 4).
- Consumption of hydrogen peroxide is directly proportional to that of phenol. The coefficient takes the value of optimum reaction stoichiometry between hydrogen peroxide over phenol, which is available from another study (Wu J. et al., 1993).
- Consumption of PEG is also directly proportional to that of phenol. Its coefficient is the ratio between minimum PEG dose and initial phenol concentration. This also is available from the other research (Wu J. et al., 1993).

The diagrammatic representation of the sequence of events is shown below:

![Diagram](image)

Figure 5-3 Schematic Description of the Proposed Model
Note: Polymers are represented by a dimer in this scheme
HRP•PEG represents activated HRP
The reaction scheme is summarized in the following three reaction pathways, which will further be used for the description of the model in the next section:

\[
\text{Phenol} + \alpha \text{H}_2\text{O}_2 \xrightarrow{HRP, PEG} \lambda \text{Polymer} \cdot \text{PEG} + \alpha \text{H}_2\text{O}
\]

\[
\text{HRP} + \text{Polymer} \cdot \text{PEG} \xrightarrow{k_a^1} \text{HRP}_{ia,p} \text{(or, Polymer} \cdot \text{PEG} \cdot \text{HRP)}
\]

\[
\text{HRP} + \text{H}_2\text{O}_2 \xrightarrow{k_a^2} \text{HRP}_{ia,h} \text{(or, HRP}_{670})
\]

The reaction to be modeled can be considered as a two-substrate enzymatic reaction. Phenol and hydrogen peroxide act as substrates and HRP is the enzyme. The activity of the HRP increases after the addition of PEG and hydrogen peroxide as discussed in Section 4.3.3. The active form of HRP (HRP_a) includes HRP-I and HRP-II. One phenol needs $\alpha$ hydrogen peroxide to complete the polymerization. The first products of enzymatic reaction are water and phenoxy radicals. The final products of the polymerization are the polymers conjugated with PEG. Extra PEG remains in the solution. HRP is inactivated during its function as a catalyst, both by polymers and by hydrogen peroxide. The HRP inactivated by polymers is connected with polymers chemically or physically, while the HRP inactivated by hydrogen peroxide takes the form of HRP_{670}.

5.2.2 Kinetics of the Model

The pseudo-steady state differential equations used to model the HRP catalyzed polymerization of phenol in the presence of polyethylene glycol are presented on the assumptions described in the previous section.
\[
- \frac{d[\text{AH}_2]}{dt} = \frac{kE_a[\text{AH}_2][\text{H}_2\text{O}_2]}{K_1[\text{AH}_2] + K_2[\text{H}_2\text{O}_2] + [\text{AH}_2][\text{H}_2\text{O}_2]} 
\]

\[
- \frac{dE_a}{dt} = k_{a1}E_a[\text{AH}_2] + k_{a2}E_a[\text{H}_2\text{O}_2] 
\]

\[
- \frac{d[\text{H}_2\text{O}_2]}{dt} = -\alpha \frac{d[\text{AH}_2]}{dt} 
\]

\[
- \frac{dC_{\text{PEG}}}{dt} = -\beta \frac{d[\text{AH}_2]}{dt} 
\]

where \( E_a \) is the enzyme activity, and

\[
\alpha = \frac{100 - [\text{AH}_2]_0}{90} \quad \text{(Wu J. et al., 1993)} 
\]

\[
\beta = \frac{C_{\text{PEG}\text{max}}}{[\text{AH}_2]_0} 
\]

\[
C_{\text{PEG}\text{max}} = 0.0065 + 0.024[\text{AH}_2]_0 \quad \text{(Wu J. et al., 1993)} 
\]

### 5.3 Model Calibration

The difficult aspect of this model was to determine the five reaction constants involved in these differential equations, i.e. \( k, k_{a1}, k_{a2}, K_1 \) and \( K_2 \). Among them, only \( k_{a1} \) and \( k_{a2} \) were determined previously through experiments by another researcher (Baynton, 1992). These were reported as 0.023 M\(^{-1}\)s\(^{-1}\) for \( k_{a1} \) and 0.0193 M\(^{-1}\)s\(^{-1}\) for \( k_{a2} \), respectively. After transforming the units, the values were 0.0014 and 0.0012 mM\(^{-1}\) min\(^{-1}\), respectively. However, when these values were applied in the model, they seemed to be too small to reflect the effect of these two kinds of inactivations. Consequently, these values were increased to fit some of the experimental data in Section 5.4.4.2 and the final values are shown in Table 5-2. The constant values derived from the data are still of the same order of magnitude as that of the measured ones. The reason for the difference may be due to the different HRP materials used in each experiment. It may also be as a
consequence of different experimental conditions that were controlled, e.g. the absence of PEG in Baynton's experiments. The final values of these constants were determined through curve fitting using the experimental data collected in this study.

<table>
<thead>
<tr>
<th>(k_{a1} \text{ (mM}^{-1}\text{min}^{-1}))</th>
<th>(k_{a2} \text{ (mM}^{-1}\text{min}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0095 ± 0.0005</td>
<td>0.0095 ((1+N))</td>
</tr>
<tr>
<td>([\text{H}_2\text{O}_2]\geq0.001 \text{ mM})</td>
<td>([\text{H}_2\text{O}_2]\geq0.001 \text{ mM})</td>
</tr>
<tr>
<td>(\text{C}_{\text{PEG}} &gt; 0 \text{ mg/L})</td>
<td>(\text{C}_{\text{PEG}} = 0 \text{ mg/L})</td>
</tr>
</tbody>
</table>

\(N\) is related to the ratio of \(\text{C}_{\text{PEG},0}\) over \(\text{C}_{\text{PEG},\text{min}}\). The larger the ratio, the larger is the value of \(N\), which means the faster is the inactivation. When the ratio is large, the enzyme is protected for a longer time and more polymers are formed during that time. This indicates from another perspective that the more polymers present, the faster is the inactivation. Also, \(N\) value is dependent on the initial concentrations of phenol and HRP. The higher the concentrations are, the larger the \(N\) value is.

In order to determine \(k\), \(K_1\) and \(K_2\), the constant values from the one-substrate Michaelis-Menten equation (Model 1) were taken as reference with the assumption that \(k\) was approximately equal to 0.6 min\(^{-1}\), and \((K_1 + K_2)\) was approximately equal to 1 mM (see Figure 5-2). Using the trial and error method, the final values of these constants were determined as given in the Table 5-3. These values were kept constant throughout the model verification.

<table>
<thead>
<tr>
<th>Table 5-3 Second-Order Michaelis-Menten equation Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k)</td>
</tr>
<tr>
<td>0.59 min(^{-1})</td>
</tr>
</tbody>
</table>

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5.4 MODEL VERIFICATION BY EXPERIMENTAL DATA

The proposed model was verified through the experimental data collected in this study.

5.4.1 Scope of Verification

The scope of verification of this model through the experimental data collected in this study was to:

- Predict phenol removal under different initial concentrations of phenol and hydrogen peroxide with different initial enzyme activities;
- Determine PEG effect on the model; and
- Predict changes in enzyme activity with time;

5.4.2 Kinetic Data Collection Procedure

The model verification included the collection of kinetic data under different reaction conditions and the comparison of these data with the model output. All data were collected from batch reactors as described in Chapter 3 under different matrices of reactants presented in Table 5-4. The pH value of buffer solutions was always set at 8. Initial concentrations of phenol and activity were measured as the reference. Hydrogen peroxide was added to initiate the reaction. One milliliter aliquots of reaction mixture were taken at different time intervals and mixed with catalase prepipetted in centrifuge tubes. After centrifugation, remaining phenol concentrations were measured with the ferricyanide colorimetric method.

<table>
<thead>
<tr>
<th>Table 5-4 Reactant Concentration Ranges for Data Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model Variable</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
</tr>
<tr>
<td>H₂O₂</td>
</tr>
<tr>
<td>PEG</td>
</tr>
<tr>
<td>HRP</td>
</tr>
</tbody>
</table>
5.4.3 Calculation Procedure

A simple numerical program was developed to solve the differential equations in the model. Initial concentrations of phenol, HRP, PEG and hydrogen peroxide were input to start the computations. Constants were set at the values in Table 5-2. Then, the amount of phenol converted and HRP inactivated in the time period of $\Delta t$ were computed and new concentrations of phenol and enzyme at time $t+\Delta t$ were computed. Constant $k_{a1}$ had to be determined for each step of the calculation. The decrease in hydrogen peroxide and PEG was also computed according to the values of $\alpha$ and $\beta$. The step calculations continued until the time was greater than the experiment duration. The flow chart for the calculation procedure is shown in Figure 5-4.

5.4.4 Results and Discussions

5.4.4.1 Effect of Changing Hydrogen Peroxide Concentration

Because the major parameter in this model was hydrogen peroxide, efforts were made first on the curve-fitting and constant-estimation with this variable. Experimental results are plotted in Figures 5-5 to 5-7 for different experimental conditions and constants were obtained from curve-fitting. These constants were applied in the subsequent analysis. Among them, $k$, $K_1$, $K_2$ were kept unchanged, while $k_{a1}$ and $k_{a2}$ were adjusted in specific cases.

The reported values for $k_{a1}$ and $k_{a2}$ (Baynton, 1992) were utilized at the beginning of the curve-fitting. However, these values were found to be too small to emulate the actual effect of hydrogen peroxide. Therefore, the values of these two constants were increased so that the output from the model could fit the data obtained from the experiments. The value of $k_{a1}$ was between 0.009 and 0.01 mM$^{-1}$min$^{-1}$, while the value of $k_{a2}$ was between 0.005 and 0.006 mM$^{-1}$min$^{-1}$. 

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START

SET:
\( k, K_1, K_2, k_{a2} \)
and \( \Delta t \)

INPUT:
Initial [Phenol], [peroxide]
[PEG] and \( E_a \)

CALCULATE:
\( C \) PEG, min
Alpha, Beta

DETERMINE:
\( k_{a1} \) (Table 5-2)

CALCULATE:
\( \Delta [\text{Phenol}] \) \( E_a \) \( \Delta [\text{PEG}] \) and \( \Delta [\text{Peroxide}] \)

CALCULATE:
[Phenol], \( E_a \), [PEG]
and [Peroxide]
at time \( t + \Delta t \)

END

Figure 5-4 Flowchart of the Program Solving the Differential Equations
Figure 5-5 Curve-Fitting Results for the Effect of H\textsubscript{2}O\textsubscript{2} (1 mM Phenol)

<table>
<thead>
<tr>
<th>Legends</th>
</tr>
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<tbody>
<tr>
<td>H\textsubscript{2}O\textsubscript{2} mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Designed Experimental Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>

Initial Activity Measured: - 0.12 U/mL
Initial Phenol Concentration Measured: 0.95 mM

<table>
<thead>
<tr>
<th>Constant Value from Curve-Fitting</th>
</tr>
</thead>
<tbody>
<tr>
<td>k</td>
</tr>
<tr>
<td>min\textsuperscript{-1}</td>
</tr>
<tr>
<td>0.59</td>
</tr>
</tbody>
</table>
Figure 5-6 Curve-Fitting Results for the Effect of $\text{H}_2\text{O}_2$ (2 mM Phenol)

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<tr>
<td>H$_2$O$_2$ mM</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<table>
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<tbody>
<tr>
<td>pH</td>
<td>Phenol</td>
</tr>
<tr>
<td>----</td>
<td>--------</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

Initial Activity Measured: - 0.14 U/mL
Initial Phenol Concentration Measured: 1.9 mM

<table>
<thead>
<tr>
<th>Constant Value from Curve-Fitting</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$k$</td>
<td>$k_1$</td>
</tr>
<tr>
<td>min$^{-1}$</td>
<td>mM</td>
</tr>
<tr>
<td>0.59</td>
<td>0.11</td>
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</table>
Figure 5-7 Curve-Fitting Results for the Effect of \( \text{H}_2\text{O}_2 \) (3 mM Phenol)

**Legends**

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<th>( \text{H}_2\text{O}_2 ) mM</th>
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<th>2.5</th>
<th>2.7</th>
<th>3.3</th>
<th>4.0</th>
<th>4.5</th>
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</table>

**Designed Experimental Conditions**

<table>
<thead>
<tr>
<th>pH</th>
<th>Phenol mM</th>
<th>HRP U/mL</th>
<th>( \text{H}_2\text{O}_2 ) mM</th>
<th>PEG mg/L</th>
<th>Aliquot Vol. µL</th>
<th>Catalase Vol. µL</th>
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</thead>
<tbody>
<tr>
<td>8</td>
<td>3</td>
<td>0.10</td>
<td>2.0 - 4.5</td>
<td>100</td>
<td>1000</td>
<td>300 - 600</td>
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</tbody>
</table>

Initial Activity Measured: 0.12 U/mL
Initial Phenol Concentration Measured: 0.95 mM

**Constant Value from Curve-Fitting**

<table>
<thead>
<tr>
<th>( k )</th>
<th>( k_1 )</th>
<th>( K_1 )</th>
<th>( K_2 )</th>
<th>( k_{31} )</th>
<th>( k_{22} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm^{-1}</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mm^{-1}</td>
<td>mm^{-2}</td>
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<td>0.50</td>
<td>0.11</td>
<td>0.90</td>
<td>0.0090</td>
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Comparisons between the output from the computer model and the experimental data are shown in Figures 5-5 to 5-7. These figures clearly indicate that the model developed in this study can reflect the effect of hydrogen peroxide on the reaction under a variety of experimental conditions. Generally, there is an excellent agreement over a wide range of hydrogen peroxide concentrations for different initial phenol concentrations. The excellent prediction of the final phenol removal efficiency, with the concentrations of hydrogen peroxide lower than the optimum, demonstrated that the assumption on the stoichiometry between hydrogen peroxide and phenol is correct. With hydrogen peroxide concentration lower than the optimum, the reaction stopped at the phenol concentration corresponding to the concentration of hydrogen peroxide multiplied by a ratio of $\alpha$. Also, the removal efficiency of phenol deteriorated in the presence of extra hydrogen peroxide, obviously, as a consequence of the inactivation effect of hydrogen peroxide on HRP. In the process of model development, it was assumed that the inactivation of HRP by hydrogen peroxide was a second-order reaction with respect to the concentrations of hydrogen peroxide and enzyme. The good agreement between the model output and the data under the conditions of extra hydrogen peroxide indicates that this assumption is valid. Therefore, the optimum ratio between hydrogen peroxide and phenol is the value of $\alpha$, which is dependent on the initial phenol concentration.

The largest deviations between the model outputs and the observed data occurred at the initial stage of the conversion in cases where hydrogen peroxide concentrations were less than the optimum. Under these conditions, the model outputs were found to be less than the actual removal. Usually, the lower the hydrogen peroxide concentration, the faster was the phenol conversion rate. It explains the effectiveness of semi-batch addition of hydrogen peroxide (Wu J. et al., 1994). This tendency might be due to the overestimation of the inactivation of enzyme by hydrogen peroxide at the initial stage. One of the assumptions made in the model was that the inactivations caused by hydrogen peroxide and polymers could be added together linearly. The comparison between the
model output and the data suggests that this assumption might have been partially correct. The hydrogen peroxide added might be utilized only for the phenol conversion and not for the inactivation. When hydrogen peroxide concentration was less than the optimum, there might be a certain threshold concentration of hydrogen peroxide at which inactivation by hydrogen peroxide became effective. However, it is hard to determine this value below which it does not cause inactivation.

It is interesting to notice the results in Figure 5-5, in which extra hydrogen peroxide seems to have little effect on the removal of phenol. Actually, this episode occurred as the consequence of an initially high concentration of enzyme. The initial concentration of HRP used in the experiment was 0.1 U/mL, which was double the amount of the minimum HRP dose (0.05 U/mL) needed to complete the phenol removal at the phenol concentration of 1 mM. This result indicates that the semibatch addition might have little effect on the phenol removal efficiency when the initial HRP concentration is high. In fact, the model could predict that hydrogen peroxide inactivation effect was more obvious at the HRP concentration of 0.05 U/mL. This is further discussed in Section 5-5.

5.4.4.2 Effect of Changing PEG Concentration

As stated in Section 5.1.2, a lot of effort was made to establish a relationship between inactivation constant $k_{a1}$ and PEG concentration. After all these attempts failed, it was realized that the reaction was not first-order with respect to phenol concentration in the absence of sufficient PEG. The presence of PEG in solution did not affect the phenol conversion rate but it did affect the value of $k_{a1}$.

The model assumed that PEG was bound on polymers during the reaction. When PEG concentrations were less than the minimum, the initial reaction rates were identical to those obtained with sufficient PEG. Thus, the values of $k_{a1}$ were also identical. PEG would be used up sometime during the reaction as PEG concentration was less than
the minimum. As soon as PEG disappeared in the solution, the phenol conversion rates would rapidly approach zero. This also meant that the $k_{a1}$ value without PEG would be much greater than that with PEG. The experimental data confirmed that PEG consumption was directly proportional to the phenol removed. For example, in Figure 5-8 when PEG was 10 mg/L, the reaction curve deviated from the curve with excess PEG after about 50 percent removal of the phenol. Similarly, when the PEG dose was 5 mg/L, the deviation occurred after about 25 percent removal. This gives 20 mg/L of PEG consumption per mM phenol removed, which is the minimum PEG dose for 1 mM phenol. It should be recognized that this value would change with change in phenol concentration. Figures 5-9 and 5-10 also confirm this conclusion when HRP concentration was increased from 0.065 to 0.13 and 0.3 U/mL. The results demonstrate that this phenomenon is independent of the initial HRP dose. Similarly, this phenomenon was not affected by the initial phenol concentrations, as shown in the Appendix C. The results also confirmed that excess PEG in the solution could not improve the reaction performance.

However, the initial concentrations of PEG and HRP did have an influence on the values of $k_{a1}$ after PEG was used up. The higher the initial concentration of HRP, the higher was the value of $k_{a1}$. In the same manner, the higher the initial concentration of PEG, the higher was the $k_{a1}$ value after PEG was used up. These results are contrary to Model 3. This can further explain that as more polymers were formed, more enzyme was inactivated, because more polymers were formed when the concentrations of phenol and HRP were high. The experimental data also suggest that minimum PEG dose can better be determined by the kinetic method because it is not affected by the HRP dose used.

5.4.4.3 Effect of Changing HRP Concentration

The effect of HRP concentration on the model is important because the degree of the fit of experimental data to model output indicates whether or not the values for model constant $k$, and $k_{a1}$, $k_{a2}$ are acceptable.
Figure 5-8 Curve-Fitting Results for the Effect of PEG (0.05 U/mL)

Legends

| PEG mg/L | 5 | 10 | 20 - 100 |

Designed Experimental Conditions

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1</td>
<td>0.05</td>
<td>1.1</td>
<td>0 - 100</td>
<td>1000</td>
<td>250</td>
</tr>
</tbody>
</table>

Initial Activity Measured: 0.065 U/mL
Initial Phenol Concentration Measured: 0.95 mM

Constant Value from Curve-Fitting

<table>
<thead>
<tr>
<th>k₁</th>
<th>K₁</th>
<th>K₂</th>
<th>kᵢ₁</th>
<th>kᵢ₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm⁻¹</td>
<td>mM</td>
<td>mM</td>
<td>mM⁻¹mm⁻¹</td>
<td>mM⁻²mm⁻¹</td>
</tr>
<tr>
<td>0.59</td>
<td>0.11</td>
<td>0.90</td>
<td>0.0100</td>
<td>0.0060</td>
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</table>
Figure 5-9 Curve-Fitting Results for the Effect of PEG (0.1 U/mL)

### Legends

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<th>PEG (mg/mL)</th>
<th>□</th>
<th>×</th>
<th>▲</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>20 - 100</td>
<td></td>
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</table>

### Designed Experimental Conditions

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<tr>
<th>pH</th>
<th>Phenol (mM)</th>
<th>HRP (U/mL)</th>
<th>H₂O₂ (mM)</th>
<th>PEG (mg/mL)</th>
<th>Aliquot Vol.</th>
<th>Catalase Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1</td>
<td>0.10</td>
<td>1.1</td>
<td>0 - 100</td>
<td>1000</td>
<td>250</td>
</tr>
</tbody>
</table>

- Initial Activity Measured: 0.13 U/mL
- Initial Phenol Concentration Measured: 0.95 mM

### Constant Value from Curve-Fitting

<table>
<thead>
<tr>
<th>k (min⁻¹)</th>
<th>K₁ (mM)</th>
<th>K₂ (mM)</th>
<th>kₐ₁ (mM/min⁻¹)</th>
<th>kₐ₂ (mM/min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.59</td>
<td>0.011</td>
<td>0.006</td>
<td>0.0100</td>
<td>0.0060</td>
</tr>
</tbody>
</table>
Figure 5-10 Curve-Fitting Results for the Effect of PEG (0.3 U/mL)

Legends

| PEG mg/L | 5  | 10  | 20–100 |

Designed Experimental Conditions

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>mM</td>
<td>U/mL</td>
<td>mM</td>
<td>mg/L</td>
<td>pL</td>
<td>pL</td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0</td>
<td>1.1</td>
<td>0–100</td>
<td>1000</td>
<td>250</td>
</tr>
</tbody>
</table>

Initial Activity Measured: - 0.30 U/mL
Initial Phenol Concentration Measured: 0.95 mM

Constant Value from Curve-Fitting

<table>
<thead>
<tr>
<th>k</th>
<th>K₁</th>
<th>K₂</th>
<th>kₐ₁</th>
<th>k₂₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>min⁻¹</td>
<td>mM</td>
<td>mM</td>
<td>mM⁻¹</td>
<td>mM⁻⁴ min⁻¹</td>
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<tr>
<td>0.59</td>
<td>0.11</td>
<td>0.96</td>
<td>0.0100</td>
<td>0.0060</td>
</tr>
</tbody>
</table>
The comparison of results is shown in Figures 5-11 to 5-13, which also include the experimental conditions and constants used in curve-fitting. The constants $k$, $K_1$, $K_2$ were kept the same as those used previously, while $k_{a1}$ and $k_{a2}$ were adjusted in each specific case. These figures clearly demonstrate that the model developed in this study reflects the effect of HRP on the reaction. In general, there is an excellent agreement over a wide range of HRP concentrations for different initial phenol concentrations. The excellent agreement in the situations of higher concentrations of HRP indicates that kinetic constant $k$ used in the model was reasonable. Without enough HRP, phenol was not converted completely. The higher the initial concentration of HRP, the faster was the phenol conversion. For instance, at 1 mM phenol, it needed more than 3 hours to reach 95 percent conversion if the initial concentration of HRP was 0.065 U/mL (Figure 5-11). In contrast, it needed only about one hour if the initial concentration of HRP was doubled (Figure 5-11). Thus, an increase in HRP concentration can greatly reduce the time for the completion of the reaction. Nevertheless, the higher the concentration of enzyme, the higher was the enzyme inactivation because the inactivation of HRP was directly proportional to the concentration of enzyme both in the polymers-inactivation and hydrogen peroxide-inactivation. Thus, the enzyme turnover was correspondingly lower when enzyme concentration was high. This can explain why semi-batch addition of HRP did not improve enzyme turnover when additive was present because the highest turnover had already been achieved.

The largest deviations in data fit appeared when the initial concentration of HRP was low under all three initial phenol concentrations. This was particularly significant for higher concentrations of phenol. These might be due to fixed values of $K_1$ and $K_2$, which were kept identical during the entire model simulation. It is evident from the model that the curve shape can be modified by varying the values of $K_1$ and $K_2$. This suggests that the estimation of the values for $K_1$ and $K_2$ should be improved further.
Figure 5-11 Curve-Fitting Results for the Effect of HRP (1 mM Phenol)

<table>
<thead>
<tr>
<th>Legends</th>
</tr>
</thead>
<tbody>
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<td>■</td>
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</tbody>
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<table>
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<tr>
<th>Designed Experimental Conditions</th>
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</thead>
<tbody>
<tr>
<td>----</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Initial Activity Measured: 0.065, 0.13, 0.30 U/mL.
Initial Phenol Concentration Measured: 0.95 mM

Constant Value from Curve-Fitting

<table>
<thead>
<tr>
<th>k</th>
<th>k₁</th>
<th>k₂</th>
<th>k₃₁</th>
<th>k₃₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm⁻¹</td>
<td>mM</td>
<td>mm</td>
<td>mM⁻¹</td>
<td>mM⁻¹</td>
</tr>
<tr>
<td>0.59</td>
<td>0.11</td>
<td>0.90</td>
<td>0.0100</td>
<td>0.0060</td>
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</tbody>
</table>
Figure 5-12 Curve-Fitting Results for the Effect of HRP (2 mM Phenol)

<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>0.081</td>
<td>0.15</td>
<td>0.32</td>
<td></td>
<td></td>
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</table>

**Designed Experimental Conditions**

<table>
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<th></th>
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<tr>
<td>2</td>
<td>2</td>
<td>0.081</td>
<td>0.15</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Initial Activity Measured: -0.081, 0.15, 0.32 U/mL
Initial Phenol Concentration Measured: 1.9 mM

**Constant Value from Curve-Fitting**

<table>
<thead>
<tr>
<th>k</th>
<th>K₁</th>
<th>K₂</th>
<th>k₁</th>
<th>k₂</th>
</tr>
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<td>mM⁻¹min⁻¹</td>
<td>mM⁻²min⁻¹</td>
</tr>
<tr>
<td>0.59</td>
<td>0.11</td>
<td>0.90</td>
<td>0.0100</td>
<td>0.0060</td>
</tr>
</tbody>
</table>
Figure 5-13  Curve-Fitting Results for the Effect of HRP (3 mM Phenol)

<table>
<thead>
<tr>
<th>pH</th>
<th>Phenol (mM)</th>
<th>HRP (U/mL)</th>
<th>H$_2$O$_2$ (mM)</th>
<th>PEG (mg/mL)</th>
<th>Aliquot Vol. (µL)</th>
<th>Catalase Vol. (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>3</td>
<td>0.11–0.34</td>
<td>3.3</td>
<td>100</td>
<td>1000</td>
<td>506</td>
</tr>
</tbody>
</table>

Initial Activity Measured: -0.11, 0.18, 0.34 U/mL
Initial Phenol Concentration Measured: 2.85 mM

<table>
<thead>
<tr>
<th>Constant Value from Curve-Fitting</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k$ (min$^{-1}$)</td>
</tr>
<tr>
<td>0.59</td>
</tr>
</tbody>
</table>
5.4.4.4 Prediction of Activity Depletion

One of the purposes of the model was to predict the activity depletion during the reaction. Therefore, activity data collected from experiments were compared with the model output to examine the fit of the model. The first test was done for a time period of 120 minutes at the phenol concentrations of 1.8 and 2.7 mM. The hydrogen peroxide concentration was a little more than the optimal stoichiometry. The corresponding phenol removal during this time was more than 90 percent.

The initial enzyme activities added to the reactor were 0.082 and 0.11 U/mL, respectively. However, the activities measured within 30 seconds were 0.14 and 0.20 U/mL, respectively, and these values were used as the initial activities in the model simulation. Experimental results and the model outputs together with the corresponding reaction conditions are presented in Figure 5-14. The comparison shows a good agreement between the measured data and the output from the model calculations. The model outputs clearly demonstrate an activity depletion in the entire course of phenol removal. In spite of the good agreement, the accuracy of the prediction is not as good as that for the phenol removal. The larger variations between the data and the outputs may be attributed partially to the larger deviations in the activity determinations than in the phenol determinations.

In order to examine if the model would work well under different initial concentrations of the reactants, experiments were carried out under different initial conditions, i.e. some of the initial concentrations of phenol, hydrogen peroxide, and HRP activity were changed while others were fixed. The experiments were conducted under the following four different conditions:

- changing the initial concentration of phenol only
- changing the initial concentration of hydrogen peroxide only
- changing the initial concentration of HRP only
- changing the initial concentration of phenol, hydrogen peroxide and HRP
Figure 5-14 Comparison of the Activity Data with the Model Output

The results are shown in Figures 5-15 to 5-18. In all these figures, results exhibit a reasonably good agreement between the model output and the data collected under a variety of initial reaction conditions. This confirms that the model was appropriate to predict the activity changes during the reaction. This also verifies, from another perspective, the assumption that enzyme was inactivated by both polymer and hydrogen peroxide at the same time. Figure 5-15 and 5-16 also show that the initial activities were different for the same amount of HRP if the initial phenol or hydrogen peroxide concentration was different. The initial activity increased with an increase in their values. However, the inactivation at higher initial phenol and hydrogen peroxide concentrations was also higher and the final activity remaining was lower. This is further discussed in the next section.
Figure 5-15 Comparison of the Activity Data with the Model Output with Different Initial Phenol Concentration

Experimental Condition:
- HRP added = 0.1 U/mL
- Peroxide : Phenol = 1:1
- PEG = 100 mg/L

Figure 5-16 Comparison of the Activity Data with the Model Output with Different Initial Hydrogen Peroxide Concentration

Experimental Condition:
- H2O2 mM
- HRP: 0.085 U/mL
- Phenol: 2.7 mM
- PEG: 100 mg/L

Legend:
- 1
- 2
- 3
- 4
Figure 5-17 Comparison of the Activity Data with the Model Output with Different Initial HRP Concentration

Figure 5-18 Comparison of the Activity Data with the Model Output with Different Initial HRP, Phenol and Hydrogen Peroxide Concentration
5.5 MODEL VERIFICATION BY INDEPENDENT STUDY

It has been shown earlier that the model can predict theoretically the values obtained for different scenarios. This model was further verified with actual experimental results from a separate study. The calculated values from the present model were in excellent agreement with the published experimental data of Wu J. *et al.* (1993, 1994). The agreement was consistent with respect to all reaction parameters such as optimum hydrogen peroxide ratio, minimum HRP dose, and the effect of PEG. It also reproduced the effect of HRP on the reaction rate. For example, when phenol concentration was set at 1 mM and 30 mg/L PEG was used as the minimum dose, the model predicted that the optimal hydrogen peroxide concentration was 1.1 mM and the minimum HRP dose was about 0.05 U/mL (this value did not include the effect of PEG). The comparison between model outputs and the reported data is illustrated in Figures 5-19 and 5-20.

Figure 5-19 Comparison between Model Output and the Reported Data (Wu J. *et al.*, 1993), Effect of Hydrogen Peroxide on the Phenol Removal
Figure 5-20 Comparison between Model Output and the Reported Data (Wu J. et al., 1993), Effect of HRP Dose on the Phenol Removal

In Figure 5-19, the model accurately predicted the hydrogen peroxide effect on the phenol removal efficiency at the minimum HRP dose for 1 mM phenol. Such prediction for the effect of HRP dose and hydrogen peroxide on the reaction is considered satisfactory.

It was reported in Section 5.4.4.1 that when HRP dose was twice the minimum dose at 1 mM phenol, extra hydrogen peroxide had reduced the removal efficiency slightly as shown in Figure 5-5. The model successfully predicted that the same amount of extra hydrogen peroxide would reduce the removal efficiency considerably when minimum HRP dose was used as shown in Figure 5-19. The model also successfully predicted the minimum HRP dose (without the effect of PEG) for 95 percent removal as shown in Figure 5-20. In all comparisons, not only did the model predict correctly the final removal efficiency, but also the reaction rate was similar.
The model can also simulate the effect of semi-batch addition of hydrogen peroxide on the phenol removal efficiency with time course. Wu J. et al. (1994) reported that semibatch addition of hydrogen peroxide could increase HRP turnover if phenol concentration was higher than 3 mM, whereas semibatch addition of HRP had no effect. The higher the phenol concentration, the more obvious was such effect. This behavior can be well reproduced by the model as shown in Section 6.2.2.1 later.

5.6 INITIAL ACTIVITY SUB-MODEL

In the absence of PEG, the initial enzyme activity remained the same as the measured one before the reaction. Because of the presence of PEG, the initial HRP activities had increased after the reaction was initiated by hydrogen peroxide. Therefore, all the initial values of activities used in the model were those obtained from actual measurements. It was essential to establish a sub-model that could predict the initial activity increase if the final model was to predict the activity change with time. Such a prediction is particularly essential when the model is to be used to evaluate the outcome of semi-batch operation with regard to hydrogen peroxide or HRP. The initial activity increase due to the presence of PEG was found to be related to the initial concentrations of HRP, phenol and hydrogen peroxide. Experiments were carried out to investigate the possible relationships. To determine the significance of phenol concentration on the activity increase, a certain amount of HRP was pipetted into different culture tubes and the initial activities associated with different initial phenol concentrations were measured. The hydrogen peroxide used was equimolar with the phenol concentration. Experimental data (Figure 5-21) show that the initial activity increase was related to both the initial phenol concentration and the original amount of HRP added. The higher the initial concentrations of phenol and HRP, the higher was the increase in initial activity.
Figure 5-21  Effect of HRP and Phenol on the Activity Increase

The increase was directly proportional to the initial HRP activity added and the constant of proportionality, $k_{ia}$, depended on phenol concentration. However, the increase due to the phenol concentration had an upper limit. According to Figure 5-22, the increase in constant $k_{ia}$ can be expressed as the function of phenol concentration according to the following relationship:

$$k_{ia} = \frac{1.7\times[\text{Phenol}]}{0.3+[\text{Phenol}]} \quad (1 \text{ mM} < [\text{Phenol}] < 10 \text{ mM})$$

5-28

Based on the experimental results presented above and discussions in Chapter 4, it is obvious that several factors influenced the activity increase, the most significant being the hydrogen peroxide concentration, the phenol concentration and the HRP amount present. The presence of PEG also had an effect as shown in Section 4.3.1.1. After statistical analysis of data, the following activity increase sub-model is proposed.
Figure 5-22 Phenol Effect on the Activity Increase Constant $k_{ia}$

\[ k_{ia} = \frac{1.7 \times [\text{PHENOL}]}{0.3 + [\text{PHENOL}]} \]

Phenol Concentration (mM)

---

\[ E_{a0} = f([\text{HRP}]_0,[\text{AH}_2]_0,[\text{H}_2\text{O}_2]_0,C_{\text{PEG}}) \]

This can be further separated into four different groups:

\[ E_{a0} = f(C_{\text{PEG}})f([\text{HRP}]_0)f([\text{AH}_2]_0)f([\text{H}_2\text{O}_2]_0) \]

Let \( f(C_{\text{PEG}}) = \Phi \),

\[ f([\text{AH}_2]) = \frac{k_A[\text{AH}_2]_0}{K_A + [\text{AH}_2]_0} \], and

\[ f([\text{H}_2\text{O}_2]) = K_H + k_H \frac{[\text{H}_2\text{O}_2]_0}{[\text{AH}_2]_0} \]

from the experimental results in Figure 5-16.

Then, the final activity sub-model can be expressed as:
\[ E_{a0} = \Phi \gamma [\text{HRP}]_0 \frac{k_A [AH_2]_0}{K_A + [AH_2]_0} (K_H + k_H \frac{[H_2O_2]_0}{[AH_2]_0}) \]

where

\[ \gamma = \frac{125U/ml}{0.0025mM} = 50000(U/ml/mM) \quad \text{(assume HRP = 40000)} \]

\[ \Phi = 1.2 \pm 0.05 \quad \text{(Based on the results in Section 4.3.1.1.)} \]

\[ k_A = 1.8 \pm 0.2, \quad K_A = 0.4 \pm 0.1 \, \text{mM} \quad \text{(Based on the results in Figure 5-22)} \]

\[ k_H = 0.15 \pm 0.01, \quad K_H = 0.85 \pm 0.05 \quad \text{(Based on the results in Figure 5-16)} \]

(Note: The unit for all concentrations in brackets is mM)

In summary, the model proposed in this study (Equations 5-21 to 5-27), together with the activity sub-model (Equation 5-31) can be used to simulate the reaction of the HRP catalyzed polymerization of phenol with different initial reaction conditions. The input units for HRP, phenol and hydrogen peroxide concentrations are in mM and initial concentration of PEG is in mg/L. The model can predict both the phenol removal efficiency and the activity change with time, which can be used to calculate enzyme turnover. In the next chapter, it is shown that the model can be used not only in batch reactors, but also in semi-batch, plug flow and continuous stirred reactors.

5.7 SENSITIVITY ANALYSIS OF THE MODEL CONSTANTS

There are five constants involved in this model. Among them, \( k \) is the first order reaction constant that determines the overall reaction rate. The inactivation constants, \( k_{a1} \) and \( k_{a2} \), control the enzyme depletion rate and indirectly affect the reaction rate. \( K_1 \) and \( K_2 \) are the half-saturation concentrations of the substrates (see Abbreviations) that define the shape of the overall reaction curve. The values determined for \( k_{a1} \) and \( k_{a2} \) in this model were 0.0095 ± 0.0005 mM⁻¹min⁻¹ and 0.0055 ± 0.0005 mM⁻¹min⁻¹, respectively.
The model analysis in Figure 5-23 shows that the maximum difference in the model output was less than 5 percent when \( k_{a1} \) and \( k_{a2} \) values were changed from the mean values (curve 1) to the lower end (curve 5) or the higher end (curve 4) of the range. This variation is equal to that caused by 7 percent change in the initial HRP activity.

![Graph showing phenol fraction remaining over time](image)

**Model Conditions:**

HRP = 0.14 U/mL, Phenol = 2 mM, \( \text{H}_2\text{O}_2 \) = 2.2 mM, PEG = 100 mg/L

<table>
<thead>
<tr>
<th>Plot</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{a1} )</td>
<td>0.0095</td>
<td>0.01</td>
<td>0.009</td>
<td>0.009</td>
<td>0.01</td>
</tr>
<tr>
<td>( k_{a2} )</td>
<td>0.0055</td>
<td>0.0055</td>
<td>0.0055</td>
<td>0.005</td>
<td>0.006</td>
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</tbody>
</table>

**Figure 5-23 Variations Caused by Changing Inactivation Constants**

Considering that \( k_{a1} \) and \( k_{a2} \) were the only constants whose values varied during the modeling and that the measured initial activity values had certain analytical errors, the values determined from curve-fitting for \( k_{a1} \) and \( k_{a2} \) are considered to be reasonable.
Although the range for $k_{a2}$ is greater than 5 percent, it is still in the same order of magnitude as the value measured by Baynton (1992). The model analysis also showed that a ten percent variation in the value of $k$ was equivalent to the variations of $k_{a1}$ and $k_{a2}$ in their ranges. This indicates that the model output was not sensitive to the change in the $k$ value.

The constants $K_1$ and $K_2$ not only define the shape of the reaction curves but also affect the removal efficiency. The effect of one tenth to ten fold changes in $K_1$ and $K_2$ on the model output is illustrated in Figure 5-24. The results show that the model is much more sensitive to the change in $K_2$ than $K_1$.

![Graph showing phenol fraction remaining over time with different plots for varying $K_1$ and $K_2$ values.]

**Model Conditions:**
- HRP = 0.14 U/mL
- Phenol = 2 mM
- $H_2O_2$ = 2.2 mM
- PEG = 100 mg/L

<table>
<thead>
<tr>
<th>Plot</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$K_1$</td>
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<td>$K_2$</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
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</tbody>
</table>

*Figure 5-24 Variations Caused by Changing $K_1$ and $K_2*
5.8 SUGGESTED IMPROVEMENTS IN THE MODEL

All the merits of the present model have been discussed in the previous sections. However, it is still possible to make further improvements in it. First of all, the determination of constants in the model was done by curve-fitting. It should be possible to estimate these constants from experimental data. Attempts were made to do so but failed because of the difficulty in determining the initial reaction rates. Therefore, efforts should be made to find an appropriate method of determining the constants through experiments. The values of $k_{a1}$ and $k_{a2}$ should become smaller with an increase in initial phenol concentration because more HRP was saved when the initial phenol concentration was higher (Wu J. et al., 1993).

Another concern about the model is the fact that phenol conversion was faster when the initial concentration of hydrogen peroxide was less than the optimal. It may be speculated that most of the hydrogen peroxide in the solution did not inactivate enzyme when its concentration was lower than the optimal value. Efforts should be made to find the mechanism for the inactivation caused by hydrogen peroxide in the presence of phenol.

Finally, all initial activity values used in the modeling were measured within 30 seconds of the reaction because of the initial increase of activity due to the addition of PEG. Although a sub-model has been developed to predict such increase, the accuracy of these predictions was far from satisfactory. These fluctuations may be attributed to several factors such as temperature and other chemicals present in the solution. The real difficulty maybe dwells in the timing for such measurement. A more systematic study should be conducted on this aspect.

It should be appreciated that this model can be simplified to Model 2 if the hydrogen peroxide concentration is fixed and it can be simplified further to Model 1 by assuming a first order constant related to the hydrogen peroxide concentration and HRP activity.
NOTE TO USERS

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that are to be changed over to continuous operation after a more advanced stage of
development (Denbigh and Turner, 1984; Butt, 1980).

The advantages of adopting continuous flow system in large scale chemical
industries are:

- Diminished labor costs owing to the elimination of operations such as the repeated
  filling and emptying of batch vessels.
- The facilitation of automatic control.
- Greater stability in reaction conditions and hence greater consistency in the quality of
  product.

Plug-flow reactors (PFR) have the same reaction efficiency curve as batch
reactors, but the compositions in the plug flow systems change in the space co-ordinate. It
is assumed that no axial mixing occurs in the vessel and the liquid passes through the
reactor as a discrete fluid element. It is a type of continuous flow reactor in which there is
a steady movement of one or all of the reagents in a chosen spatial direction. The reagents
enter at one end of the system and leave at the other with no induced mixing between
elements of fluid at different points along the direction of flow.

In continuous stirred tank reactors (CSTR), the reactor vessel is assumed to be
perfectly mixed such that the concentration of components in the outlet stream is equal to
that in the bulk of the vessel. This type of reactor consists of a well-stirred tank into which
there is a continuous flow of reacting material and from which the reacted material flows
out continuously. Its advantages over the plug flow system are its cheaper cost, simplicity
of construction, better temperature control and ease of cleaning the internal surfaces of the
reactor.

Modification can be made in these reactors if any one reactant is added
continuously into the system during the time or space course of the reaction. Semi-batch
reactors are stirred tank reactors in which there is a non-steady flow through the system.
In their normal application, one or more reactants are contained initially in the tank and a
selected reactant is continuously added into it, with no flow out of the vessel. It is assumed that perfect mixing occurs in the reactors, also the reaction volume changes with time of operation due to the introduction of the reactant. The method of processing is under unsteady state.

In real reactors, deviations from the ideal flow may occur due to channeling in the reactor, dead volume within the reactor and bypassing through the reactor (Denbigh and Turner, 1984). Separation of the precipitate formed during the reaction has the same importance as the reactor design itself because it can be time-consuming and costly.

In this chapter, different reaction scenarios are examined initially using the model developed in Chapter 5. Then, different types of reactors are evaluated and compared with one another under the same concentrations of reaction components. The computer model is applied to compare its outputs with the experimental results to confirm the model application of PFR and CSTR systems. Finally, a reaction system is recommended for the general design.

6.2 REACTOR CONFIGURATION AND OPERATION

6.2.1 Batch Reactor Operation

Parameters affecting the batch operation have been discussed previously in Chapter 4. These include pH, reaction stoichiometry between hydrogen peroxide and phenol, use of additives and HRP dose. The results showed that phenol derivatives were removed from solution by HRP catalyzed polymerization in the presence of PEG, gelatin or PEs. All batch studies in Chapter 4 were carried out with mixing as described in Figure 3-1. In this phase of the study, the model predictions for different reaction conditions are discussed. Then, the effects of mixing and recycling of the precipitate are examined.
6.2.1.1 Prediction of the Reaction under Different Scenarios

As mentioned in the introduction of Chapter 5, an acceptable model must satisfy boundary conditions based on the combination of various reaction parameters. These conditions include insufficient or excess concentration of any of the reactants involved.

The phenol polymerization reaction catalyzed by HRP is influenced not only by HRP but also by hydrogen peroxide and PEG. Several possible scenarios and the corresponding experimental limits, based on previous studies, are tabulated in Table 6-1. As reported in Chapter 4, none of the reactants were left in reaction solution after separation of polymer products if the minimum amounts of HRP, hydrogen peroxide and PEG were used. If the concentration of any reactants (except phenol) was less than the minimum required, the phenol was not removed completely. Excess HRP speeded up the reaction and some activity was left in the solution. On the other hand, extra hydrogen peroxide reduced the phenol removal efficiency and extra PEG made no further improvement.

All expected reactions listed in Table 6-1 are predictable from the model. The model outputs for all seven scenarios are illustrated in Figure 6-1. If the line of the first scenario is defined as the baseline, then it is clearly shown that phenol removal efficiency is lower than the baseline without enough HRP (Scenario 2), or with excess hydrogen peroxide (Scenario 6). On the other hand, the reaction line goes below the baseline if excess HRP is used (Scenario 5). The reaction curve deviates from the baseline curve at some point when there is not sufficient hydrogen peroxide or PEG (Scenario 3, 4). The corresponding activity depletion curves are shown in Figure 6-2. It can be seen that, if PEG dose was less than the minimum requirement, HRP activity proceeds rapidly towards zero as soon as PEG is used up (Scenario 4).
Table 6-1 Summary of the Reaction Scenarios

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Reaction Condition</th>
<th>Expected Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HRP</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>1</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>2</td>
<td>ins</td>
<td>min</td>
</tr>
<tr>
<td>3</td>
<td>min</td>
<td>ins</td>
</tr>
<tr>
<td>4</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>5</td>
<td>excess</td>
<td>min</td>
</tr>
<tr>
<td>6</td>
<td>min</td>
<td>excess</td>
</tr>
<tr>
<td>7</td>
<td>min</td>
<td>min</td>
</tr>
</tbody>
</table>

Note: min = minimum; ins = insufficient; incomp = incomplete

![Graph showing different scenarios of phenol removal](image_url)

Figure 6-1 Model Output for Different Scenarios: Phenol Removal
6.2.1.2 Effect of Mixing on the Reactor Performance

Mixing is usually a prerequisite for chemical reactions involving two or more reactants or two processes occurring simultaneously. Mixing can make a difference in the detention time required to complete the reaction. The effect of mixing on detention time can be determined by dye tests. Proper mixing leads to uniformity of the reaction mixture and the product. It is recognized that mixing processes can significantly alter the outcome of chemical reactions. Inadequate mixing can result in spatial concentration and temperature gradients, which can cause nonuniform products and runaway reactions (Denbigh and Turner, 1984). On the other hand, complete mixing may reduce reactor efficiency under certain circumstances as shown later in the case of CSTR.

The effect of mixing on HRP catalyzed polymerization of phenol was evaluated in batch reactors. Experiments were carried out with the phenol concentrations ranging from
1 to 10 mM. The results in Figures 6-3 and 6-4 show a very small difference in terms of the phenol removal efficiency over the entire range of phenol concentration studied. Without mixing, most of the polymer products formed as solids during the reaction settled during the course of the reaction. The particles remaining in the solution after the completion of reaction appeared to be very fine and settled slowly. However, the total time needed for the separation of these particles was much less than that with mixing. This behavior can significantly reduce the detention time needed in the settling tank. It was especially true for low concentration of phenol (1 mM) with minimum HRP, because it needed 5 hours to complete the reaction. The corresponding time needed for the settling of precipitate in the stirred reactors was also long. The particles formed without mixing had different characteristics, possibly due to different final product. They attached together to form huge flocs compared to the small uniformly sized particles obtained with mixing. Experimental data also showed no difference in the solution activities with or without mixing at any stirring speed. Therefore, it is concluded that mixing is not necessary in the enzymatic reaction.

![Graph](image)

**Figure 6-3 Effect of Mixing on the Removal Efficiency (1 mM Phenol)**
6.2.1.3 Effect of Recycling Precipitate on Reactor Performance

Precipitate was separated from solution of the reaction and added into new reactions with PEG to examine its effect on the polymerization. The precipitate was added at 0 and 7 minutes of reaction time. The results in Figure 6-5 show that recycling precipitate had no effect on the phenol removal irrespective of when precipitate was added. A small amount of HRP had been immobilized on the precipitate as shown in Table 4-7, but it was too small to influence the HRP activity significantly. Similar experiments showed no difference in the removal efficiency whether or not the precipitate was formed in the presence of PEG. It was also found that the amount of precipitate recycled back into the solution had little effect on the outcome of the reaction, as shown in Figure 6-6. The Equivalent Unit of Recycled Precipitate (EURP) is defined as the amount of precipitate formed after more than 95 percent of 1 mM phenol is removed from a certain volume of solution.
Figure 6-5 Effect of Recycling Precipitate on the Phenol Removal

Figure 6-6 Effect of the Amount of Recycled Precipitate
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7. Under these conditions, semi-batch addition of hydrogen peroxide can save HRP. Thus, the model is able to predict all the observed results.

Semi-batch operation should be considered only when the optimal hydrogen peroxide concentration is higher than a certain value. The model simulation showed this value to be approximately 1.5 mM. The model simulation also showed that the performance of continuous semi-batch addition of hydrogen peroxide was not better than that of discontinuous semi-batch operation.

<table>
<thead>
<tr>
<th>Plot</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol (mM)</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HRP (U/mL)</td>
<td>0.065</td>
<td>0.065</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$ (mM)</td>
<td>1.1 at 0 min + 0.5 at 30 min</td>
<td>0.6 at 0 min + 3.3 at 0 min</td>
<td>1.7 at 0 min + 1.6 at 30 min</td>
<td>1.1 at 0 min + 1.1 at 20 min +</td>
<td>1.1 at 40 min</td>
</tr>
</tbody>
</table>

Figure 6-7 Model Prediction for Semi-batch Addition of Hydrogen Peroxide
6.2.2.2 Advantages of Semi-batch Operation

Experiments were conducted to compare semi-batch reactors with batch reactors. The results illustrated in Figure 6-8 verify the results from an earlier study that the semi-batch operation would increase the turnover of the enzyme when initial phenol concentration was higher than 3 mM (Wu J. et al., 1994). The results in Figure 6-8 are further used to compare with plug flow and step-addition operations of PFR.

![Graph showing phenol fraction against time for batch and semi-batch operations]

**Reaction Conditions:**

<table>
<thead>
<tr>
<th></th>
<th>Batch</th>
<th>Semi-B1</th>
<th>Semi-B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$ (mM)</td>
<td>3.3 at 0 min</td>
<td>1.7 at 0 min</td>
<td>1.1 at 0 min +</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>1.1 at 20 min +</td>
</tr>
<tr>
<td></td>
<td>1.6 at 30 min</td>
<td>1.1 at 40 min</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6-8 Comparison between Semi-batch and Batch Operation**

In the presence of additives, semi-batch addition of hydrogen peroxide showed an increase in the enzyme turnover while semi-batch addition of HRP showed no benefit. The
computer model developed in Chapter 5 revealed that both the addition mode and addition timing were vital to the success of such operation:

- Mode: discontinuous (how many spikes) or continuous
- Timing: in what time points or in what time period

The model simulation shows that if time period is short, the semi-batch effect is reduced; whereas if the time period is long, the time needed to complete the reaction is extended.

6.2.3 Plug Flow Reactor Operation

As with batch reactors, detention time in plug flow reactors will have a significant effect on the removal efficiency. In addition, the flow pattern and the reactor configuration will also have influence on the reactor performance. Ideally, there should be no back mixing in these reactors. However, the settling of precipitate in the up-flow reactors can have a mixing effect in the reactor. The influences of non-ideal conditions are studied and discussed in the following sections.

6.2.3.1 Effect of Mixing on Reactor Performance

A small CSTR unit with a 2 mL volume mixing zone was installed in place of the Y-connector shown in Figure 3-2. Experiments were conducted under the same flow rates and concentrations of reaction components (3 mM phenol, 0.1 U/mL HRP, 3.3 mM hydrogen peroxide) to evaluate if the Y-connector could sufficiently mix the reaction components. The results proved that the Y-connector served as a mixer, and a separate mixing unit was not necessary in the system. The reason for this intense mixing might be the small diameters (less than 1 mm) of the Teflon-tube which connected the Y-connector to the system. The reaction components were mixed due to the sheer flow gradient and dispersion in these tubes. It is suggested that a mixing unit should be added to a full scale plug operation.
6.2.3.2 Effect of Flow Direction

The flow direction effects were also evaluated under the same experimental conditions as in Section 6.3.3.1. The result demonstrated that a down-flow pattern greatly degraded the function of the reactor. The removal efficiency for the same reactor was reduced by 13% to 27.5%. Although a down-flow system helped the particles to settle faster, it also induced a shorter detention time for part of the reaction flow, because of short circuiting due to the settling particles.

6.2.3.3 Effect of the Reactor Configuration

Another reactor with a different shape was tested. Its diameter was 27 mm with a height of 110 mm. The results from this reactor were compared with those from the reactor described in Section of 6.3.3.1, with an identical reactor volume. The removal efficiency for the reactor was reduced by 13% to 20.5% due to change in configuration. The 7% loss in efficiency was probably caused by the dead-end in the reactor.

6.2.3.4 Effect of Detention Time

The effect of detention time, $T_d$, was examined by using the system shown in Figure 3-2. The experimental conditions were exactly the same as those in the batch and semi-batch reactors in Section 6.2.2.2. The detention time for each reactor was set at 40 to 50 minutes. Samples were taken from the sampling points to measure remaining phenol concentration and the corresponding activities over a period of time. The results, plotted in Figure 6-9, reveal an almost identical pattern of reaction in terms of phenol removal for the plug-flow system as that for the batch reactor. This suggests that the computer model developed for batch reactors can be used for plug-flow reactors also. It is interesting to see from Table 6-2 that the activities for these two different systems were similar to those obtained from the model.
It was observed that no precipitate was formed in the first 30 minutes in the batch reactors. The color of the reaction solution changed from light yellow to dark brown during that period. Similarly, almost no precipitate was formed in the first reactor of the plug flow system. The reactor showed a spectrum of color change from bottom to the top. In contrast, a lot of precipitate, gathering together as flocs of large size, were formed and settled in the second reactor. In the third reactor, only a small amount of precipitate settled. Thus, the sludge collected in the second reactor accounted for most of the settled precipitate in the entire system. This phenomenon indicates that most of the polymerization of the high oligomers occurred during the period from 40 minutes to 100
minutes under these experimental conditions. Settling of the precipitate obviously caused mechanical mixing to some extent in the plug flow reactor system.

6.2.4 Step-Addition of Hydrogen Peroxide in Plug Flow System

Step-addition of hydrogen peroxide along the flow path in the plug flow system was also tested. The results showed its effectiveness by improving the enzyme usage as observed with semi-batch operation. As shown in Figure 6-10, the removal efficiency for phenol had increased with step addition of hydrogen peroxide. This is the expected output from the computer model when the model is applied to plug flow reactors.

---

**Figure 6-10 Results of the Step-Addition of H₂O₂ in Plug Flow System**

<table>
<thead>
<tr>
<th>Reaction Conditions:</th>
<th>Plug</th>
<th>Step-addition 1 point at Reactor B</th>
<th>Step-addition 2 points at Reactor B &amp; C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂ (mM)</td>
<td>3.3 at Rₐ</td>
<td>2.5 at Rₐ</td>
<td>1.8 at Rₐ</td>
</tr>
<tr>
<td></td>
<td>0.8 at Rₜ</td>
<td>+</td>
<td>0.9 at Rₜ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.6 at Rₜ</td>
</tr>
</tbody>
</table>
The extent of this improvement is related to the detention time of the reactions. For instance, as shown in Figure 6-11, the behavior of the reactors with 30 minutes detention was better than those with 40 minutes, when the reaction components were identical. This was due to the low concentration of hydrogen peroxide at the end of first reactor, which resulted in ineffective end volume. This, again, confirms the result from semi-batch experiments that timing of hydrogen peroxide addition was important to its effectiveness. The computer model can simulate this phenomenon as it did in the case of semi-batch operation.

![Bar chart showing phenol fraction remaining for different detention times](image)

**Figure 6-11 Effect of Detention Time on the Step-Addition**

### 6.2.5 Continuous Stirred Tank Reactor

Finally, the performance of a continuous stirred tank reactor (CSTR) was examined using the system illustrated in Figure 3-4. Under exactly the same concentrations of the reaction components as in the batch and plug flow systems, the time needed for the
completion of the reaction increased significantly as expected while the enzyme turnover showed little improvement (Figure 6-12). The data demonstrate that when the detention time was extended beyond 200 minutes, the activities in the reaction solutions became low (below 0.04 U/mL). This, combined with the low concentrations of phenol and hydrogen peroxide, led to a slow phenol conversion rate. At the same time, the phenol removal efficiency was higher than that from the model output if the detention time was less than 100 minutes. This suggests that the inactivation of enzyme was reduced and the corresponding enzyme turnover had improved. Although the low activity in the solution reduced the inactivation, it also reduced the activity increase according to the activity sub-model. This might explain why the HRP turnover showed little improvement when the concentration of enzyme was low. The precipitates formed in these experiments had similar characteristics to those in the batch or plug flow experiments. The longer the detention time, the more precipitate was formed and faster it settled.

6.3 COMPARISON AND DESIGN CONSIDERATIONS

As discussed in the previous sections, batch, plug flow and CSTR systems together with semi-batch and semi-plug flow systems were studied under similar reaction conditions, i.e. 3 mM phenol and 0.1 U/mL HRP. The comparison showed that the plug flow reactor had a reaction behavior similar to that of the batch reactor. The benefits from the semi-plug flow operation were also similar to those from the semi-batch operation. All of these reaction conditions can be simulated by the computer model developed in Chapter 5 without any modification. The reaction in the CSTR system can also be simulated approximately by the same computer model. The CSTR system greatly increased the reaction time needed for the completion of the reaction. The detention time needed for CSTR system was so long that the saving in HRP did not compensate for the
large reactor volume. Batch operation involves in the time axis, while the plug flow system is on the space axis. The CSTR system represents a time point in a batch or a space section in plug flow system. Because the enzyme turnover had shown little improvement in the CSTR system, plug flow or batch reactors should be considered first in the reactor design in most cases. When the initial HRP or phenol concentrations are very high, a CSTR system can be considered over a plug flow system.

Because the objective of reactor design is to minimize the overall cost, it is necessary to discuss the important parameters in the design procedure. The most important parameter is the phenol concentration to be handled which subsequently determines the minimum HRP and PEG doses and the optimum hydrogen peroxide dose. The minimum HRP dose can greatly influence the time needed for the completion of the
reaction or the detention time. In the case of pure phenol, it was 5 hours for 1 mM phenol, and 2 hours for 10 mM (Wu J. et al., 1993). In the case of real wastewater, it usually needed more enzyme and hydrogen peroxide (Dantas, 1995) which meant a faster reaction rate and shorter reaction time. If HRP and hydrogen peroxide concentrations are high (e.g. HRP > 2 U/mL, H₂O₂ > 1.5 mM), semi-batch operation should be considered.

The flowrate of the wastewater is another very important parameter. Usually, the higher it is, the better is the possibility of using plug flow systems. Oppositely, the lower it is, the more realistic is it to adopt batch or CSTR systems.

On the basis of the above discussion, a general design protocol is suggested as follows:

- Decide if pretreatment, such as pH adjustment and solid removal, are needed.
- Run batch experiments to determine
  (a) the minimum HRP dose,
  (b) the reaction stoichiometry,
  (c) the corresponding detention time, and
  (d) the settling characteristics of the precipitates.
- Choose a reactor configuration or a combination of reactors according to (a) and (c) above. If the HRP dose is high or the detention time is very short, a CSTR system may be considered. Otherwise, semibatch or plug flow systems should be preferred.
- Run semi-batch experiments to determine if semi-batch operation should be used when HRP and hydrogen peroxide concentrations are high.

### 6.4 RECOMMENDED SYSTEM

A plug flow system (Figure 6-13) is recommended for both high and low phenol concentrations based on the results obtained in this study. This system consists of a mixing tank, a reaction and settling tank, and a settling tank or filtration system with a
possible supernatant recycling. The experiments in Section 6.3.3.1 showed that a mixer was not needed, so the mixing tank can be replaced by an in-line mixer provided that the enzyme activity is not inactivated by the high velocity in the mixer.

The influent is combined with hydrogen peroxide and then mixed in the mixing tank with the HRP and additive from the storage tank. The detention time for this mixing tank can range from 5 minutes to 30 minutes depending on the flowrate. The larger the flowrate, the shorter is the detention time required. Even when the size of the mixing tank is fairly small, up to 50 percent conversion may be expected in this tank. In addition, the mixing tank can reduce HRP dose and consequently improve the enzyme turnover. Subsequently, the reaction mixture flows into a tank in which phenol conversion and polymerization as well as the settling of polymerization product occurs simultaneously. Finally, the slow-settling particles are removed in the settling tank. A filtration system can
be considered in place of the settling tank. If the initial phenol concentration is higher than 5 mM (e.g. 10 mM), the supernatant from the settling tank may be recycled back to the mixing tank because the enzyme turnover is a maximum at 5 mM (Wu J. et al., 1993)*. This approach will also recycle the remaining activity in the effluent which will provide additional improvement in the turnover of the enzyme.

The advantages of this system are proper mixing, improvement in the turnover of enzyme, simple operation, minimum maintenance and saving on the settling space, thereby satisfying the primary consideration of minimum overall cost in the design while satisfying the treatment requirements at the same time.

* Note: According to Wu J. et al., (1993), minimum HRP dose can be expressed as a function of phenol concentration, which suggests that enzyme turnover would be maximized at phenol concentration of 4 - 5 mM, as shown in the following table:

<table>
<thead>
<tr>
<th>Phenol (mM)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP_{min} (U/mL)</td>
<td>0.05</td>
<td>0.065</td>
<td>0.09</td>
<td>0.112</td>
<td>0.14</td>
<td>0.184</td>
<td>0.23</td>
<td>0.338</td>
<td>0.40</td>
</tr>
<tr>
<td>HRP_{min}/Phenol</td>
<td>0.050</td>
<td>0.032</td>
<td>0.030</td>
<td>0.028</td>
<td>0.030</td>
<td>0.032</td>
<td>0.037</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>Phenol/HRP_{min}</td>
<td>20</td>
<td>30.8</td>
<td>33.3</td>
<td>35.7</td>
<td>35.7</td>
<td>32.6</td>
<td>30.4</td>
<td>26.7</td>
<td>25</td>
</tr>
</tbody>
</table>
CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

On the basis of the experiments conducted in this study, several conclusions are drawn:

7.1.1 Additives

1. Additives have certain distinct impacts on the reaction conditions. For the low concentrations (1 mM, about 100 mg/L) of phenol derivatives (2-, 3-, 4-chlorophenol, o-, m-, p-cresol and 2,4-dichlorophenol):

- The additives can significantly reduce the minimum amount of HRP needed for the complete polymerization of phenolic compounds. The enzyme turnover can be improved by more than 100-fold by using additives. Addition of extra enzyme beyond the minimum dose does not improve the removal efficiency. The minimum amount of HRP needed for the completion of reaction varies with the phenolic compounds.

- The optimum pH range becomes wider in the presence of additives and the optimal pH is closer to neutral except for 2-chlorophenol. The influence of pH is independent of the amount of enzyme or additive used.

- The minimum additive dose depends on the nature of the additive and the phenolic compound. There is no general relationship between the minimum additive dose and the minimum HRP dose for the phenolic compounds studied.

- The reaction time required for completing polymerization is usually less than 3 hours when minimum HRP and additive doses are used. Most of the phenolic compounds (eighty to ninety percent) are removed in the first hour.

- The optimum \([\text{H}_2\text{O}_2]/[\text{Phenol}]\) is around 1. Addition of PEG\(_{3550}\) has a little influence on the reaction stoichiometry, while gelatin does not change it. Deficiency in hydrogen peroxide reduces the removal efficiency.

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• Coprecipitation can occur in the presence of additive.

2. In addition to polyethylene glycol and gelatin, some polyelectrolytes are effective as additives. PEG is the best additive for saving HRP. Gelatin and PE₁ show similar effectiveness and are better than PE₂.

• In terms of operation, PEG₃₅₅₀ has a high solubility and it is much easier to make 40 g/L PEG₃₅₅₀ solution than to make 1 g/L gelatin and PE solutions.

• PEG is also better than the other additives in terms of Practical Additive Dose Range (PADR). Excess PEG has no negative effect on the reaction. In contrast, excess gelatin and PE can lower the removal efficiency and also result in no formation of particles. In addition, their PADR ranges are narrow.

• Excess hydrogen peroxide can also lower removal efficiency if gelatin or PEs are used and also result in no formation of particles.

• In terms of the effluent quality, at minimum PEG dose, there is little PEG left in solution after completion of reaction. Most of the PEG combines with the particles and separates from solution as precipitate. However, a considerable amount of gelatin remains in solution even at the minimum gelatin dose. Also, gelatin produces more precipitate than PEG.

Therefore, PEG is the best choice as an additive.

3. Additives can be used in the HRP catalyzed polymerization of phenolic compounds to appreciably increase the enzyme turnovers. The HRP saving is contingent on the nature of additives and phenolic compounds, which indicates that the protective mechanism of additives on HRP lies in the interaction of additive and the polymerization product. Additives are activators of HRP, but, this activation is not the reason why they can save HRP. Because they can not protect the HRP inactivation caused by temperature
or hydrogen peroxide, the inactivation must be due to the reaction products. In the absence of additives, inactivated HRP remains in solution in large proportion. Polymer products separated from that reaction mixture can inactivate fresh HRP. Additives in the HRP solution prevent the inactivation. In contrast, polymer products collected in the presence of additive do not inactivate HRP. Most of the additives are incorporated covalently with the polymer product during the reaction and can not be separated from the reaction.

Based on the above observations, a protection mechanism has been proposed in terms of the interaction among polymer product, HRP and additives. This interaction can be described as partition affinity. Different polymer products from different phenolic compounds have different affinities for HRP and so do different additives. That is why different phenolic compounds require different minimum HRP doses in the absence of additives. In the presence of additive, HRP and additive interact with polymer product according to their ratio of affinities for the polymer product; The smaller the ratio, the bigger is the HRP saving.

7.1.2 Kinetic Model

A computer model has been developed to simulate the reaction based on the following kinetics:

- The phenol conversion expression is a second order Michaelis-Menten equation with respect to the concentrations of phenol and hydrogen peroxide.
- The enzyme inactivation is attributed to the polymers and hydrogen peroxide simultaneously. The constant for the inactivation caused by polymers \( k_{a1} \) is a function of concentration of PEG and hydrogen peroxide.
- The rates of consumption of hydrogen peroxide and PEG are directly proportional to the rate of conversion of phenol. The coefficients can be obtained from experiments.
A sub-model is also developed for the increase in initial activity of HRP which is a function of the concentration of HRP, phenol, hydrogen peroxide and PEG.

Experimental data have proven that the model output can predict the phenol depletion and the activity decrease realistically over a variety of reaction conditions. The model has been verified by predicting some independent experimental results on reaction stoichiometry, HRP dose effect and semi-batch operation of hydrogen peroxide.

### 7.1.3 Reactor

1. Experiments confirmed that:
   - Batch mixed reactors and plug flow reactors are identical in terms of reaction kinetics.
   - Semi-batch operation and step-addition operation of plug flow reactor with respect to hydrogen peroxide produce similar output.
   - In presence of additives, continuous stirred tank reactors need a long time to complete the reaction at the minimum HRP dose.

   Although the computer model was developed from batch operation in this study, it can predict batch and plug flow reactions and CSTR reaction very well.

2. Experiments proved that:
   - There is no need to stir the reaction mixture continuously during the reaction. Mixing at the beginning of the reaction is sufficient.
   - Recycling of the precipitate is not beneficial either to the removal efficiency or to the operation of the settling tank.

   A plug flow reactor system is recommended in the presence of additive. The system should consist of a small mixing tank followed by a tank in which reaction and settling occur simultaneously.
7.2 RECOMMENDATIONS

The following recommendations are made for further study:

- Before the treatment method can be applied to real wastewater, the separation of solids from the reaction must be studied in detail. The settling characteristics of the polymer solids are influenced by enzyme type and amount, additive type and amount, concentrations of aromatic compounds and hydrogen peroxide, degree of agitation, salinity. In addition to gravity settling, other separation methods such as filtration can be considered for use independently or in combination with sedimentation. The effect of coagulants on the settling should also be determined.

- The recommended system should be used to test the real wastewater. The effect of pretreatment on the enzyme demand and additive requirement should be investigated.

- A crude form of HRP from horseradish root can be considered to replace pure HRP. The maximum activity from a specific amount of horseradish root should be determined before that replacement and a cost comparison should be carried out.

- Other peroxidases such from Arthromyces Ramosus (ARP) and soybean (SBP) can be considered in this application.

- The effect of low concentrations of hydrogen peroxide by computer modeling should be studied further. This will determine if hydrogen peroxide at low concentrations will significantly inactivate enzyme.

- A systematic method should be developed to determine the model constants through experiments.

- A detailed cost analysis sub-model can be added to the computer model. The model should include at least the cost of enzyme, additive and hydrogen peroxide, reactor costs and operating and maintenance costs.

- Experiments should be conducted at higher concentrations of phenolic compounds.
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APPENDIX A-1 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 phenol, AAP and appropriate concentration of hydrogen peroxide, the initial rate is measured by observing the rate of color formation in a solution in which a reaction between phenol and hydrogen peroxide is catalyzed by HRP such that the products of the reaction react with AAP to form a red colored 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 (200 mL)
   phosphate buffer to 200 mL
   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 H₂O₂)
   226.7 μL of 30% H₂O₂ in flask (100 mL)
   then remove 10 mL of which into another flask (100 mL), dilute it to 100 mL.
   remake it daily

3. Procedure

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

50 μL Sample (diluted)
500 μL NaPP buffer
100 μL 0.1 M Phenol
250 μL 9.6 mM AAP
100 μL 1.0 mM H₂O₂
The total volume in the cuvette must be 1 mL and the rate of color 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. 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. Calculate the activity in the cuvette

\[ \text{Activity (U/mL)} = \frac{\text{slope(au/min)}}{6000 \text{au/L/mol}} \times 10^6 \times \frac{\text{1 mol}}{\text{1000 mL}} \]

Thus, the activity is in terms of micromoles of hydrogen peroxide converted per minute at 20 °C, pH 7.4 with 10 mM Phenol, 2.4 mM AAP and 0.2 mM H₂O₂.

c. Calculate the activity of the sample

\[ \text{Activity in Sample (U/mL)} = \frac{\text{Activity in Cuvette (U/mL)}}{\frac{1000 \mu L}{\text{sample volume(\mu L)}}} \]

5. Interferences

Aromatics which are substrates of HRP may interfere with this assay. Because phenol is used as the color generating agent, the interference is less than that by HDCBS.
APPENDIX A-2 Aromatic Substrate Assay
(Enzyme Based)

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 color generating cosubstrate in combination with the aromatic substrate in the sample. In this end-point assay, the amount of aromatic introduced into the assay sample is the only limiting reactant; therefore, the degree of color 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 (200 mL)
   phosphate buffer to 200 mL
   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 H₂O₂)
   113.3 μL of 30% H₂O₂ in flask (100 mL)
   then remove 10 mL of which into another flask (100 mL), 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 prepare 25 mL solutions of aromatic substrate ranging in concentration from 0 to 0.25 mM. In a semi-micro cuvette place solutions in the following order:

200 μL Aromatic Sample
200 μL NaPP Buffer
100 µL HRP Stock  
250 µL 9.6 mM AAP  
250 µL 1.0 mM H₂O₂  

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

4. Measurement of Aromatic Substrate

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

50-200 µL Aromatic Sample  
200-350 µL NaPP Buffer  
100 µL HRP Stock  
250 µL 9.6 mM AAP  
250 µL 1.0 mM H₂O₂

The total volume in the cuvette must be 1 mL. Keep the aromatic concentration in the cuvette below 50 µM. Immediately after the addition of the hydrogen peroxide, shake the cuvette and then wait for the color to develop fully. Read the maximum amount of absorbance at the peak wavelength at 510 nm. For samples of low concentration after the HRP catalyzed reaction, the maximum color might be formed in less than 5 minutes.

5. Calculations

Calculate the sample aromatic concentration from:

\[
[Aromatic]_{\text{in sample}} = \frac{[Aromatic]_{\text{in cuvette}} \times 1000\mu\text{L}}{\text{sample volume}} \times (\mu\text{L})
\]

where \([Aromatic]_{\text{cuvette}}\) is determined from the calibration curve.
APPENDIX A-3 Aromatic Substrate Assay
(Ferricyanide Based)

1. General

This is a colorimetric assay used to measure the concentration of aromatic substrate in a given sample. It uses potassium ferricyanide as catalyst and 4-aminoantipyrine as a color generating cosubstrate in combination with the aromatic substrate in the sample. In this assay, the amount of aromatic introduced into the assay sample is the only limiting reactant; therefore, the degree of color developed in the reaction at the peak wavelength is proportional to the aromatic concentration at a peak wavelength of 510 nm. The assay can deal with a dilute sample.

2. Reagents

a. Ferricyanide reagent (83.4 mM of K$_3$Fe(CN)$_6$ in 0.25 M NaHCO$_3$)
   2.75 g K$_3$Fe(CN)$_6$ and
   2.1 g NaHCO$_3$
   distilled water to 100mL

b. AAP reagent (20.8 mM of AAP in 0.25 M NaHCO$_3$)
   0.423 mg AAP and
   2.1 g NaHCO$_3$
   distilled water to 100mL

Use 100 μL each of the reagents in the assay. The remaining could be sample or sample diluted with deionized water. First, add samples in cuvettes, then AAP, and finally ferricyanide. Mix, and let it stand for a couple of minutes. Measure the absorbance at 510 nm versus reagent blank. Standard curves for the phenolic compounds studied were established for determining their concentrations.

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, add solutions in the following order:

100 μL Aromatic Sample
700 μL Distilled Water
100 μL AAP
100 μL K$_3$Fe(CN)$_6$
The total volume in the cuvette must be 1 mL. Keep the aromatic concentration in the cuvette below 50 μM. Immediately after the addition of the K$_3$Fe(CN)$_6$, shake the cuvette and then wait a couple of minutes for the color to develop fully. Read the maximum amount of absorbance at the peak wavelength at 510 nm. 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:

50-800 μL Aromatic Sample
0-750 μL Distilled Water
100 μL AAP
100 μL K$_3$Fe(CN)$_6$

The total volume in the cuvette must be 1 mL. Keep the aromatic concentration in the cuvette below 50 μM. Immediately after the addition of the K$_3$Fe(CN)$_6$, shake the cuvette and then wait a couple of minutes for full color development. Read the maximum amount of absorbance at the peak wavelength at 510 nm.

5. Calculations

Calculate the sample aromatic concentration from:

\[
[Aromatic]_{\text{in sample}} = \frac{[Aromatic]_{\text{in cuvette}} \times 1000 \mu L}{\text{sample volume (μL)}}
\]

where $[Aromatic]_{\text{cuvette}}$ is determined from the calibration curve.
NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

UMI
200 µL 9.6 mM AAP  
100 µL 0.1 M phenol  
50 µL Sample

The total volume in the cuvette must be 1 mL. Keep the hydrogen peroxide concentration in the cuvette below 50 µM. Immediately after the addition of the phenol, shake the cuvette and then wait until the color is fully developed (mostly after 30 minutes). Read the maximum amount of absorbance at the peak wavelength at 510 nm. Make a plot of absorbance versus concentration of hydrogen peroxide in the cuvette.


In a semi-micro cuvette add solutions in the following order:

50-100 µL Aromatic Sample  
510-460 µL NaPP Buffer  
130 µL HRP Stock  
210 µL 9.6 mM AAP  
100 µL 100 mM phenol

The total volume in the cuvette must be 1 mL. Keep the hydrogen peroxide concentration in the cuvette below 50 µM. Immediately after the addition of the phenol, shake the cuvette and then wait for the color to develop fully. Read the maximum amount of absorbance at the peak wavelength at 510 nm.

5. Calculations

Calculate the sample aromatic concentration from:

\[
[H_2O_2]_{\text{in sample}} = \frac{[H_2O_2]_{\text{in cuvette}} \times 1000 \mu L}{\text{sample volume} (\mu L)}
\]

where \([H_2O_2]_{\text{cuvette}}\) is determined from the calibration curve.
APPENDIX B-1 Standard Curves for Phenol derivatives

The following is for Appendix A-3 Aromatic substrate Assay (Ferricyanide Based)

phenol:

--- Standard Calibration Report ---

Date: 3/19/1995
Time: 12:05:33
Operator: Not Entered

FILE NAME: C:\\C BMCWDATA\phytofda.STD

Sample Name: phenol
Solvent Name: ddW
Conc Units: um

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

Beer's Law Fit

![Graph showing Beer's Law fit with absorbance and concentration data]
2-Chlorophenol:

--- Standard Calibration Report ---

File Name: C:\\USER\DATA\\2cpstdfcts.STD

Sample Name: 2cp
Solvent Name: dw
Conc Units: uM

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

Beer's Law Fit

Absorbance

3.439
2.292
1.146
0.000

Concentration (uM)

0.000 1.000 2.000 3.000 4.000 5.000 6.000 7.000 8.000 9.000 10.000 11.000 12.000 13.000 14.000 15.000 16.000 17.000 18.000 19.000 20.000 21.000 22.000 23.000 24.000 25.000 26.000 27.000 28.000 29.000 30.000 31.000 32.000 33.000 34.000 35.000 36.000 37.000 38.000 39.000 40.000 41.000 42.000 43.000 44.000 45.000 46.000 47.000 48.000 49.000 50.000 51.000 52.000 53.000 54.000 55.000 56.000 57.000 58.000 59.000 60.000 61.000 62.000 63.000 64.000 65.000 66.000 67.000 68.000 69.000 70.000

Date: 05-15-93
Time: 17:10:46
Operator: Not Entered
3-Chlorophenol:

--- Standard Calibration Report ---

File Name: C:\UV\DATA\Scpstdofc.STD

Sample Name: Scp
Solvent Name: dw
Conc Units: uM

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

Beer's Law Fit

Absorbance

Concentration (uM)

Data: 01-16-335
Time: 22:16:34
Operator: Not Entered
4-Chlorophenol:

--- Standard Calibration Report ---

File Name: C:\\W:\DATA;3ppdfefo.STD

Sample Name: 3pp4
Solvent Name: dw
Conc Units: µM

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

Beer's Law Fit
o-Cresol:

--- Standard Calibration Report ---

Date: 05-27-1993
Time: 10:42:00
Operator: Not Entered

File Name: C:\UV\DATA\ompstofc.STD

Sample Name: omp
Solvent Name: dw
Conc Units: µM

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

--- Standard Calibration Report ---

File Name: C:\VUV\DATA\mmmpolstfc.STD

Sample Name: mmp
Solvent Name: dw
Conc Units: um

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

Beer's Law Fit

Absorbance vs Concentration (um)
p-Cresol:

--- Standard Calibration Report ---

Date: 01-16-1996
Time: 22:24:27
Operator: Not Entered

File Name: C:\UV\DATA\pcstdfc.STD

Sample Name:
Solvent Name: 
Conc Units: 

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

Beer's Law Fit

Absorbance

Concentration (ppm)

0.000 0.030 0.059 0.089

+1.40E+62 +2.10E+62

0.008 0.006 78.008
2,4-Dichlorophenol

--- Standard Calibration Report ---

File Name: C:\D:\DATA\24cpstdf.STD

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

Analytical Wavelength: 510 nm
Reference Wavelength: None Selected
Confirmation Wavelengths: None Selected
Integration Time: 7 seconds

Beer's Law Fit

![Absorbance vs Concentration Graph]

Absorbance

Concentration (uM)
The following is for Appendix A-2 Aromatic substrate Assay (Enzyme Based)

2-Chlorophenol:

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

File Name: C:\UV\DATA\2cp4Smin.570

Sample Name: 
Solvent Name: 
Conc Units: 
Analytical Wavelength: 510 nm
Reference Wavelength: None Selected
Confirmation Wavelengths: None Selected
Integration Time: 1 seconds

Beer's Law Fit

Absorbance

0.000  0.185  0.370  0.555

Concentration (uM)
0   10   20   30   40   50

185
3-Chlorophenol:

--- Standard Calibration Report ---

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

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

Analytical Wavelength: 374 nm
Reference Wavelength: None Selected
Confirmation Wavelengths: None Selected
Integration Time: 7 seconds

Beer's Law Fit

Absorbance

0.650
0.433
0.217
0.000

Concentration (µM)

0.000 +1.31E+02 +2.63E+02 +3.94E+02

186
4-Chlorophenol:

--- Standard Calibration Report ---

Date: 09-04-1993
Time: 16:29:57
Operator: Not Entered

File Name: C:\UV\DATA\cpl45min.STD

Sample Name: 
Solvent Name: 
Conc Units: 

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

Beer's Law Fit

Absorbance

Concentration (μM)

0.000

0.000

0.000

0.208

0.416

0.623

0.000

26.250

52.500

78.750

Analytical
o-Cresol:

--- Standard Calibration Report ---

File Name: o-cresol.SFC

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

Analytical Wavelength: 510 nm
Reference Wavelength: None Selected
Confirmation Wavelengths: None Selected
Integration Time: 4 seconds

Beer's Law Fit
m-Cresol:

--- Standard Calibration Report ---

File Name: aimmparone.STO

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

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

Beer's Law Fit

Absorbance

0.000 0.117 0.235 0.352

Concentration (µM)

0.000 17.500 35.000 52.500

Date: 05-01-1992
Time: 16:27:10
Operator: Not Entered
p-Cresol:

--- Standard Calibration Report ---

File Name: a CMP278 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

Beer's Law Fit

Absorbance

0.000  0.173  0.347  0.520

Concentration (UM)

0.000  +1.05E+02  +2.10E+02  +3.15E+02
2,4-Dichlorophenol

--- Standard Calibration Report ---

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Solvent Name</th>
<th>Conc Units</th>
<th>Analytical Wavelength</th>
<th>Reference Wavelength</th>
<th>Confirmation Wavelengths</th>
<th>Integration Time</th>
</tr>
</thead>
<tbody>
<tr>
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<td>dw</td>
<td>uM</td>
<td>510 nm</td>
<td>None selected</td>
<td>None selected</td>
<td>1 seconds</td>
</tr>
</tbody>
</table>

![Beer's Law Fit](image)
APPENDIX B-2 Standard Curves for TOC

CORELATION BETWEEN TOC AND PHENOL
CORRELATION BETWEEN TOC & m-CRESOL
CORELATION BETWEEN PEG AND TOC
PEG 300

TOC (mg/L)

PEG 300 (mg/L)
CORELATION BETWEEN TOC AND HRP
CORRELATION BETWEEN TOC AND GELATIN

![Graph showing the correlation between TOC (Total Organic Carbon) and Gelatin concentrations. The graph has a linear trend with data points indicating a positive correlation.](image)

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APPENDIX C Additional Experimental Results

HRP FATE IN REACTION WITHOUT ADDITIVE
5 mM PHENOL

- added
- remain TOC
- consumed TOC
- remain Act.
Comparison among the Additives

Phenol 1 mM
Peroxide 1.1 mM
PEG 20 mg/L
Gelatin 80 mg/L
PE1 15 mg/L
PE2 10 mg/L
Comparison among the Additives

Phenol 3 mM
Peroxide 3.3 mM
PEG 100 mg/L
Gelatin 250 mg/L
PE1 60 mg/L
PE2 25 mg/L

Phenol Concentration Remaining (%)

HRP dose (U/mL)

PEG
Gelatin
PE1
PE2
PEG Dose Effect on Phenol Removal
1.8 mM PHENOL, 2.2 mM H2O2

![Graph showing phenol fraction remaining over time with PEG concentration on the y-axis and time in minutes on the x-axis.]
PHENOL REMOVAL WITH TIME
2.7 mM PHENOL, 3.3 mM H2O2

PEG
mg/L
0  15  30  50

Phenol remaining

Time (min)
0  20  40  60  80  100  120  140  160
EFFECT OF STIRRING ON PHENOL REMOVAL
3 mM PHENOL

PHENOL CONCENTRATION REMAINING (%)

REACTION TIME (minute)

- STIRRING
- NO STIRRING

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EFFECT OF STIRRING ON PHENOL REMOVAL
6 mM PHENOL

PHENOL CONCENTRATION REMAINING (%)

REACTION TIME (minute)

STIRRING  NO STIRRING
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