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Arthromyces ramosus Peroxidase Catalyzed Phenol Removal

by

Yiseon Han

A thesis submitted to the Faculty of Graduate Studies and Research in
partial fulfillment of the requirements for
the degree of Master of Science.

in

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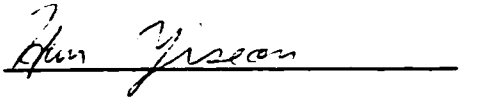

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THE UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***Arthromyces ramosus* peroxidase catalyzed phenol removal** submitted by **Yi Seon Han** in partial fulfillment of the requirements for the degree of **Master of Science** in Environmental Science.

Ian Buchanan

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Date. August 21 1998

Abstract

The potential of *Arthromyces ramosus* peroxidase (ARP) to catalyze the polymerization and precipitation of phenol from aqueous solution was investigated.

A simplified kinetic model was applied to ARP catalyzed phenol removal. The model was calibrated and validated by a global optimization method using data from batch tests. The model showed good predictive ability for treatment both with and without polyethylene glycol (PEG), a protective additive.

The permanent enzyme inactivation rate was greatly decreased in the presence of PEG. According to the model prediction, the predominant inactivation mechanism for ARP is found to be; 1) adsorption by end-products polymer for reaction without PEG; and 2) enzyme interaction with free radicals for reaction in the presence of PEG. The model predicted a very slow rate of the compound III decomposition while direct spectrophotometric observations indicated a faster decomposition of compound III.

Dedicated to my parents

who supported me with love.

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1. Introduction

Phenol and phenolic compounds are classified as one of the major groups of pollutants in the United States. Phenols are listed as priority pollutants regulated under the Clean Water Act (Patterson, 1985), and also listed as hazardous constituents in hazardous waste under the Resource Conservation and Recovery Act (RCRA) and its amendments (Aitken et al., 1994).

Phenols and phenolic compounds are present in the wastewaters from various industries such as adhesives manufacturing, aluminum forming, iron and steel production, petroleum refining, pulp mills and paper plants, among others. Typical concentrations of phenols in wastewaters range between 100 and 1000 mg/L (Wu et al., 1993).

Due to increasing industrial activities, the inflow rate of xenobiotics and recalcitrant organic pollutants into the environment is increasing, and it is becoming more difficult to gain the required degree of removal of these pollutants using conventional chemical and biological processes. Moreover, many organic compounds in industrial wastes including phenol can be inhibitory to mixed culture biological processes (Allsop et al., 1990). The regulations for the maximum acceptable concentration of toxic material in industrial effluents may become stricter in the future because of the increasing awareness of related health-concerns.

Therefore, effective, cost efficient treatment alternatives should be developed for industrial wastewaters.

1.1. Phenols in the Environment

Some phenols are known or suspected as carcinogens (Klibanov, 1982), and phenol concentrations over 2 mg/L are toxic to fish (Lanouette, 1977). With respect to drinking water quality, undesirable production of chlorophenols, which causes unpleasant taste and odour, is one of the most significant problems. According to the Guidelines for Canadian Drinking Water Quality, maximum acceptable phenol concentration is 0.002mg/L in drinking water (Canadian Council of Ministers of the Environment, 1992). Since the generation of chlorophenols still occurs with the maximum acceptable concentration of phenols in drinking water, the objective concentration is recommended as "less than" 0.002mg/L. Therefore, the concentration of phenol and phenolic compounds in wastewater need to be reduced before discharge.

According to the amended Canadian guidelines for recreational water quality, the total phenol is limited to 0.001 mg/L, and it may become lower in the future with increasing health concern (Canadian Council of Ministers of the Environment, 1992).

1.2. Enzymatic Wastewater Treatment

There are a number of methods currently being used to remove the phenolic compounds from wastewaters, including: microbial degradation, adsorption on activated carbon, chemical oxidation, and solvent extraction (Atlow et al., 1984). Despite the importance of treating phenol-containing wastewater, current methods have many drawbacks.

Since physical and chemical processes are not highly selective of the contaminants removed during treatment, they may not be adequate for high-strength wastewater treatment. Thus, large amounts of oxidant or adsorbant would be required to remove targeted pollutants from moderately high strength wastewaters, even when the targeted compounds are present in relatively low concentrations. Microbial degradation processes are typically used to reduce bulk organic loadings, but this method has difficulties removing toxic pollutants to consistently low levels (Aitken, 1993).

Some of the limitations of conventional treatment were summarized by Nicell (1991), and are listed in Table 1.1. Consequently, new approaches that are highly selective for aromatic removal from wastewater are essential.

The concept of enzymatic pollutant removal was developed in the 1970's (Aitken, 1993), and a peroxidase-catalyzed method for removal of aromatic compounds has been proposed by Klibanov's group (Klibanov, 1982; Klibanov et al., 1980; Klibanov and Morris, 1981). Peroxidases have the capacity to catalyze a large number of biochemical reactions once they are oxidized by a peroxide. Using this characteristic, a large number of research projects have studied the application of various enzyme-catalyzed treatment processes to the selective removal of a number of compounds frequently contained in industrial wastewaters.

Table 1.1. Disadvantages of Conventional Methods for Phenol Removal (Nicell, 1991).

Conventional processes	Disadvantages
Solvent extraction	Incomplete purification High cost of operation
Microbial degradation	Hard to remove specific chemicals to low levels consistently Susceptible to inhibition by one or more compounds in the waste
Physical and chemical processes	Not highly selective in items of range of pollutant removed Cost becomes prohibitive as the total concentration of organic compounds in the waste increases or as the required final pollutant concentration decreases Chemical oxidation can cause the formation of hazardous by-products Activated carbon process requires low inlet phenol concentration and high retention time in the reactor

Some of the advantages of enzymatic processes over biological treatment methods were explained by Nicell (1993 c) as follows:

- Removal of broad range of compounds, including biorefractory compounds and those which are toxic to microorganisms;
- Selective removal of a specific single class of contaminant;

- Quick response to shock loadings;
- Short hydraulic retention times;
- Easy control processing due to the use of isolated enzymes;
- Less sludge produced (since no biomass is involved); and
- Effective over a wide range of pH, temperature, and salinity.

The potential advantages of enzymatic treatment as compared to conventional physico-chemical treatment could be (Nicell et al., 1993 c):

- Ability to remove organic material which is not removed by chemical oxidation methods;
- Highly selectivity;
- Less oxidant required than for conventional chemical oxidation methods:
- Effective at high and low substrate concentrations;
- Economic use of adsorbent materials such as activated carbon ; and
- Operation under milder, less corrosive conditions than chemical oxidation methods.

However, potential disadvantages associated with the peroxidase-based process include the formation of trace amounts of hazardous soluble byproducts in some cases, the cost of the enzyme, and the ultimate disposal of solid reaction products.

According to Aitken and coworkers (1994), the characteristics of reaction product mixtures depend on the parent phenol and the enzyme used. The authors reported pH affects the product distributions for some combinations of enzyme-substrate. Griffin (1991) noted that the rate of radical formation, molar ratio of hydrogen peroxide to

phenolic substrate, and dissolved oxygen concentration can also affect the distribution of final products in peroxidase systems. Therefore, it is recommended the toxicity of reaction products should be studied case by case.

Since peroxidase enzymes are currently produced in relatively small quantities for predominantly laboratory analytical purposes, the cost is currently high. Large-scale production of the peroxidase enzymes and the concomitant reduction in cost should be achieved before the process can be applied to wastewater treatment. During the removal process, the enzyme is susceptible to inactivation via interactions with the free radicals and end-product polymers. While this remains the significant drawback of the enzymatic process, Nakamoto and Machida (1992) investigated the effect of using additives such as polyethylene glycol (PEG) and gelatin to reduce the amount of peroxidase needed for phenol removal. Wu and coworkers (1993) continued the work of Nakamoto and Machida, and reported that the addition of PEG effectively suppressed the inactivation of peroxidase. Empirical models have been developed by Wu and coworkers (1993), Saadi (1993), and Nicell and coworkers (1995).

The satisfactory methods for disposition of solid reaction products have not been determined. However, incineration and disposal in landfills are possible options to be chosen (Buchanan, 1996). Further research should be conducted to resolve this issue.

1.3. Enzymes Applied to Phenol Removal

A number of enzymes from various sources of plants and microorganisms have been studied for phenol removal from an aquatic environment. Among them, horseradish peroxidase (HRP) is the most studied enzyme so far. Other than HRP, *Arthromyces ramosus* peroxidase (ARP), cauliflower peroxidase, soybean peroxidase, lignin peroxidase, manganese peroxidase, tyrosinase, hemoglobin, laccase, and many others have been studied for phenol removal. These enzymes have shown a great potential for a large number of waste treatment areas. If one of these enzymes can be produced economically in large enough quantities to apply to the real wastewater treatment, the enzymatic process may become one of the most efficient methods for removal of toxic aromatic compounds from wastewater.

Shinmen and coworkers (1986) reported that a large amount of peroxidase is produced extracellularly by a soil fungus named *Arthromyces ramosus*. Recently, ARP has been shown to have specific activity and substrate aspects similar to HRP (Kjalke et al., 1992). Since extracellular enzymes require far less processing to achieve commercially useful forms than do intracellular enzymes (Aitken, 1993), ARP has the strong potential of being a cost effective alternative to HRP in wastewater treatment.

A detailed list of enzymes applied to phenol removal is contained in Table 1.2.

Table 1.2. Enzymes Applied to Phenol Removal.

Enzymes	Researchers
Arthromyces ramosus peroxidase	(Al-Kassim et al., 1993); (Ibrahim et al., 1997)
Coprinus macrorhizus peroxidase	(Al-Kassim et al., 1994); (Heck, 1995)
Coprinus cinereus peroxidase	(Andersen, 1991)
Cauliflower peroxidase	(Lee et al., 1984)
Chloroperoxidase	(Aitken et al., 1994); (Carmichael et al., 1985)
Hemoglobin (bovine)	(Chapsal et al., 1986)
Horseradish peroxidase	(Klibanov and Morris, 1981); (Klibanov et al., 1980); (Alberti, 1982); (Danner et al., 1973); (Nakamoto and Machida, 1992); (Arseguel and Baboulene, 1994); (Baynton et al., 1994); (Nicell, 1991); (Sawahata and Neal, 1982); (Wu et al., 1993), 1994 ; (Yu et al., 1994); (Buchanan, 1996); (Cooper and Nicell, 1996); (Tong, 1997)
Laccase	(Hoff et al., 1985); (Bollag et al., 1988); (Milstein et al., 1988); (Kersten et al., 1990); (Roy-Arcand and Archibald, 1991)
Lignin peroxidase	(Aitken et al., 1989)
Polyphenol oxidase (known also as tyrosinase)	(Atlow et al., 1984); (Estrada et al., 1991); (Sun et al., 1992); (Wada and Ichikawa, 1993);
Soybean peroxidase	(Wright and Nicell, 1995 b)

1.4. Objective and Scope

The objective of this research is to investigate the potential of *Arthromyces ramosus* peroxidase (ARP) to catalyze the polymerization and precipitation of phenol from aqueous solution.

The scope of this research includes:

- Characterization of *Arthromyces ramosus* peroxidase(ARP) with respect to its catalytic activity, thermal stability, and ability to remove phenols from a synthetic wastewater.
- Investigation of the inactivation of ARP by reaction products with and without polyethylene glycol (PEG).
- Estimation of the kinetic rate constants.

2. Literature Review

2.1. Peroxidase Enzymes

Peroxidase enzymes are capable of catalyzing the oxidation of a variety of organic and inorganic compounds after being activated by hydrogen peroxide or some other peroxides (Dunford and Stillman, 1976). The primary function of these enzymes is to mediate the oxidation of a variety of organic and inorganic compounds by peroxides such as hydrogen peroxide (H_2O_2). Peroxidases and isoperoxidases have been investigated intensively, because they are widely distributed in living organisms, mostly in plants. It appears that they have masses from 40,000 to 50,000 Daltons (Saunders et al., 1964). Isoperoxidases, or isoenzymes are different molecular forms of an enzyme which catalyze the same reaction within the same species (Bailey and Ollis, 1986). Isoperoxidase C from horseradish may consists four distinct components - a heme group, amino acid residues, carbohydrate side chains, and calcium ions (Dunford, 1990). Among these components, the ferric heme prosthetic group is known to be the center of peroxidase activity in HRP. The sap of the fig tree and the root of the horseradish are particularly rich in peroxidase, and reports have shown the presence of peroxidases in various fungi and bacteria (Saunders et al., 1964).

The concept of the enzymatic removal of pollutants was developed in the 1970's (Aitken, 1993). Peroxidases that have been studied for the laboratory-scale treatment of aqueous aromatic compounds include horseradish peroxidase (HRP), *Arthromyces*

ramosus peroxidase (ARP), soybean peroxidase (SBP), lignin peroxidase, and a number of others from various sources (see Table 1.2).

HRP is the most studied enzyme for application to wastewater treatment. Once activated by H_2O_2, HRP catalyzes the oxidation of a wide variety of toxic aromatic compounds such as phenols and aromatic amines. It is reported that HRP can precipitate phenol in a wide range of pH values and temperatures. HRP is also known to coprecipitate certain hard-to-remove contaminants (Klibanov et al., 1980; Nicell et al., 1993).

The mechanism of HRP reaction is relatively well understood and has been described in many reports. Recently, Nicell (1994) developed and compared steady-state, transient-state, and pseudo-steady-state models to understand HRP reaction. Buchanan and Nicell (1998 b) developed a simplified model of HRP-phenol polymerization.

Soybean peroxidase is found in the mature seed coat of the soybean from Glycine max (Gillikin and Graham, 1991). Wright (1995) reported that seven phenolic compounds were removed up to 99% by an excess dose of soybean peroxidase at alkaline reaction pH values.

Kjalke and coworkers (1992) found that peroxidases from *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus* are most likely identical in their primary structure, but deviate in N-linked glycosylation. The authors mentioned glycan differences had no relation to catalytic activity. Specific activity of ARP with a number of substrates was the same order of magnitude as that of HRP. Ibrahim and coworkers (1997) reported the optimum pH for phenol removal with ARP at room temperature to be pH 7.5 with

sodium phosphate buffer, and pH 7.0 with dechlorinated tap water. In their experiments, ARP achieved 94% phenol removal efficiency when alum and PEG were used under the optimum conditions.

2.2. Stability of Peroxidases

In order to apply enzymes to industrial waste treatment, the enzymes should retain their activity over expected temperature and pH ranges. Since enzymes have complex tertiary molecular structures, their structures are very delicate and fragile. If the enzyme molecule absorbs too much energy (high temperature), the tertiary structure will be deformed and the enzyme will be denatured and lose its catalytic activity (Segel, 1993).

Enzymes may contain both positively and negatively charged groups at any given pH. Such ionizable groups are often included in the active site, since acid-base reactions have been linked closely to several enzyme mechanisms. The catalytically active enzyme exists in only certain particular ionization states. Thus, changing pH affects the activity of the enzyme. The effects of pH and temperature for several enzymes will be discussed briefly below.

2.2.1. Horseradish Peroxidase (HRP)

The thermal stability of HRP was studied by Nicell and co-workers (1993 b). They reported that the relative activity of HRP increased with temperature up to 50 °C and then

decreased rapidly thereafter. HRP showed a stable activity at neutral pH and 4 °C for one month. The dry enzyme retains its activity for several years when stored at -15°C, and for many weeks when stored at 37 °C. It was also found that the HRP retains its activity between pH values of 4 and 10 although in the pH range of 6 to 9 HRP showed the highest activity (Nicell, 1991).

Saadi (1993) studied HRP catalyzed phenol removal over a pH range from 6 to 10, and found the optimum pH to be 9.2. In addition, significantly decreased removal efficiency was observed for pH values greater than 10. He also reported that the pH dependence of HRP efficiency is more significant at low enzyme concentrations.

2.2.2. Soybean Peroxidase (SBP)

Soybean peroxidase (SBP) incubated at 25 °C showed practically stable activity within the range of pH 7 to 10.7, and in unbuffered distilled water over a 12 day period. However, at 25 °C, SBP was observed to have almost zero activity at pH 2 and pH 3, and less than 70% of the initial activity at pH 5.0, pH 6.0 and pH 6.4 after several days incubation period (Wright, 1995).

Wright (1995) observed thermal inactivation of SBP was first order and modeled it using:

$$A(t) = A_0 e^{-kt} \qquad (2.1)$$

where A(t) is activity at time t, A_0 is the activity at time t=0, and k is the inactivation decay constant. He also reported that SBP shows various thermostability depending on the incubation pH. Wright (1995) concluded that SBP is much more stable than HRP and shows optimal stability at pH 6.0 for temperatures up to 90.8 °C.

Thermal stability of SBP was also studied by McEldoon and Dordick (1996). They reported that SBP is thermally very stable at pH 8.0 in the presence of 1 mM $CaCl_2$ and that SBP holds onto its heme much more tightly than does HRP.

2.2.3. Cauliflower Peroxidase

Lee and co-workers (1984) investigated the heat inactivation of cauliflower peroxidase isoenzyme over a range from 30 to 55°C for incubation the times from 0 to 60 minutes, and found that the activity increased with increasing temperature up to 40 °C and rapidly decreased thereafter. When the enzyme was heated to 50 °C, approximately 98% of the activity was lost within 15 minutes.

2.2.4. *Arthromyces ramosus* Peroxidase (ARP)

Since *Arthromyces ramosus* peroxidase is comparatively new, limited information is available on the stability of the enzyme.

Shinmen and coworkers (1986) reported that purified ARP was stable in the pH range of 5.0 to 9.0 at 30 °C for 16 hours, and below 50 °C at pH 7.0 for 30 minutes.

Andersen and coworkers (1991) reported that *Coprinus cinereus* peroxidase (CiP), which is identical to ARP, dialyzed in Milli Q water lost less than 10 % of its activity within a month when stored at 5 °C.

The thermostability of ARP (at pH 8.8 with Tris-HCl buffer) was compared with that of HRP (at pH 8.5 with Tris-HCl buffer) by Kim and coworkers (1991 a) over various incubation time periods at 45 °C. In their studies, ARP showed over 90 % loss of activity within 25 minutes while the relative activity of HRP increased to almost 100 % over a 50 minute period, and they concluded that ARP is thermally less stable than HRP. However, this result disagrees with data reported by Nicell (1991) which showed approximately 30% loss of HRP activity at 45 °C and pH 7.4 over a 60 minute period.

2.3. The Catalytic Cycle of Peroxidase

The kinetics of the entire enzymatic process were established by Nicell in 1991. The complete cycle of the reaction is shown in Figure 2.1.

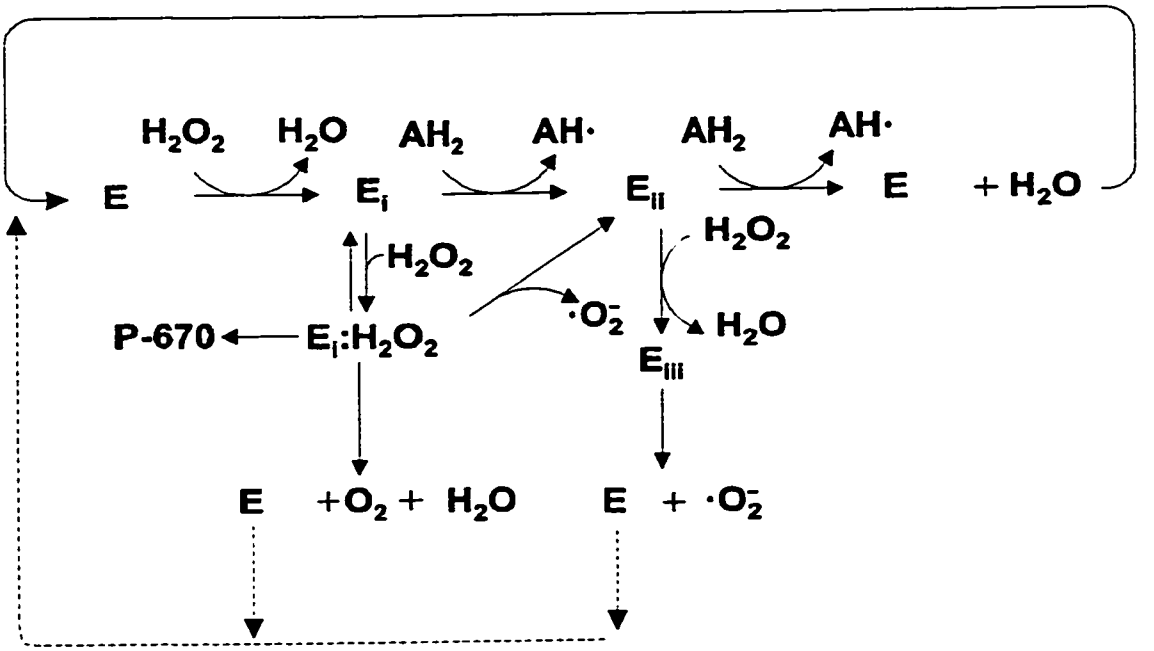

Figure 2.1. Peroxidase catalytic cycle and side reactions (After Nicell, 1991).

The one-electron oxidation of organic substrates (AH_2) catalyzed by peroxidase is depicted by the well-known Chance-George mechanism (Chance, 1943; Chance, 1952; George, 1952; George, 1953) .This process is usually represented as follows :

$$E + H_2O_2 \xrightarrow{k_1} E_i + H_2O \qquad (2.2)$$

$$E_i + AH_2 \xrightarrow{k_2} E_{ii} + AH\cdot \quad (2.3)$$

$$E_{ii} + AH_2 \xrightarrow{k_3} E + AH\cdot + H_2O \quad (2.4)$$

The native form of peroxidase, compound I, and compound II are represented by E, E_i, E_{ii}, respectively, while the oxidizable substrate has been represented by AH_2. Hydrogen peroxide oxidizes the native enzyme to make compound I (E_i). Then, compound I oxidizes the substrate which is an aromatic compound (AH_2) to produce a free radical (AH•), leaving the enzyme in the compound II (E_{ii}) state. Compound II, also called ferryl peroxidase, oxidizes another aromatic molecule, producing one more radical and returning the enzyme to its native form, thus completing the cycle.

The overall reaction can be expressed as :

$$H_2O_2 + 2AH_2 \longrightarrow 2AH\cdot + 2H_2O \quad (2.5)$$

These free radicals combine to form dimers and as the reaction continues eventually make even larger polymers as shown conceptually in equation (2.6). These polymers tend to be less soluble in water than their monomeric precursors and can precipitate from solution.

$$\text{Free radical} + \text{Free radical} \xrightarrow{k_p} \text{Polyaromatic Product} \downarrow \quad (2.6)$$

While reactions (2.2), (2.3), and (2.4) proceed in the reaction mixture of enzyme, hydrogen peroxide and aromatic substrate, a number of side reactions take place, some of

which are related to inactivation and inhibition of the enzyme causing limitation of its catalytic life. Much of what is known of the mechanisms of peroxidase reactions has come from the study of HRP. Thus, the following discussion will be focused on HRP. Other peroxidases are likely to be susceptible to these side reactions in varying degrees which have yet to be established.

The compound II (E_{ii}) state of HRP can be oxidized by hydrogen peroxide and transformed to the compound III state (E_{iii}) which is a catalytically inactive form (Dunford and Stillman, 1976). This reaction can be represented by the following scheme:

$$E_{ii} + H_2O_2 \xrightarrow{k_{app}} E_{iii} + H_2O \qquad (2.7)$$

Compound III is structurally and electronically similar to oxymyoglobin and oxyhemoglobin, and for this reason, compound III is often called an oxyperoxidase (Wittenberg et al., 1967). It is largely accepted that compound III is relatively inactive (Smith et al., 1982), and decomposes slowly to the native form of HRP (E) (Nakajima and Yamazaki, 1987) according to :

$$E_{iii} \xrightarrow{k_{eff}} E + \cdot O_2^- \qquad (2.8)$$

The detailed mechanism of compound III formation was studied by Nakajima and Yamazaki (1987) and Adediran and Lambeir (1989). Another pathway for the return of HRP from compound III to native enzyme in the presence of a substrate was suggested

by Tamura and Yamazaki (1972). However, neither the rate constants nor the pathways for these reactions have been confirmed by any other researchers.

2.4. Permanent Inactivation of Peroxidases

Inactivation of the enzyme is a very significant aspect since it results in increased quantities of the enzyme being required for a given level of treatment, which increases the cost of treatment. Inactivation occurs via several routes in an enzymatic process. Modest heating, changing pH, or exposure to sunlight and to ultra-violet radiation can change the activity of the enzyme. Since enzyme activity is limited by these chemical, thermal, and mechanical processes, optimization of the process is needed for maximizing the enzyme catalytic life.

The main mechanisms of the permanent enzyme inactivation are thought to be caused by interactions between phenoxy radicals and enzymes (Klibanov et al., 1983), adsorption of enzyme by polymerized phenol , and blocking the active site of the enzyme by phenolic polymers (Nakamoto and Machida, 1992). Klibanov (1983) reported the interactions of the phenoxy radicals with the enzyme's active center as the main cause of the permanent inactivation of peroxidase. Inactivation by a free radical may be expressed as:

$$\mathbf{E_{active}} + \mathbf{Free\ Radical} \xrightarrow{k_r} \mathbf{E_{inactive}} \qquad (2.9)$$

Peroxidase also shows suicide inactivation with excess hydrogen-peroxide in the absence of organic substrates, in which a permanently inactive form of enzyme, verdohemoprotein (P-670) is produced according to (Arnao et al., 1990):

$$\begin{array}{c} E_{ii} + \cdot O^-_2 \\ \uparrow \\ E_i + H_2O_2 \longleftrightarrow (E_i \cdot H_2O_2) \longrightarrow E + O_2 + H_2O \\ \downarrow \\ P\text{-}670 \end{array} \qquad (2.10)$$

where the "670" refers to the peak wavelength at which this compound absorbs light. Nakajima and Yamazaki (1980) reported the formation of the verdohemoprotein occurred via two intermediates which have an absorbance peak 965 or 940 nm.

$$\textbf{Compound I} \rightarrow \textbf{965 compound} \rightarrow \textbf{940 compound} \rightarrow \textbf{P} - \textbf{670} \qquad (2.11)$$

The complex (E_i-H_2O_2) also can be converted to compound II through:

$$(E_i \cdot H_2O_2) \longrightarrow E_{ii} + \cdot O_2^- \qquad (2.12)$$

Adediran (1996) claimed P-670 is produced from compound III in two parallel reactions according to:

$$\begin{array}{l} \textbf{compound III} + H_2O_2 \longrightarrow \textbf{compound II} \rightarrow \textbf{Ferriperoxidase} \\ \downarrow \\ \textbf{P-670} \end{array} \qquad (2.13)$$

Thus, there is not agreement in the literature as to pathways which lead to the formation of P-670. Nicell (1994) showed that less than 0.1 % of the HRP dose in batch tests was

converted to P-670 in the presence of an aromatic substrate and less than 0.02 % of the initial hydrogen peroxide was consumed in these side reactions. Thus, the amount of HRP inactivated via this pathway would be negligible during treatment.

2.4.1. Chemical Additives

Additives, such as gelatin and polyethylene glycol (PEG), are reported to hamper the adsorption of peroxidase by polymerized phenol, resulting in a great reduction in the required amount of peroxidase by up to 200 fold. Nakamoto and Machida (1992) reported that the apparent enzyme inactivation occurs mainly by the adsorption of peroxidase molecules onto polymerized phenol.

Besides gelatin and PEG, certain polyelectrolytes, milk casein, borate, bovine serum albumin and polyvinyl alcohol were also effective (Nakamoto and Machida, 1992; Wu et al., 1997). Arseguel and Baboulene (1994) found talc was also effective to prolong the enzymatic life by adsorption of polymeric products onto the talc.

Among these additives, polyethylene glycol is found to be the best choice since it saved more peroxidase and had no negative overdose effect. PEG is also more competitive than the others in terms of cost; is safe for human consumption; and can yield a low residual PEG concentration. Other additives have critical drawbacks which limit their application to the enzymatic process. The removal efficiency may be reduced with Gelatin and polyelectrolytes in the presence of excess hydrogen peroxide (Wu et al., 1997). Borate should not be used since it is a pesticide.

2.5. Kinetic Studies for HRP and ARP

2.5.1. Horseradish Peroxidase

The values of kinetic rate constants for HRP catalyzed treatment have been reported by several authors. The rate constant associated with the oxidation of HRP by hydrogen peroxide, k_1, was reported at 25°C and pH 7.0 by Yamazaki and Nakajima (1986). Values for k_2 and k_3 for HRP oxidation of various substrates have also been reported in literature (Job and Dunford, 1976; Dunford and Adeniran, 1986; Sakurada et al., 1990). Nakajima and Yamazaki (1987) reported k_{app} value at 20°C and pH 7.0, and k_{eff} at 20°C and pH 7.0 in the absence of a reductant substrate (AH_2). The k_{app} value was also reported by Adediran and Lambier (1989) at 25°C and pH 7.0.

A summary of reported rate constant values for HRP catalyzed reactions is presented in Table 2.1.

Table 2.1. Kinetic Rate Constants Studied Previously with HRP.

Constant	pH, Temperature	Values	Literature source
k_1 ($M^{-1}s^{-1}$)	7.0 , 25 °C	$2x10^7$	(Yamazaki and Nakajima, 1986)
* k_2 ($M^{-1}s^{-1}$)	7.0 , 25 °C	$2.76x10^6$	(Job and Dunford, 1976)
* k_3 ($M^{-1}s^{-1}$)	7.0 , 25 °C	$3.15x10^5$	(Sakurada et al., 1990)
		$2.86x10^5$	(Dunford and Adeniran, 1986)
k_{app} ($M^{-1}s^{-1}$)	7.0 , 25 °C	20	(Adediran and Lambeir, 1989)
	7.0 , 20 °C	25	(Nakajima and Yamazaki, 1987)
k_{eff} (s^{-1})	7.0 , 20 °C	2.2×10^{-3}	(Nakajima and Yamazaki, 1987)

* Rate constant is quoted specifically for phenol as substrate.

2.5.2. *Arthromyces ramosus* Peroxidase

Kjalke and coworkers (1992) reported k_1 as being $3.8 \pm 0.2 \times 10^6$ $M^{-1}s^{-1}$ in pH 7.5 for guaiacol at 25 °C. The authors also showed the k_1 value depends on the pH.

However, Abelskov and coworkers (1997) evaluated the rate constant k_1 for compound I formation at 25 °C and pH 7.0 by transient-state analysis as being $9.9 \pm 0.6 \times 10^6$ $M^{-1}s^{-1}$ which is about 2.5 times larger than Kjalke's value. The k_1 rate constant evaluated by steady-state analysis was $8.8 \pm 0.6 \times 10^6$ $M^{-1}s^{-1}$ in the presence of ferulic acid and $6.7 \pm 0.2 \times 10^6$ $M^{-1}s^{-1}$ in the presence of ABTS (2,2'-azinobis). Both of these values were measured at 25 °C and pH 7.0. The authors also reported that the values of k_2 and k_3 were identical at pH 5 to 6, and the ratio k_2/k_3 increases to 10 at pH 10 using transient-state analysis with ferulic acid. Both k_2 and k_3 were observed to decrease with increasing pH (Abelskov et al., 1997).

Kim and coworkers (1991 a) studied pre-steady-state kinetics of ARP-catalyzed luminol oxidation at 25 °C, in pH 8.8 borate buffer and evaluated the rate constant k_1, k_2 and k_3 as $2.7 \times 10^6 (M^{-1}s^{-1})$, $2.4 \times 10^7 (M^{-1}s^{-1})$ and $2.2 \times 10^6 (M^{-1}s^{-1})$.

Andersen and coworkers (1991) reported k_1 to vary with various pH at 25 °C according to:

$$k_1 = \frac{7.1 \times 10^6}{1 + \frac{[H^+]}{10^{-4.9}}}$$

According to this expression, at pH 7.0 and 25 °C, $k_1 = 7.04 \times 10^6$ ($M^{-1}s^{-1}$).

The summary of the rate constants for ARP is indicated in Table 2.2.

Table 2.2. Literature Values of Kinetic Constants for *Arthromyces ramosus* Peroxidase (all values are determined at 25 °C).

Kinetic parameter	pH	Reductant substrate	Value	Literature source
k_1 ($M^{-1}s^{-1}$)	7.5	Guaiacol	$3.8 \pm 0.2 \times 10^6$	(Kjalke et al., 1992)
	7	ABTS*	$6.7 \pm 0.2 \times 10^6$	(Abelskov et al., 1997)
	7	Ferulic acid	$8.8 \pm 0.6 \times 10^6$	(Abelskov et al., 1997)
	7	None	$9.9 \pm 0.6 \times 10^6$	(Abelskov et al., 1997)
	8.8	Luminol	2.7×10^6	(Kim et al., 1991 a)
	NA	None	$7.1 \pm 0.1 \times 10^6$	(Andersen et al., 1991)
k_2 ($M^{-1}s^{-1}$)	8.8	Luminol	2.4×10^7	(Kim et al., 1991 a)
k_3 ($M^{-1}s^{-1}$)	8.8	Luminol	2.2×10^6	(Kim et al., 1991 a)
	8	Guaiacol	$2.5 \pm 0.1 \times 10^5$	(Kjalke et al., 1992)
	5	Iodide	$2.5 \pm 0.1 \times 10^4$	(Kjalke et al., 1992)

* ABTS : 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
NA : Not applicable

2.6. Enzymatic Removal of Phenol

2.6.1. Horseradish Peroxidase

A number of enzymes have been studied for phenol removal processes by a number of research groups as previously mentioned, and among the enzymes HRP is the most studied enzyme.

The Klibanov group (1980) investigated removal efficiencies of over 30 different phenols and aromatic amines with HRP. Among 16 different phenols, several phenols (e.g. o-, p-chlorophenols, m-methoxyphenol, and 2,3-dimethylphenol) showed high removal efficiencies up to 99.8 % and the others showed lower percent of removal in the range 70 to 95 %. The authors also reported enhanced removal by coprecipitation of hard-to-remove compounds with HRP. Nicell and coworkers (1993 b) also demonstrated, when 4-methylphenol, 2,4-dichlorophenol and phenol were treated together, a lower residual was achieved than when they were treated separately. Since real wastewaters may contain many different aromatic pollutants from various sources, the coprecipitation aspect can be a good advantage for enzymatic wastewater treatment.

Nicell and coworkers (1991; 1992) investigated the removal of phenols as a function of pH, and reported that the optimum pH for various aromatic substrates occurs within a range of 7 to 9. It was demonstrated that decreasing the rate of HRP addition to the reaction mixture increased the removal efficiency for a fixed total HRP dose in the absence of a protective additive. However, no improvement in catalytic lifetime was observed with the gradual addition of peroxide.

Nakamoto and Machida (1992) demonstrated that HRP was effective at treating even very high phenol concentrations, removing over 90 % of 30 g phenol/L in the presence of PEG. Even though 30 g phenol/L is not encountered in real wastewaters , the result showed the ability of HRP to treat strong wastewaters. They also tested a real wastewater sample (pH 7.6) from a semiconductor manufacturing plant containing 9.1 g/L phenol, and the phenol removal efficiency reached 97 % with gelatin as an additive to suppress the inactivation of HRP.

According to Wu and co-workers (1993), the optimum pH for phenol removal is 8.0 with phosphate buffer and the optimum molar ratio of hydrogen peroxide to phenol is near 1.0 for the HRP catalyzed phenol removal process in the presence of PEG.

2.6.1.1. Empirical Models

The relationships between the minimum amount of HRP required to achieve 90 % removal efficiency for phenol in the presence of PEG or the absence of PEG were established by Saadi (1993).

$$[HRP]_{\text{with PEG}} = 0.0644\,[\text{phenol}]_0 \qquad (2.14)$$

$$[HRP]_{\text{without PEG}} = 1.431\,[\text{phenol}]_0 \qquad (2.15)$$

where [HRP] is enzyme concentration (U/mL) and $[phenol]_0$ is initial phenol concentration (mM). The minimum amount of PEG (mg/L) required to achieve the 90 % removal efficiency for the initial phenol concentration was formulated as:

$$\mathbf{[PEG]\ (mg/L)\ =\ 19.16\ [phenol]_0} \tag{2.16}$$

where $[phenol]_0$ is the initial concentration of phenol in mM.

Wu and coworkers (1993) also correlated the minimum HRP dose required to remove 95 % of initial phenol in the presence of PEG , and the minimum dose of PEG (mg/L) under the minimum dose of HRP (U/mL) for at least 95 % phenol (mM) removal as follows:

$$\mathbf{[HRP]_{min}\,(U/mL) = 0.0435 + 0.0048\,[phenol]_0 + 0.0031\,[phenol]_0^{\,2}} \tag{2.17}$$

$$\mathbf{[PEG]_{min}\,(mg/L) = 0.0065 + 0.0241\,[phenol]_0} \tag{2.18}$$

2.6.2. *Arthromyces ramosus* Peroxidase (ARP)

Arthromyces ramosus peroxidase (ARP) has attracted growing interest due to the ease of production, high specific activity, and substrate profiles similar to HRP.

Ibrahim and co-workers (1997) reported that the ARP-catalyzed polymerization and precipitation is an effective method for the removal of phenol from wastewater. They also found that polyethylene glycol (PEG) was effective in improving the removal efficiency by

28 tc 70% for 1 and 10 mM phenol solutions, respectively. The minimum PEG dose of 30 mg/L for 1 mM phenol and 120 mg/L for 10 mM phenol were reported. Minimum ARP dose was found to be 0.6 U/mL for 95% removal of 1 mM phenol. The optimum pH for phenol removal was reported to be 7.0, and the optimum molar ratio of H_2O_2 to phenol was reported as 1.15:1.0.

2.6.3. Soybean Peroxidase (SBP)

Soybean peroxidase, which is present in the mature seed coat of soybean, catalyzes removal of phenolic compounds. A 1mM initial concentration of phenol was treated over a wide pH range from 2.0 to 10.0. A 99.7 % removal efficiency was observed at pH 6.0 and 99% removal was observed over the pH range from 5 to 9 with a SBP dose of 3.31 U/mL in a 3-hour batch reaction (Wright, 1995).

Information concerning phenol removal with SBP and additives is not available in literature. Since the phenol removal efficiency is limited by inactivation of SBP, additives such as PEG may help to prevent the inactivation of SBP, resulting in reduced SBP requirements.

2.7. Kinetic Models

Steady-state kinetic models have been developed as typical methods for evaluation of enzyme rate constants. However, steady-state models have limitations for reactor development because they express only the initial conditions of a reaction (Nicell, 1991).

These models have difficulties to accurately predict the reaction under non-ideal or unsteady-state conditions.

Nicell (1994) developed steady-state, fully transient, and pseudo-steady-state models of the HRP system to obtain a better understanding of the inactivation mechanisms and to aid in the selection of the best reactor system.

The steady-state model was developed with the following assumptions: (1) Inactivation reactions are insignificant due to their comparatively small rate constants relative to those in the enzymatic cycle. (2) Since the rate constants k_1 and k_2 which express the rate constants for the formation of compound I from native enzyme and peroxide, and subsequent production of compound II are extremely rapid, compound I and II are present at steady-state levels. (3) The stoichiometric ratio between peroxide and aromatic substrate is 1:1. The steady-state model was found to be inadequate in modeling the transient behavior of the reaction, and indicated the importance of modeling enzyme inactivation (Nicell, 1991).

The transient state model was based on the reactions shown in Figure 2.1 and modeled permanent inactivation of HRP as being directly proportional to substrate removal. The model showed a good predicting ability for the treatment of 4-chlorophenol in batch reactors. However, the transient state model needed excessive time for the computation. Application of the transient state model showed the reactions involving the enzyme-peroxide complex shown in equation (2.10) to have an insignificant effect on substrate removal and enzyme activity for reactions in the presence of a reductant

substrate, such as phenol. Thus, this system of side reactions could be neglected without significantly affecting the model's predictive ability. For these reasons, the pseudo-steady-state model which incorporated a dynamic steady-state assumption into the transient state model was developed (Nicell, 1991).

2.7.1. The Pseudo-Steady-State Model

The pseudo-steady-state model showed great reduction of computation time by a factor of 1/20000 in comparison with transient state model, while still maintaining the good predictive ability (Nicell, 1994). This kinetic model is based on the reactions given in Figure 2.2, which contains all reactions shown in Figure 2.1 except for the enzyme-hydroxide complex, E_i-H_2O_2, related reactions.

The pseudo steady-state model was developed based on the following assumptions: (1) The quantity of inactivated enzyme is directly related to the quantity of aromatic substrate removed from solution. (2) In the presence of an aromatic substrate, the catalytic reaction with which the HRP-peroxide complex is associated and its corresponding peroxide consumptions are insignificant. (3) Compound II is formed only via the reaction of compound I with an aromatic molecule. (4) Compound I and II exist at a dynamic steady-state. (5) Aromatic substrates and peroxide are consumed with a 1:1 stoichiometric ratio (Nicell, 1991).

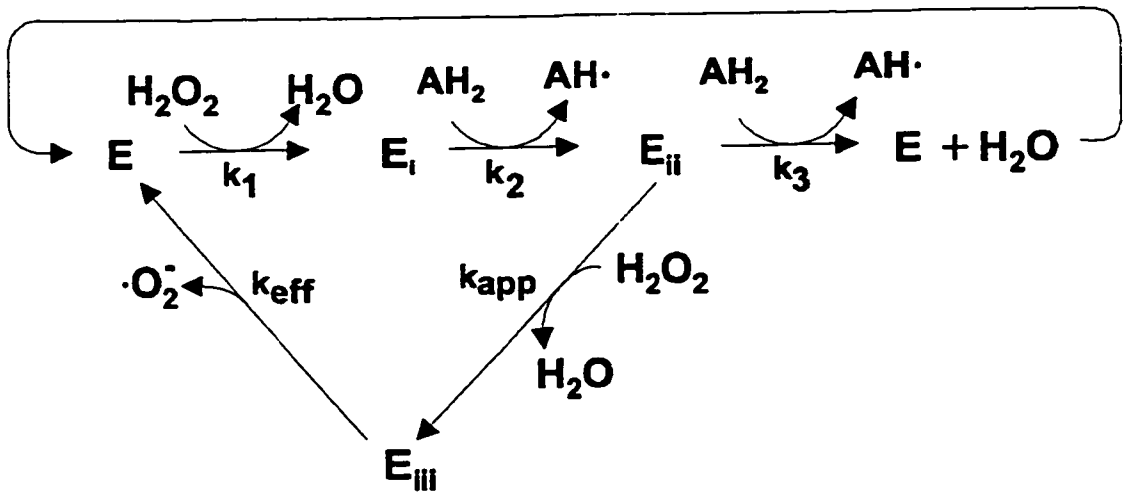

Figure 2.2. The main reactions that occur in the presence of an aromatic substrate.

The pseudo-steady-state model includes the following reactions:

$$E + H_2O_2 \xrightarrow{k_1} E_i + H_2O \qquad (2.2)$$

$$E_i + AH_2 \xrightarrow{k_2} E_{ii} + AH\cdot \qquad (2.3)$$

$$E_{ii} + AH_2 \xrightarrow{k_3} E + AH\cdot + H_2O \qquad (2.4)$$

$$E_{ii} + H_2O_2 \xrightarrow{k_{app}} E_{iii} + H_2O \qquad (2.7)$$

$$E_{iii} \xrightarrow{k_{eff}} E + \cdot O_2^- \qquad (2.8)$$

$$E + K_s AH_2 \longrightarrow E_{inact} + \text{polymer products} \qquad (2.19)$$

where K_s is the number of molecules of AH_2 removed per enzyme molecule provided to the reaction mixture.

Nicell (1994) noted two major inadequacies in the modeling of HRP inactivation: (1) the assumption of a fixed number of substrate molecules removed per enzyme molecule inactivated; and (2) the over prediction of compound III production.

2.7.2. Modified Pseudo Steady-State Model

Buchanan and Nicell (1997) modified the pseudo-steady-state model to incorporate enzyme inactivation mechanisms resulting in improved predictive ability. This modified model accounted for permanent enzyme inactivation by free radicals as well as interaction with end-product polymers as they form.

Based on the Law of Mass Action, the following differential equations were written to describe the rate of compound formation:

$$\frac{dEi}{dt} = k_1 E[H_2O_2] - k_2 E_i[AH_2] \qquad (2.20)$$

$$\frac{dE_{ii}}{dt} = k_2 E_i[AH_2] - k_3 E_{ii}[AH_2] - k_{app} E_{ii}[H_2O_2] \qquad (2.21)$$

$$\frac{dE_{iii}}{dt} = k_{app}E_{ii}[H_2O_2] - k_{eff}E_{iii} \quad (2.22)$$

Based on the observed 1:1 stoichiometry, the rate of aromatic substrate removal, ($-d[AH_2]/dt$), can be equated to the rate of peroxide consumption, ($-d[H_2O_2]/dt$).

$$-\frac{d[AH_2]}{dt} = -\frac{d[H_2O_2]}{dt} = k_1E[H_2O_2] + k_{app}E_{ii}[H_2O_2] \quad (2.23)$$

The value of k_1 is many orders of magnitude greater than that of k_{app}, thus $k_1E[H_2O_2] >> k_{app}E_{ii}[H_2O_2]$ and (2.23) can be simplified as:

$$\frac{d[AH_2]}{dt} \approx -k_1\,E\,[H_2O_2] \quad (2.24)$$

The peroxide concentration may be calculated from the observed 1:1 stoichiometry as:

$$[H_2O_2] = [H_2O_2]_0 - ([AH_2]_0 - [AH_2]) \quad (2.25)$$

An enzyme mass balance is

$$E = E_0 - E_i - E_{ii} - E_{iii} - E_{inact} \quad (2.26)$$

where E_{inact} represents permanently inactive enzyme, and E_0 represents the initial amount of enzyme provided to the reaction mixture.

The formation and conversion of compounds I and II are very rapid reactions compared to the other reaction pathways. Thus, the concentrations of these compounds

respond very quickly to changes in the reaction conditions and may be modeled as being at a pseudo steady-state. Therefore, the rates of change of these compounds may be equated to zero to give:

$$\frac{dE_i}{dt} \approx 0 \text{ and } \frac{dE_{ii}}{dt} \approx 0 \tag{2.27}$$

Equating (2.20) and (2.21) to zero, and solving for E_i and E_{ii}, respectively, gives:

$$E_i = \frac{k_1 E[H_2O_2]}{k_2[AH_2]} \tag{2.28}$$

$$E_{ii} = \frac{k_1[H_2O_2]E}{k_3[AH_2] + k_{app}[H_2O_2]} \tag{2.29}$$

Substitution of equation (2.28) and (2.29) into the enzyme mass balance and solving for the native enzyme concentration gives:

$$E = \frac{E_0 - E_{iii} - E_{inact}}{\left[1 + \frac{k_1[H_2O_2]}{k_2[AH_2]} + \frac{k_1[H_2O_2]}{k_3[AH_2] + k_{app}[H_2O_2]}\right]} \tag{2.30}$$

Equation(2.30) can be substituted into equation (2.24) to give an expression for the aromatic compound removal:

$$\frac{d[AH_2]}{dt} = \frac{-(E_0 - E_{iii} - E_{inact})}{\left[\frac{1}{k_1[H_2O_2]} + \frac{1}{k_2[AH_2]} + \frac{1}{k_3[AH_2] + k_{app}[H_2O_2]}\right]} \tag{2.31}$$

The rate of compound III formation can be obtained by substitution of equation (2.30) into (2.29) and the resulting expression into (2.22) to give:

$$\frac{dE_{iii}}{dt} = \frac{k_{app}(E_0 - E_{iii} - E_{inact})[H_2O_2]}{1 + \left((k_3[AH_2] + k_{app}[H_2O_2]\right)\left[\frac{1}{k_1[H_2O_2]} + \frac{1}{k_2[AH_2]}\right]} - k_{eff}E_{iii} \qquad (2.32)$$

Since the rate of reaction of compound II with aromatic substrate (k_3) is much greater than the rate of compound III formation from compound II and peroxide (k_{app}), i.e. $k_3[AH_2] >> k_{app}[H_2O_2]$, equation (2.32) can be simplified as:

$$\frac{dE_{iii}}{dt} = \frac{k_{app}(E_0 - E_{iii} - E_{inact})}{\left[1 + \frac{k_3}{k_2} + \frac{k_3[AH_2]}{k_1[H_2O_2]}\right]}[H_2O_2] - k_{eff}E_{iii} \qquad (2.33)$$

The overall rate of permanent enzyme inactivation is modeled by Buchanan and Nicell (1997) as follows:

$$\frac{dE_{inact}}{dt} = k_r(E_0 - E_{inact})\sqrt{(-\frac{d[AH_2]}{dt})} - k_e\frac{d[AH_2]}{dt} \qquad (2.34)$$

in which k_r is a lumped parameter ($M^{-0.5}S^{-0.5}$) associated with enzyme inactivation by free radicals, and k_e is a dimensionless proportionality constant associated with enzyme inactivation by end-product polymer.

2.7.3. A Simplified Kinetic Model

The values of the rate constants associated with the system of reactions outlined in Figure 2.2 depend on the type of peroxidase and substrate, as well as reaction temperature and pH. Thus, a comparison on a kinetic basis of the efficiencies of alternative peroxidases to accomplish the treatment of various aromatic substrates would require the specification of kinetic constants for each peroxidase, substrate, pH, and temperature, which are rarely available in the literature. The evaluation of these rate constants has been typically accomplished using highly purified enzyme stocks and sophisticated and time-consuming analytical techniques. Recently, Buchanan and Nicell (1997) employed a global optimization procedure, based on a genetic algorithm, to estimate the values of the rate constants k_3, k_{eff}, k_{app} (see Figure 2.2) as well as those associated with permanent enzyme inactivation in the presence or the absence of PEG. This methodology represents a vast improvement on the traditional techniques for rate constant evaluation. However, this procedure was not appropriate to evaluate the rate constants, k_1 and k_2, which are associated with the two most rapid steps in the catalytic cycle: the oxidation of the enzyme by hydrogen peroxide, and the first of two subsequent reactions with the reductant substrate, as indicated in Figure 2.2. This problem was partially overcome by Nicell and Wright (1997) who developed a methodology for estimating the value of k_1, based on simple spectrophotometric techniques and a standard enzyme assay. However, a reliable value of k_2 still cannot be obtained using widely available analytical techniques.

Therefore, a simplified model in which compounds I and II are lumped together and expressed as the active enzyme (E_a), was produced by Buchanan and Nicell (1998 b). This simplification allows all kinetic constants of the model to be estimated using widely available analytical techniques. The simplified model has been shown to predict HRP catalyzed phenol removal from batch reactors with accuracy comparable that of the more complex model presented in Buchanan and Nicell (1997). The scheme of simplified reactions is indicated in Figure 2.3.

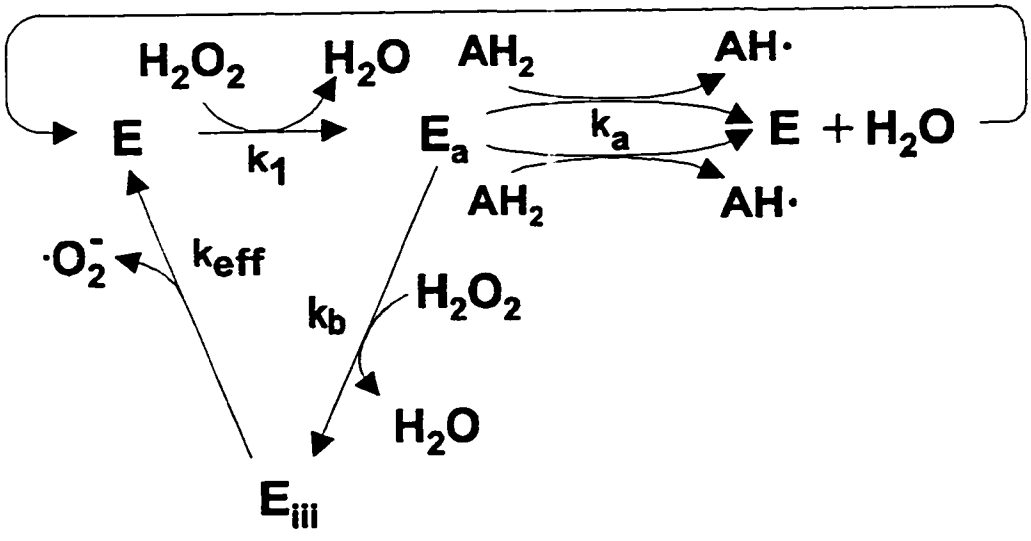

Figure 2.3. Simplified peroxidase enzyme catalytic cycle.

The formation rate of active enzyme in a system could be written as:

$$\frac{dE_a}{dt} = k_1[H_2O_2]E - 2k_a[AH_2]E_a - k_b[H_2O_2]E_a \qquad (2.35)$$

in which E and E_a represent the native and active enzyme concentrations, respectively. Since $2k_a[AH_2] >> k_b[H_2O_2]$, the equation (2.35) may be simplified to:

$$\frac{dE_a}{dt} = k_1[H_2O_2]E - 2k_a[AH_2]E_a \qquad (2.36)$$

The formation of compound I and II are so rapid with respect to all other reactions that the rates of change can be assumed to be in a pseudo steady-state (Nicell, 1994). Due to this assumption, equation (2.36) may be equated to zero and the concentration of active enzyme (E_a) may be expressed as a function of the native enzyme concentration (E), to give:

$$E_a \approx \frac{k_1[H_2O_2]}{2k_a[AH_2]} E \qquad (2.37)$$

The enzyme mass balance may be expressed as :

$$E = E_0 - E_a - E_{iii} - E_{inact} \qquad (2.38)$$

where E_{iii} and E_{inact} represent the concentrations of compound III and inactive enzyme, respectively, and E_0 represents the initial total concentration of enzyme added to the system. Substitute equation (2.37) into (2.38), and subsequently solving for the native enzyme concentration yields :

$$E = \frac{(E_0 - E_{iii} - E_{inact})}{1 + \frac{k_1[H_2O_2]}{2k_a[AH_2]}} \tag{2.39}$$

The net rate of compound III formation may be given by,

$$\frac{dE_{iii}}{dt} = k_b E_a [H_2O_2] - k_{eff} E_{iii} \tag{2.40}$$

Expressions for the rates of removal of aromatic compound and formation of inactive enzyme are given by equations (2.24) and (2.34), respectively. The instantaneous peroxide concentration is given by equation (2.25).

This simplified model makes it possible to calculate the concentrations of aromatic substrate, peroxide, native enzyme, active enzyme, inactive enzyme, and compound III enzyme from the initial concentrations of enzyme, aromatic substrate, and hydroxide as a function of time (Buchanan and Nicell, 1998 b).

3. Materials and Methods

3.1. Materials

ARP (*Arthromyces ramosus* peroxidase), HRP (horseradish peroxidase) and catalase enzymes were purchased from Sigma Chemical Co., St. Louis, MO. and stored in a freezer at -15°C until needed. Polyethylene glycol (PEG) which has a molecular weight of 3,350 was also purchased from Sigma Chemical Co., St. Louis, MO. based on the results of Nakamoto and Machida (1992), who reported that PEG with an average molecular weight less than 400 is ineffective and with molecular weight above 1000 is most effective for enzyme protection. ACS grade solid phenol (MW 94.11, purity 99% or greater), ACS grade hydrogen peroxide (30% w/v), 98% 4-aminoantipyrine, ACS grade potassium ferricyanide, ACS grade sodium bicarbonate, and ACS grade monobasic and dibasic sodium phosphate were purchased from Fisher Scientific Co., Edmonton, Alberta. Hydrogen peroxide was stored at 4 °C, and diluted solutions were prepared using deionized water on the day of their use. Aqueous solutions of potassium ferricyanide and 4-aminoantipyrine were prepared using deionized water and stored at 4°C. Reaction solutions were buffered to pH 7.0 using a monobasic-dibasic sodium phosphate buffer prepared according to the method of Gomori (1955). 3,5-dichloro-2-hydroxy-benzenesulfonic acid (HDCBS) was purchased from Aldrich Chemical Co., Milwaukee,WI. Chromogen and sulfuric acid were purchased from Fisher Scientific Co., Edmonton, Alberta, to make chromic acid for the purpose of cleansing cuvettes.

3.2 . Equipment

Colourimetric assays and UV absorbance were monitored using a Pharmacia Biotech Ltd. Ultrospec 2000 UV/Visible spectrophotometer (wavelength range 190 to 1100 nm with 1 nm resolution) operated from a DECpc 320sxLP computer. Glass and quartz semi-micro cuvettes with a 1 cm optical path length and a 1.5 mL volume were obtained from Hellma (Canada) Ltd. In order to ensure the cuvettes have no residue of chemicals, all cuvettes were soaked in a chromic acid for few minutes prior to each assay and washed with deionized water.

Reaction pH was measured using a Fisher Accumet pH meter. Two point calibrations bracketing the pH ranges under investigation were performed with pH standard solutions purchased from Fisher Scientific Co., Alberta, Canada.

Reaction temperature was controlled using a respirometer water bath, model IGRP-20, manufactured by Gilson Medical Electronics Co.

An International Clinical centrifuge manufactured by International Equipment Co. Neeham Hts. Mass. was used to centrifuge samples at 3000 rpm for 30 minutes to separate precipitate.

Liquid volumes ranging from 40 μL to 10 mL were measured using Eppendorf micropipetters, Eppendorf Maxipettor and tips.

3.3. Analytical Techniques

In order to determine the concentrations of the various chemicals in solution, the following assays were used. A complete description of the procedures, theories and calculations may be found in Appendix A. In order to provide a measure of repeatability, all assays were performed in triplicate to ensure a maximum measurement error of 5%.

3.3.1. *Arthromyces ramosus* Peroxidase (ARP) Activity Assay

A colourimetric assay involving the reaction of *Arthromyces ramosus* peroxidase, hydrogen peroxide (H_2O_2), phenol, and 4-aminoantipyrine (AAP) was used to measure the enzymatic activity of ARP. With excess amounts of phenol, H_2O_2, and AAP, the initial rate of reaction is directly proportional to the amount of active enzyme in the assay. The rate of colour development of non-precipitating product, which absorbs light at a peak wavelength of 510 nm with an extinction coefficient of 7100 $M^{-1}cm^{-1}$ (based on hydrogen peroxide), was measured and converted to enzyme activity in the cuvette. This assay should be conducted under well controlled conditions of substrate concentration, pH, and temperature due to the dependence of the activity measurements on these variables. One unit of activity (U) is defined as the number of micromoles of peroxide utilized in one minute at pH 7.4 and 25 °C in an assay mixture consisting of 10 mM phenol, 2.4 mM AAP, and 0.2 mM H_2O_2. ARP concentrations are expressed as ARP activity measured under standard assay conditions.

Since the molar concentration of active enzyme is directly proportional to the enzyme activity, a constant of proportionality, C_a, has been defined during this investigation. For the ARP stock used in this investigation, 1 nanomole of ARP (based on the heme absorbance at 403 nm and a peroxidase extinction coefficient of 102000 $M^{-1}cm^{-1}$) corresponded to 13.6 ± 1.4 U as measured by this assay (i.e., C_a=73.7x10 $^{-9}$ M/(U/mL)).

3.3.2. Total Phenol Assay

Phenol concentrations were measured using a colourimetric assay in which the phenolic compounds within a sample react with 2.08 mM AAP in the presence of 8.34 mM potassium ferricyanide. When the assay reaction is limited only by phenol, the extent of colour development in the cuvettes is directly proportional to the phenol concentration. When the color has fully developed after 8 to 10 minutes reaction time, the absorbance at 510 nm was measured and converted to total phenol concentration in the cuvette (mM) with the following calibration:

$$[\text{phenol}] = 0.0924(A_{510})$$

in which A_{510} is the absorbance at 510 nm. The result of the phenol assay calibration is presented in Figure 3.1.

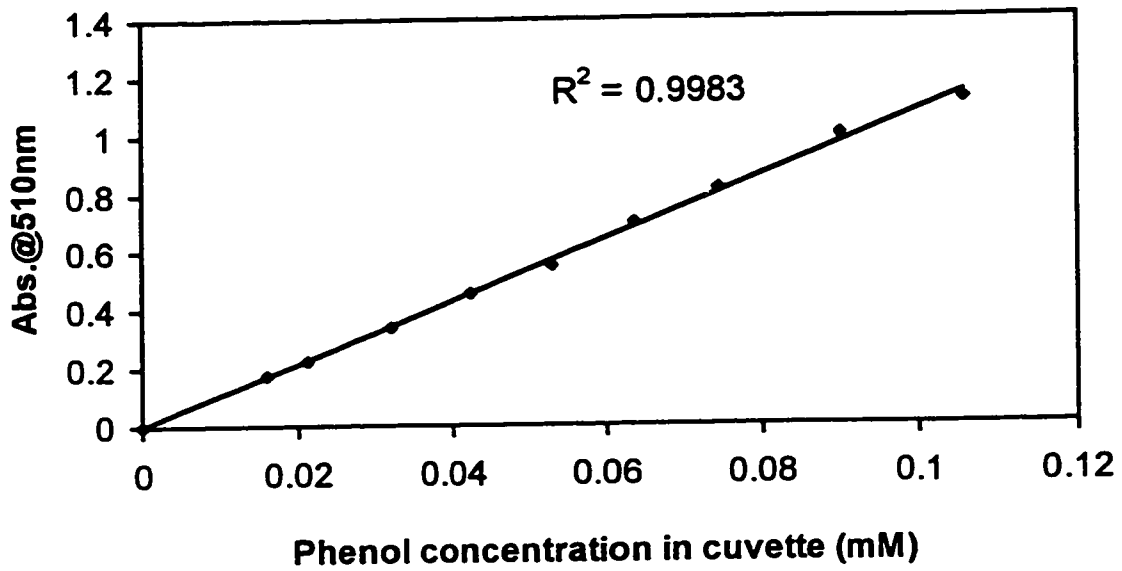

Figure 3.1. Calibration curve used for measuring phenol using the colourimetric method.

3.3.3. Hydrogen Peroxide Assay

A colourimetric assay was applied to measure the hydrogen peroxide concentrations using horseradish peroxidase (HRP) as catalyst, 3,5-dichloro-2-hydroxybenzenesulfonic acid (HDCBS) and 4-aminoantipyrine (AAP) as substrates at pH 7.4. For hydrogen peroxide to remain the limiting substrate, its concentration in the cuvette should not exceed 50 μM. After the full colour development, the absorbance at 510 nm is measured and converted to the hydrogen peroxide concentration (mM) in the cuvette by the following calibration result:

$$[H_2O_2] = 0.033(A_{510})$$

The concentration in the sample was calculated according to the dilution the sample underwent in the cuvette. The result of hydrogen peroxide calibration is presented in Figure 3.2.

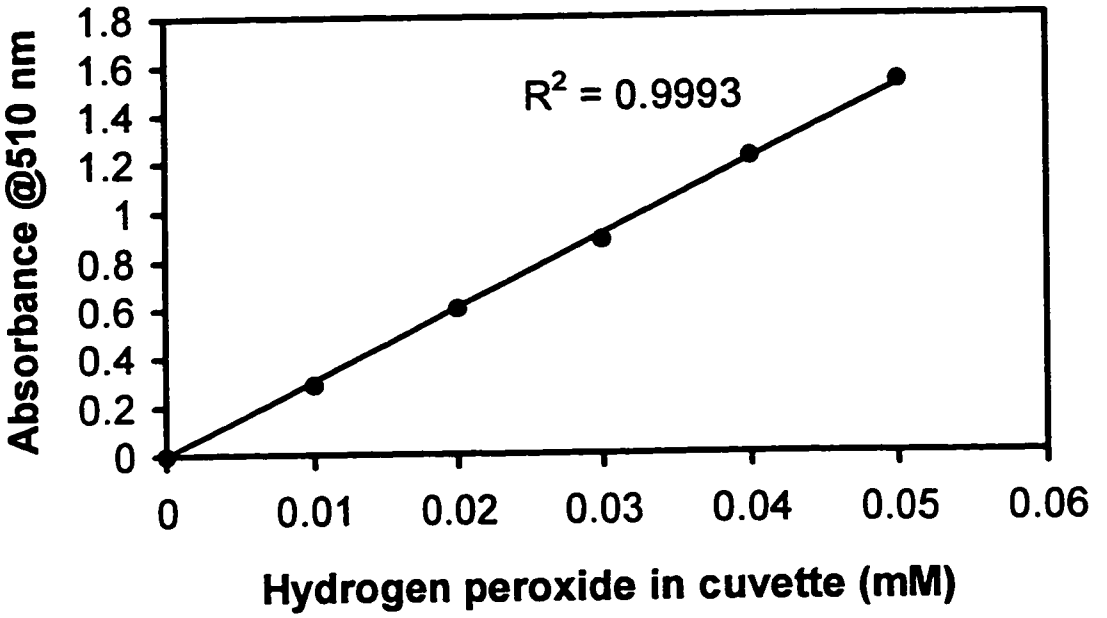

Figure 3.2. Hydrogen peroxide calibration curve.

3.4. Experimental Procedures

Batch reactors were used to collect data. All experiments were conducted in a constant temperature water bath maintained at 25 °C ± 0.5 °C. The reaction mixtures were buffered to pH 7.0 using 0.1 M monobasic-dibasic sodium phosphate buffer and were allowed to reach thermal equilibrium before each test was begun. Initial samples of reaction mixtures were taken before the initiation of reaction, and analyzed for phenol and hydrogen peroxide concentrations. Two control batch reactions, alternatively without ARP or without hydrogen peroxide, are conducted to check if phenol removal occurs without one of these items. All assays were performed in triplicate and the results were analyzed for outliers as recommended by Standard Methods for the Examination of Water and Wastewater (APHA et al., 1995).

3.4.1. Treatment of Samples

Samples were taken from reactors and mixed with a concentrated catalase solution immediately in order to stop the reaction. Catalase catalyses the rapid conversion of hydrogen peroxide to oxygen and water (Nicell, 1994) according to :

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O$$

At the end of each test, samples were centrifuged at 3000 rpm for 30 minutes before their phenol concentrations were measured.

3.4.2. Batch Reactor Operation

Batch reactors used to determine reaction stoichiometry consisted of 30 mL capped vials, while those used during the collection of kinetic data consisted of 500 mL capped bottles. The reaction mixtures were completely mixed by a Teflon-coated stir-bar rotated by a magnetic stirrer. Batch tests were conducted alternatively in the presence of 400 mg L^{-1} polyethylene glycol (PEG) or in the absence of PEG. Initial tests indicated 200 mg PEG/L to be required to provide maximum enzyme protection in the presence of the highest phenol concentration considered during this investigation (8 mM phenol). The presence of PEG in excess to that needed for maximum enzyme protection was found to have no negative effect on phenol removal.

Reactions were initiated by adding a measured dose of ARP. During kinetic testing, at each predetermined time interval, a sample of the reaction mixture was quickly withdrawn and treated as indicated in section 3.4.1.

3.4.3. Thermostability of *Arthromyces ramosus* Peroxidase

Thermostability of ARP was investigated by incubating the enzyme at pH 7.00 in sodium phosphate buffer over a range of incubation temperatures from 5 °C to 55 °C.

All measurements were performed at room temperature (22 °C ± 0.5). ARP activity in the incubation mixture was measured over time using the standard activity assay and

aliquots obtained from the incubation mixture. Aliquots at elevated temperature were quickly cooled down to near room temperature using a cold water bath and then allowed to reach room temperature before each assay was begun.

3.4.4. Spectrophotometric Measurements

Spectral changes of ARP upon addition of H_2O_2 in sodium phosphate buffer pH 7.0 at room temperature were monitored by recording the absorbance within a range between 360 and 700 nm.

4. Experimental Results and Discussion

The ability of *Arthromyces ramosus* peroxidase to treat phenolic wastewater was investigated using batch reactors. The experiments were conducted alternatively in the presence or absence of PEG. 400 mg PEG L^{-1} was added to the reactor mixture in the experiments for which the additive was used. All experiments were performed at 25 °C and pH 7.0.

No appreciable permanent enzyme inactivation occurs over the course of the enzyme assay. Therefore, in order to confirm that the additive affects only the permanent enzyme inactivation rate and not the rate associated with other reactions, the enzyme activities in the presence and absence of PEG were compared over a range of hydrogen peroxide concentrations. The results of this test are presented in Figure 4.1. This graph shows larger differences between the activity measurements in the absence and presence of PEG in the range where a small change of peroxide concentration has a big effect on the activity assay measurement. These differences may be caused by a slight change in peroxide concentration from one assay to another, independent of the presence or absence of PEG. Thus, it can be concluded that PEG suppresses only the rate of permanent inactivation of the enzyme. This is in agreement with previous studies in which it has been found that PEG affects only the enzyme inactivation rate (Buchanan, 1996). Thus, from a modelling perspective, only the kinetic constants associated with permanent enzyme inactivation (k_e, k_r) would depend on the presence or absence of PEG.

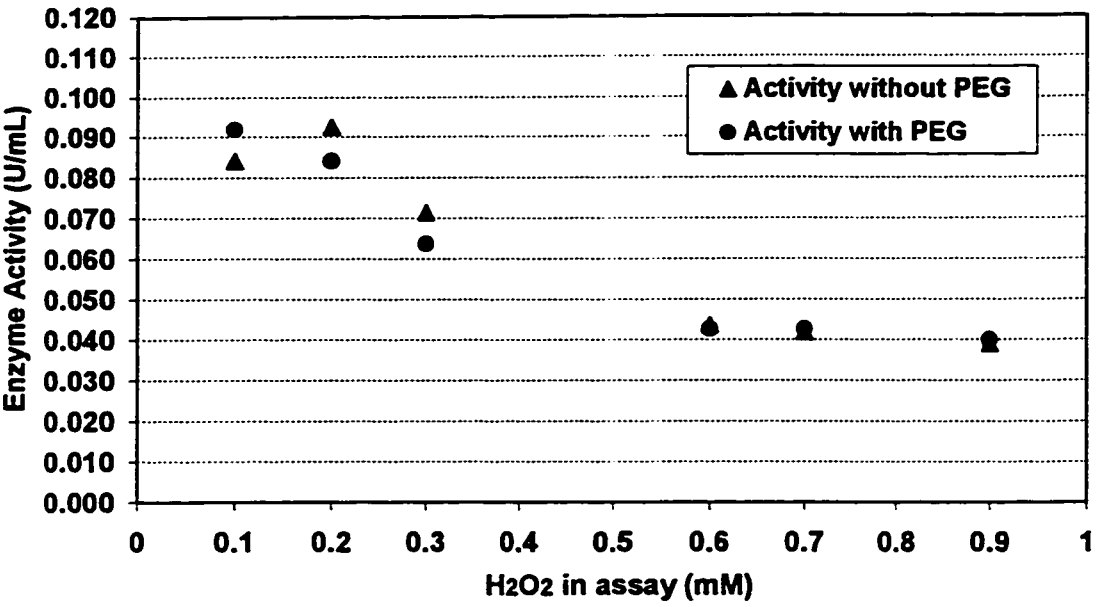

Figure 4.1. Enzyme activities in the presence (400 mg/L) and the absence of PEG.

4.1. Effect of PEG Dose

Various phenol concentrations treated in the presence of various doses of PEG. The purpose was twofold: (1) to investigate the amount of PEG needed to give maximum protection against permanent enzyme inactivation; and (2) to determine if excess PEG reduces the efficiency of phenol removal. Phenol concentrations from 2.01 mM to 8.05 mM were tested, and the results are shown in Figure 4.2.

As can be seen in Figure 4.2, PEG doses greater than 100 mg/L and 200 mg/L have little effect on phenol removal for initial phenol concentrations up to 4.81 mM and 8.05 mM, respectively. In some cases the excess PEG dose showed a slightly reduced

efficiency for phenol removal. This may be caused by experimental error rather than a real negative effect, since the results do not show this trend for the higher PEG concentrations.

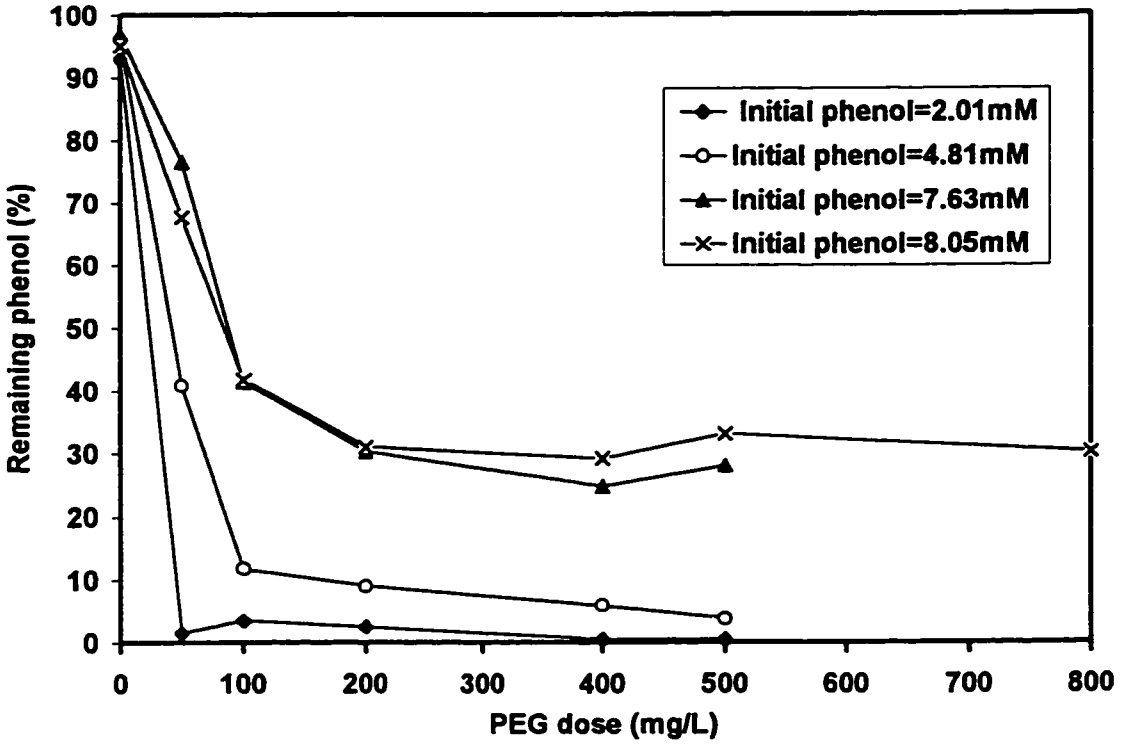

Figure 4.2. The effect of PEG on phenol removal for various initial phenol concentrations (♦- ARP=0.4 U/mL, H_2O_2= 2.2 mM; ○- ARP=1.0 U/mL, H_2O_2= 5.5 mM; ▲ - ARP=1.3 U/mL, H_2O_2=8.8 mM; × - ARP = 1.3 U/mL, H_2O_2= 8.8 mM).

This is in a good agreement with Saadi's report (1993) which indicates at least 200 mg/L of PEG would be required for maximum enzyme protection in the presence of 10 mM phenol. Wu and coworkers (1997) reported that various minimum PEG doses from 10 to 100 mg/L were needed depending on different phenolic compounds. They also confirmed that any excess PEG (up to 5 g/L) does not reduce or improve the phenol removal efficiency.

4.2. Stoichiometry

The stoichiometry between phenol removed from solution and hydrogen peroxide consumed was investigated.

According to reaction (2.5), two free radicals are generated for each molecule of peroxide consumed, so the stoichiometric ratio of phenol removed per peroxide consumed would be 2:1. However, the experimental results reported in literature showed 1:1 stoichiometry between phenolic substrate removed and peroxide consumed (i.e. $d[AH_2] / d[H_2O_2]$) for treatment with HRP (Buchanan, 1996; Nicell, 1991; Wu et al., 1993; Yu et al., 1994). Hewson and Dunford (1976) suggested the mechanism of 1:1 stoichiometric reactions as following:

$$\begin{aligned} HRP_N + H_2O_2 &\longrightarrow HRP_i + H_2O \\ HRP_i + AH_2 &\longrightarrow \cdot AH + HRP_{ii} \\ \cdot AH &\longrightarrow \frac{1}{2}\,dimer \\ HRP_{ii} + \frac{1}{2}\,dimer &\longrightarrow HRP_N + Product \end{aligned} \qquad (4.1)$$

which can be summed to yield:

$$H_2O_2 + AH_2 \longrightarrow H_2O + Product \qquad (4.2)$$

Yu and coworkers (1994) observed a 1:1 molar ratio and explained the reason as some dimers which are formed during phenol oxidation (such as p-phenoxyphenol and p,p'-biphenol) being preferred substrates of peroxidase. Thus, the dimers generate an additional peroxide demand which reduces the observed stoichiometry to 1:1.

Nicell (1994) explained the observed 1:1 stoichiometry as being due to the formation of dimers which remain substantially soluble and participate in the catalytic reactions to form trimers, or even bigger polymers until they precipitate. It was demonstrated that in theory the stoichiometry approaches unity as larger polymers are formed. Dec and Bollag (1990) investigated the molecular mass of end products from the HRP catalytic reaction with 2,4-dichlorophenol, and reported average molecular masses of polymers of up to 800 for the fraction soluble in dioxane. Other precipitable polymers may contain higher molecular mass and it is possible to approach the unity stoichiometry when high molecular mass polymers are generated.

Al-Kassim and coworkers (1993) observed various stoichiometric ratios of phenol removed per peroxide consumed from 0.5 to 0.83 as phenol concentration was increased from 0.5 to 1.0 mM in phenol removal with ARP. Al-Kassim and coworkers (1994 b) obtained 1:0.85 stoichiometry for 4-chlorophenol to peroxide and 1:1 stoichiometry for phenol to peroxide with *Coprinus macrorhizus* peroxidase.

Recently, the stoichiometry of p-cresol:peroxide with *Coprinus macrorhizus* peroxidase was found to be 1:0.7 and 1:1.1 depending on the initial ratio of p-cresol:peroxide (Heck, 1995). It has been known that *Coprinus cinereus*, *Coprinus macrorhizus* and *Arthromyces ramosus* peroxidases are most likely identical (Kjalke et al., 1992). Thus, it is likely that ARP will exhibit a variable stoichiometry of phenol removed per peroxide consumed.

Two series of batch tests were conducted to study the relationship between moles phenol removed per mole peroxide consumed. In order to get complete reactions, over three hours of reaction time were given. In the first set of tests, all batch reactors contained 1.77 mM initial phenol, various concentrations of H_2O_2, and fixed excess concentrations of PEG (400 mg/L) and ARP (0.38 U/mL). The resulting data indicated a 1:1 stoichiometry. The best fit line to these data is shown in Figure 4.3.

Further experiments were conducted with various higher phenol and hydrogen peroxide concentrations. The initial phenol concentrations in this set of tests were from 0.47 to 5.99 mM and initial peroxide concentrations ranged from 0.23 to 3.64 mM. It is assumed and that the peroxide was completely consumed during the 3 hour reaction time, and thus the extent of all reactions was limited by peroxide. Initial ARP concentrations of up to 4.85 U/mL were used to ensure that an excess amount of enzyme was present.

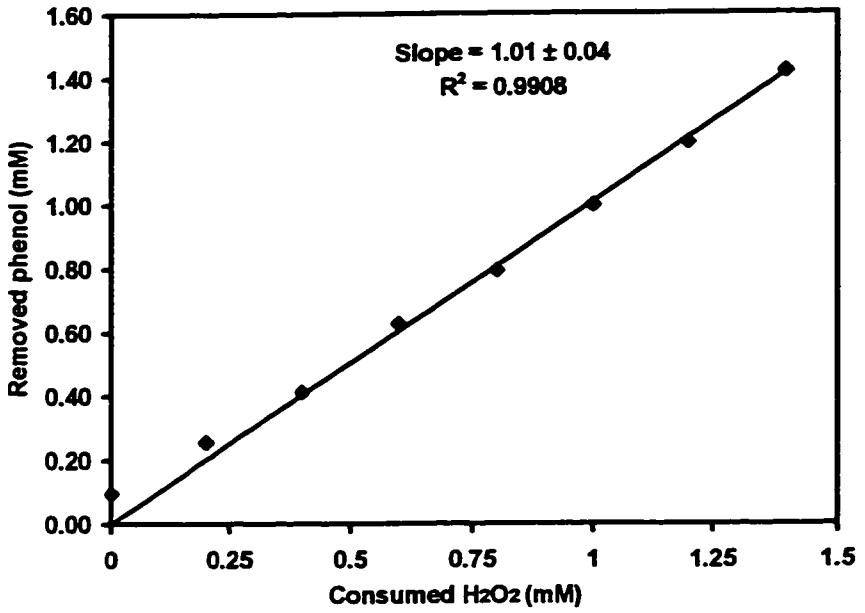

Figure 4.3. The stoichiometry between removed phenol and consumed H_2O_2 (with 1.77 mM of initial phenol).

Attempts to model the variation of stoichiometry as a function of initial phenol and peroxide concentrations were unsuccessful. However, linear regression of moles phenol removed on mole peroxide consumed yielded a best fitting line with a slope of 1.24±0.11, and a coefficient of determination (R^2) of 0.98, which indicates a stoichiometry of 1.24:1. The result is depicted graphically in Figure 4.4. According to Nicell (1991), the average size of precipitating polymer decreases with increasing stoichiometry. Therefore, a possible reason for the increase in the observed stoichiometry may be that the higher ARP dose used in the second set of tests produced higher instantaneous free radical concentrations, which favoured the formation of polymers with smaller average sizes than did the lower ARP dose used in the first set of tests.

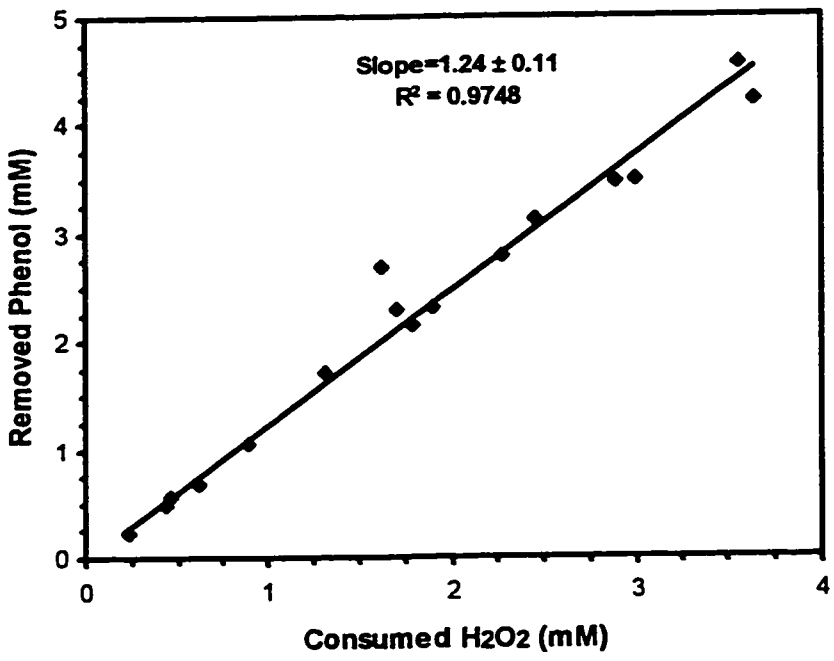

Figure 4.4. The stoichiometry between hydrogen peroxide and various phenol concentrations.

Because of the differences in observed stoichiometry values reported in literature for phenol removal by ARP, and the variable stoichiometry observed in this investigation, further research is required to resolve this issue.

The stoichiometry ratio of phenol removed to peroxide consumed was also estimated from kinetic data shown in Figure 4.7 using a genetic algorithm package. This yielded a stoichiometry very close to 1:1. This lower stoichiometry may be due to the lower ARP concentrations used to obtain these kinetic data. In these tests, a maximum enzyme dose of 1.42 U/mL (104.7 nM) was used, compared to the maximum ARP dose

of 4.85 U/mL (357.4 nM) used to obtain the data represented in Figure 4.4. Therefore, a 1:1 stoichiometry is likely to be appropriate when modelling phenol removal in the absence of excess quantities of ARP. Thus, 1:1 stoichiometry was used to model the removal process and estimate kinetic parameters.

4.3. Thermostability of ARP

The thermal stability of *Arthromyces ramosus* peroxidase was studied by measuring the activity of an *Arthromyces ramosus* peroxidase solution over time as it was incubated at a specific temperature. All ARP solutions were buffered to pH 7.0 using 0.1 M sodium phosphate buffer, and all ARP activities were measured at room temperature (22 ± 0.5 °C).

A slight increase in activity was observed one day after the dry enzyme was added to the buffer solution regardless of the incubation temperature. This is commonly observed, and may be due to the dry enzyme unfolding into its active configuration in the buffer. Storage of the aqueous enzyme at room temperature for 5 days produced about 25% loss in activity. In addition, storage of the enzyme at 5 °C for one month produced essentially no loss of activity, while storage for 9 months at 5 °C produced about a 30% loss in activity.

This result differs slightly from that of Andersen and coworkers (1991) who reported a 10% loss of activity when *Coprinus cinereus* peroxidase (CiP) was stored in Milli Q water at 5 °C for one month. Since CiP and ARP have been shown to be essentially the

same enzyme, the difference in thermostability may be due to different incubation pH values in this and the Andersen study as the pH of Milli Q water is typically near 6.0.

The short term thermostability was also studied during this investigation. Figure 4.5 illustrates the inactivation of the enzyme over a one hour time period as it is incubated at temperatures between 5 °C and 55 °C in a buffered solution at pH 7.0. The results indicate that the enzyme is susceptible to inactivation at elevated temperatures. ARP retained almost 100 % of its activity when incubated for one hour at 5 °C and 25 °C and 60 % or more of its activity after incubation at 35 °C and 45 °C for one hour. ARP incubated at 55 °C for one hour retained only 30 % of its activity.

This result differs from Kim and coworker (1991 a). They reported ARP lost 90 % of its activity in 25 minutes when it was incubated at pH 8.8, 45 °C. One possible explanation would be the different pH and buffer since they used Tris-HCl buffer pH 8.8.

The loss of activity resulting from incubation at 35 °C or more was modelled by the following first order relationship:

$$A(t) = A_0 e^{-Kt} \qquad (2.1)$$

where t= time in minute, K= first order rate constant (min^{-1}), A(t)= time dependent activity (U/mL) and A_0= initial activity (U/mL). The temperature dependence of the rate constant, K, may be described using the van't Hoff-Arrehenius relationship:

$$\frac{d(\ln K)}{dT} = \frac{E_a}{R} \cdot \frac{1}{T^2} \qquad (4.3)$$

where E_a=activation energy (KJ $mole^{-1}$), R=ideal gas constant (8.314×10^{-3} KJ $mole^{-1}$

degree^{-1}), and T= absolute temperature (K). A general expression for the rate constant is obtained by integrating equation (4.3) between the limits of an arbitrary temperature, T, and infinity, yielding (De Cordt et al., 1992):

$$K = K_{\infty} e^{-E_a/RT} \tag{4.4}$$

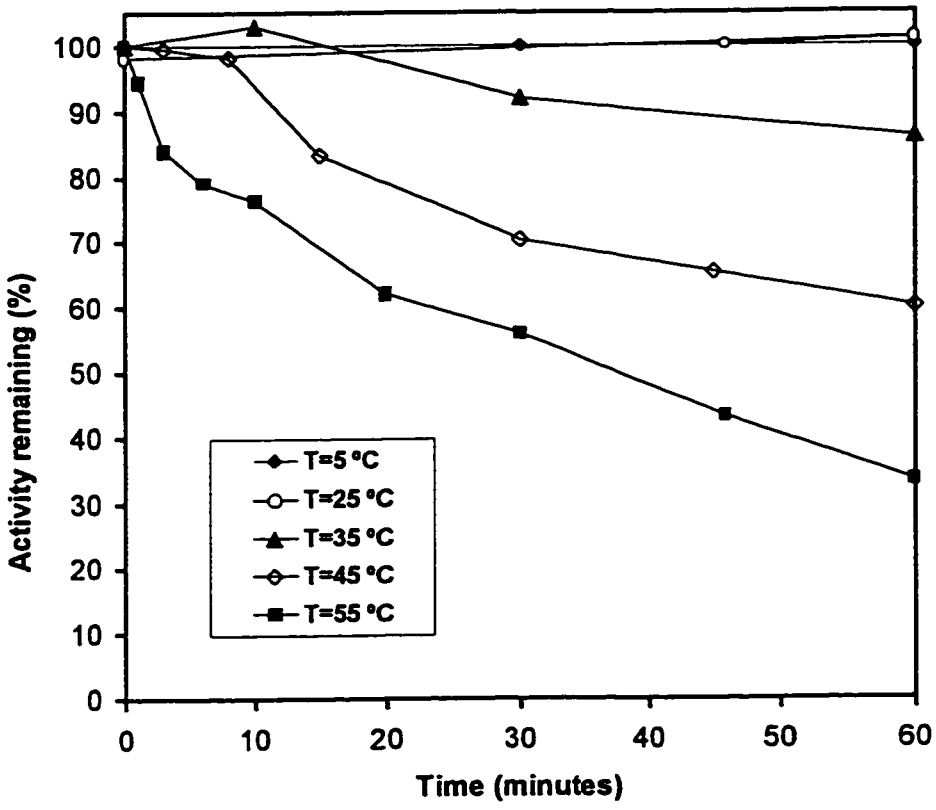

Figure 4.5. Thermostability of *Arthromyces ramosus* peroxidase incubated from 5 to 55 °C in sodium phosphate buffer pH 7.0.

The reaction rate constant, K, at 35 °C, 45 °C, and 55 °C was evaluated from experimental data using the Solver tool in Microsoft Excel according to equation (2.1). The estimated temperature-dependent rate constants are shown in Table 4.1. The activation energy, E_a and K_∞ were then estimated from equation (4.4) using Excel's solver tool. This resulted in estimates of 76.9 KJ $mole^{-1}$ for E_a and 3.8×10^{10} for K_∞. The fit of modelled temperature dependence of ARP to experimental data is shown in Figure 4.6.

Nicell (1991) studied the thermostability of HRP at pH 7.4 over a range of temperatures between 5 and 50 °C and found no loss of activity when stored at 5 °C, and 30 % or less reduced activity when incubated from 25 °C to 45 °C for one hour. He concluded that the thermal inactivation of HRP is not first-order with respect to enzyme concentration. He suggested that this may be the result of a mixture of isoenzymes which may have various heat-resistances. Wright (1995) reported the thermal inactivation of SBP to be first order with respect to enzyme concentration and that SBP was more stable than HRP at high temperature. Since isoenzymes of ARP have not been reported, first order thermal inactivation was expected and the results in this investigation show the first-order decay model fits the data well. A comparison of the results of the current investigation to those reported by Nicell (1991) and Wright (1995) indicates that ARP is less stable than both HRP and SBP at high temperature.

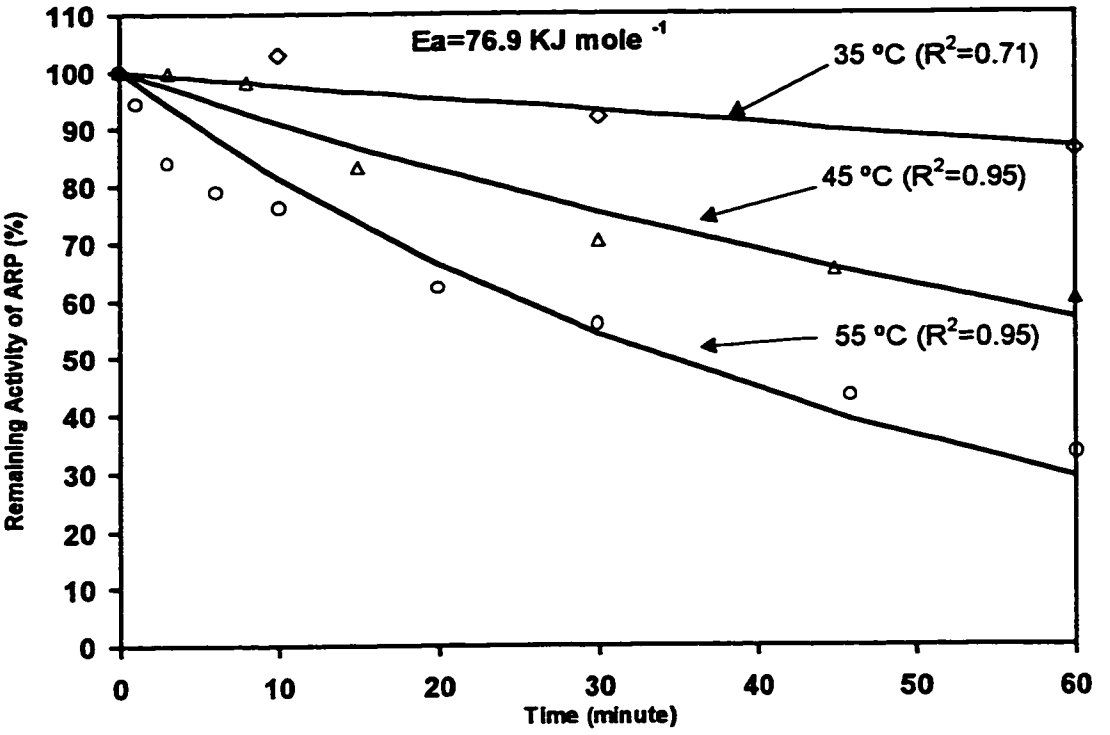

Figure 4.6. Overlay of experimental thermostability data onto the modelled thermal inactivation.

Table 4.1. Calculated Reaction Rate Constants Depend on Temperature.

Temperature (°C)	Reaction rate constant, K(minute $^{-1}$)
35	0.0024
45	0.0094
55	0.0205

4.4. Batch Reactor Studies

Experiments were conducted using bench-scale batch reactors alternatively with or without PEG. The objective was twofold: (1) to determine the savings in enzyme dosage that can be realized in the presence of PEG; and (2) to collect kinetic data necessary to test the ability of the Buchanan and Nicell (1998 b) simplified model to predict ARP catalyzed phenol removal for various reaction conditions. Two control batch reactions were also conducted during each series of tests alternatively without ARP or without hydrogen peroxide. Phenol removal was observed only in the tests which included both ARP and peroxide. Thus, the phenol removal observed during this investigation can be associated with only the combined action of ARP and peroxide on phenol.

4.4.1. Phenol Removal with PEG

In order to ensure maximum ARP protection, 400 mg/L of PEG was used for this series of batch reactor tests based on the results from the section 4.1. The initial phenol and peroxide concentrations ranged between approximately 0.5 and 8.7 mM in this phase of the investigation. The results of these batch reactor experiments are shown in Figure 4.7, and the experimental data are tabulated in Appendix B.

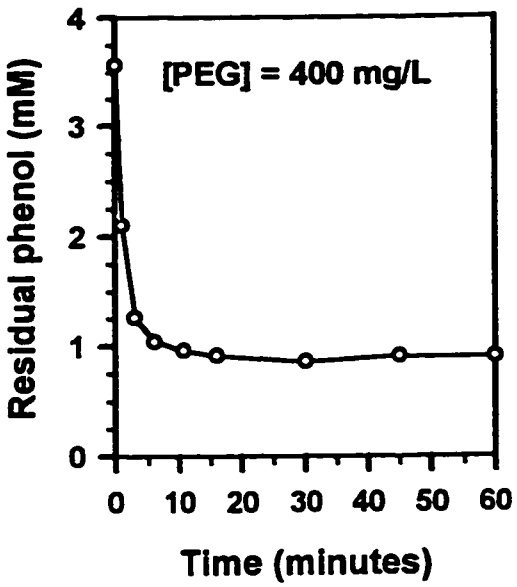

Figure 4.7a. Batch reactor kinetic data. $[phenol]_0$= 3.56 mM, $[H_2O_2]_0$= 4.11 mM, $[ARP]_0$ = 0.61 U/mL

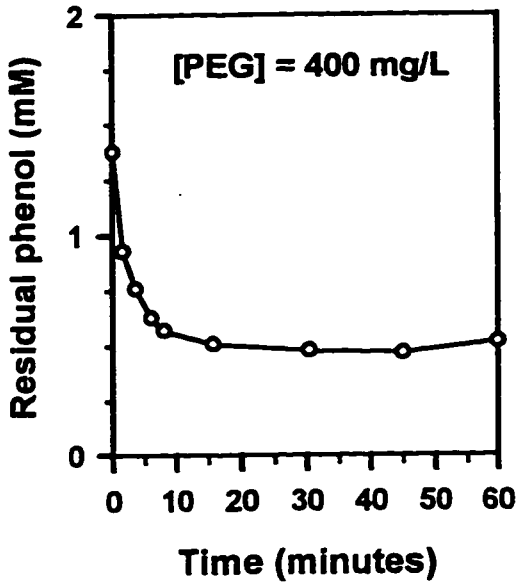

Figure 4.7b. Batch reactor kinetic data. $[phenol]_0$= 1.38 mM, $[H_2O_2]_0$= 1.00 mM, $[ARP]_0$ = 0.21 U/mL

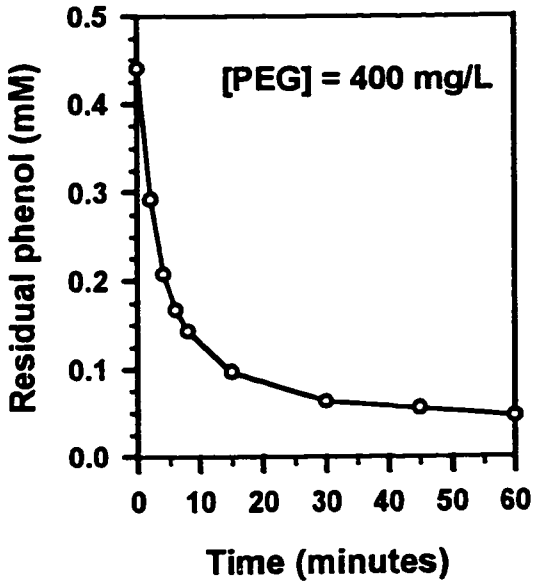

Figure 4.7c. Batch reactor kinetic data. $[phenol]_0$= 0.44 mM, $[H_2O_2]_0$= 0.59 mM, $[ARP]_0$ = 0.21 U/mL

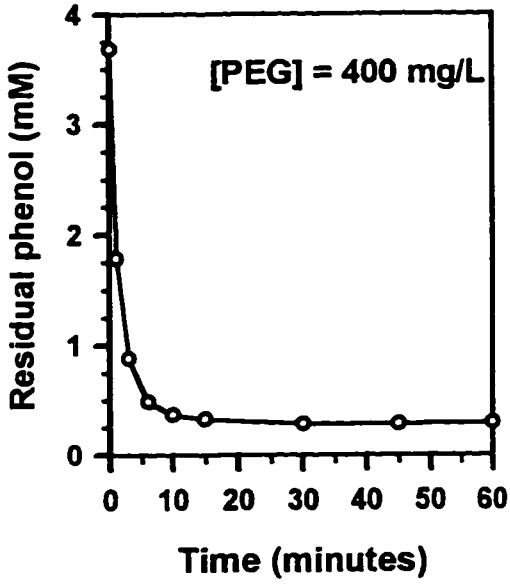

Figure 4.7d. Batch reactor kinetic data. $[phenol]_0$= 3.68 mM, $[H_2O_2]_0$= 3.99 mM, $[ARP]_0$ = 0.81 U/mL

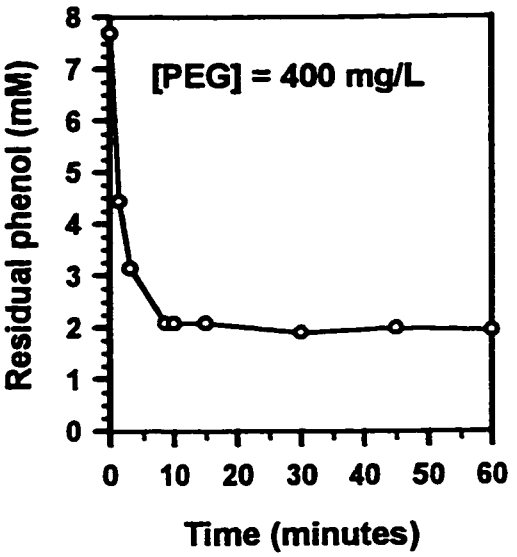

Figure 4.7e. Batch reactor kinetic data. $[phenol]_0$= 7.69 mM, $[H_2O_2]_0$= 8.73 mM $[ARP]_0$= 1.41 U/mL

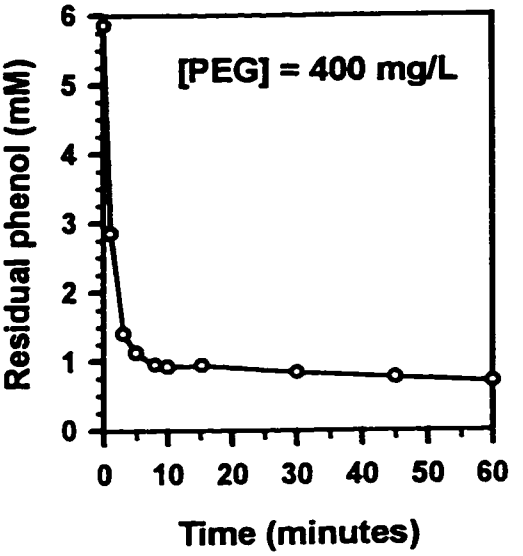

Figure 4.7f. Batch reactor kinetic data. $[phenol]_0$= 5.86 mM, $[H_2O_2]_0$= 6.43 mM, $[ARP]_0$ = 1.42 U/mL

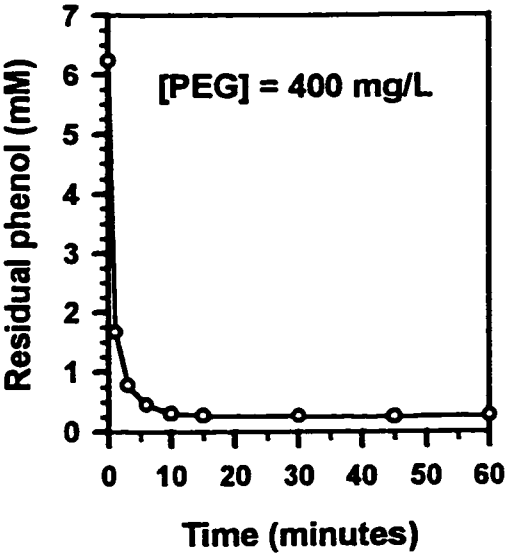

Figure 4.7g. Batch reactor kinetic data. $[phenol]_0$= 6.24 mM, $[H_2O_2]_0$= 6.30 mM, $[ARP]_0$ = 1.47 U/mL

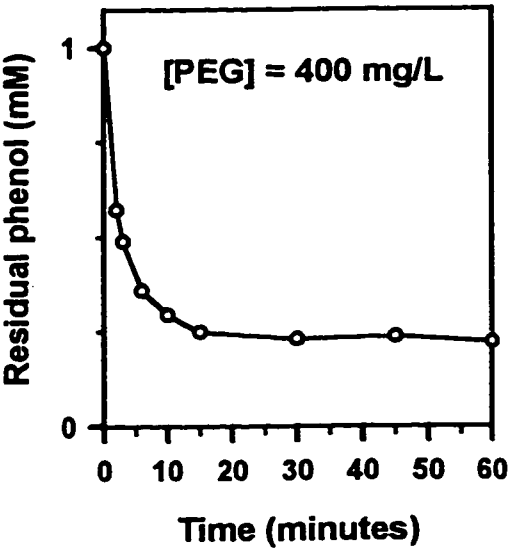

Figure 4.7h. Batch reactor kinetic data. $[phenol]_0$= 1.00 mM, $[H_2O_2]_0$= 1.07 mM, $[ARP]_0$ = 0.18 U/mL

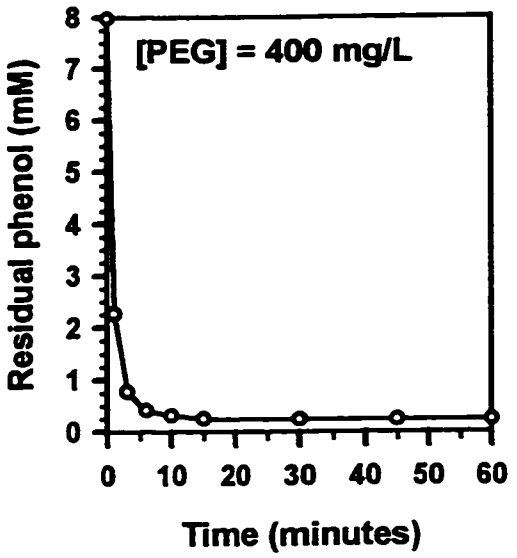

Figure 4.7i. Batch reactor kinetic data. $[phenol]_0$= 7.98 mM, $[H_2O_2]_0$= 8.31 mM $[ARP]_0$ = 1.88 U/mL

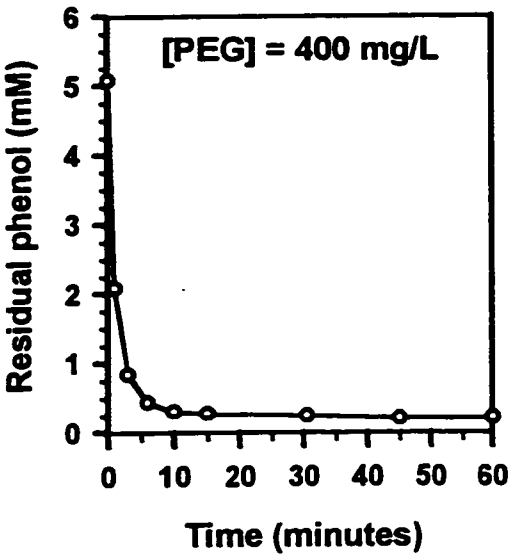

Figure 4.7j. Batch reactor kinetic data. $[phenol]_0$= 5.09 mM, $[H_2O_2]_0$= 5.05 mM, $[ARP]_0$ = 0.98 U/mL

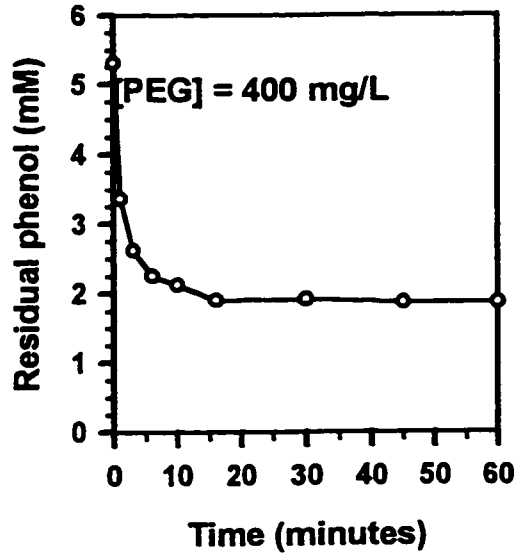

Figure 4.7k. Batch reactor kinetic data. $[phenol]_0$ = 5.31 mM, $[H_2O_2]_0$= 4.85 mM, $[ARP]_0$ = 0.60 U/mL

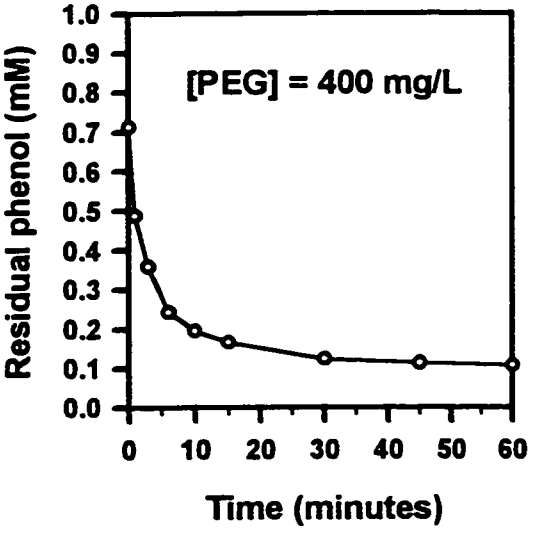

Figure 4.7l. Batch reactor kinetic data. $[phenol]_0$ = 0.71 mM, $[H_2O_2]_0$= 0.68 mM, $[ARP]_0$ = 0.14 U/mL

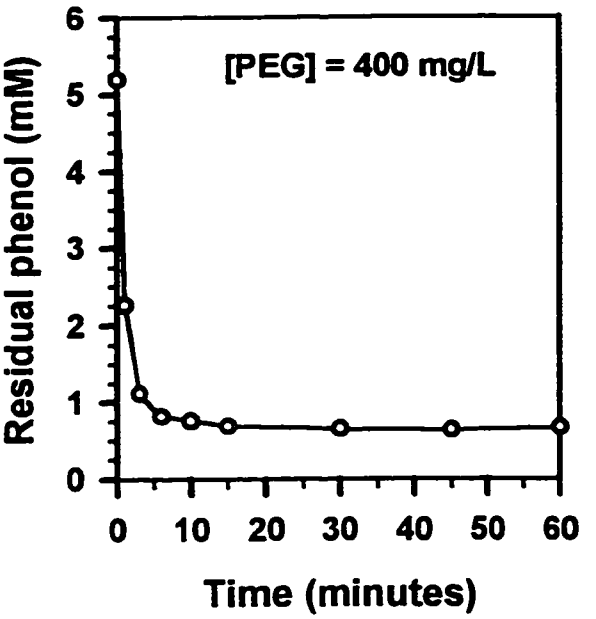

Figure 4.7m. Batch reactor kinetic data. $[phenol]_0$ = 5.20 mM, $[H_2O_2]_0$= 5.33 mM, $[ARP]_0$ = 0.99 U/mL

4.4.2. Phenol Removal Without PEG

A series of batch tests was also conducted without PEG. In these tests the initial phenol and peroxide concentrations ranged from approximately 0.5 to 5.2 mM. The results of this experiment are shown in Figure 4.8 and the experimental data are tabulated in Appendix B.

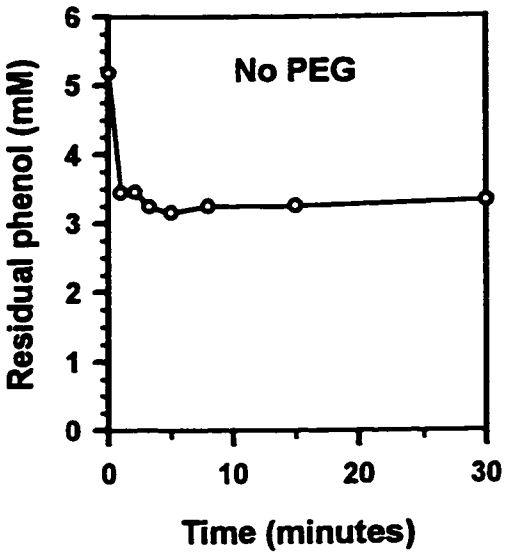

Figure 4.8a. Batch reactor kinetic data. $[phenol]_0$= 5.18 mM, $[H_2O_2]_0$= 4.56 mM $[ARP]_0$ = 7.07 U/mL

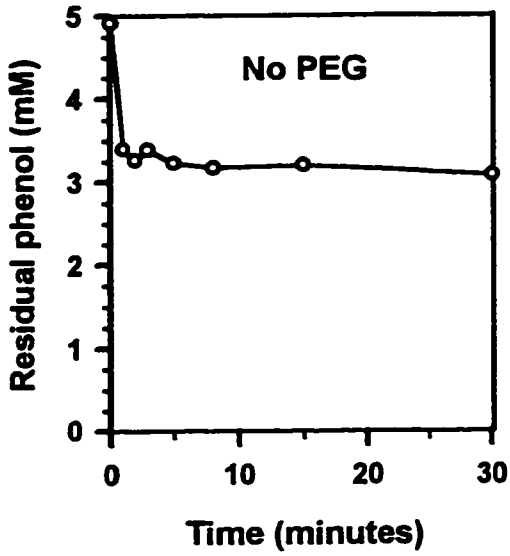

Figure 4.8b. Batch reactor kinetic data. $[phenol]_0$= 4.92 mM, $[H_2O_2]_0$= 4.43 mM, $[ARP]_0$ = 2.96 U/mL

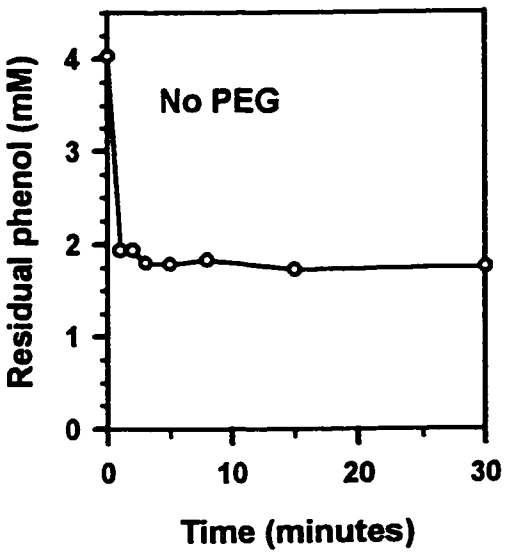

Figure 4.8c. Batch reactor kinetic data. $[phenol]_0$ = 4.03 mM, $[H_2O_2]_0$ = 4.07 mM, $[ARP]_0$ = 8.01 U/mL

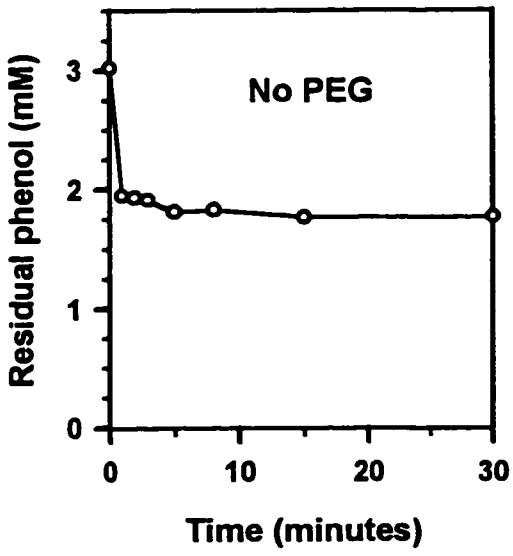

Figure 4.8d. Batch reactor kinetic data. $[phenol]_0$ = 3.02 mM, $[H_2O_2]_0$ = 3.09 mM, $[ARP]_0$ = 4.00 U/mL

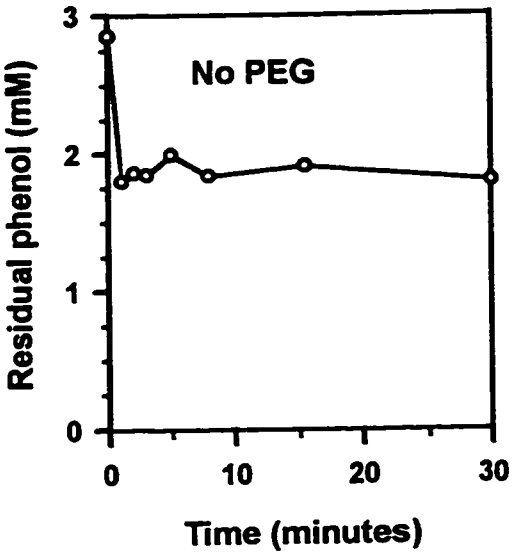

Figure 4.8e. Batch reactor kinetic data. $[phenol]_0 = 2.85$ mM, $[H_2O_2]_0 =$ 2.73 mM, $[ARP]_0 = 2.96$ U/mL

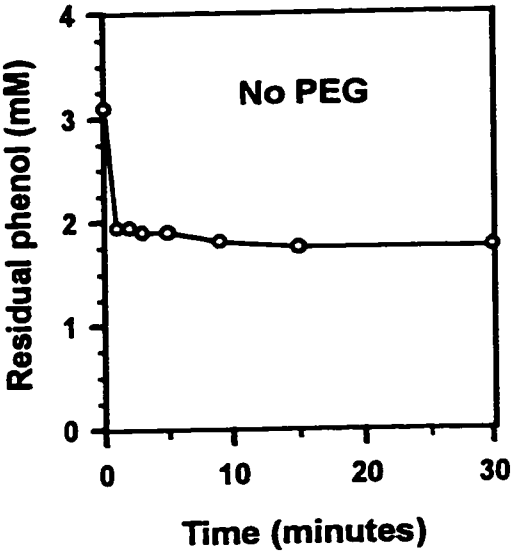

Figure 4.8f. Batch reactor kinetic data. $[phenol]_0 = 3.10$ mM, $[H_2O_2]_0 =$ 3.15 mM, $[ARP]_0 = 4.78$ U/mL

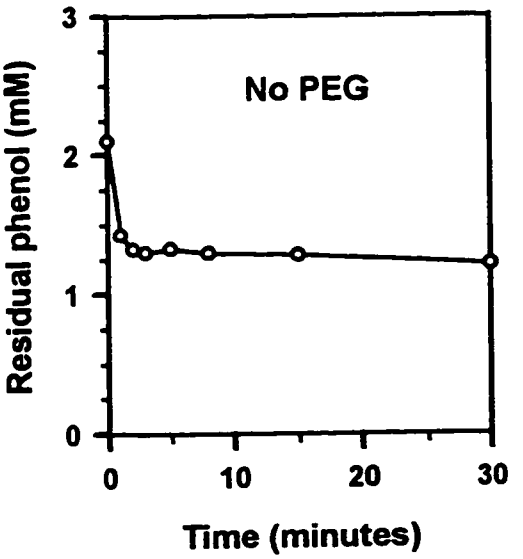

Figure 4.8g. Batch reactor kinetic data. $[phenol]_0 = 2.10$ mM, $[H_2O_2]_0 =$ 1.86 mM, $[ARP]_0 = 3.01$ U/mL

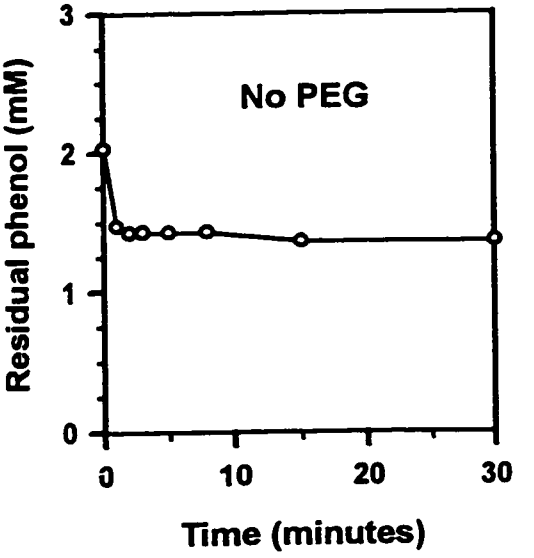

Figure 4.8h. Batch reactor kinetic data. $[phenol]_0 = 2.03$ mM, $[H_2O_2]_0 =$ 2.06 mM, $[ARP]_0 = 1.84$ U/mL

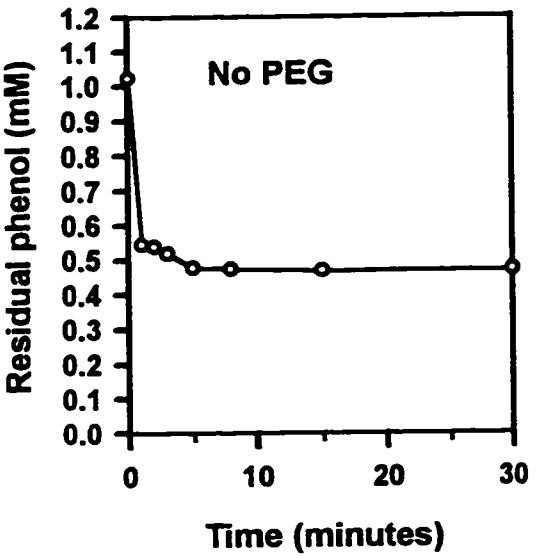

Figure 4.8i. Batch reactor kinetic data. $[phenol]_0 = 1.02$ mM, $[H_2O_2]_0 =$ 0.99 mM, $[ARP]_0 = 2.15$ U/mL

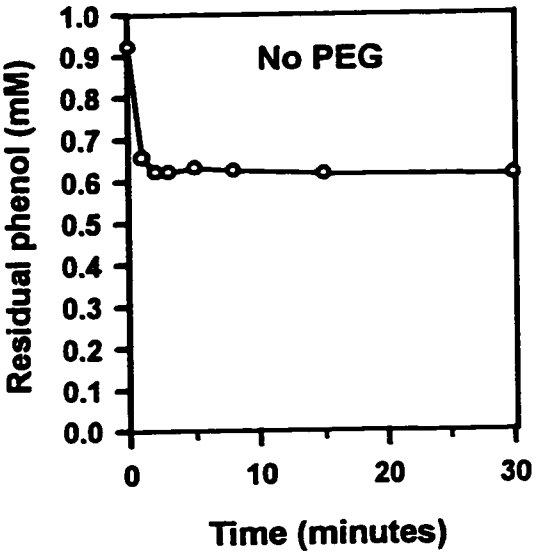

Figure 4.8j. Batch reactor kinetic data. $[phenol]_0 = 0.92$ mM, $[H_2O_2]_0 =$ 0.98 mM, $[ARP]_0 = 1.25$ U/mL

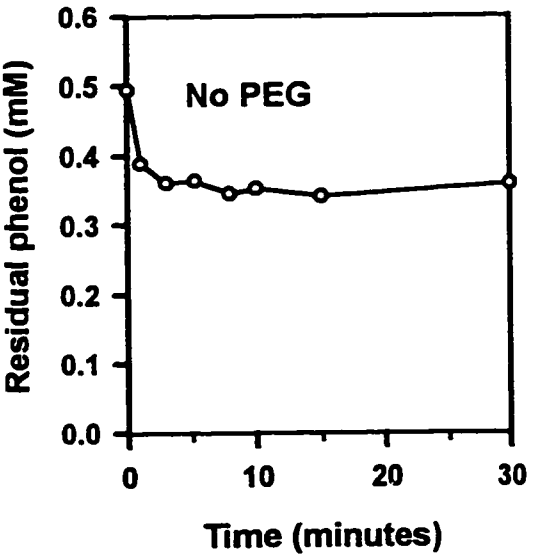

Figure 4.8k. Batch reactor kinetic data. $[phenol]_0 = 0.50$ mM, $[H_2O_2]_0 =$ 0.62 mM, $[ARP]_0 = 0.34$ U/mL

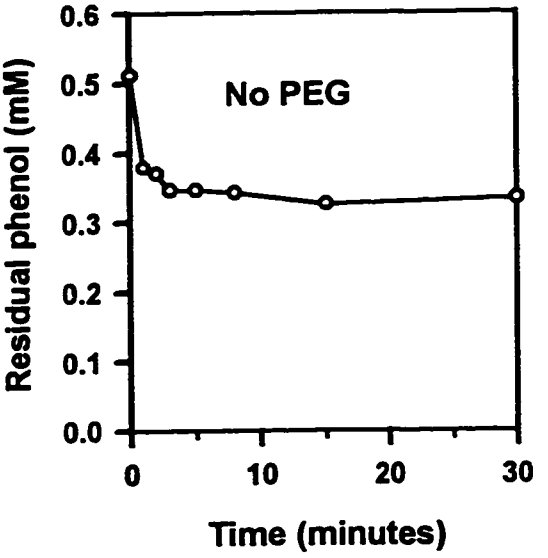

Figure 4.8l. Batch reactor kinetic data. $[phenol]_0 = 0.51$ mM, $[H_2O_2]_0 =$ 0.52 mM, $[ARP]_0 = 0.41$ U/mL

4.4.3. Turnover Number

It may be noted from Figures 4.7 and 4.8 that a smaller amount of ARP is needed to achieve a given level of phenol removal in the presence of PEG than in its absence.

Nicell (1993 b) defined the turnover number as the number of molecules of aromatic compound removed from solution per molecule of enzyme provided to the system based on the assumption that all enzyme is consumed or inactivated. The turnover number is calculated according to (Nicell, 1994):

$$\textbf{Turnover Number} = \frac{[AH_2]_0 - [AH_2]_f}{[ARP]_0} \qquad (4.5)$$

where $[AH_2]_0$ and $[AH_2]_f$ are the initial and final concentrations of phenol (M), $[ARP]_0$ is the initial concentration of ARP (M). A higher turnover number means less enzyme is needed for the same amount of phenol removal.

The average turnover number was calculated from the batch test data illustrated in Figures 4.7 and 4.8. For reaction in the presence of excess PEG the average turnover number was 59,000. For reaction in the absence of PEG, an average turnover number of 4,300 was calculated. Thus, 13.7 times less ARP was required for a given phenol removal in the presence of PEG.

Nicell and coworkers (1993) reported a turnover number of 15,600 for HRP catalyzed phenol removal without PEG at pH 7.0 and 25 °C. Buchanan and Nicell (1998 a) reported a turnover number in excess of 400,000 for HRP catalyzed phenol removal in the presence of excess PEG at pH 7.0 and 25 °C. Thus, approximately 3.6 and 7 times

more ARP would be required for any given phenol removal in the absence and the presence of PEG (respectively) than would be needed if HRP were used.

Wright (1995) reported a turnover number of 4,875 for soybean peroxidase catalyzed phenol removal in the absence of PEG at 25 °C and pH 7.0. Therefore, ARP appears to be as efficient as SBP, but less efficient than HRP under the given set of reaction conditions.

4.5. Applicability of the Simplified Kinetic Model

The ability of the simplified kinetic model of the peroxidase-peroxide-phenol system, which was developed by Buchanan and Nicell (1998 b) and summarized in section 2.6.2, to predict ARP catalyzed phenol removal from batch reactors was tested.

4.5.1. Model Calibration and Validation

The simplified model was calibrated for ARP catalyzed phenol removal at 25° C and pH 7.0. The method of Nicell and Wright (1997) was applied to estimate the value of k_1 for the oxidation of the native enzyme by hydrogen peroxide. In accordance with this method, a series of ARP activity assays were performed, and the measured activities were converted to peroxide consumption rates by applying the definition of activity (see section 3.3.1). Two ARP cuvette concentrations, 1.7 nM and 0.7 nM, were used together with initial peroxide cuvette concentrations ranging from 0.05 to 3.0 mM. The results of this series of assays are shown in Figure 4.9.

The assay model developed by Nicell and Wright (1997) assumes that changes in aromatic substrate (phenol) and hydrogen peroxide concentrations are negligible over the course of the assay because they are present in excess quantity. Thus, the concentrations of these two compounds are treated as constant and the rate of peroxide consumption is related to enzyme concentration according to:

$$\frac{d[H_2O_2]}{dt} = \frac{-E_0}{\left(A + B/[H_2O_2] + C[H_2O_2]\right)} \tag{4.6}$$

in which

$$A = \frac{1}{2[AH_2]}\left(\frac{1}{k_2} + \frac{1}{k_3}\right),\ B = \frac{1}{2k_1} \text{ and } C = \frac{k_{app}}{2k_3 k_{eff}[AH_2]} \tag{4.7}$$

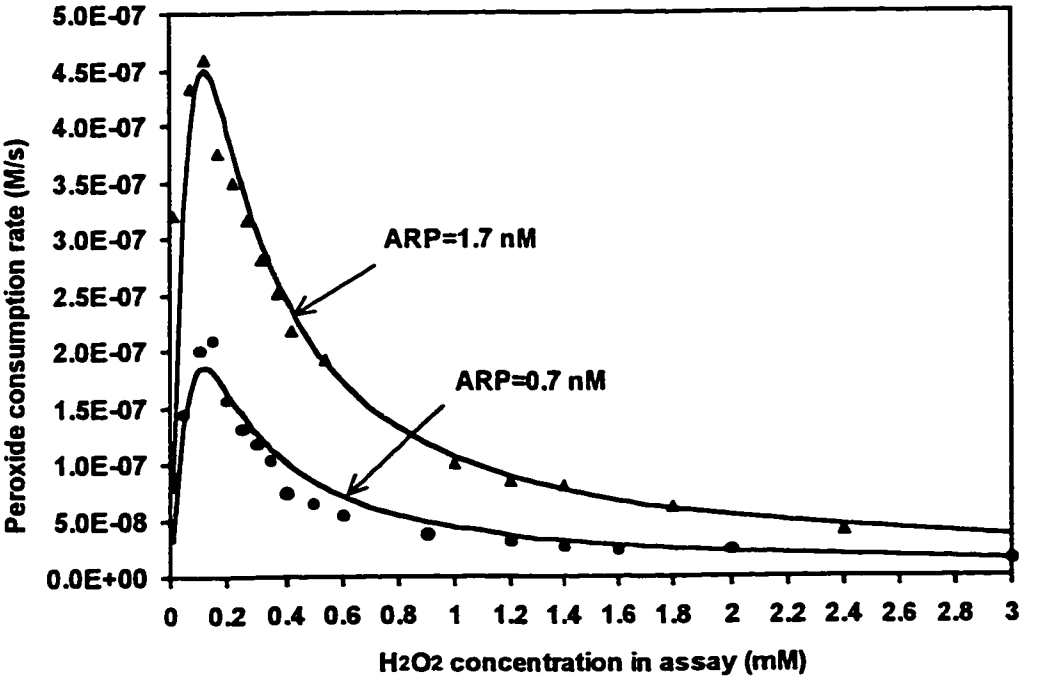

Figure 4.9. Activity of ARP as a function of peroxide concentration in the assay mixture.

The constants A, B and C were estimated using a genetic algorithm package as 3.9×10^{-5}, 2.15×10^{-7} and 15.4. These values were included in equation (4.6) to obtain the best fit lines shown in Figure 4.9. Based on, $k_1 = 1/(2B)$, the value of k_1 was calculated as 2.33×10^6 $M^{-1}s^{-1}$. This value is in close agreement with estimates reported in previous studies (see Table 2.2).

Calibration of the simplified model also requires the estimation of the model constants k_a, k_b, k_{eff}, k_c, and k_f. A stoichiometry of 1:1 between moles of phenol removed and moles of peroxide consumed was assumed for modelling purposes. The model was first calibrated for phenol removal at 25°C and pH 7.0 in the presence of excess PEG. Six of the thirteen experimental data sets shown in Figure 4.7 were used for this purpose. The initial phenol concentrations of the selected calibration data sets ranged from 0.6 to 8.3 mM (see Tables B1 and B2 in Appendix B).

Parameter estimation was accomplished using a genetic algorithm package. This program needs a user supplied subroutine to define the system to be optimized. In this instance, this corresponds to the fit of the modelled time dependent phenol concentrations to the calibration data. Thus, the objective function to be minimized is the sum of the squared differences between modelled and experimental phenol concentrations. This objective function can be formulated by:

$$SSR = \sum_{i=1}^{n} \sum_{j=1}^{m_i} \left([AH_2]_{i,j} - [AH_2]_{i,j_{model}} \right)^2 \qquad (4.8)$$

where n represents the number of calibration data sets, SSR represents the sum of squared

residuals, m_i represents the number of observations in the i^{th} data set, and $[AH_2]_{i,j}$ and $[AH_2]_{i,j\,model}$ are experimental and modelled phenol concentrations respectively. The module of program code written for this purpose is included in Appendix C. Model equations were solved using a Fourth Order Runge Kutta numerical method.

The value of the rate constant, k_1, was fixed at the previously determined value of 2.33×10^6 $M^{-1}s^{-1}$ during this procedure. The results from the calibration procedure are shown as Group A in Table 4.2. The fit of the model to the six calibration data sets is shown in Figures 4.10, 4.11 and 4.12. For the validation of the model calibration, the remaining seven data sets were used (see Tables B3 to B5 in Appendix B). The constants shown as Group A in Table 4.2 were used together with the initial conditions of each of the seven batch tests to generate the predicted phenol concentrations. These are overlaid on experimental data in Figures 4.13 through 4.16. The largest discrepancy between measured and predicted phenol concentrations, which is indicated in Figure 4.16, is approximately 0.46 mM (7.8% of the 5.86 mM initial phenol concentration). An inspection of Figures 4.13 through 4.16 indicates that the model calibration does not systematically under- or over-predict the time dependent phenol removal.

In order to obtain a set of refined parameter estimates to be used in calibrating the model for phenol removal in the absence of PEG, the calibration procedure was repeated using all thirteen data sets. The result is shown in Table 4.2 as Group B.

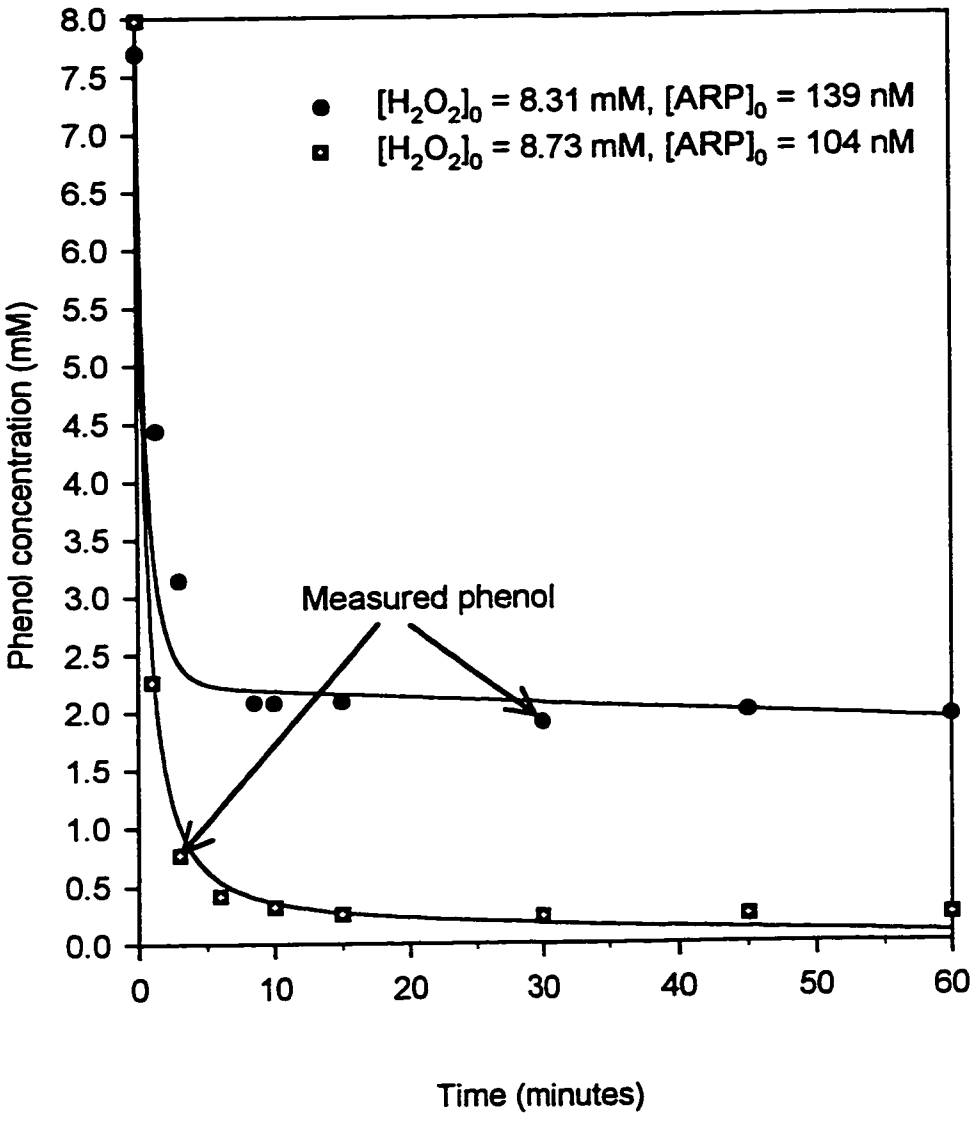

Figure 4.10. Calibration data overlaid on model output ($[phenol]_0$ = 7.98, and 7.69mM). [PEG] = 400 mg L-1, ——— Model fit.

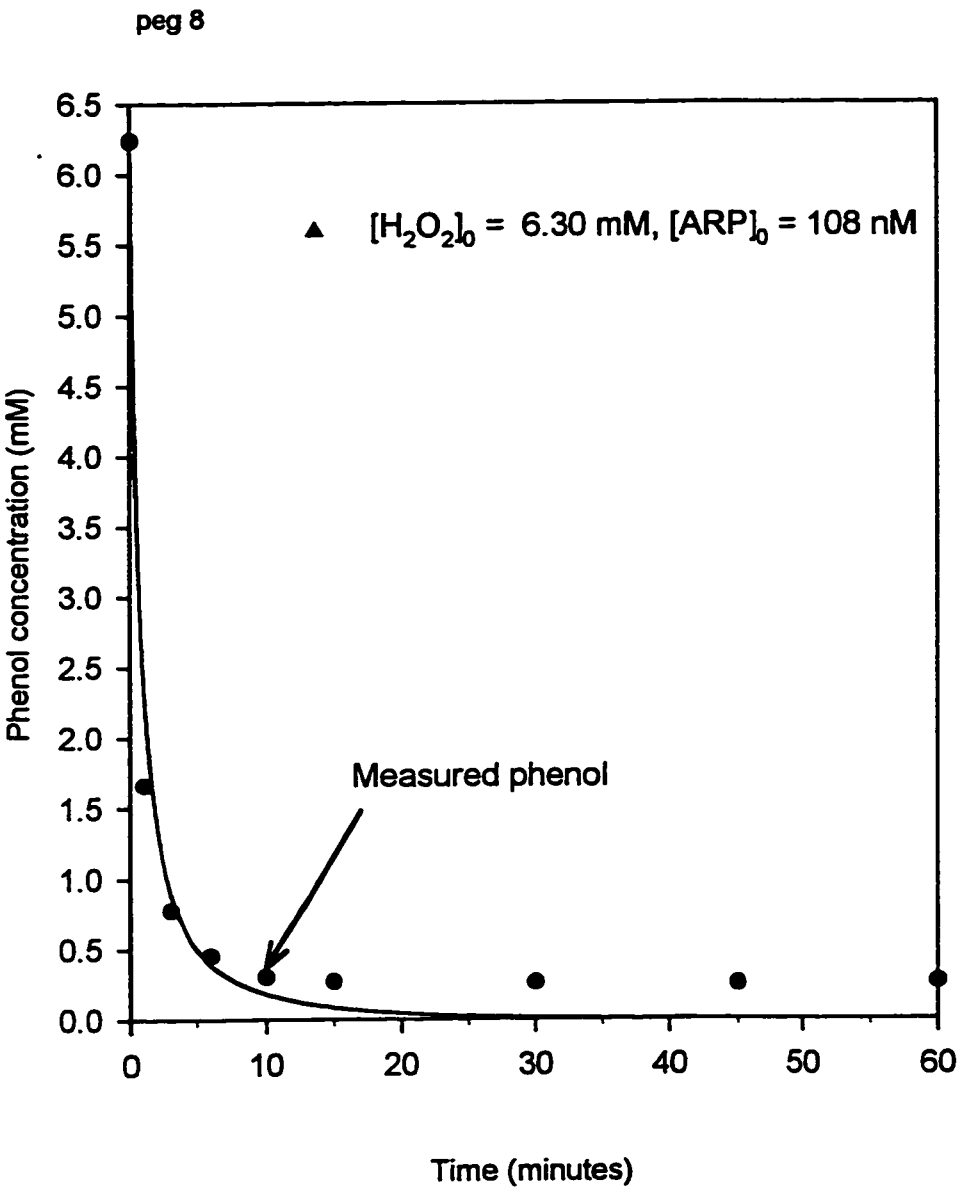

Figure 4.11 Calibration data overlaid on model output($[phenol]_0 = 6.24$ mM). [PEG] = 400 mg L-1, —— Model fit.

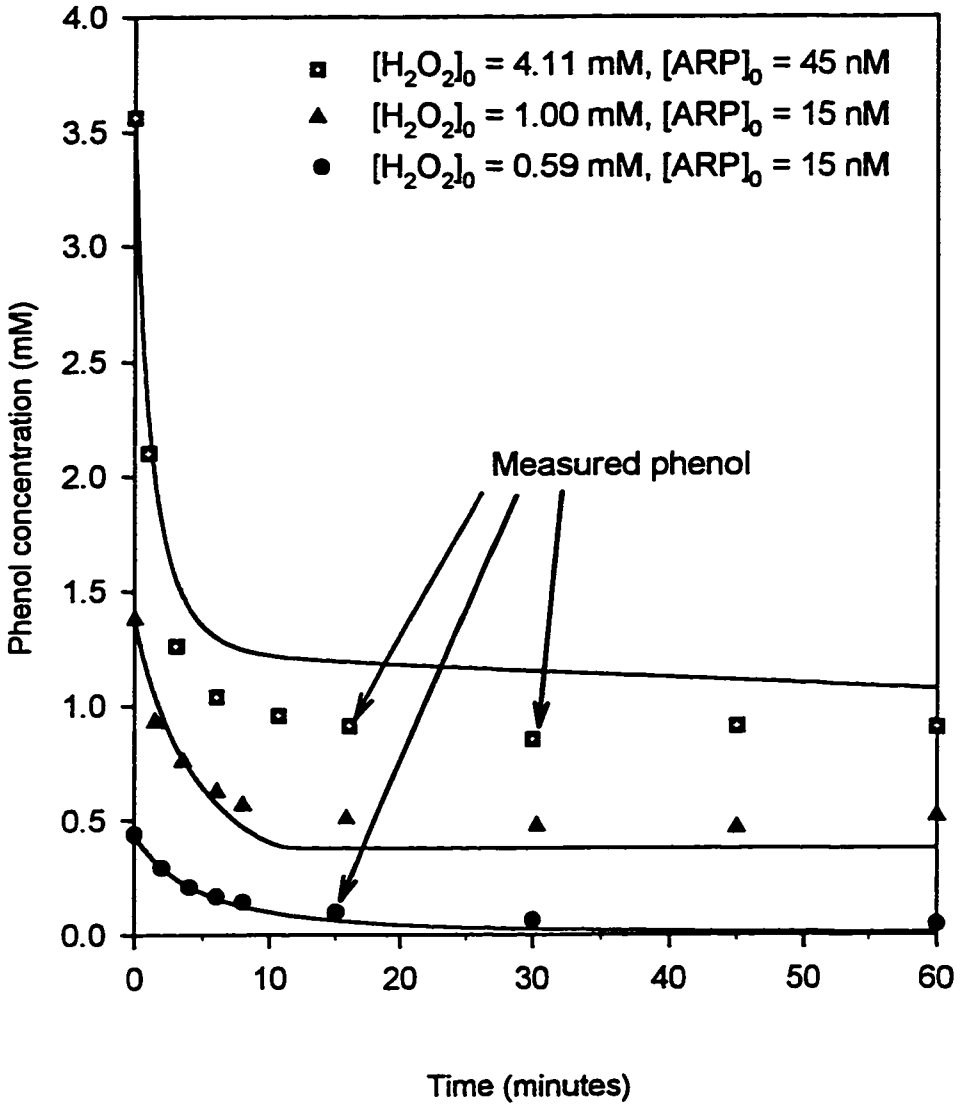

Figure 4.12. Calibration data overlaid on model output ($[phenol]_0$ = 3.56, 1.38 and 0.44 mM). [PEG] = 400 mg L-1, ——— Model fit.

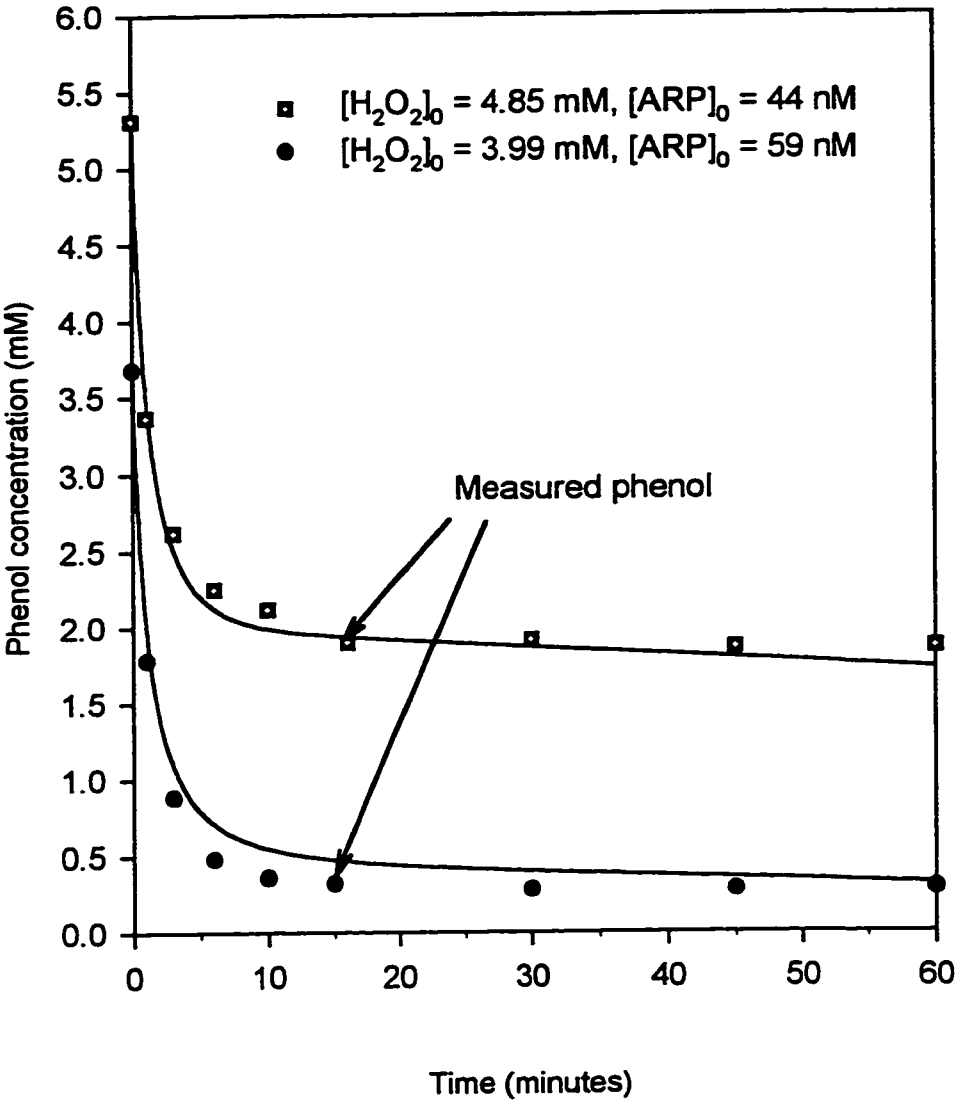

Figure 4.13. Validation data overlaid on model output ($[phenol]_0$ = 5.31 and 3.68 mM). [PEG] = 400 mg L^{-1}, —— Model fit.

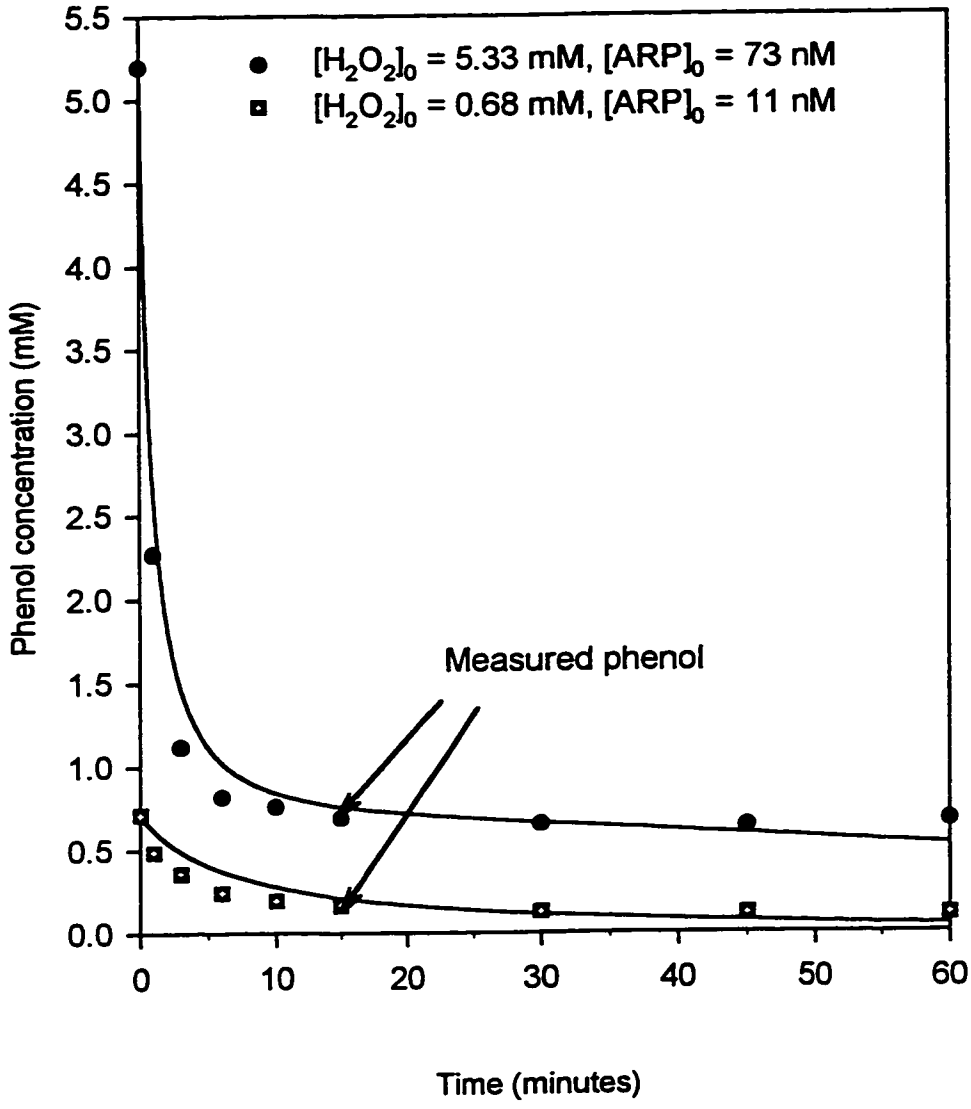

Figure 4.14. Validation data overlaid on model output ($[phenol]_0$ = 5.20 and 0.71 mM). [PEG] = 400 mg L^{-1}, —— Model prediction.

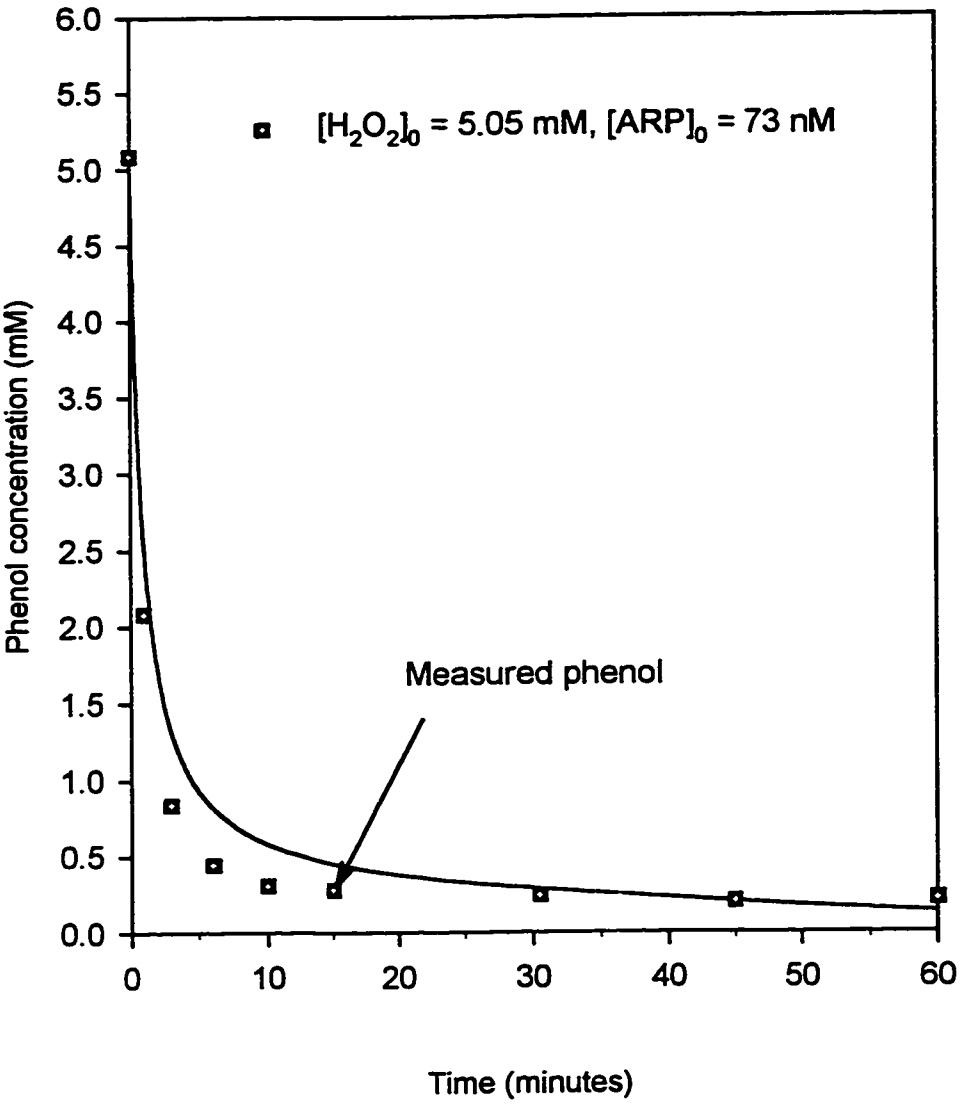

Figure 4.15. Validation data overlaid on model output ($[phenol]_0$ = 5.09 mM). [PEG] = 400 mg L^{-1}, —— Model prediction.

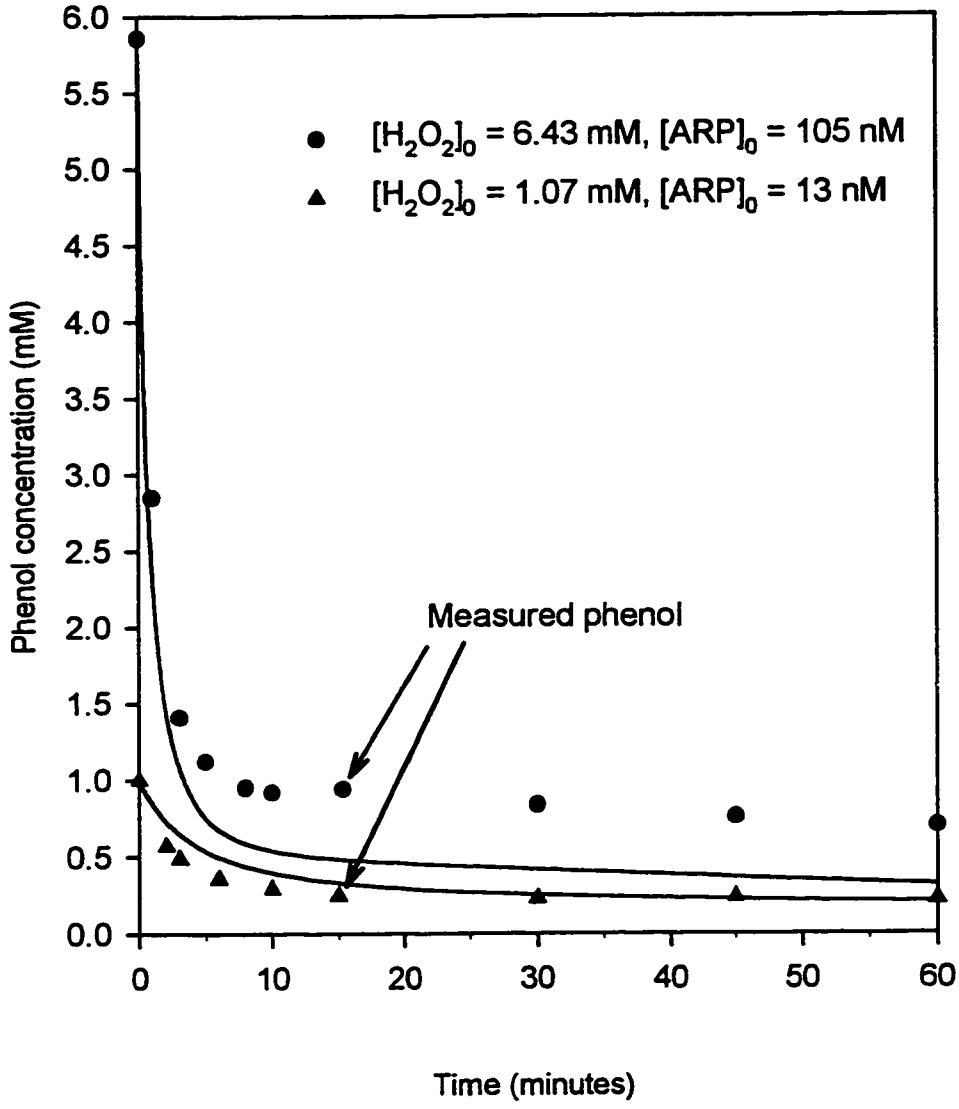

Figure 4.16. Validation data overlaid on model output ($[phenol]_0$ = 1.00 and 5.86 mM). [PEG] = 400 mg L^{-1}, —— Model prediction.

Table 4.2. Kinetic Rate Constant Values in the Presence of PEG (25 °C, pH 7.0).

Constant	Group A (6 data sets)	Group B (13 data sets)
k_1 ($M^{-1}s^{-1}$)	$2.33x10^6$	$2.33x10^6$
k_a ($M^{-1}s^{-1}$)	$1.39x10^5$	$1.49x10^5$
k_b ($M^{-1}s^{-1}$)	4.23	4.43
k_{eff} (s^{-1})	$4.68x10^{-5}$	$3.72x10^{-5}$
k_e (dimensionless)	0	0
k_r ($M^{-0.5}s^{-0.5}$)	$3.36x10^{-2}$	$5.38x10^{-2}$
Sum of Squared Residuals $x10^6$ (M^2)		
Calibration	3.69	6.53
Validation	3.12	N/A
All sets	6.81	6.53

As indicated in Table 4.2, the value of the constant k_e, which is associated with enzyme inactivation by end-product polymer, is zero. Inspection of the parameter values shown in Groups A and B of Table 4.2 shows the two groups to be in close agreement with each other. This is also demonstrated by the similar sum of squared residuals for the two groups.

The model was then calibrated for phenol removal at 25°C and pH 7.0 in the absence of PEG. Only the rate constants associated with permanent enzyme inactivation are affected by the presence of PEG. Therefore, the values of the kinetic constants k_1, k_a, k_b and k_{eff} were held fixed at those indicated in Table 4.2 for Group B during this calibration. Data from six of the twelve batch tests conducted without PEG were used for

calibration (see Tables B.6 and B.7 in Appendix B). The initial phenol concentrations ranged from 0.51 to 5.18 in the calibration data sets. This calibration process yielded the parameter estimates shown in Table 4.3 for Group C. The fit of model output to the calibration data sets may be found in Figures 4.17 and 4.18. The model was then used to predict the time dependent phenol concentrations using the Group C parameter values and the initial phenol, peroxide and ARP concentrations of the six validation data sets. Figures 4.19, 4.20 and 4.21 show the predicted phenol concentrations overlaid on the experimental data used for validation the calibration.

The parameter estimation procedure was applied again using all twelve data sets to obtain a "best estimate" of the inactivation rate constants for reaction without PEG. The resulting values of the enzyme inactivation rate constants are contained in Group D of Table 4.3.

Groups C and D in Table 4.3 have similar estimates for k_e. However, the Group C estimate of k_r is 38% larger than that of Group D. Nevertheless, the sums of squared residuals associated with the Groups C and D parameter values are essentially the same. This indicates that the model is quite insensitive to changes in the constant k_r. This is also borne out by a sensitivity analysis contained in Appendix D.

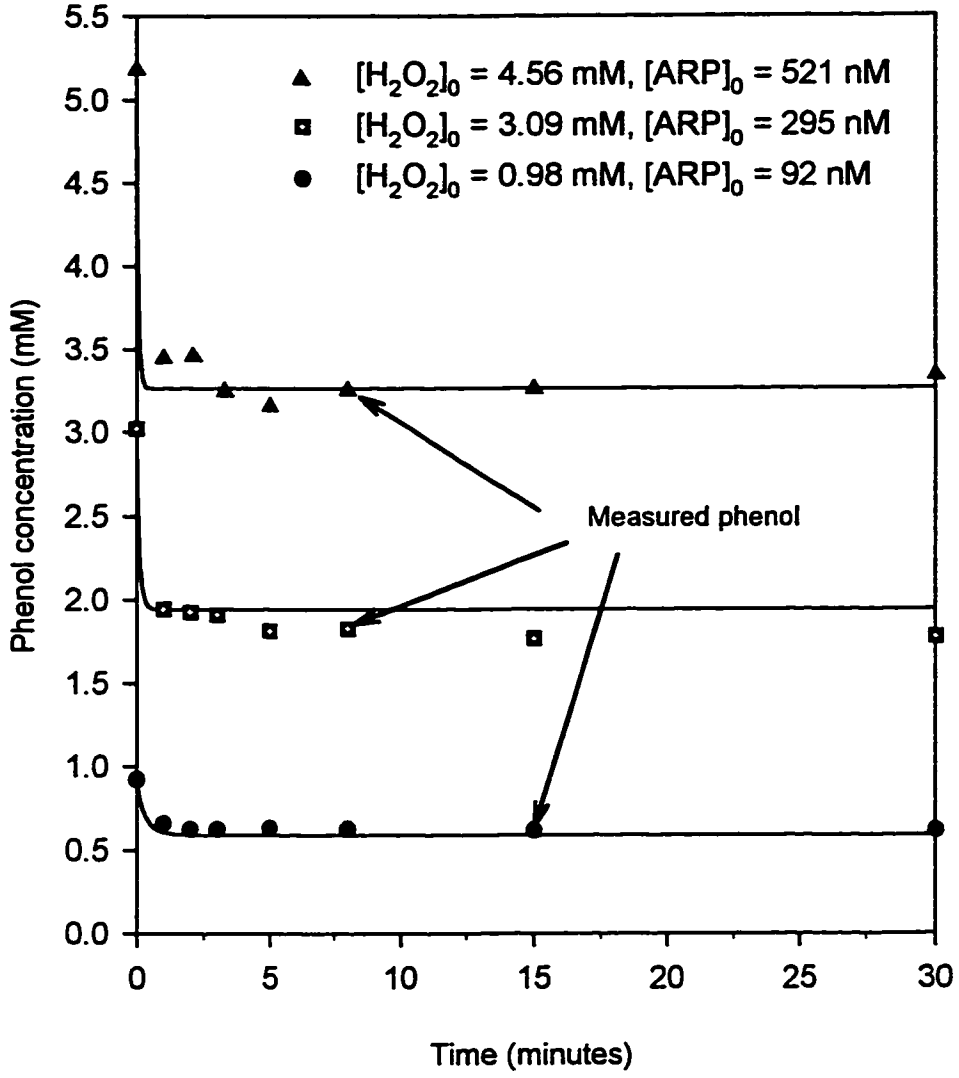

Figure 4.17. Calibration data overlaid on model output ($[phenol]_0$ = 5.18, 3.02 and 0.92 mM). [PEG] = 0 mg L^{-1}, —— Model fit.

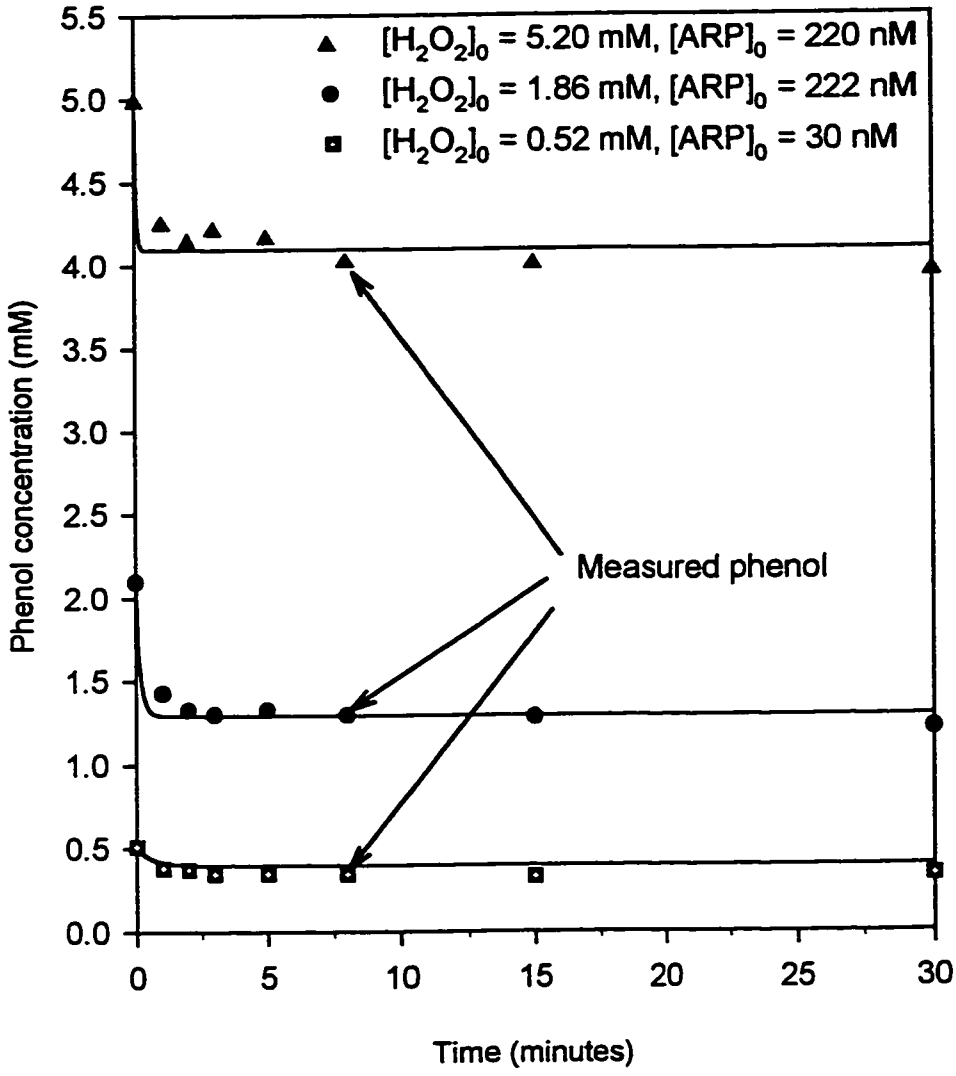

Figure 4.18. Calibration data overlaid on model output ($[phenol]_0$ = 4.98, 2.10 and 0.51 mM). [PEG] = 0 mg L^{-1}, —— Model fit.

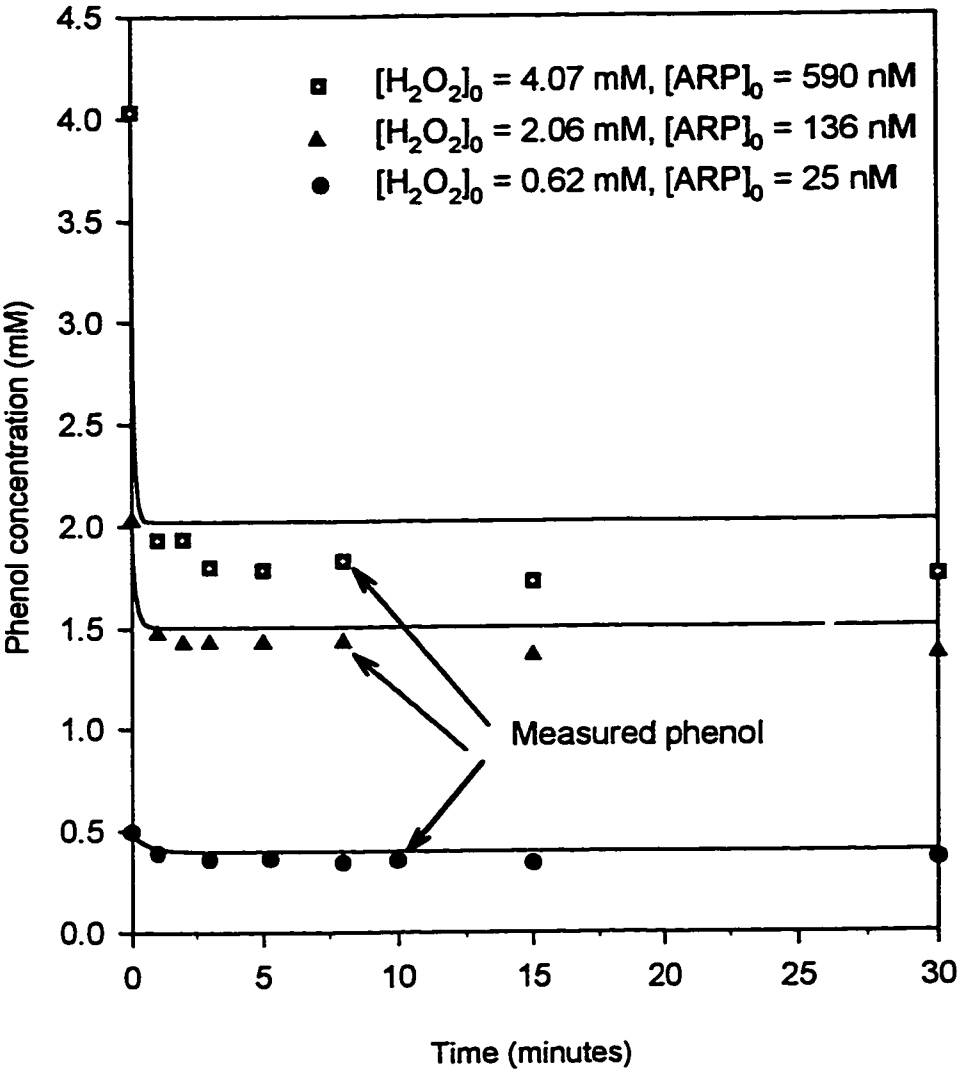

Figure 4.19. Validation data overlaid on model output ($[phenol]_0$ = 4.03, 2.03 and 0.50 mM). [PEG] = 0 mg L^{-1}, —— Model prediction.

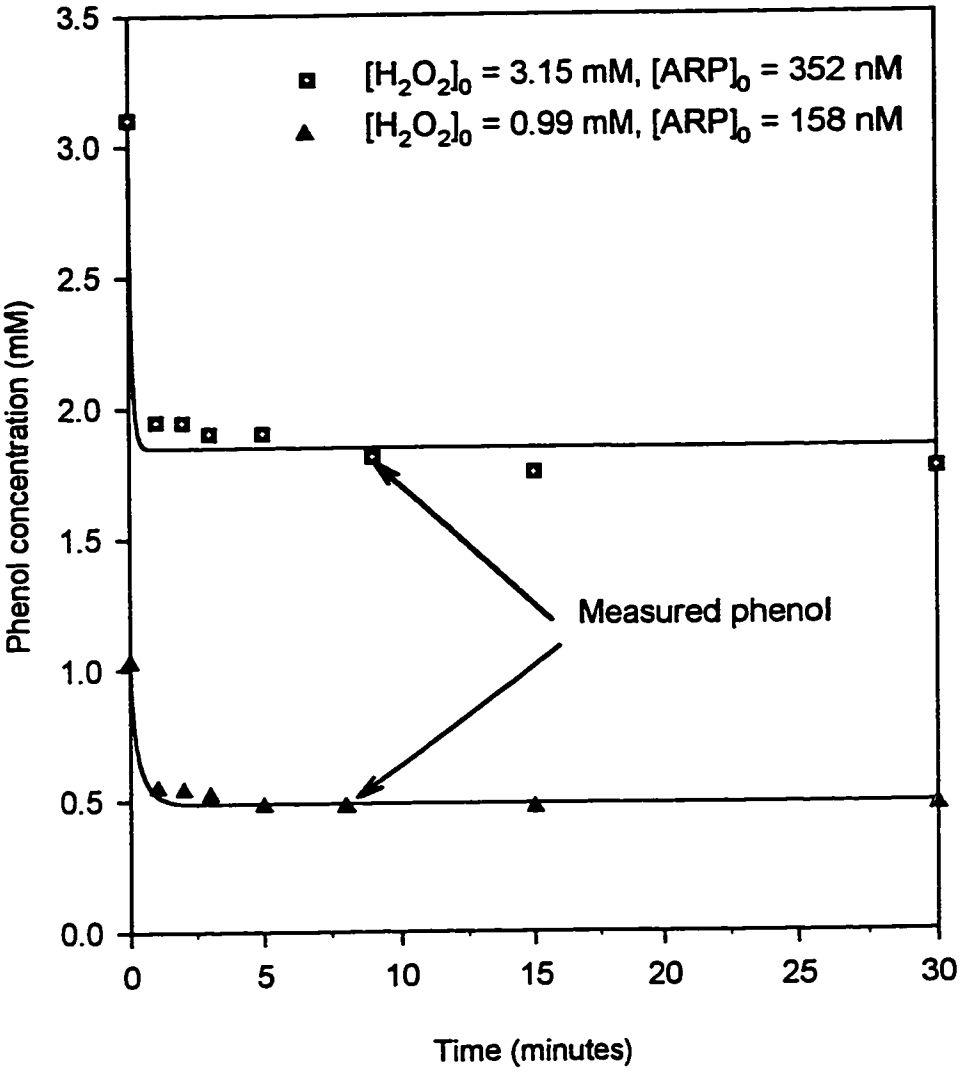

Figure 4.20. Validation data overlaid on model output ($[phenol]_0$ = 3.10 and 1.02 mM). [PEG] = 0 mg L^{-1}, —— Model prediction.

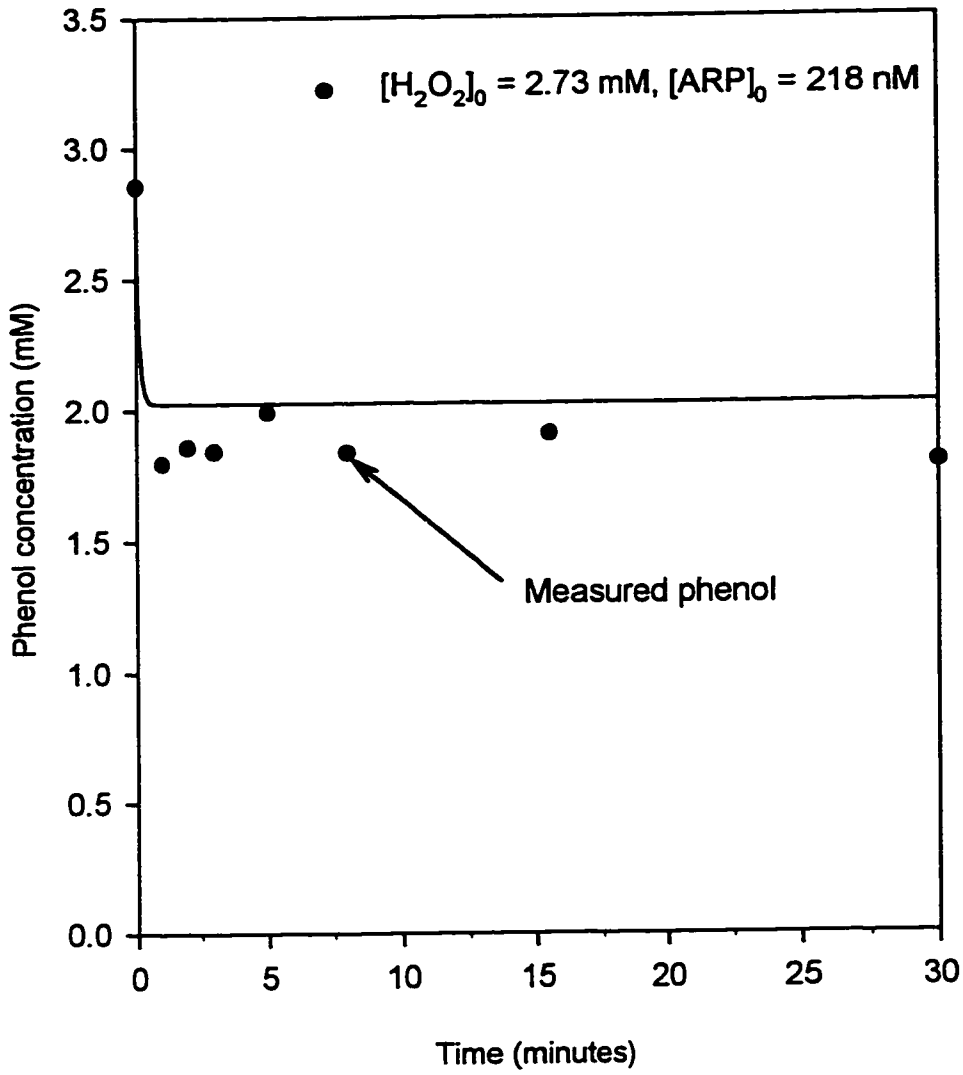

Figure 4.21. Validation data overlaid on model output ($[phenol]_0$ = 2.89 mM). [PEG] = 0 mg L^{-1}, —— Model prediction.

Table 4.3. Kinetic Rate Constant Values in the Absence of PEG (25 °C, pH 7.0).

Constant	Group C (6 data sets)	Group D (12 data sets)
k_1 ($M^{-1}s^{-1}$)	$2.33x10^6$	$2.33x10^6$
k_a ($M^{-1}s^{-1}$)	$1.49x10^5$	$1.49x10^5$
k_b ($M^{-1}s^{-1}$)	4.43	4.43
k_{eff} (s^{-1})	$3.72x10^{-5}$	$3.72x10^{-5}$
k_e (dimensionless)	$1.96x10^{-4}$	$2.04x10^{-4}$
k_r ($M^{-0.5}s^{-0.5}$)	4.03	2.92
Sum of Squared Residuals $x10^6$ (M^2)		
Calibration	0.31	0.79
Validation	0.61	N/A
All sets	0.92	0.79

4.6. Distribution of Enzyme Forms

The best estimates of the parameter values obtained during this investigation, are Group B for reaction in the presence of excess PEG and Group D for reaction without PEG. The ability of the model to predict phenol removal under a variety of experimental conditions has been demonstrated. Therefore, the model may be used to gain an insight into the probable distribution of enzyme forms over the course of batch reactions alternatively in the presence of excess PEG or in its absence.

The model was applied to predict the time dependent concentrations of phenol, active enzyme, compound III, and permanently inactive enzyme. One typical set of

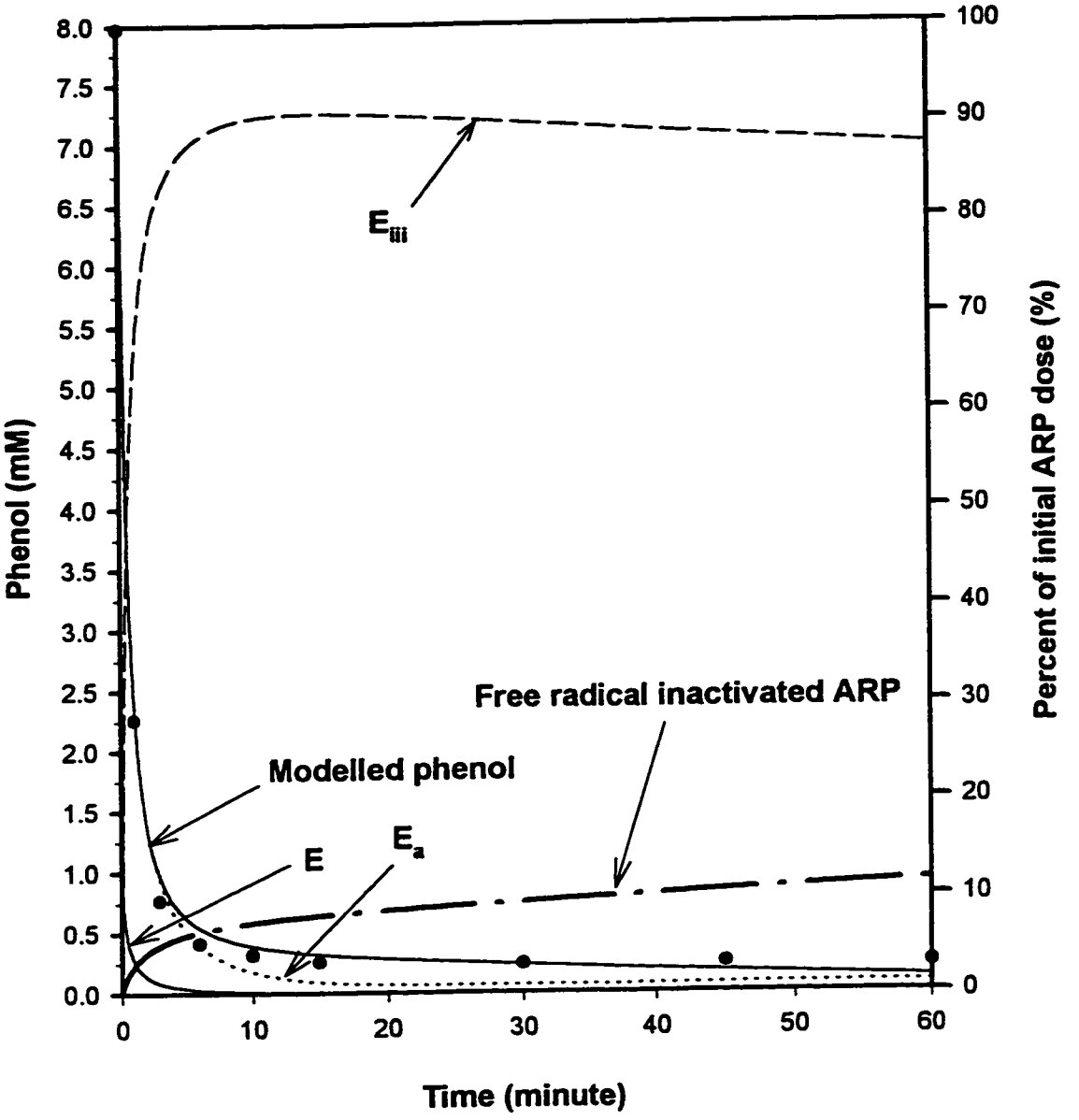

Figure 4.22. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, $[phenol]_0$ = 7.98 mM, $[H_2O_2]_0$ = 8.31 mM and $[ARP]_0$ = 139 nM.

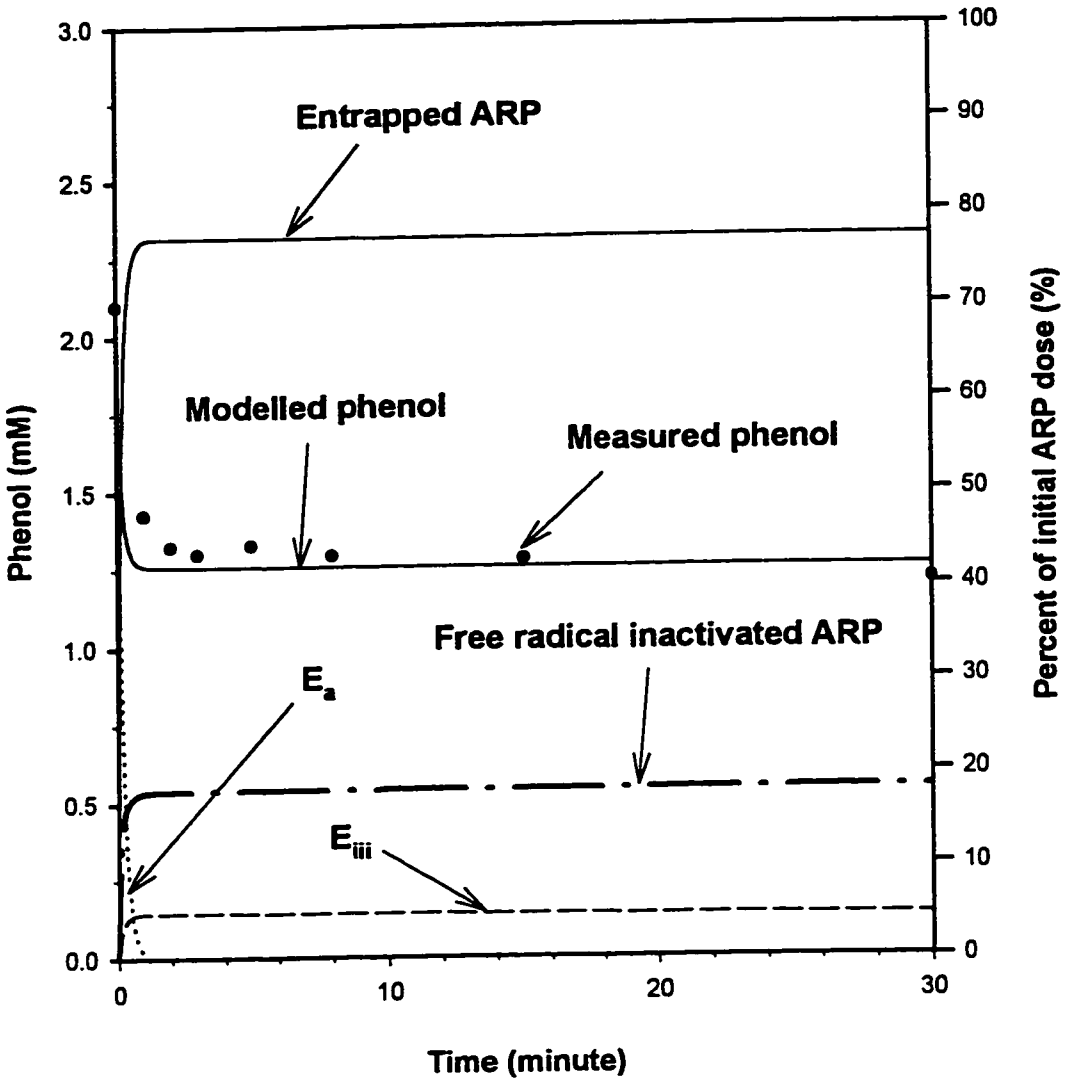

Figure 4.23. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, $[phenol]_0$ = 2.10 mM, $[H_2O_2]_0$ = 1.86 mM and $[ARP]_0$ = 222 nM.

predictions is shown in Figure 4.22 for reaction in the presence of excess PEG, and one simulation is shown in Figure 4.23 for reaction the absence of PEG. The remaining simulations are contained in Appendix E.

The predictions shown in Figure 4.22 were generated using the parameter values indicated as Group B in Table 4.2. This simulation indicates that in the presence of PEG, the majority of the ARP enzyme is converted very rapidly to compound III. This is an inactive form of the enzyme which slowly reverts to the native form. However, modelling indicates that the conversion to the native state occurs so slowly that compound III formation is essentially a permanent inactivation from a practical treatment perspective. This is in contrast with the relatively rapid conversion of the compound III form of HRP suggested by Buchanan and Nicell (1998 a), and indicates the need for process optimization to limit the formation of the compound III form of ARP. Additionally, modelling by Buchanan and Nicell (1998 a) suggests that HRP inactivation by free radicals is negligible for reaction in the presence of PEG.

Figure 4.23 contains the distribution of enzyme forms during reaction in the absence of PEG generated using the Group D parameter values (Table 4.3) and the initial phenol, peroxide and ARP concentrations indicated in the figure. This simulation indicates that the predominant form of enzyme inactivation is due to interactions between the enzyme and end-product polymer for reaction without PEG. This prediction is at variance with a simulation reported by Buchanan and Nicell (1998 a) for HRP catalyzed phenol removal in which the authors found that HRP was inactivated predominantly by free radicals, and that the compound III form of HRP decomposes quickly in the absence of PEG.

Further research is required to clarify the predominant inactivation mechanisms for both HRP and ARP in the presence or absence of PEG.

4.6.1. Spectrophotometric Observation

The changes in the absorption spectrum of a 17.9 μM ARP solution were monitored over time within the region of 360 to 700 nm in an attempt to detect the formation of compounds III and P-670 upon the addition of hydrogen peroxide. As indicated in Figure 4.24, the native form of ARP shows an absorbance peak at 403 nm. After the addition of 1 mM of H_2O_2, the largest absorbance peak shifted from 403 to 418, and two smaller peaks appeared at 548 and 582 nm within a few seconds. These double peaks at 548 and 582 are may be characteristic of the compound III form of ARP (Andersen et al., 1991). Andersen and coworkers (1991) reported that compound III of *Coprinus cinereus* peroxidase, which is identical to ARP, shows maximum absorption at 417, 547, 582 and 649 nm. These peaks, indicating the compound III form of ARP, disappeared within 20 minutes, and the maximum absorbance wavelength reverted to 407 nm indicating the conversion of compound III. This result appears to contradict the simplified model's prediction of a very slow return of compound III to the native form. The model predicted that most of the enzyme would remain in the compound III form for over 60 minutes. This disagreement may indicate a deficiency in either the model or the method of parameter estimation. Thus, further study is need to address this issue.

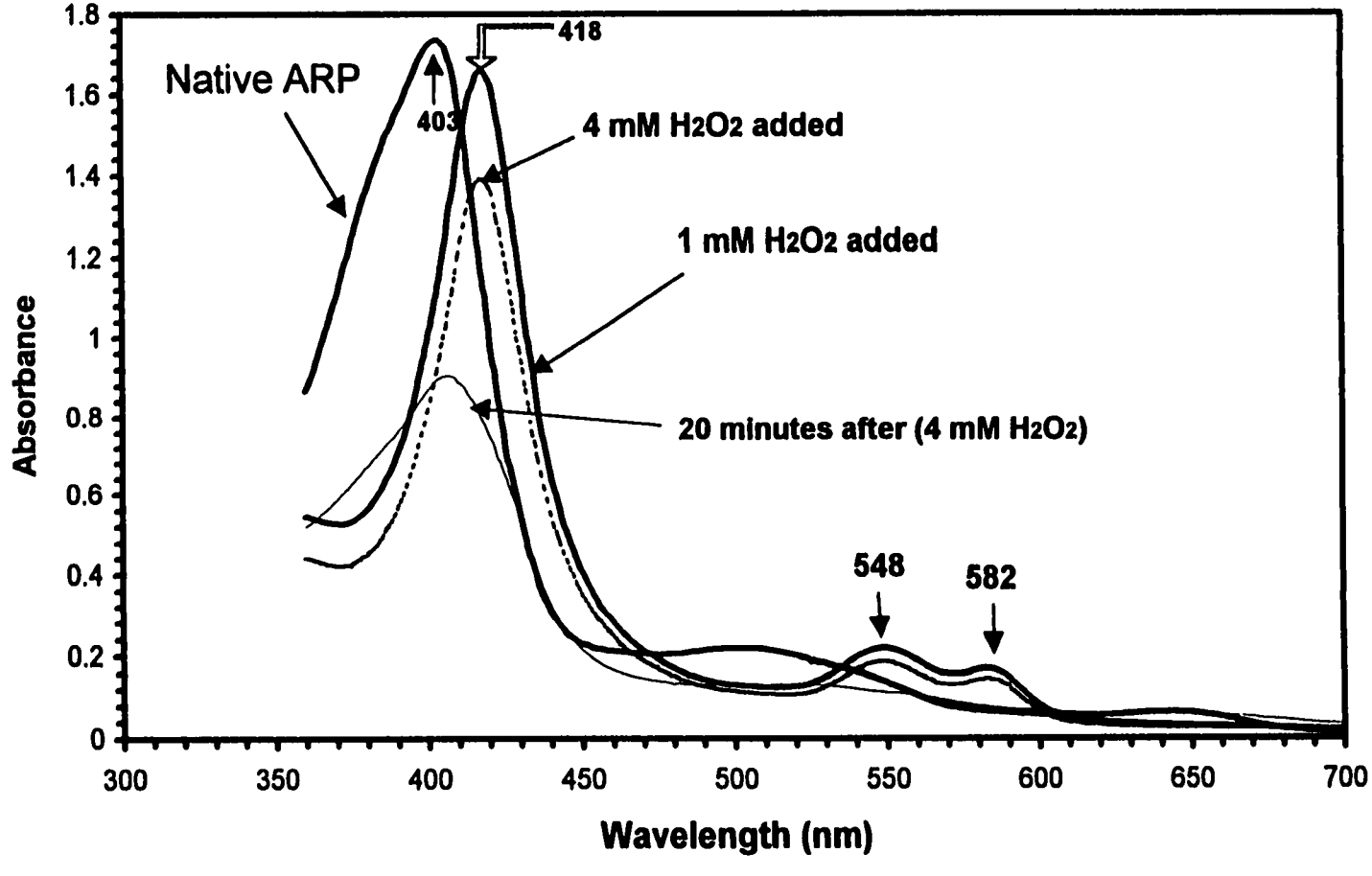

Figure 4.24. Spectrophotometric distribution of ARP performed by hydrogen peroxide.

In the current investigation, no peak was observed at 670 nm in spite of the addition of an excess amount of peroxide. This indicates that the inactive enzyme form P-670 was not formed. However, an inactive form of ARP which exhibits maximum absorbance outside of the 360 to 700 nm region may have formed. This is suggested by the area under the absorbance trace decreasing with time.

Further investigation using a diode array spectrophotometer would allow the change in absorbance at specific wavelengths to be monitored over time, and yield an improved understanding of the formation of the various forms of ARP.

5. Conclusions and Recommendations

5.1. Stoichiometry

Two series of batch tests, containing different initial concentrations of phenol, enzyme and H_2O_2, resulted in variable stoichiometric ratios. Attempts to model the variation of stoichiometry as a function of initial phenol and peroxide concentrations were unsuccessful. This variation may be related to enzyme concentration, however, further investigation will be required to resolve this issue.

5.2. Thermostability

ARP appeared to be very stable when it was stored in temperature up to 25 °C. Thermal inactivation follows first-order kinetics at temperatures higher than approximately 25 °C. The thermal inactivation rate constants were estimated at 35, 45 and 55 °C, and the activation energy was estimated to be 77 $KJmol^{-1}$. It may be concluded ARP is less stable than both HRP and SBP at high temperature.

5.3. Turnover Number

The enzyme inactivation rate was greatly decreased in the presence of PEG. The average turnover numbers were calculated as 59,000 for reaction with PEG and 4,300 for reaction without PEG. In comparison with the turnover number of HRP which is calculated as

400,000 with PEG and 15,600 without PEG in literature, approximately 3.6 and 7 times more ARP would be required for any given phenol removal in the absence and the presence of PEG respectively. The lower efficiency of ARP relative to HRP must be taken into account when a cost effectiveness comparison is made.

5.4. Application of Simplified Kinetic Model

The simplified kinetic model was applied to predict ARP catalyzed phenol removal from batch reactors. The model showed quite good predictions of phenol removal with ARP as a function of time for both with PEG and without PEG. However, in many cases of the model predictions obtained with PEG, a trend of little under-prediction at the first period of reaction and over-prediction at the end of reaction observed. Model calibration indicates that the permanent inactivation of ARP is caused predominantly by end-products polymer for reaction without PEG and by inactivation by free radicals for reaction in the presence of PEG. The model indicated the rate of compound III formation is very fast and its rate of decomposition is extremely slow. Thus, the model predicted most of enzyme was transformed to the compound III form and retained in the form for more than a 60-minute time period.

5.5. Spectrophotometric Study

Spectrophotometric study indicated that in the presence of peroxide and the absence of aromatic compound most of ARP is transformed into the compound III form, which is then

converted within 20 minutes. The formation of the permanently inactive form, P-670, was not observed. This result is different from the model predictions that most of enzyme remains in the compound III form for at least 60 minutes. This disagreement may indicate a deficiency in either the current understanding of ARP behaviour or the method of parameter estimation. Thus, further study of the life-cycle of ARP is needed.

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APPENDIX A

Analytical Methods

A.1. *Arthromyces ramosus* Peroxidase Activity Assay

This assay measures the amount of active enzyme in a sample. The assay uses saturation concentrations of phenol, 4-aminoantipyrine (AAP),and hydrogen peroxide (H_2O_2) such that the initial reaction rate will be proportional to the amount of active ARP present. The rate of reaction is proportional to the formation rate of the non-precipitable product which absorbs light at a peak wavelength of 510 nanometers (nm) with an extinction coefficient of 7 100 $M^{-1}cm^{-1}$. One unit of ARP activity (U) is defined as the number of micromoles of peroxide utilized in one minute at pH 7.4 and 25 °C in an assay mixture consisting of 10 mM phenol, 2.4 mM AAP, and 0.2 mM H_2O_2.

A.1.1. Preparation of Reagents

a) 0.1 M NaPP Buffer (pH 7.4)

Add 19 mL of 0.2 M monobasic sodium phosphate to 81 mL of 0.2 M dibasic sodium phosphate and dilute to 200 mL with deionized water.

b) 20 mM phenol

Dissolve 941.1 mg of phenol in 500 mL of 0.1 M NaPP buffer (pH 7.4).

c) 9.6 mM 4-aminoantipyrine (AAP)

Dissolve 390 mg of 4-aminoantipyrine in 200 mL of 0.1 M NaPP buffer (pH 7.4).

Store in refrigerator.

d) 2.0 mM H_2O_2

Dilute 113.3 µL of 30% (w/v) hydrogen peroxide to 100 mL with deionized water (10mM). Mix well, withdraw 2 mL and dilute to 100 mL with deionized water (2 mM). This reagent is to be prepared daily.

A.1.2. Procedure

The total assay volume is 1 mL, therefore in a semi-micro cuvette place:

- 500 µL of 20 mM phenol
- 250 µL of 9.6 mM AAP
- 100 µl of 2.0 mM H_2O_2
- 0 to 100 µL of 100 mM NaPP (pH 7.4)
- 50 to 150 µL of enzyme sample

Before the substrate depletion becomes significant, the activity of the ARP in the cuvette is proportional to the rate of change of the absorbance at 510 nm. As soon as the ARP has been added, the cuvette was capped and inverted several times and then the change in absorbance was monitored with time at 510 nm. The ARP activity in the cuvette must not exceed 0.05 U/mL for the color development to remain linear over the 1 minute duration of the assay.

A.1.3. Calculation

One unit of activity is defined as the number of micromoles of peroxide converted per minute at pH 7.4 and 25 °C. The activity of the ARP in the cuvette is obtained from the average slope (absorbance units per minute) within the linear range. Therefore, the activity within the cuvette, in units of mol min^{-1} mL^{-1} (i.e. U/mL), is calculated according to:

$$\textbf{Activity in cuvette (U / mL)} = \frac{\textbf{slope (au / min.)}}{\textbf{7100 (au L / mol)}} \cdot \frac{\textbf{10}^{6}\ \mu\textbf{mol}}{\textbf{mol}} \cdot \frac{\textbf{1L}}{\textbf{1000mL}}$$

in which au represents absorbance units and 7100 au·L/mol relates color development to peroxide consumption. The sample activity is calculated according to:

$$\textbf{Sample Activity (U / mL)} = \textbf{Activity in cuvette (U / mL)}\ \frac{\textbf{1000}\ \mu\textbf{L}}{\textbf{sample}\ \mu\textbf{L}}$$

A.2. Total phenol assay

This assay is used to measure the concentration of phenol substrate in a sample by the colourimetric method. The phenol in a sample reacts with 2.08 mM AAP in the presence of 8.34 mM potassium ferricyanide and generates color. After enough time for full color-generation, 8 to 10 minutes, the absorbance at 510 nm is measured and converted to total phenol concentration in the cuvette using a calibration curve. This assay is a modified method of the direct photometric method, which is a standard analytical procedure for phenols (APHA, 1995). This modified assay enables measurement of higher phenol concentrations than under the standard method, while also using smaller sample volumes.

A.2.1. Preparation of Reagents

a) 0.25 M Sodium bicarbonate

Add 21.0 g of sodium bicarbonate ($NaHCO_3$) into 1 L of deionized water.

b) 83.4 mM Potassium ferricyanide

Mix 13.73 g of potassium ferricyanide ($K_3Fe(CN)_6$) with 500 mL of 0.25 M $NaHCO_3$ and store in refrigerator.

c) 20.8 mM 4-aminoantipyrine

Dissolve 2.115 g of AAP in 500 mL of 0.25 M $NaHCO_3$. Store in refrigerator.

d) 1.0 mM phenol

Place 94.11 mg of phenol in 500 mL of 0.1 M of NaPP buffer (pH7.4). Prepare standard solutions ranging from 0 to 1 mM with this 1 mM stock solution.

A.2.2. Calibration Procedure

Mix these reagents in a semi-microcuvette in the following order:

- 700 µL of 0.25 M sodium bicarbonate
- 100 µL of standard
- 100 µL of 20.8 mM AAP reagent
- 100 µL of 83.4 mM potassium ferricyanide

The assay sample volume must be 1 mL. The phenol concentration in the cuvette should not exceed 0.1 mM. Tip over cuvette several times to mix reagents and place in

the spectrophotometer. When the maximum amount of color is developed (within 8 to 10 minutes), record the absorbance at 510 nm. Continue the measurement for all standards in triplicate. Plot absorbance versus phenol concentration in the cuvette, and calculate the slope of the graph by linear regression.

A.2.3. Assay Procedure

In a semi-micro cuvette place:

- 0 to 700 μL of 0.25 M sodium bicarbonate
- 100 to 800μL of sample
- 100 μL of 20.8 mM AAP
- 100 μL of potassium fericyanide

Wait 8 to 10 minutes for the full-color development and read the absorbance at 510 nm.

A.2.4. Calculations

The concentration of phenol in the cuvette is determined from the calibration curve. Dilute the sample if the concentration exceeds 0.1 mM. The phenol concentration of sample is calculated from:

$$[\text{phenol}]_{\text{sample}} = [\text{phenol}]_{\text{cuvette}}\left(\frac{1000\,\mu L}{\text{sample volume }\mu L}\right)$$

A.3. Hydrogen Peroxide Assay

Hydrogen peroxide concentration is measured by an end-point colourimetric assay which uses 9.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid (HDCBS) and 2.4 mM 4-aminoantipyrine (AAP) as ARP substrates at a pH of 7.4. For hydrogen peroxide to remain the limiting substrate, its concentration in the cuvette should not exceed 50 μM. Once the maximum amount of color has developed, the absorbance (at 510 nm) is converted to the hydrogen peroxide concentration in the cuvette by means of a calibration curve. The hydrogen peroxide concentration in the sample is then calculated according to the dilution the sample had undergone in the cuvette.

A.3.1. Preparation of Reagents

a) pH 7.4 phosphate buffer (0.1 M NaPP)

Mix 19 mL of 0.2 M monobasic sodium phosphate with 81 mL of 0.2 M dibasic sodium phosphate and make 200 mL with deionized water.

b) 3,5-dichloro-2-hydroxybenzenesulfonic acid (HDCBS)

Dissolve 954.18 mg of HDCBS in 200 mL of phosphate buffer (pH 7.4) and store in refrigerator.

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

Add 390 mg of AAP in 200 mL phosphate buffer (pH 7.4). Store in refrigerator.

d) Stock HRP (1 mg/mL)

Mix 10 mg of HRP solid 10 mL of phosphate buffer. Accuracy is not required in making this solution since the only requirement is that the enzyme be in excess so that the endpoint can be reached. The stronger this solution is, the faster the endpoint will be reached.

e) Hydrogen peroxide

Place 113.3 µL of 30% w/v hydrogen peroxide in a 100 mL volumetric flask and add deionized water. From this 10 mM solution make standard solutions with concentrations ranging from 0 to 1.0 mM.

A.3.2. Calibration Procedure

In a semi-micro cuvette place:

- 500 µL of 18 mM HDCBS
- 250 µL of 9.6 mM AAP
- 100 µL of stock HRP
- 50 µL of NaPP buffer
- 100 µL of standard peroxide solution

Invert the cuvette several times and place it in the spectrophotometer. When the maximum amount of colour has developed, record the absorbance at 510nm. Repeat the same procedure for all standards, using triplicate measurements. The assay sample

volume must be 1 mL. The peroxide concentration in the cuvette should not exceed 50 μM. Make the calibration graph by plotting absorbances versus peroxide concentrations in the cuvette.

A.3.3. Assay Procedure

The total volume in the semi-micro cuvette is 1 mL, contains:

- 500 μL of 18 mM HDCBS
- 250 μL of 9.6 mM AAP
- 100 μL of stock HRP
- 0 to 100μL of NaPP buffer
- 50 to 150 μL of sample

Measure the maximum absorbance at 510 nm. Since the peroxide concentration should not exceed 50 μM, use more NaPP buffer and less sample maintaining total volume 1 mL if necessary. Calculate the H_2O_2 concentration in the cuvette from a calibration curve.

A.3.4. Calculations

The sample peroxide concentration can be calculated from:

$$[H_2O_2] = [H_2O_2]_{cuvette}\left(\frac{1000\ \mu L}{\text{sample volume}\ \mu L}\right)$$

APPENDIX B

Batch Reactor Kinetic Data

Tables B.1 to B.4 contain data from experiments in which 400 mg PEG L^{-1} was added.

Table B.1. Batch reactor data for model calibration with 400 mg PEG L^{-1}.

Compound	Test 1		Test 2		Test 3	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	1.0	0	0.587	0	4.11
[ARP]	0	15.5×10^{-6}	0	15.5×10^{-6}	0	44.9×10^{-6}
[phenol]	0	1.377	0	0.440	0	3.564
	1.5	0.930	2	0.292	1	2.099
	3.5	0.758	4	0.207	3	1.258
	6	0.627	6	0.167	6	1.037
	8	0.568	8	0.143	10.67	0.957
	15.75	0.506	15	0.097	16	0.912
	30.33	0.475	30	0.063	30	0.856
	45	0.465	45	0.055	45	0.912
	60	0.516	60	0.046	60	0.906

Table B.2. Batch reactor data for model calibration with 400 mg PEG L^{-1}.

Compound	Test 4		Test 5		Test 6	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	8.73	0	6.30	0	8.31
[ARP]	0	103.9×10^{-6}	0	108.3×10^{-6}	0	138.6×10^{-6}
[phenol]	0	7.694	0	6.24	0	7.977
	1.33	4.435	1	1.662	1	2.264
	3	3.140	3	0.778	3	0.772
	8.5	2.081	6	0.454	6	0.416
	10	2.078	10	0.304	10	0.319
	15	2.088	15	0.268	15	0.256
	30	1.905	30	0.262	30	0.236
	45	1.994	45	0.250	45	0.240
	60	1.945	60	0.264	60	0.239

Table B.3. Batch reactor data for model validation with 400 mg PEG L^{-1}.

Compound	Test 7		Test 8		Test 9	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	3.99	0	1.07	0	0.68
[ARP]	0	$59.7x10^{-6}$	0	$13.3x10^{-6}$	0	$10.5x10^{-6}$
[phenol]	0	3.68	0	1.00	0	0.713
	1	1.783	2	0.572	1	0.488
	3	0.881	3	0.488	3	0.359
	6	0.482	6	0.356	6	0.244
	10	0.361	10	0.293	10	0.195
	15	0.323	15	0.247	15	0.166
	30	0.282	30	0.228	30	0.124
	45	0.282	45	0.235	45	0.113
	60	0.290	60	0.220	60	0.107

Table B.4. Batch reactor data for model validation with 400 mg PEG L^{-1}.

Compound	Test 10		Test 11		Test 12	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	5.048	0	4.85	0	5.33
[ARP]	0	72.5×10^{-6}	0	44.3×10^{-6}	0	72.6×10^{-6}
[phenol]	0	5.085	0	5.313	0	5.196
	1	2.083	2	3.367	1	2.265
	3	0.838	3	2.618	3	1.115
	6	0.443	6	2.248	6	0.817
	10	0.306	10	2.117	10	0.758
	15	0.278	16	1.897	15	0.692
	30.5	0.243	30	1.915	30	0.653
	45	0.204	45	1.864	45	0.640
	60	0.217	60	1.863	60	0.673

Table B.5. Batch reactor data for model validation with 400 mg PEG L^{-1}.

Compound	Test 13	
	Time (min)	Concentration (mM)
$[H_2O_2]$	0	6.43
[ARP]	0	104.7×10^{-6}
[phenol]	0	5.86
	1	2.849
	3	1.405
	5	1.124
	8	0.951
	10	0.920
	15.33	0.941
	30	0.838
	45	0.759
	60	0.699

Table B.6 to B.9 contain data from experiments in which no PEG was added.

Table B.6. Batch reactor data for model calibration without PEG (25 °C and pH 7.0).

Compound	Test 1		Test 2		Test 3	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	0.98	0	3.088	0	4.56
[ARP]	0	$92.1x10^{-6}$	0	$295.0x10^{-6}$	0	$521.1x10^{-6}$
[phenol]	0	0.924	0	3.021	0	5.181
	1	0.658	1	1.944	0.95	3.444
	2	0.623	2	1.927	2.1	3.456
	3	0.623	3	1.909	3.25	3.245
	5	0.632	5	1.811	5	3.156
	8	0.626	8	1.825	8	3.252
	15	0.618	15	1.765	15	3.258
	30	0.615	30	1.773	30	3.336

Table B.7. Batch reactor data for model calibration without PEG (25 °C and pH 7.0).

Compound	Test 4		Test 5		Test 6	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	1.859	0	0.523	0	5.201
[ARP]	0	228.5×10^{-6}	0	29.9×10^{-6}	0	220.1×10^{-6}
[phenol]	0	2.099	0	0.511	0	4.979
	1	1.426	1	0.379	1	4.247
	2	1.323	2	0.370	2	4.139
	3	1.299	3	0.346	3	4.212
	5	1.325	5	0.346	5	4.162
	8	1.293	8	0.342	8	4.017
	15	1.280	15	0.326	15	4.002
	30	1.211	30	0.334	30	3.949

Table B.8. Batch reactor data for model validation without PEG (25 °C and pH 7.0).

Compound	Test 7		Test 8		Test 9	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	0.62	0	4.066	0	2.06
[ARP]	0	$25.1x10^{-6}$	0	$590.0x10^{-6}$	0	$135.6x10^{-6}$
[phenol]	0	0.495	0	4.032	0	2.027
	1	0.389	1	1.931	1	1.473
	3	0.361	2	1.935	2	1.426
	5.25	0.363	3	1.798	3	1.429
	8	0.345	5	1.783	5	1.428
	10	0.352	8	1.827	8	1.431
	15	0.341	15	1.725	15	1.362
	30	0.359	30	1.752	30	1.363

Table B.9. Batch reactor data for model validation without PEG (25 °C and pH 7.0).

Compound	Test 10		Test 11		Test 12	
	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)	Time (min)	Concentration (mM)
$[H_2O_2]$	0	2.73	0	3.149	0	0.985
[ARP]	0	218.2×10^{-6}	0	352.3×10^{-6}	0	158.5×10^{-6}
[phenol]	0	2.853	0	3.100	0	1.023
	1	1.796	1	1.946	1	0.544
	2	1.858	2	1.944	2	0.538
	3	1.843	3	1.900	3	0.518
	5	1.991	5	1.900	5	0.477
	8	1.837	9	1.808	8	0.473
	15.5	1.907	15	1.748	15	0.468
	30	1.796	30	1.759	30	0.471

Appendix C

Kinetic Parameter Estimation Subroutines and Configuration File

This code was linked to the SUGAL genetic algorithm software package in order to evaluate the objective function to be minimized.

```
/*
 File: EaCal.C
*/
#include <stdio.h>
#include "sugal.h"
#include <stdlib.h>
#include <math.h>
FILE *dataout;

int Dset, run, last[15];
float Eo[15], Ho[15],S[15][10], t[15][10];
const double dt = 1.0;
const float k1 = 2.33e6;
//const float keff = 3.58e-5;
//const float kb = 4.42;
//const float ka = 1.48e5;
//const float kr = 0.0;
float  ke, kr, keff, kb, ka;
float E0, H0, S0, tt, res, low;
float X[3], Xdot[3];
int Error;

/* Function prototypes */
int bitwise_crossover(int length);
int SuGetCrossoverPoint(int length);
int evaluate(SuChromosome *chrom, double *fitness);

void FitEval();
void rungekutta();
void vector();

SuActionPair my_crossover_actions[] =
{
 "bitwise", bitwise_crossover,
 "normal", SuGetCrossoverPoint,
 NULL, NULL
};

SuAction my_crossover_action =
{
 1, &SuaGetCrossoverPoint, my_crossover_actions
};

SuParameter my_parameters[] =
{
 "crossover_granularity", &my_crossover_action, NULL, SU_ACTION,
```

```
 /* NULL-terminate the list; otherwise, program crash
  guaranteed! */
 NULL, NULL, NULL, NULL
};
/* bitwise crossover routine */
int bitwise_crossover(int length)
{
 return ( SuRandIntUpTo(length) );
}
        /*****************************************************
                                The Main Function
        *****************************************************/
main(int argc, char *argv[])

{
float minute, phen, ARP, H2O2;
int Cset, i;
        /*************************************************************
          Reads Experimantal Measurements from a Data File
        *************************************************************/
FILE *datain;
        run = 0;
        low = 10000.0;
        Dset = -1;
        datain = fopen("PEG-c2.CPP", "r");
        fscanf(datain, "%f", &minute);
         while (minute >= 0.0)
        {
                if (minute == 0)
                {
                        i = 0;
                        Dset = Dset + 1;
                        last[Dset] = 0;
                        fscanf(datain, "%f %f %f", &H2O2, &ARP, &phen);
                        Ho[Dset] = H2O2/1000.0;
                        Eo[Dset] = ARP*73.7e-9;
                }
                else
                {
                fscanf(datain, "%f", &phen);
                }
                last[Dset] = i;
                S[Dset][i] = phen/1000.0;
                t[Dset][i] = minute*60.0;
                i = i + 1;
        fscanf(datain, "%f", &minute);
        }
        fclose(datain);
        dataout = fopen("kr1.out", "w");
        /*************************************
                Call to Sugal
        *************************************/
        SuRegisterParameters (my_parameters);
        SuaEvaluationFunction = evaluate;
        SuRun( "Eacal.cfg" , argc, argv );
        fclose(dataout);
 return (0);
}
/*************************************************************
          Evaluates Differential Functions
```

```
**********************************************************/
void vector()


        /*****************************************
                X[0] = Inactive Enzyme
                X[1] = Substrate
                X[2] = Compound III
        *****************************************/
{
double rs, H, E, Ea;
        H  = H0 - (S0 - X[1]);
        E = (E0 - X[0] - X[2])/(k1*H/(2*ka*X[1]) + 1);
        Ea = k1*H*E/(2*ka*X[1]);
        rs = k1*E*H;
         if (rs < 0.0)
          {
           Error = 1;
           rs = 0.0;
          }
        Xdot[0] = ke*rs + kr*(E0-X[0])*sqrt(rs);
        Xdot[1] = -rs;
        Xdot[2] = kb*H*Ea - keff*X[2];
}
        /*********************************************************
                The Runge Kutta Procedure
        *********************************************************/
void rungekutta()
{
int L;
double static ts, SavX[3], K[3][4];
double sec;
        Error = 0;
        ts = tt;
        for (L=0; L<=2; L++)
        {
                SavX[L] = X[L];
        }
        vector();
        for (L=0; L<=2; L++)
        {
                K[L][1] = Xdot[L]*dt;
                X[L] = SavX[L] + K[L][1]/2;
        }
        tt = ts + dt/2;
        vector();
        for (L=0; L<=2; L++)
        {
                K[L][2] = Xdot[L]*dt;
                X[L] = SavX[L] + K[L][2]/2;
        }
        vector();
        for (L=0; L<3; L++)
        {
                K[L][3] = Xdot[L]*dt;
                X[L] = SavX[L] + K[L][3]/2;
        }
        tt = ts + dt;
        vector();
```

```
            for (L=0; L<=2; L++)
            {
                        K[L][4] = Xdot[L]*dt;
                        X[L] = SavX[L] + (K[L][1] + 2.0*(K[L][2] + K[L][3]) + K[L][4])/6.0;
            }

}
/*******************************************************
              The Fitness Evaluation Procedure
*******************************************************/
void FitEval()
{
int Cset, i;
  res = 0.0;
            for (Cset = 0; Cset < (Dset+1); Cset++)     // for each data set
            {
             i = 0;
             tt = t[Cset][0];
             X[0] = 0.0;       // inactive enzyme
             X[1] = S[Cset][0];  // phenol
             X[2] = 0.0;       // compound III
             S0 = X[1];
             H0 = Ho[Cset];
             E0 = Eo[Cset];
              do
              {
              if (abs(t[Cset][i] - tt) < dt)
               {
                res = res + 1.0e6*(S[Cset][i] - X[1])*(S[Cset][i] - X[1]);
                i = i + 1;
               }
              rungekutta();
              if (Error != 0)
                        {
                                  res = 10000.0;
                                  tt = t[Cset][last[Cset]] + 20;
                                  Cset = Dset+1;
                         }
             } while (tt <= t[Cset][last[Cset]]);
            }
  }
int evaluate(SuChromosome *chrom, double *fitness)
{

double a[5];
int i;
   for (i=0; i < SuGenesInChromosome(chrom); ++i)
             a[i] = SuGetGeneAsInt (chrom->string, i);
            ke  = 0.0 + a[0]/255.0 * 1.0e-8;
            keff = 4.5e-5 + a[1]/255.0 * 1.0e-5;
            kb = 3.8  + a[2]/255.0 * 0.6;
            ka  = 1.1e5  + a[3]/255.0 * 0.4e5;
            kr  = 5.0e-2 + a[4]/255.0 * 5.0e-2;
  FitEval();
            if (res < low)
            {
             low = res;
             fprintf(dataout,"\n \n ka = %e  res = %e",ka,res);
             fprintf(dataout,"\n keff = %e  kb = %f",keff,kb);
             fprintf(dataout,"\n ke = %e  kr = %e  k1 = %e",ke,kr,k1);
```

```
        }
  *fitness = res;
return 0;
}
```

The following is the configuration file which accompanies the above subroutines used in parameter estimation.

```
# ------------------------------------------
# File: NewCal.cfg
#
# Lines beginning with a hash '#' characters are comments
# ------------------------------------------

# In this particular program, you change the datatype to
# ANY of the numeric types without significant ill-effects.

datatype unsigned_integer

# Set initialisation range for unsigned_integer
init_minimum 0
init_maximum 255

generations    20
population     80
length 5

autoseed on
bias 0.01
normalisation invert

replacement ranked
replacement_condition if_improved

elitism off

mutation gaussian
mutation_rate 1.0
mutation_rate_type per_gene
mutation_size 3.0
mutation_decay_rate 0.99

#screen_output on
#file_output off
#report_every_pool on
#report_interval 1

report_final_pool on
file_output on
output_file Kr1.sug
```

The SUGAL software package was written by Dr. Andrew Hunter at the University of Sunderland, England.
It is available at no cost at http://www.trajan-software.demon.co.uk/sugal.htm
Email : andrew@trajan-software.demon.co.uk

Appendix D

Sensitivity Test

A sensitivity test was performed to determine the effect of changes in kinetic model parameters on model output. The initial conditions were fixed at 6 mM phenol, 6 mM hydrogen peroxide, 36.9 nM ARP, and 400 mg PEG L^{-1}. The base values of the constants are:

$k_1 = 2.33 \times 10^6$ ($M^{-1}s^{-1}$)

$k_a = 1.49 \times 10^5$ ($M^{-1}s^{-1}$)

$k_b = 4.43$ ($M^{-1}s^{-1}$)

$k_{eff} = 3.72 \times 10^{-5}$ (s^{-1})

$k_c = 0.0$

$k_r = 5.38 \times 10^6$ ($M^{-0.5}s^{-0.5}$)

Figure D.1 demonstrates that the model is relatively insensitive to change in k_1 because the rate constant k_1 is not the rate limiting constant in the catalytic cycle. Thus, a moderate amount of error in the estimation of k_1 would not affect model performance.

Figures D.2 and D.3 show the model to be very sensitive to changes in the values k_b and k_a. The k_b describes the rate of compound III formation. Decreasing k_b increases the rate of phenol removal. Figure D.3 shows increasing k_a greatly increases the phenol removal rate, because k_a contains the rate limiting step.

Figure D.4 indicates that the model is sensitive to an increase in the value of k_{eff}. However, decreasing the value of k_{eff} has minimal effect. This is because k_{eff} relates to the

speed at which compound III reverts to the native form of ARP, and this rate has already been shown to be very slow (see section 4.6).

Changing k_r showed relatively small changes in phenol removal rate, and changing k_e was not tested since its estimated value was essentially zero for reaction in the presence of PEG.

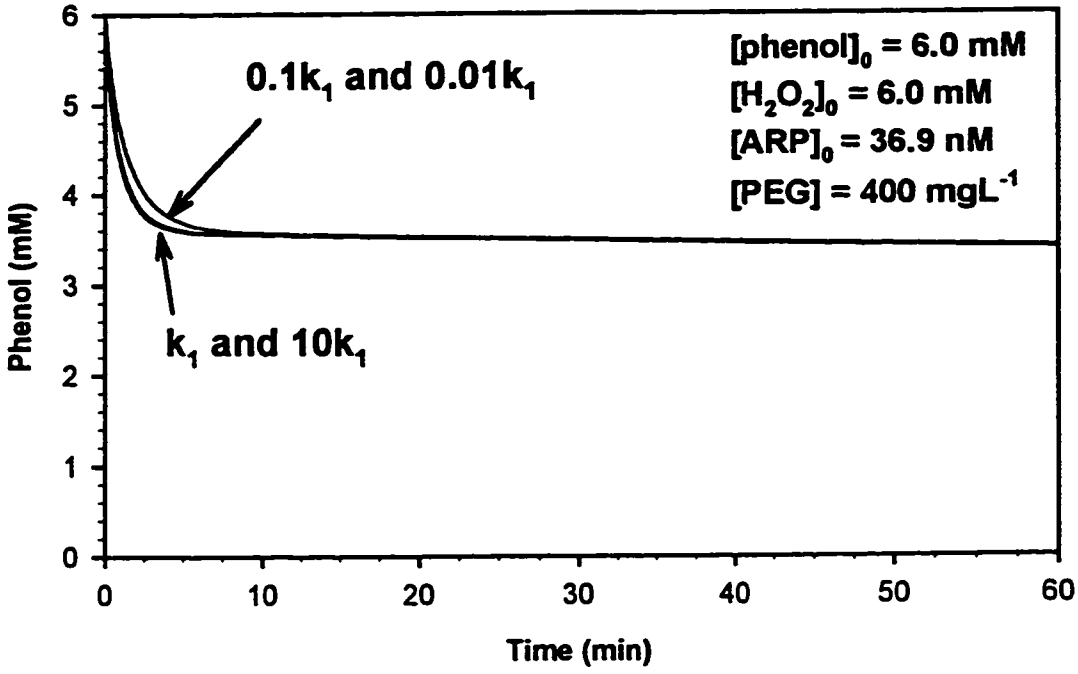


Figure D.1. Effect of changes k_1 on model output.

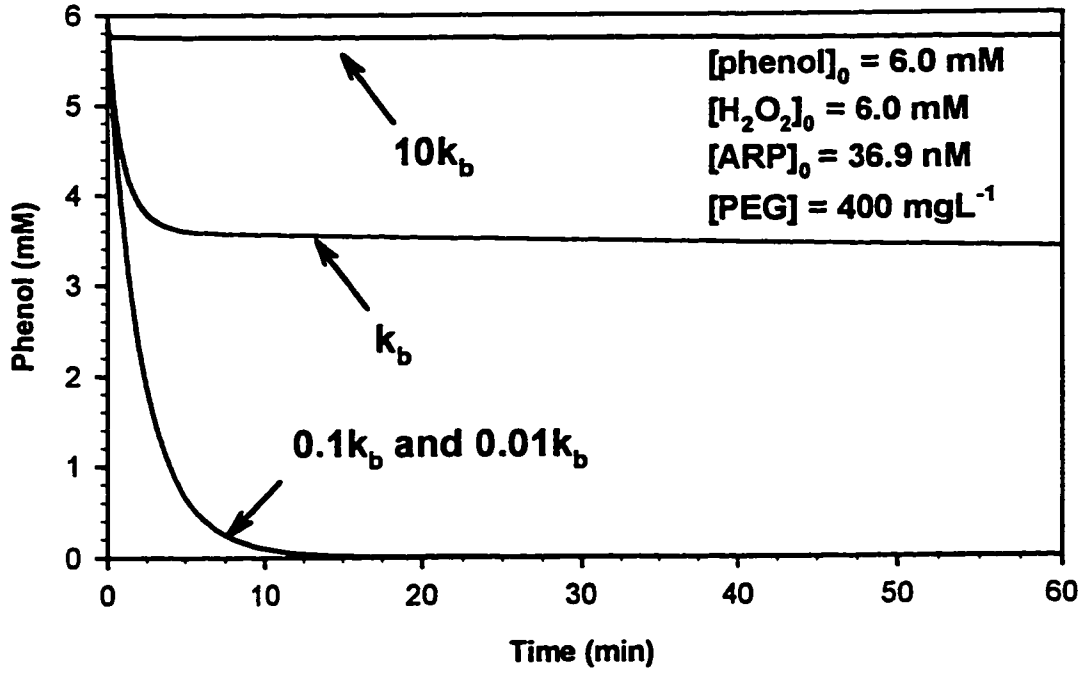
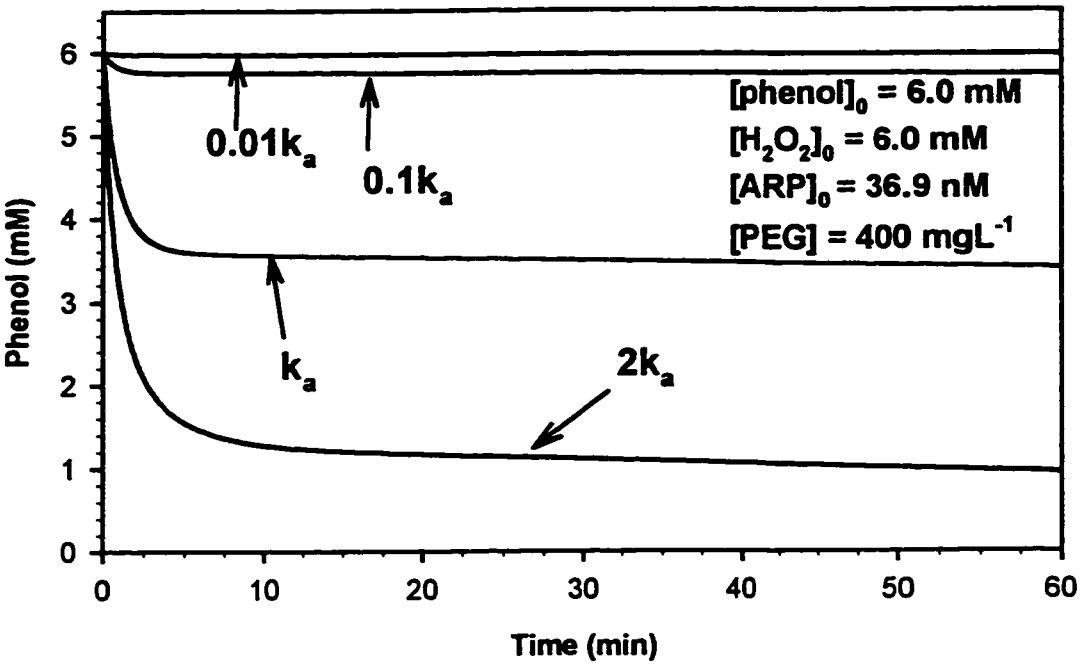


Figure D. 2. Effect of changes k_b on model output.



FigureD.3. Effect of changes k_a on model output.

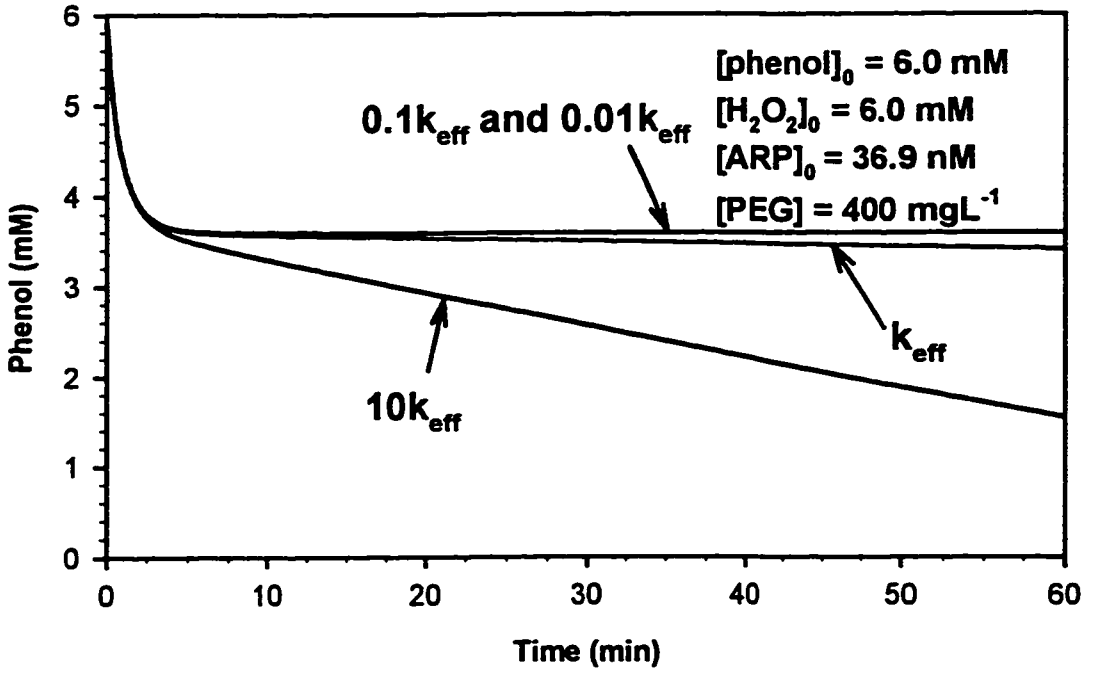


Figure D. 4. Effect of changes k_{eff} on model output.

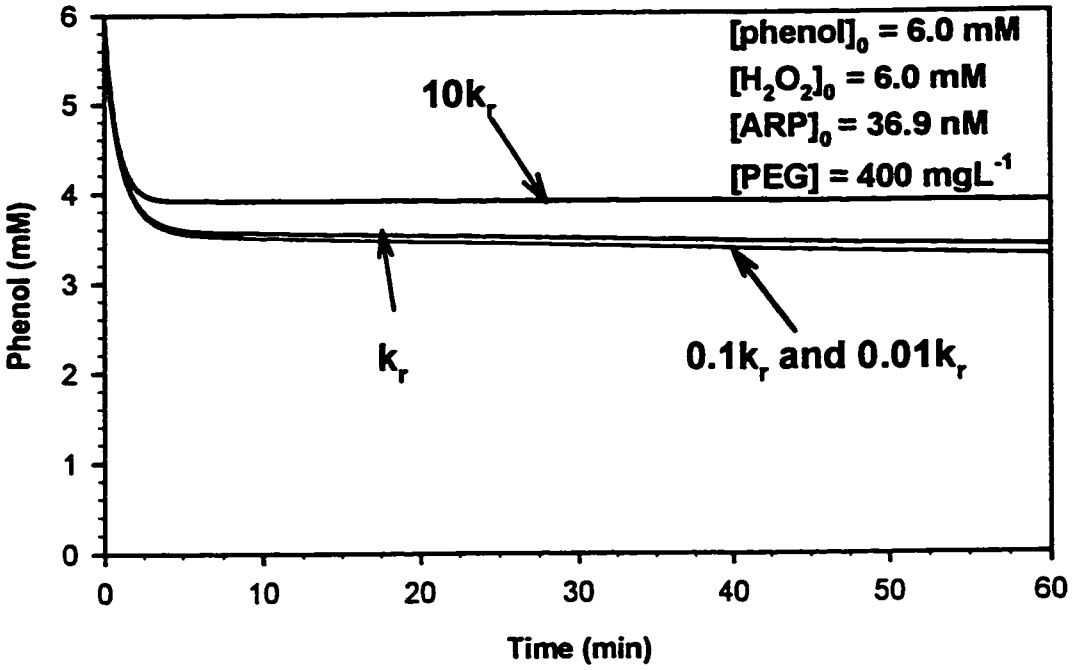


Figure D.5. Effect of changes k_r on model output.

APPENDIX E

Predicted Distribution of Enzyme Forms During Batch Test

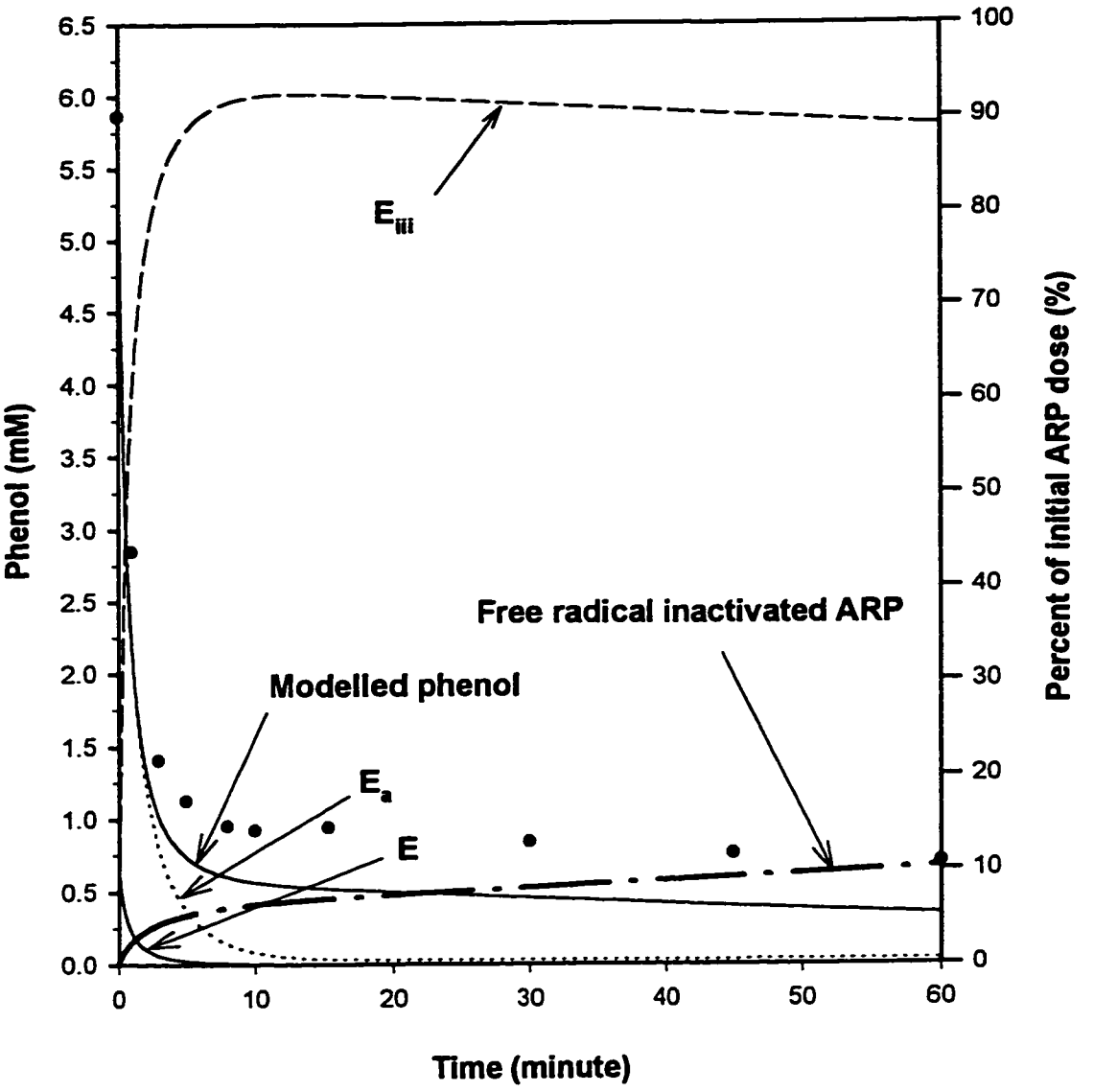


Figure E.1. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 5.86 mM, $[H_2O_2]_0$ = 6.43 mM, and $[ARP]_0$ = 105 nM.

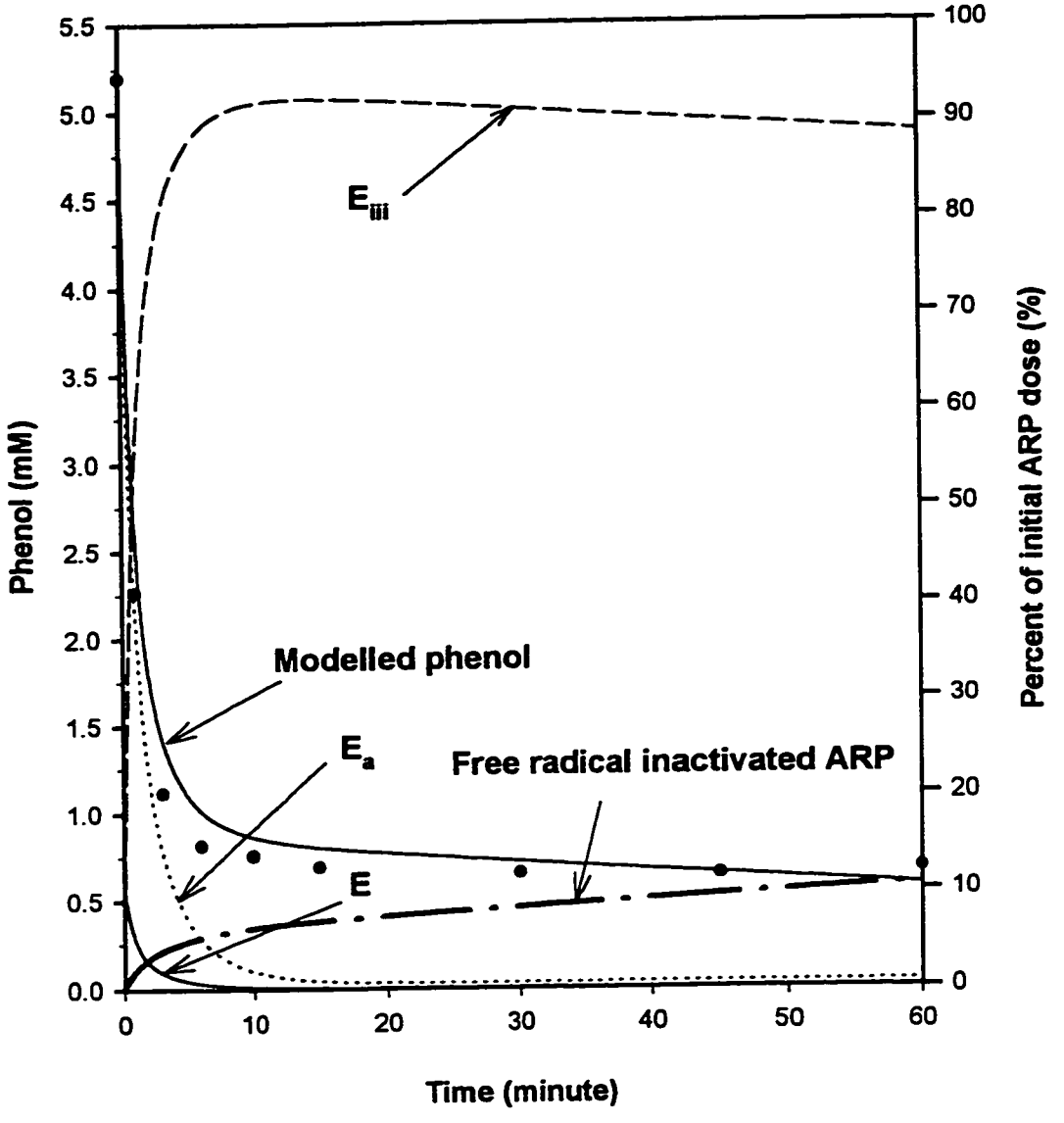


Figure E.2. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 5.20 mM, $[H_2O_2]_0$ = 5.33 mM, and $[ARP]_0$ = 73 nM.

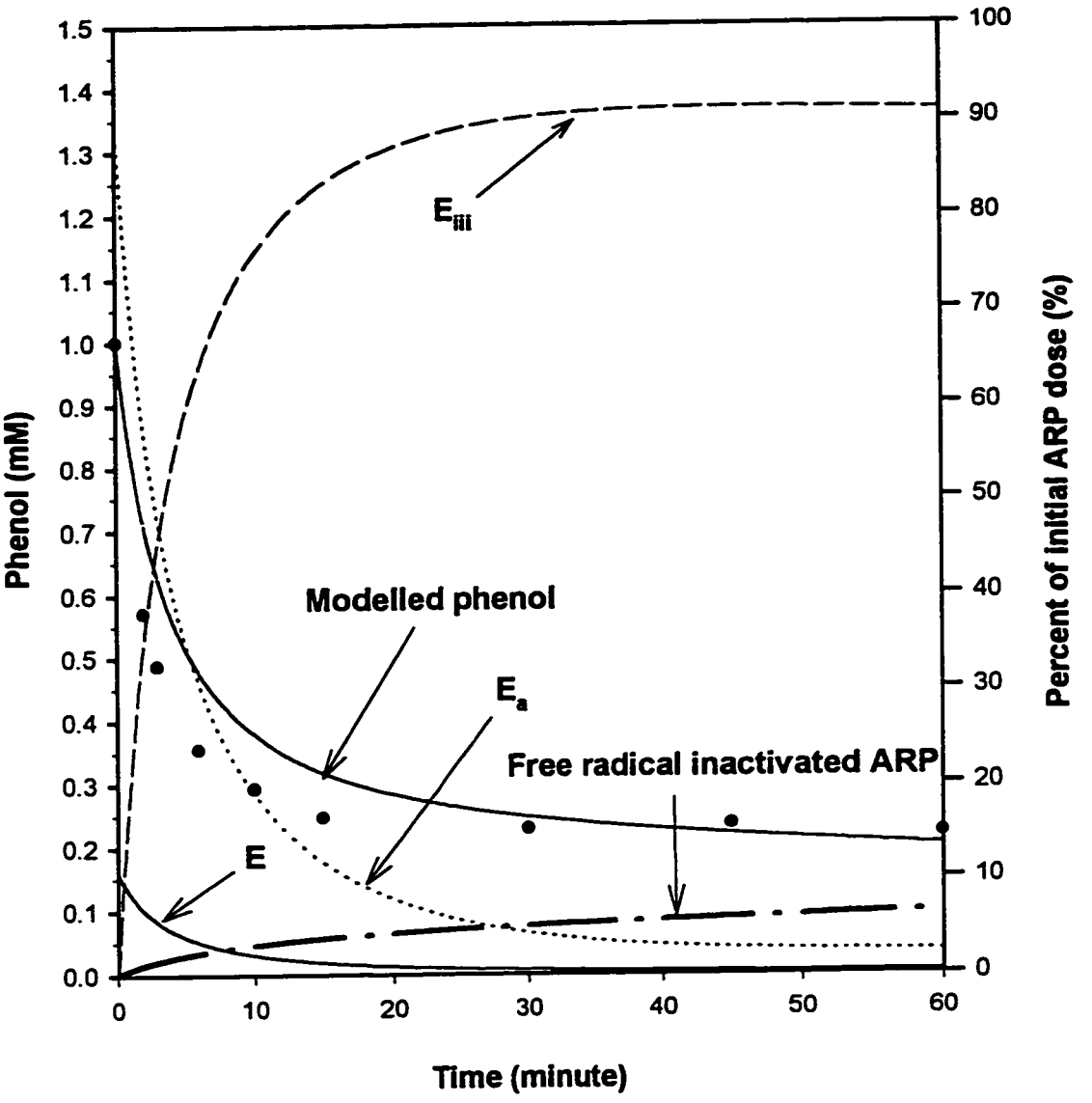


Figure E.3. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 1.00 mM, $[H_2O_2]_0$ = 1.07 mM, and $[ARP]_0$ = 13 nM.

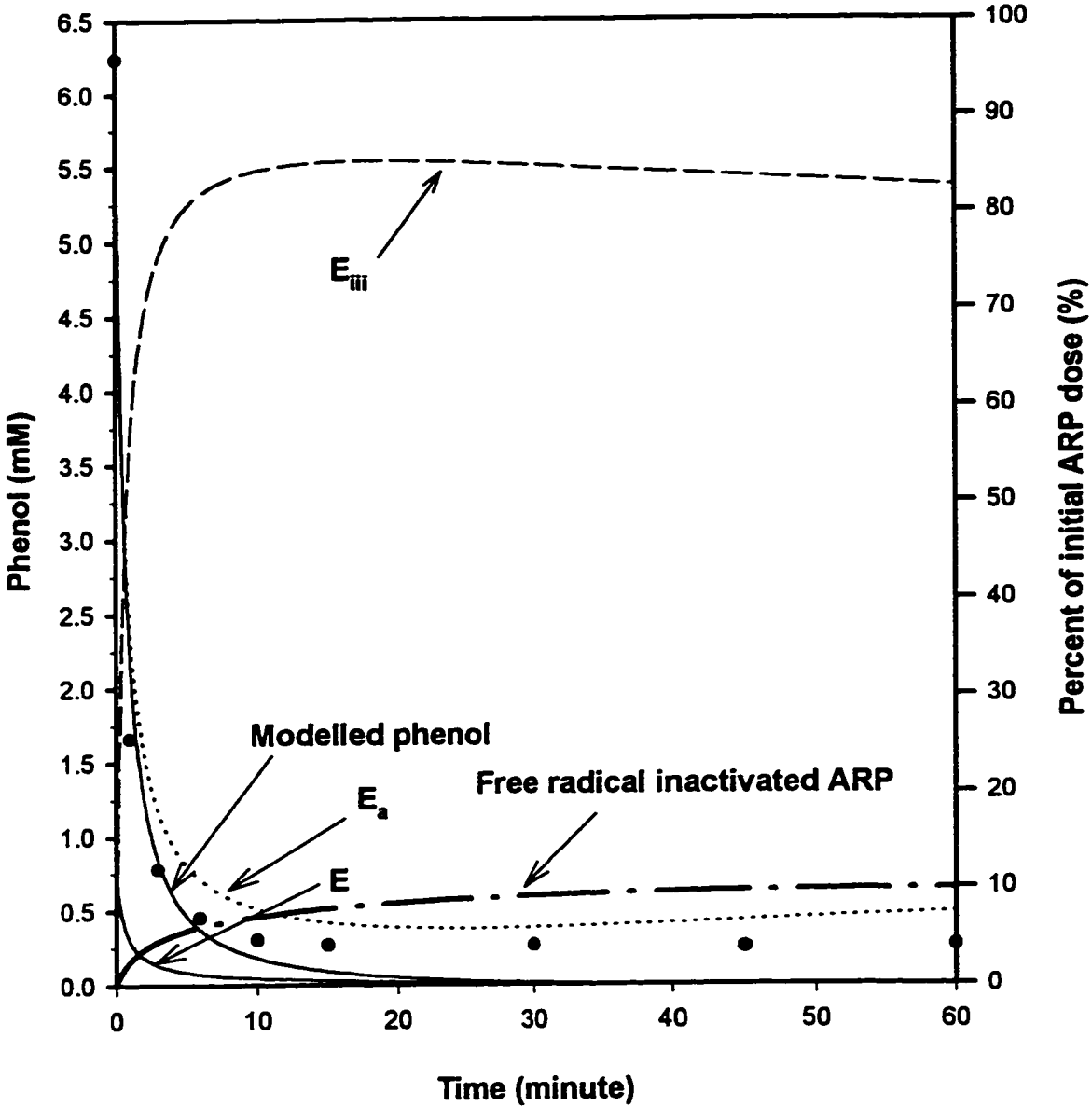


Figure E.4. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 6.24 mM, $[H_2O_2]_0$ = 6.30 mM and $[ARP]_0$ = 108 nM.

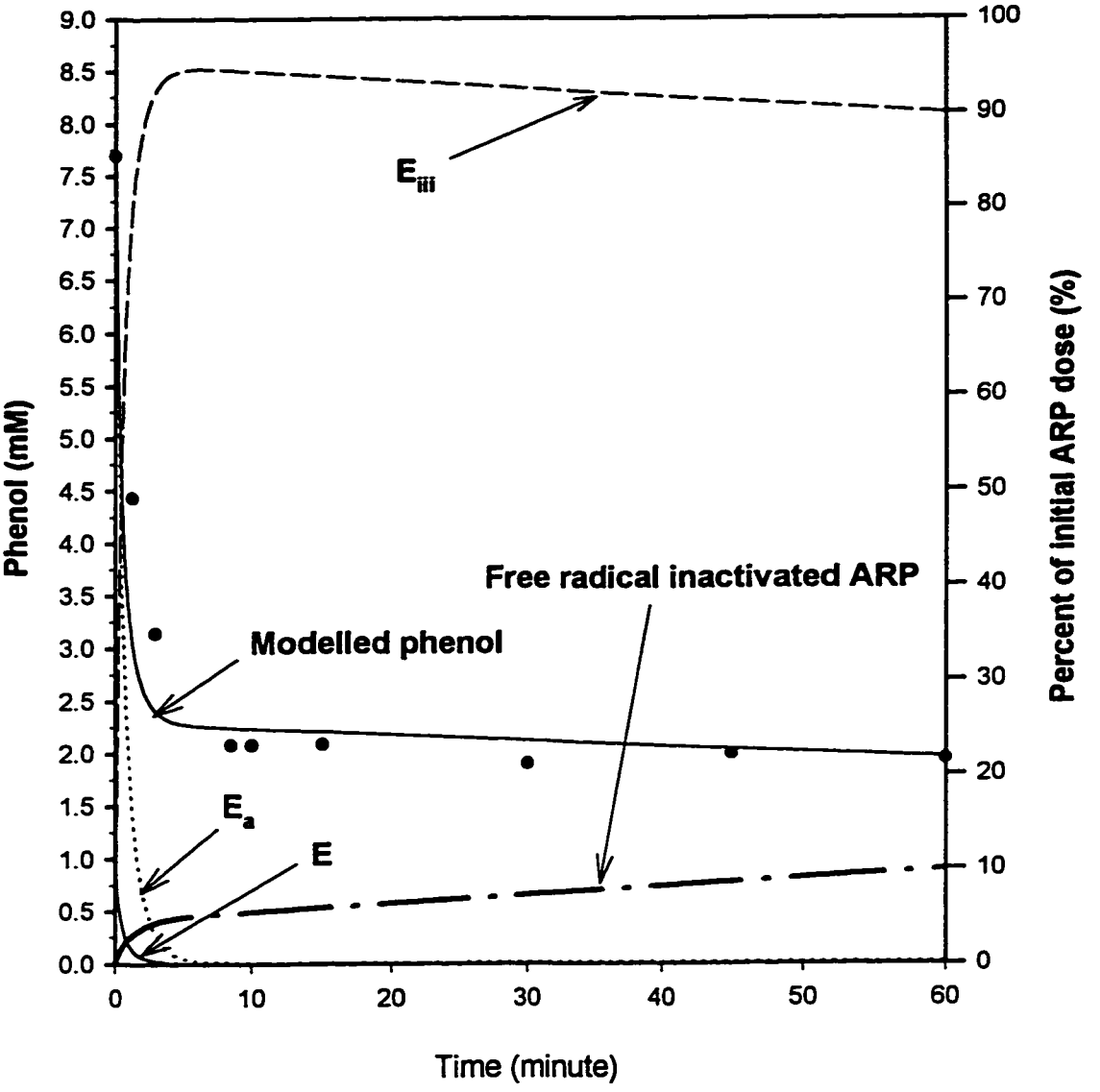


Figure E.5. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 7.69 mM, $[H_2O_2]_0$ = 8.73 mM and $[ARP]_0$ =103.9 nM.

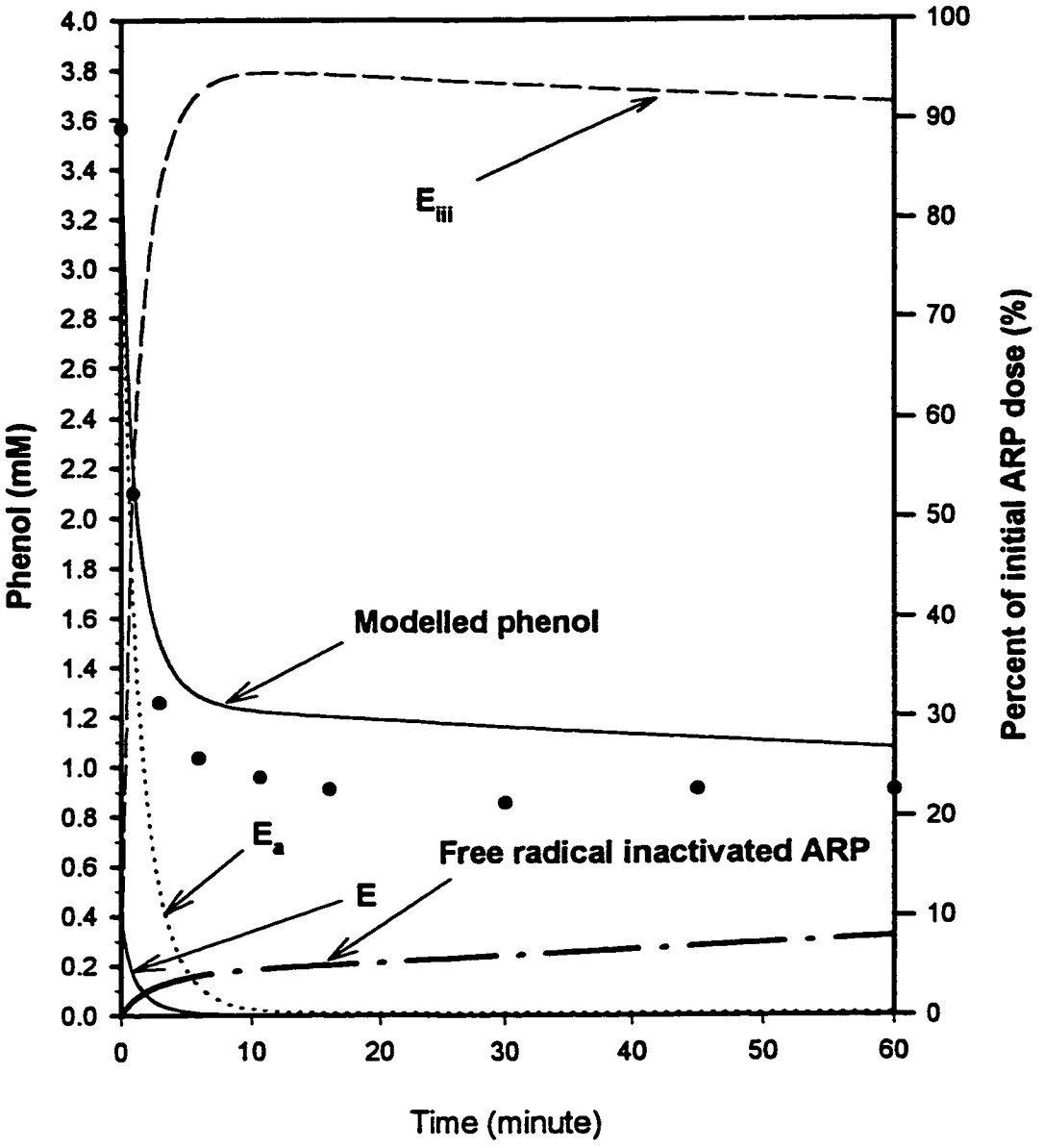


Figure E.6. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 3.56 mM, $[H_2O_2]_0$ = 4.11 mM and $[ARP]_0$ = 45 nM.

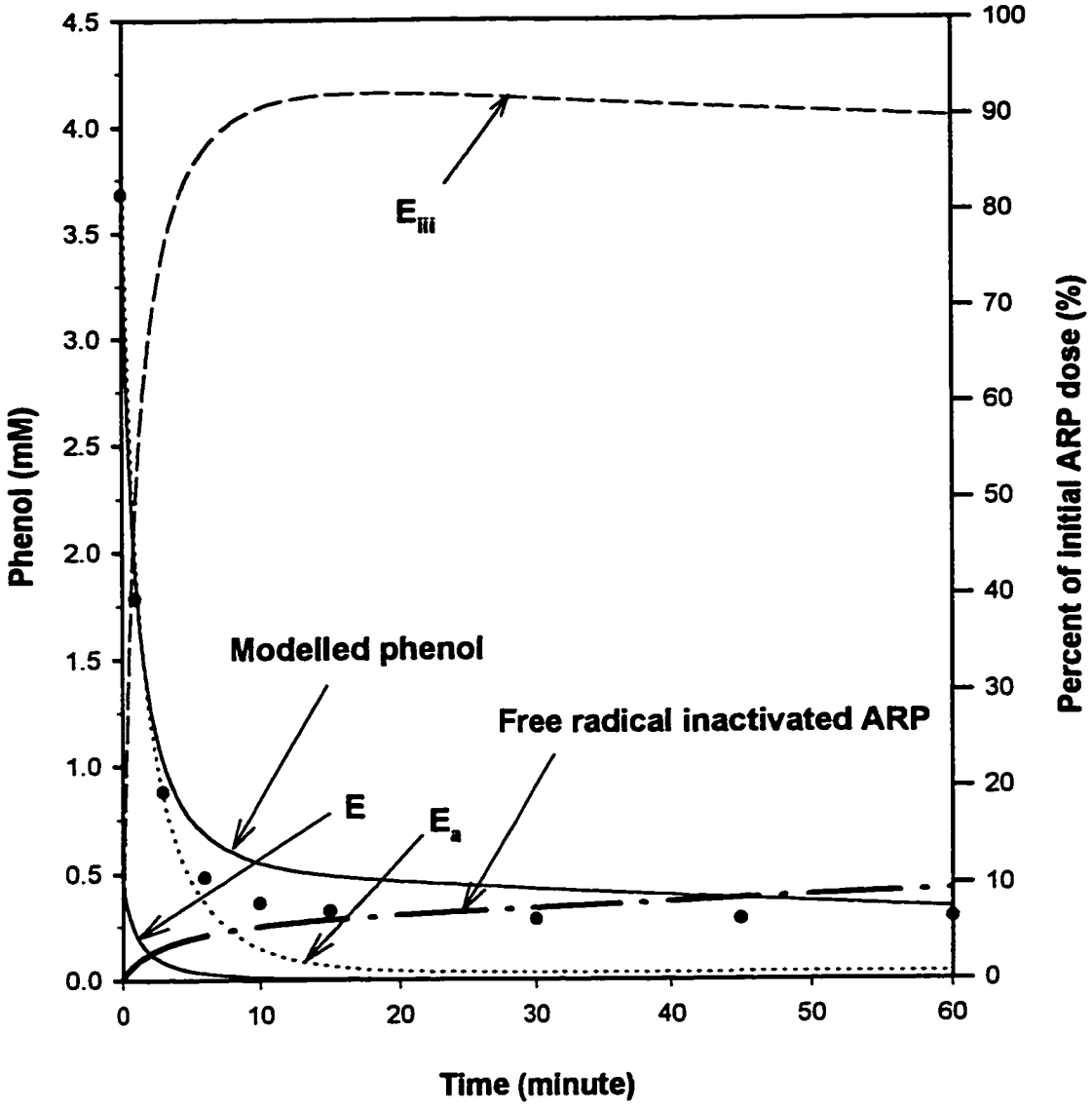


Figure E.7. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 3.68 mM, $[H_2O_2]_0$ = 3.99 mM and $[ARP]_0$ = 59.7 nM.

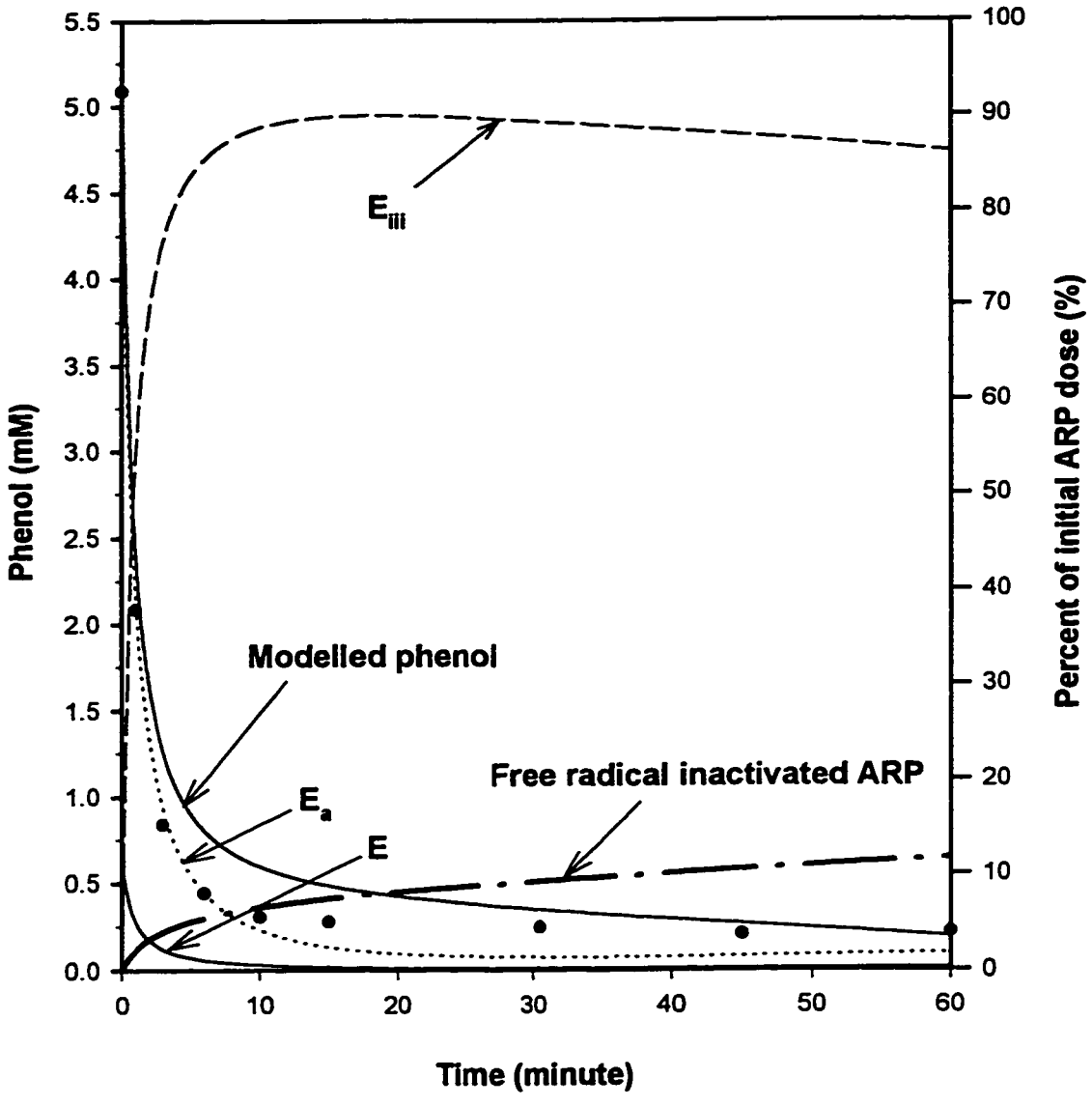


Figure E.8. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 5.09 mM, $[H_2O_2]_0$ = 5.05 mM, and $[ARP]_0$ = 73 nM.

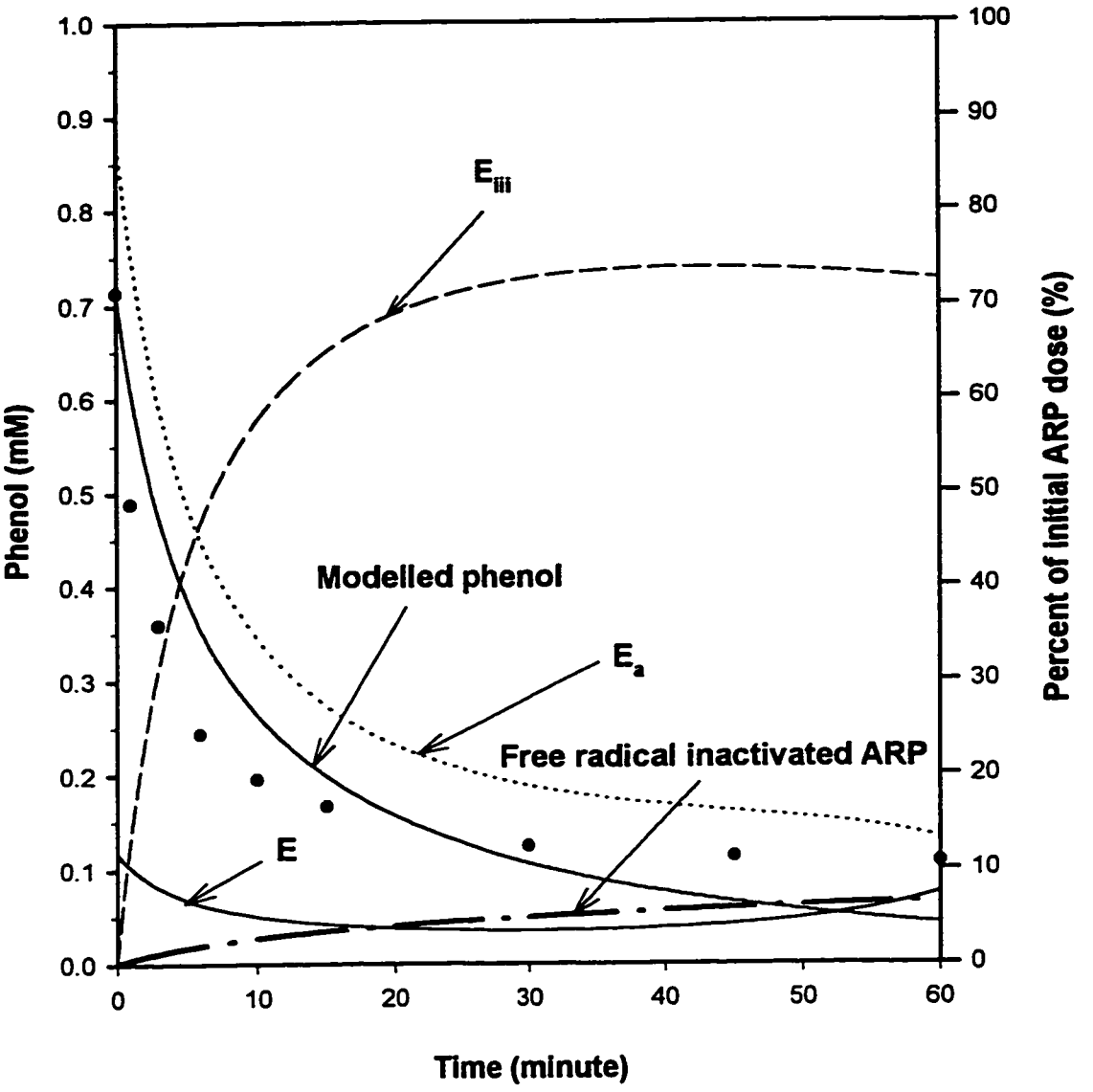


Figure E.9. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 0.71 mM, $[H_2O_2]_0$ = 0.68 mM, and $[ARP]_0$ = 10.5 nM.

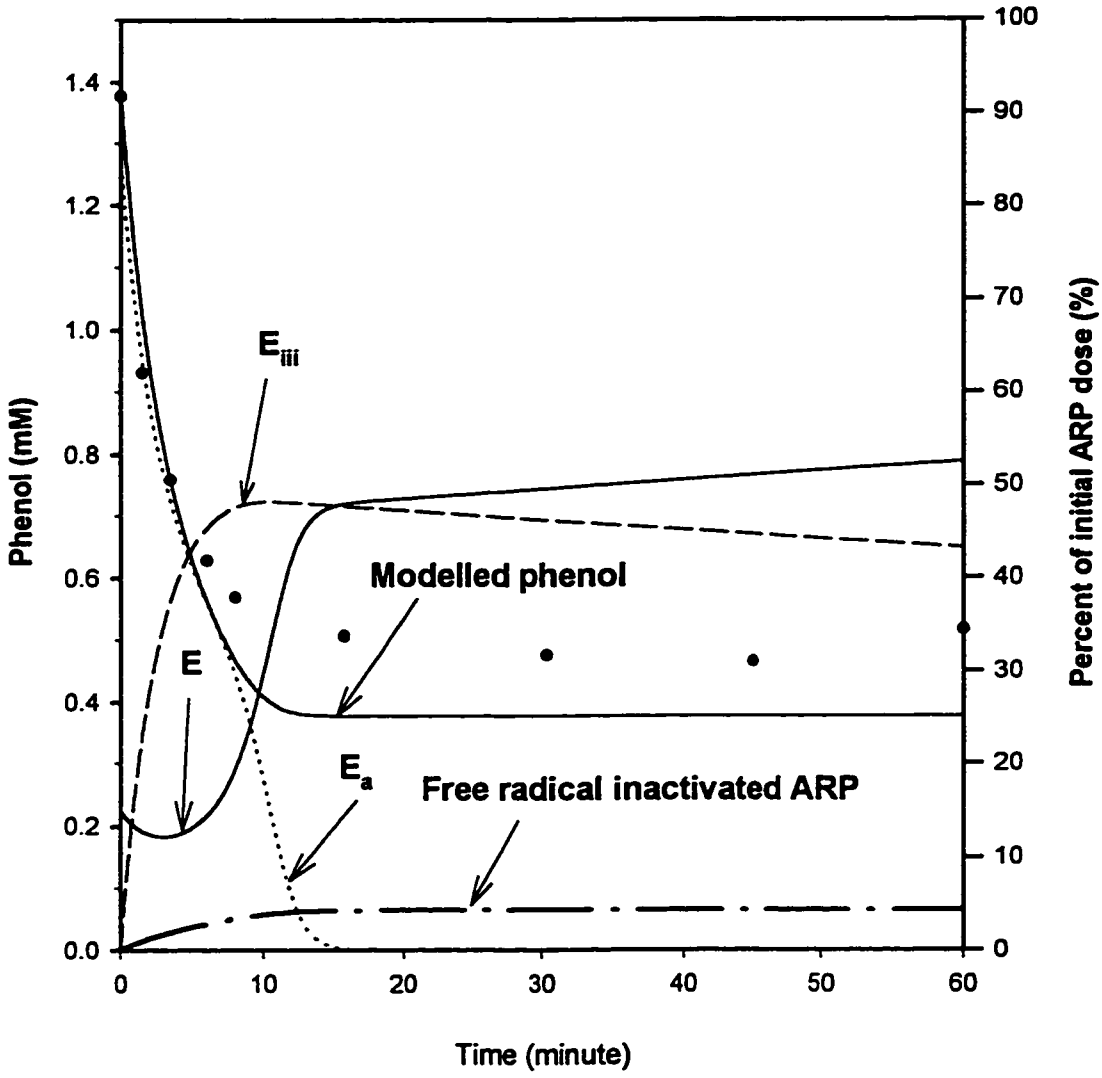


Figure E.10. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 1.38 mM, $[H_2O_2]_0$ = 1.00 mM and $[ARP]_0$ = 15.5 nM.

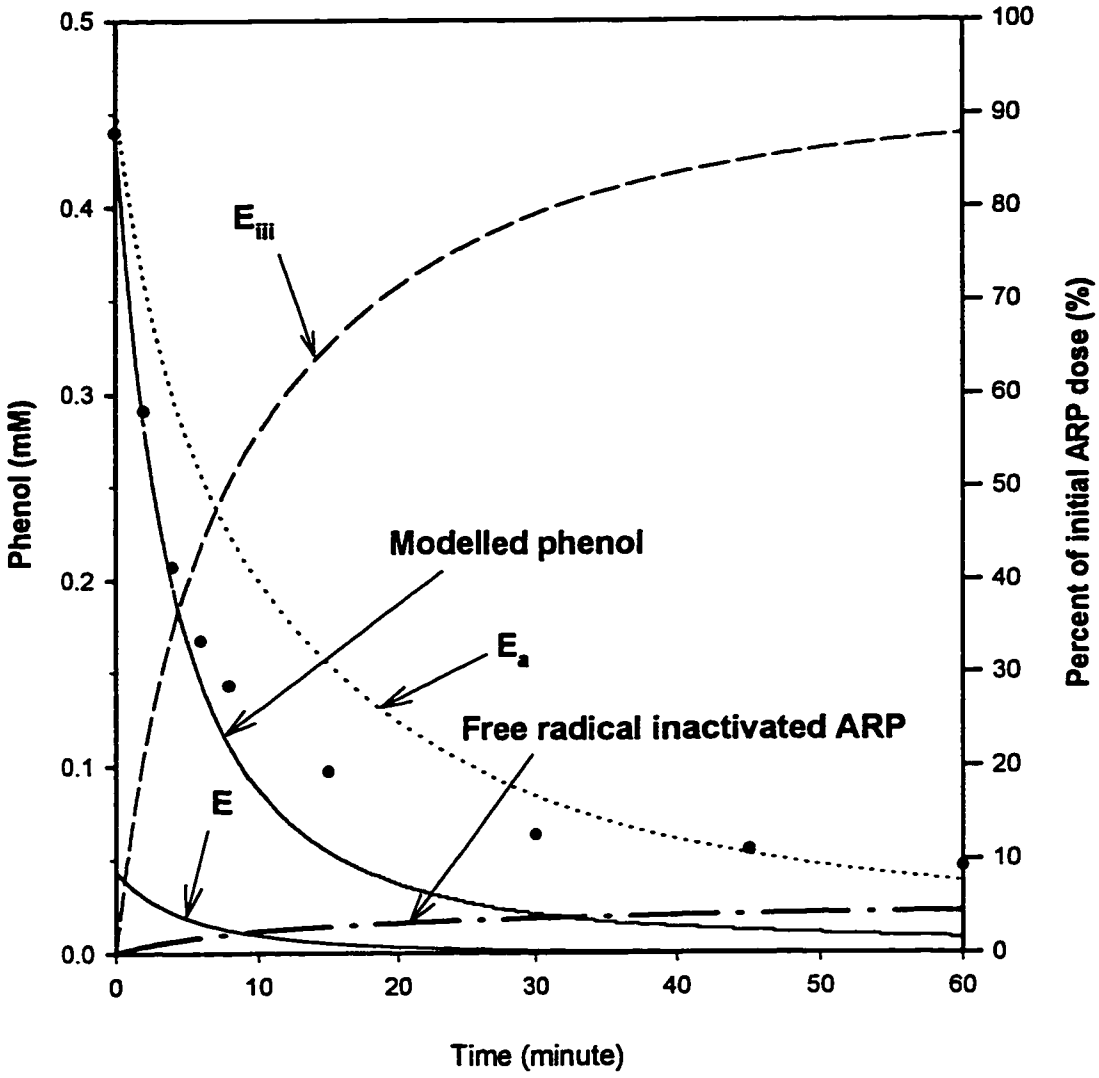


Figure E.11. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 0.44 mM, $[H_2O_2]_0$ = 0.59 mM and $[ARP]_0$ = 15.5 nM.

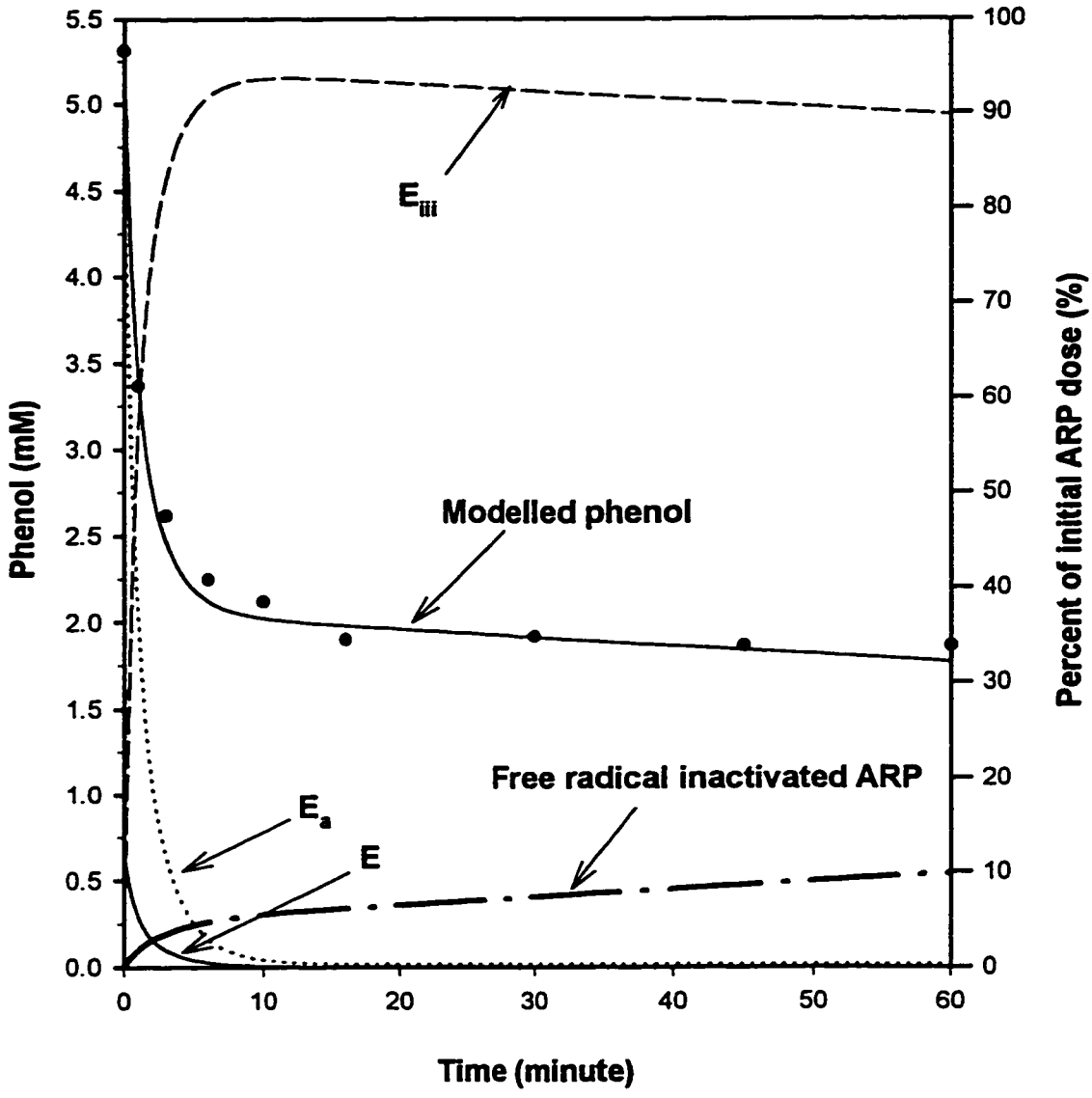


Figure E.12. Measured phenol concentrations and model output using Group B parameters with [PEG]= 400 mgL^{-1}, [phenol] = 5.31 mM, $[H_2O_2]_0$ = 4.85 mM, and $[ARP]_0$ = 44.3 nM.

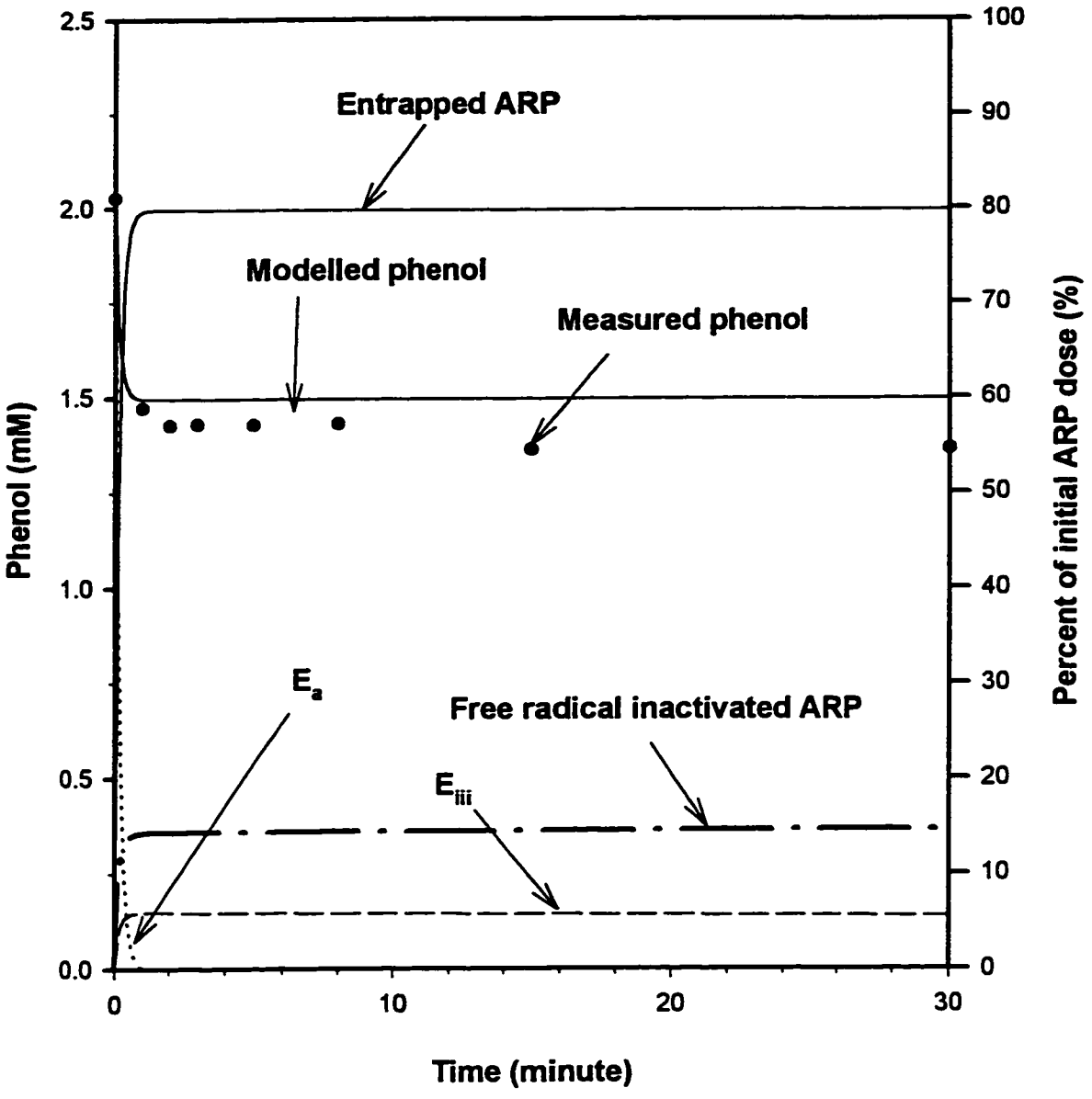


Figure E.13 . Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] =2 .03 mM, $[H_2O_2]_0$ = 2.06 mM and $[ARP]_0$ = 136 nM.

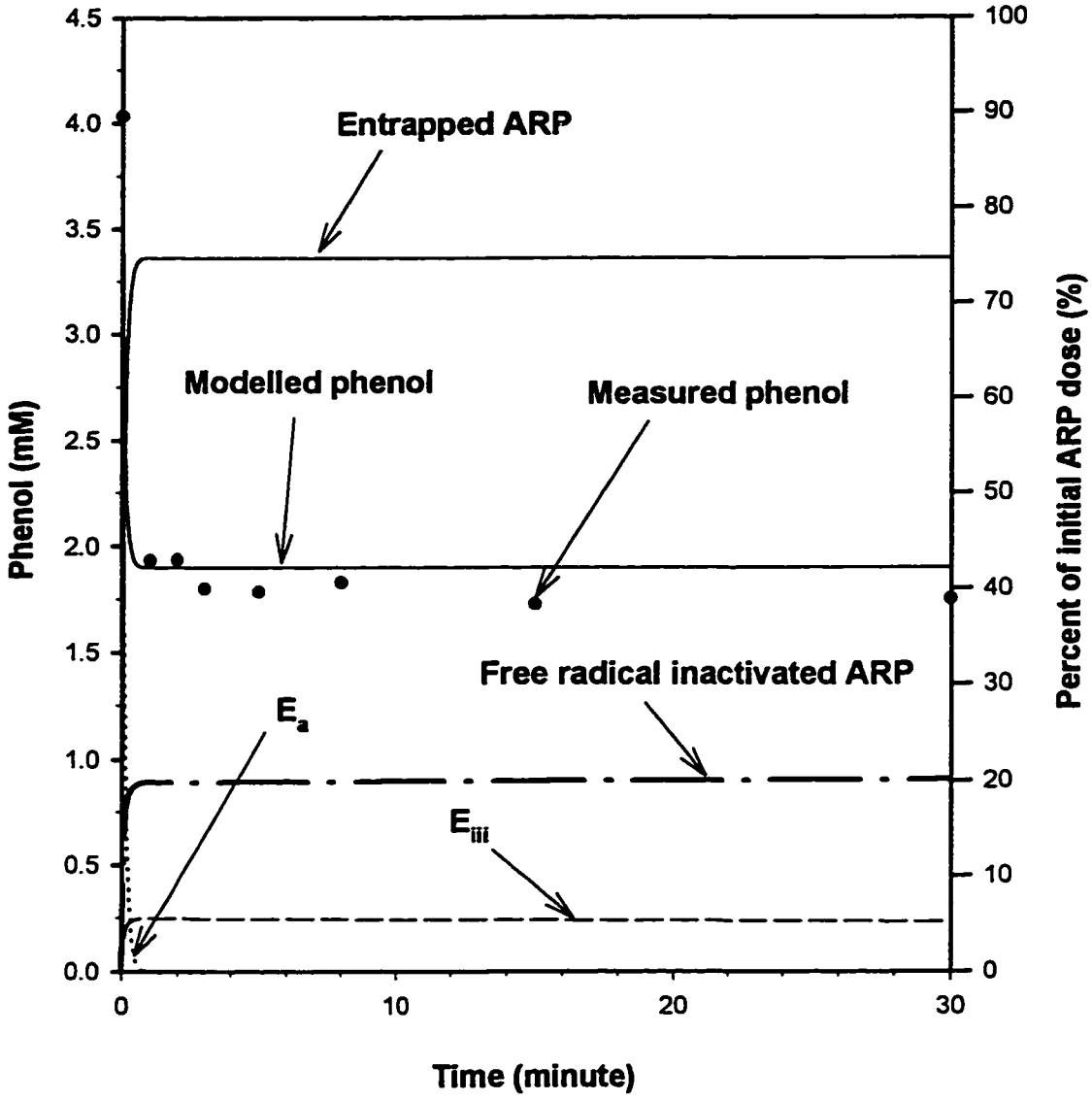


Figure E.14. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 4.03 mM, $[H_2O_2]_0$ = 4.07 mM and $[ARP]_0$ = 590 nM.

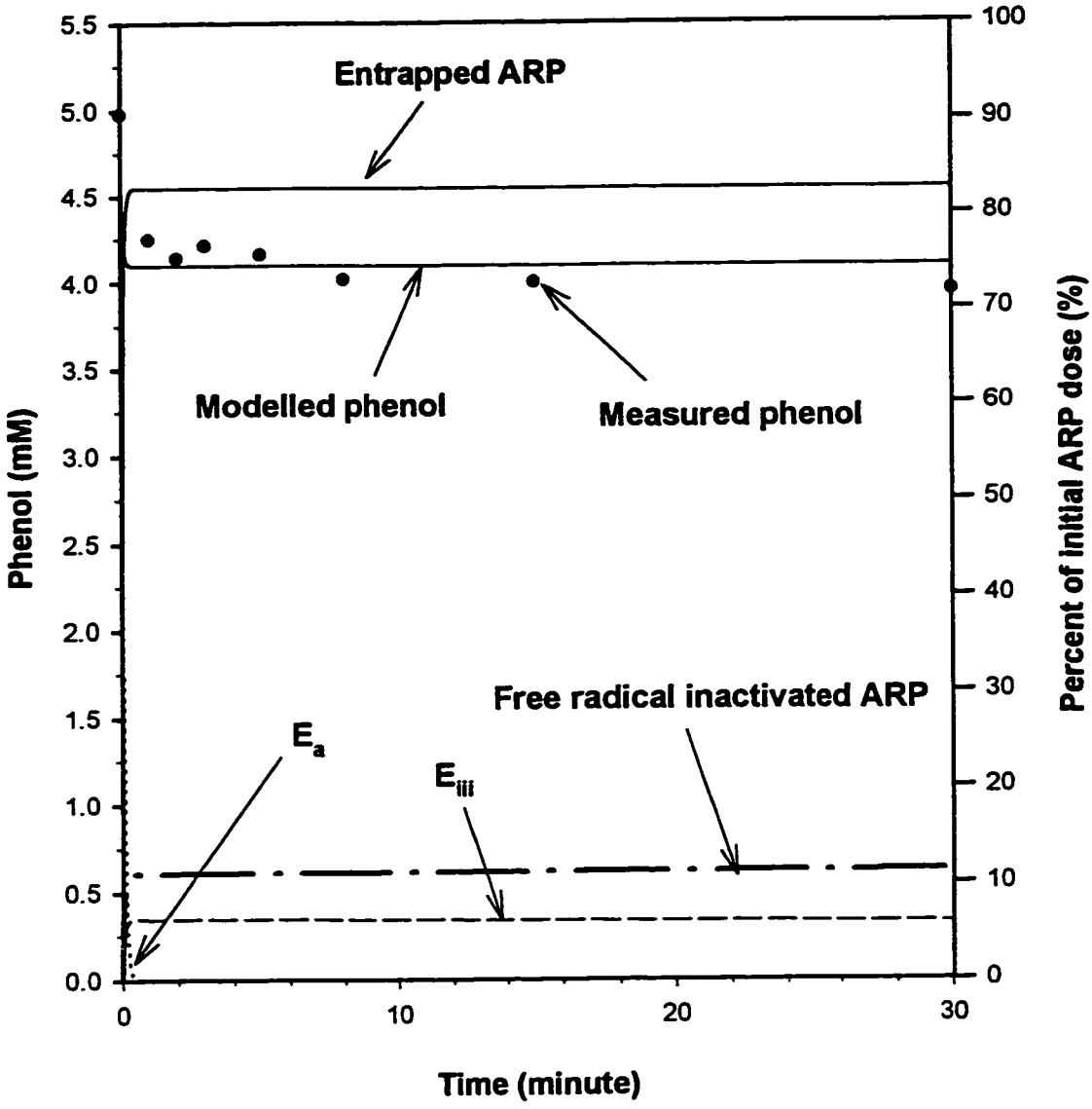


Figure E.15. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 4.98 mM, $[H_2O_2]_0$ = 5.20 mM and $[ARP]_0$ = 220 nM.

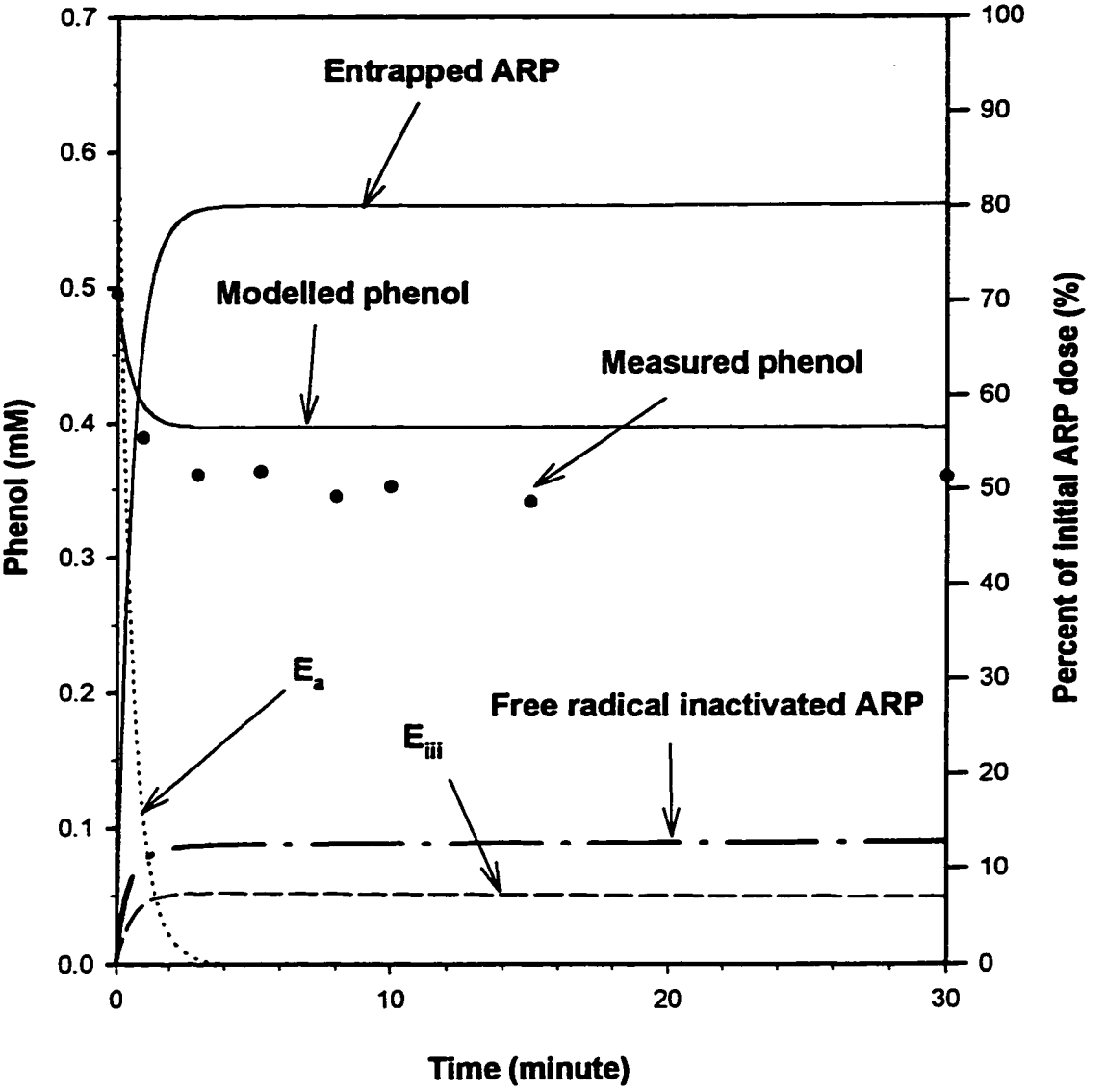


Figure E.16. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 0.50 mM, $[H_2O_2]_0$ = 0.62 mM and $[ARP]_0$ = 25 nM.

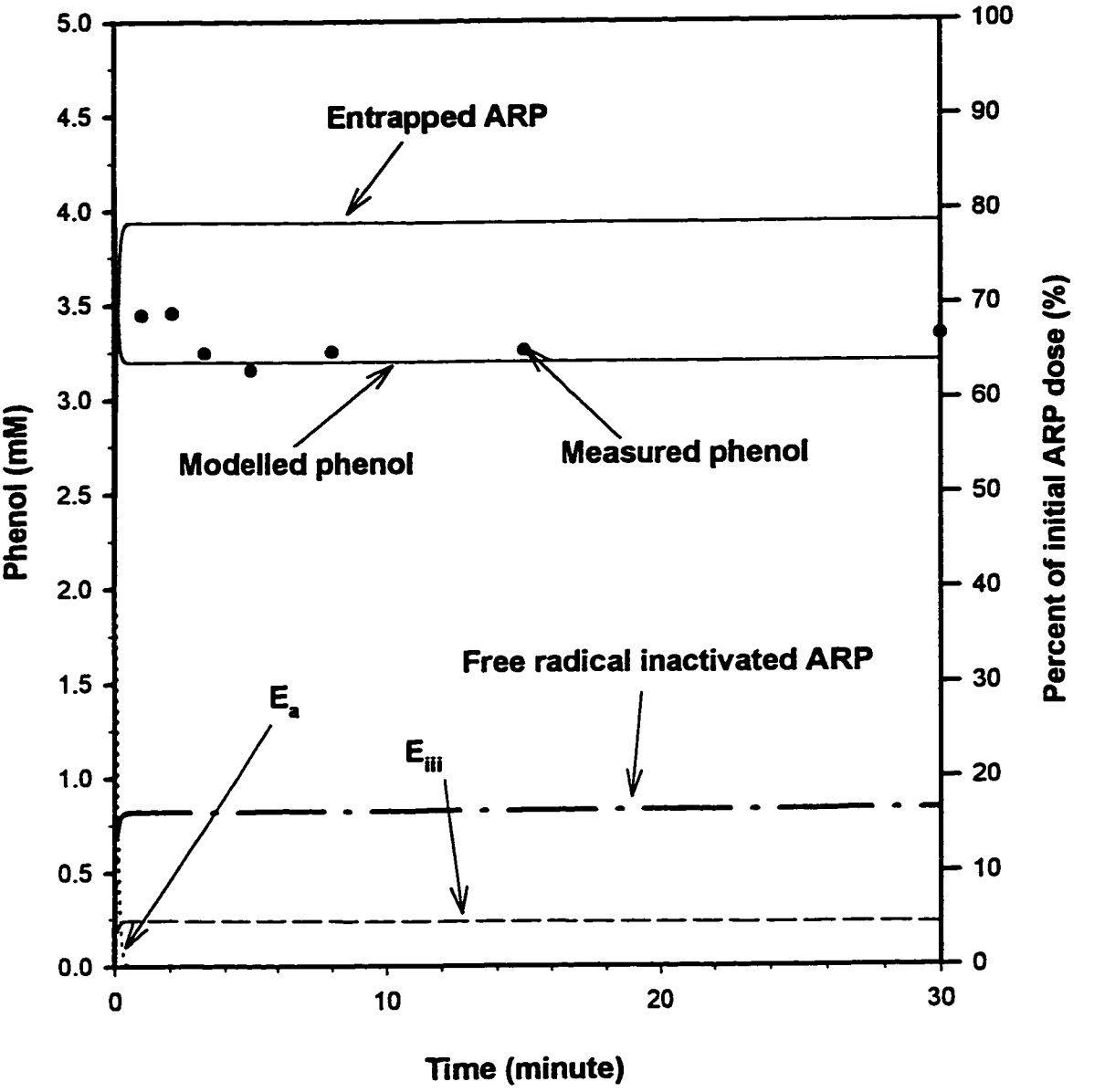


Figure E.17. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 5.18 mM, $[H_2O_2]_0$ = 4.56 mM and $[ARP]_0$ = 521 nM.

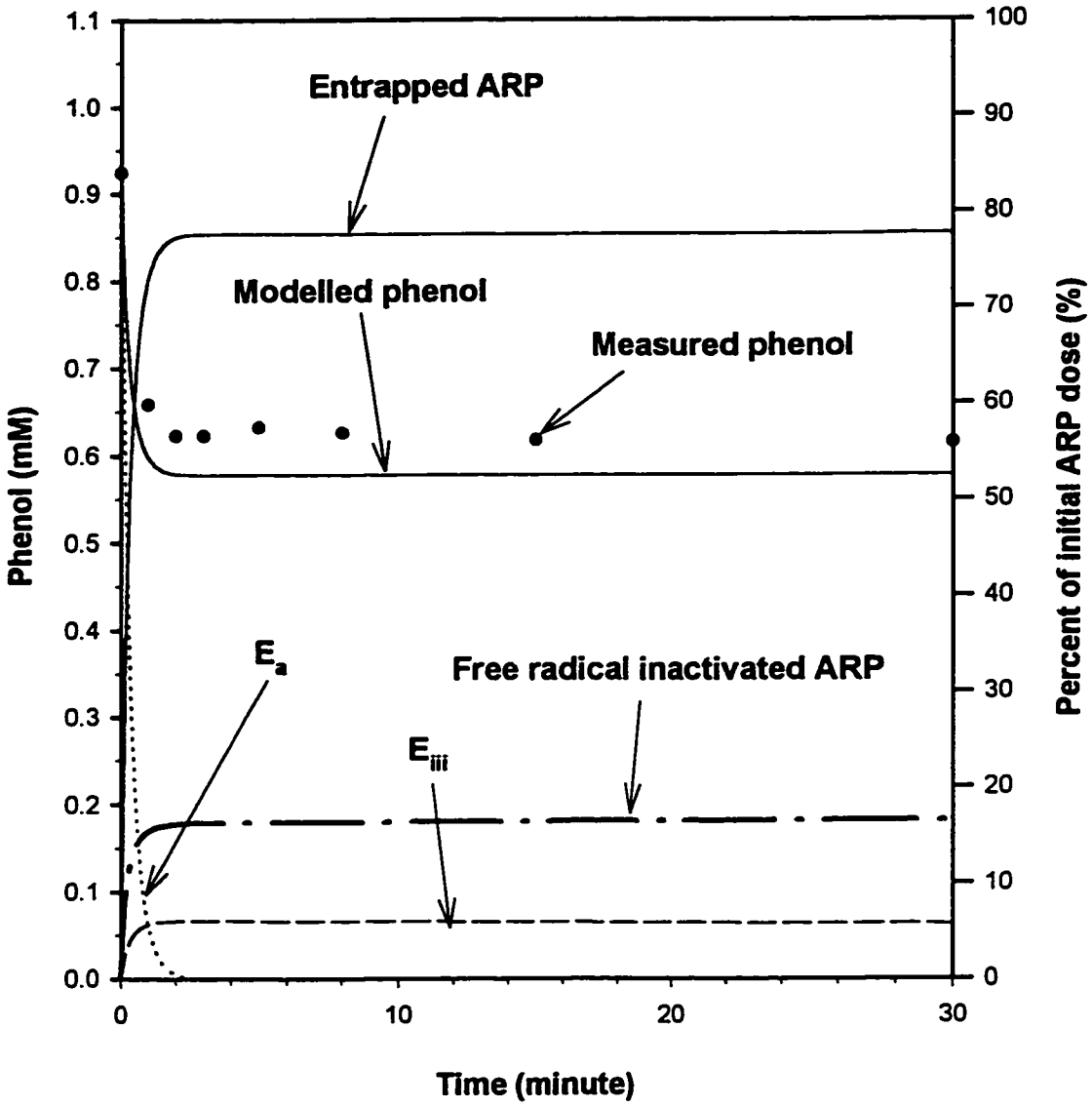


Figure E.18. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 0.92 mM, $[H_2O_2]_0$ = 0.98 mM and $[ARP]_0$ = 92 nM.

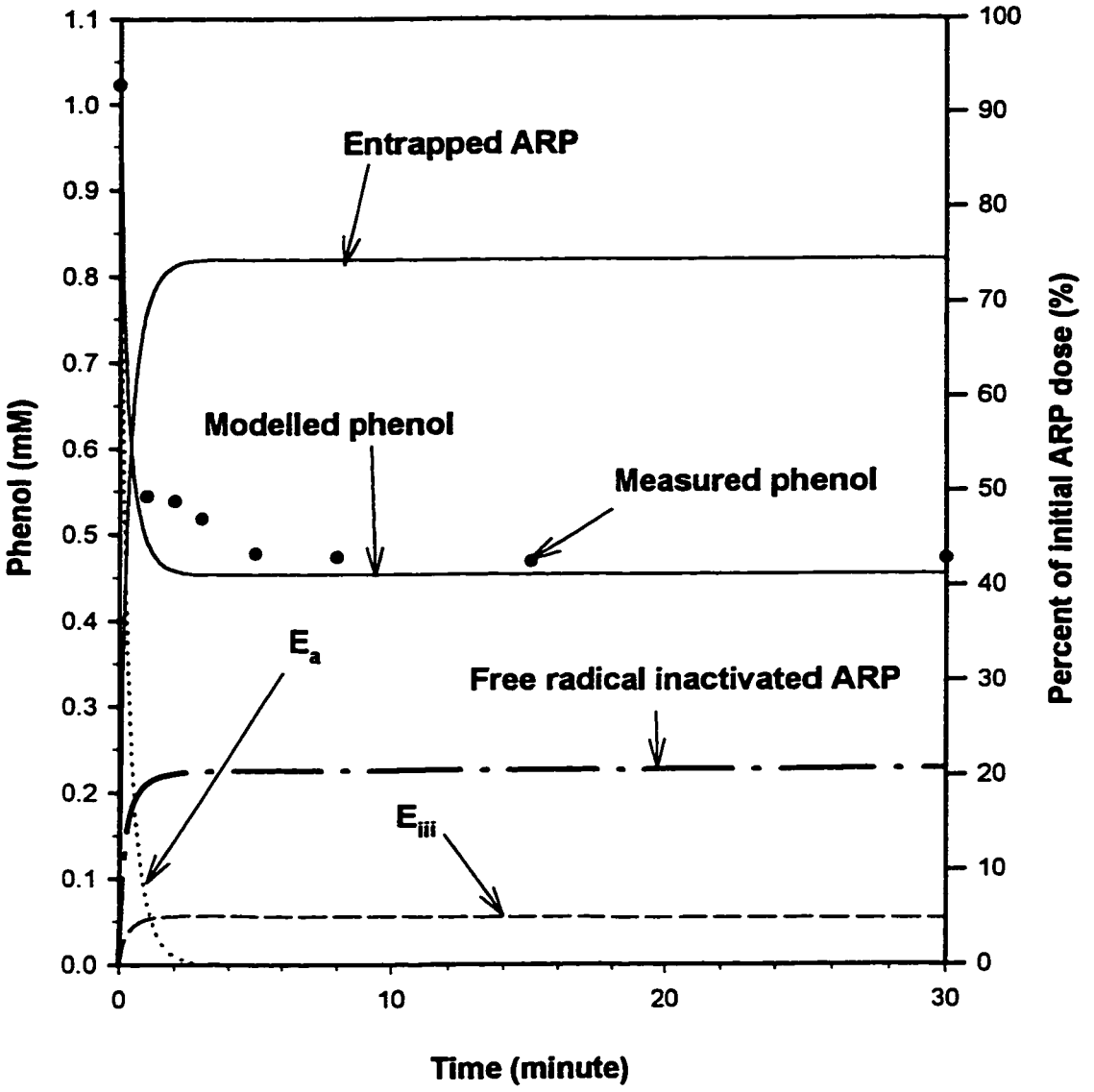


Figure E.19. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 1.02 mM, $[H_2O_2]_0$ = 0.99 mM and $[ARP]_0$ = 158 nM.

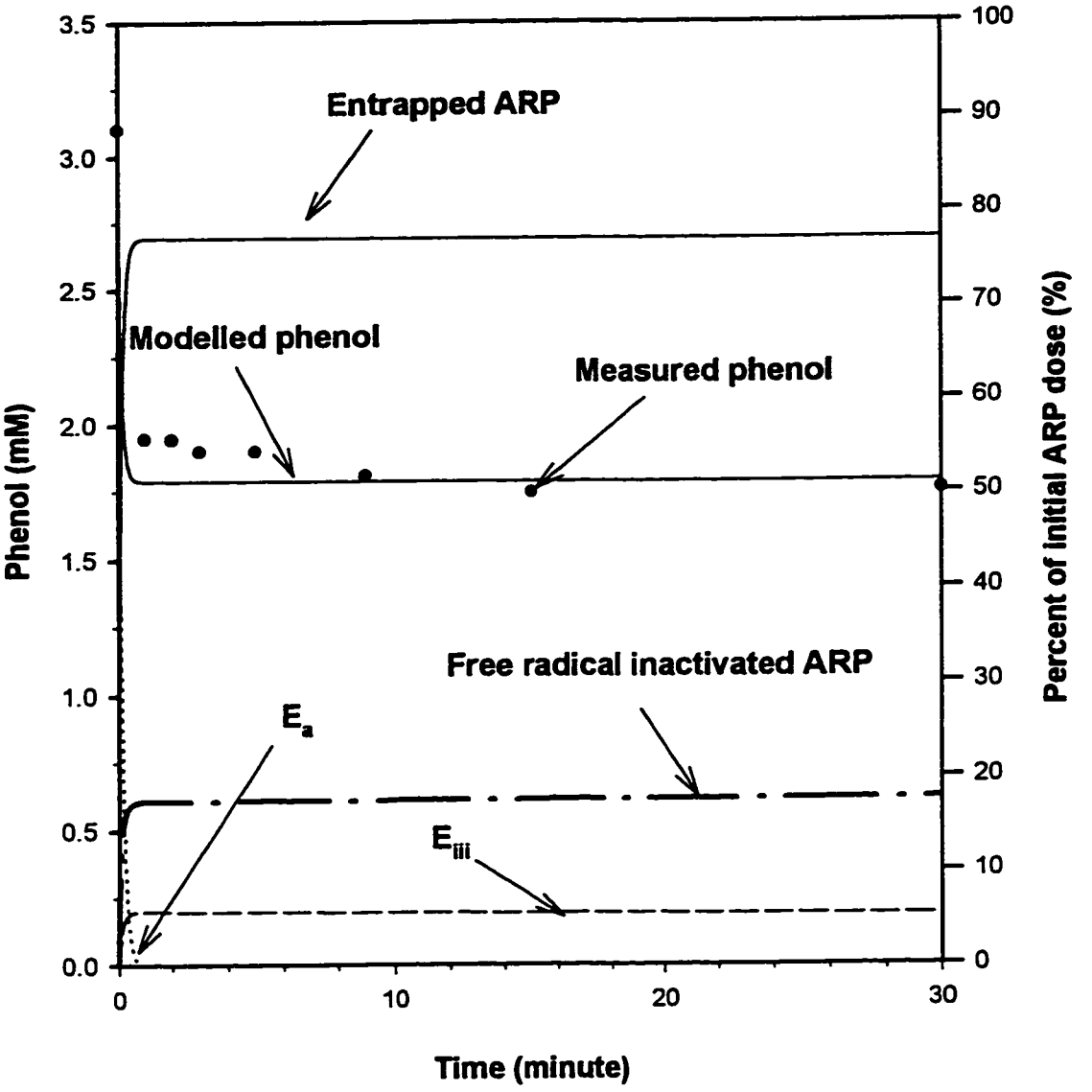


Figure E.20. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 3.10 mM, $[H_2O_2]_0$ = 3.15 mM and $[ARP]_0$ = 352 nM.

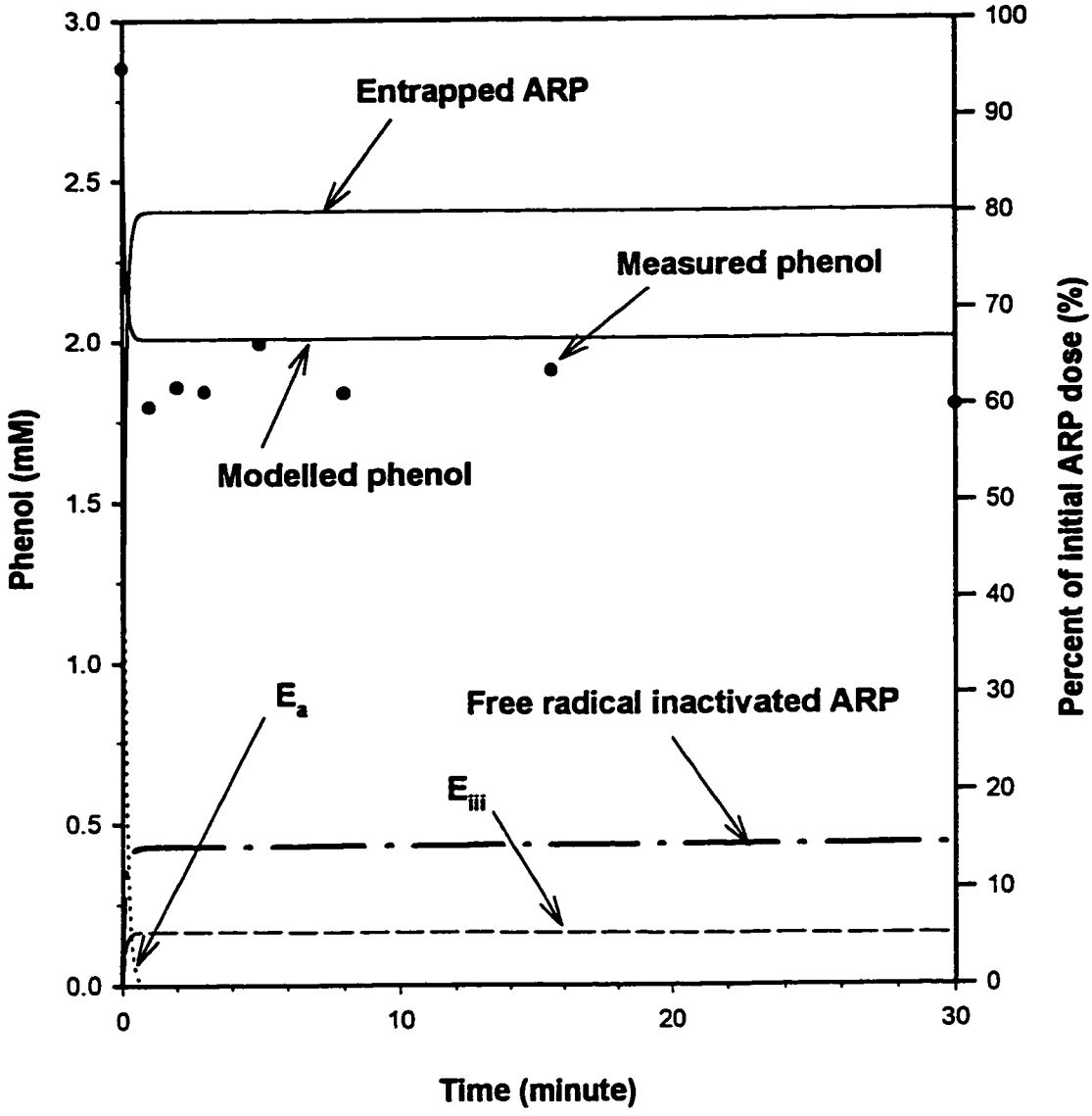


Figure E.21. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 2.85 mM, $[H_2O_2]_0$ = 2.73 mM and $[ARP]_0$ = 218 nM.

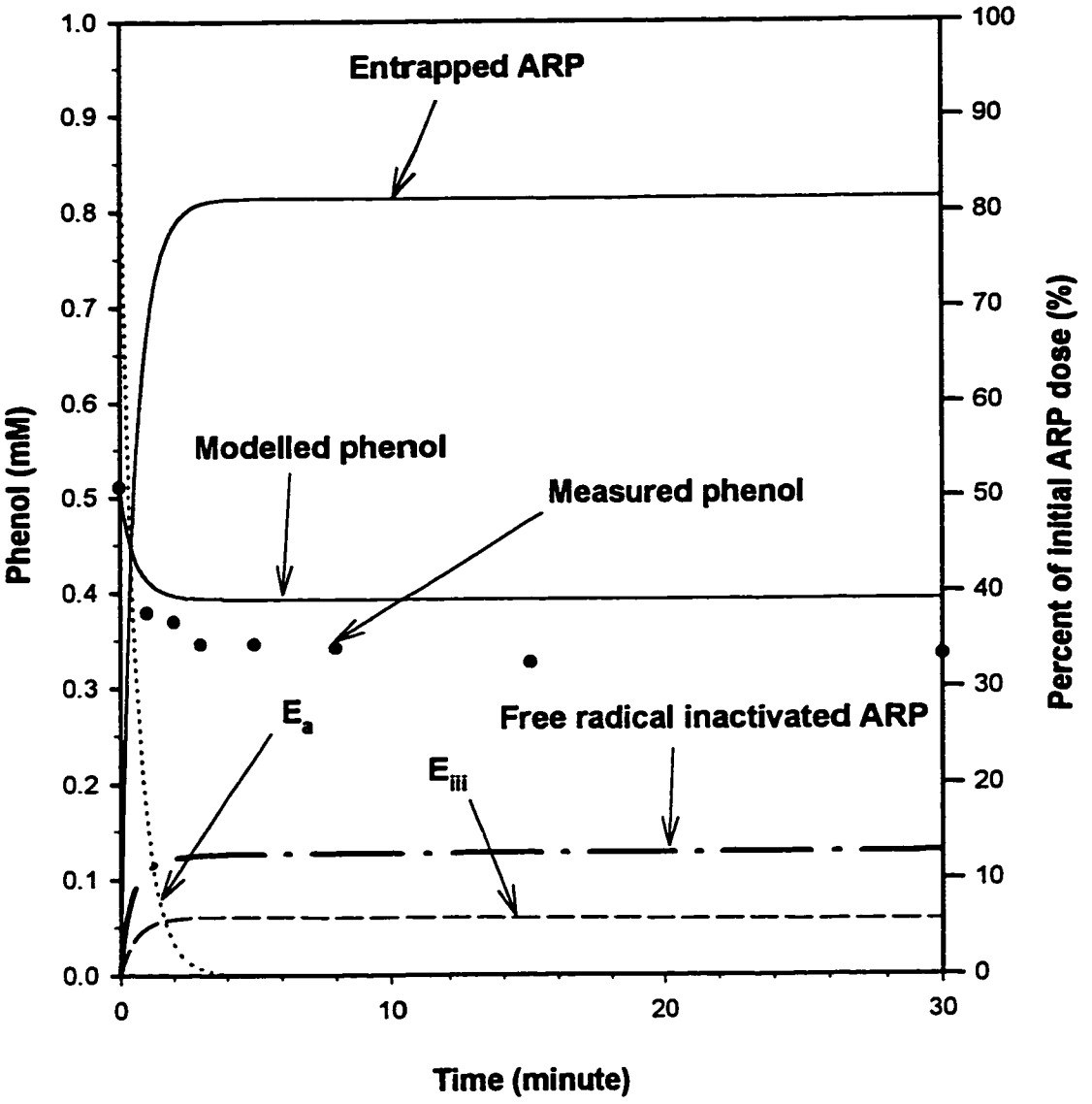


Figure E.22. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 0.51 mM, $[H_2O_2]_0$ = 0.52 mM and $[ARP]_0$ = 30 nM.

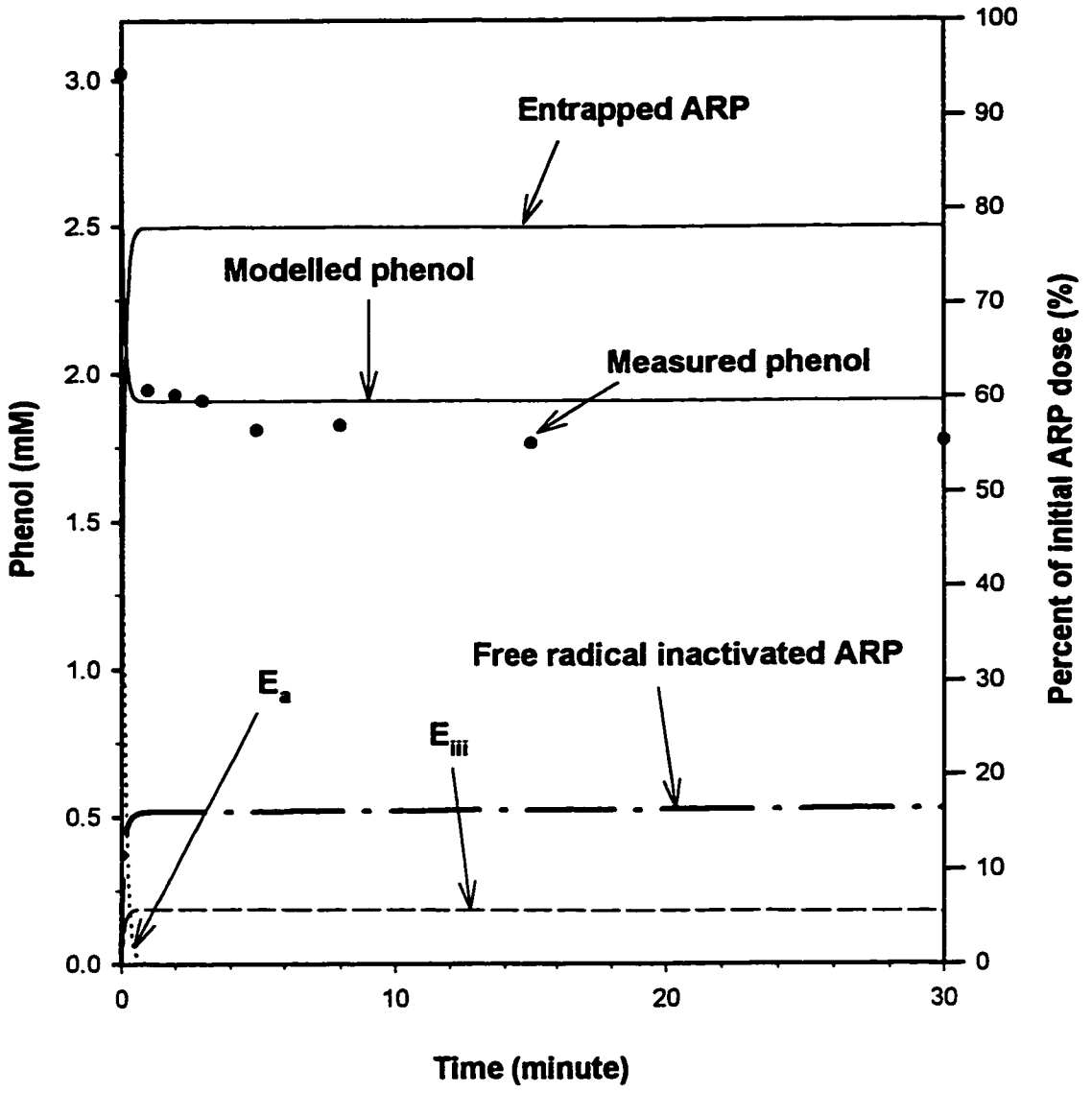
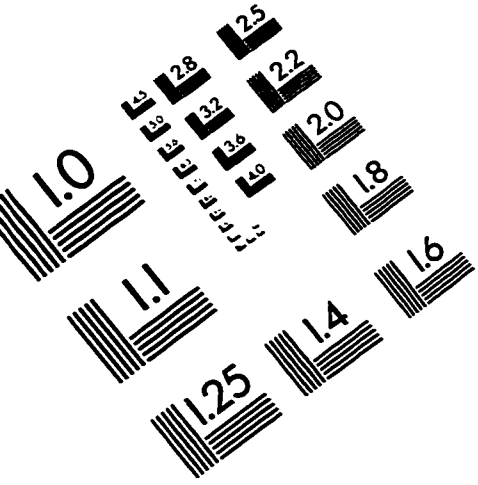
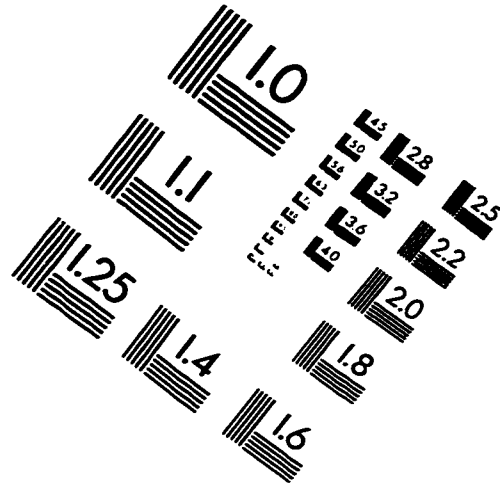
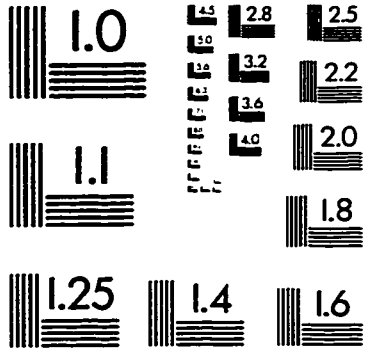


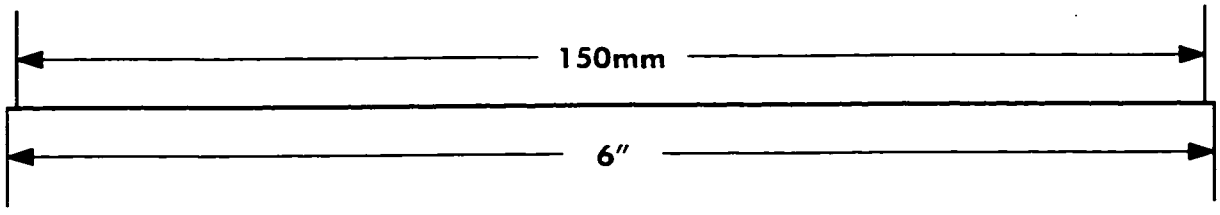
Figure E.23. Measured phenol concentrations and model output using Group D parameters with [PEG]= 0 mgL^{-1}, [phenol] = 3.02 mM, $[H_2O_2]_0$ = 3.09 mM and $[ARP]_0$ = 295 nM.

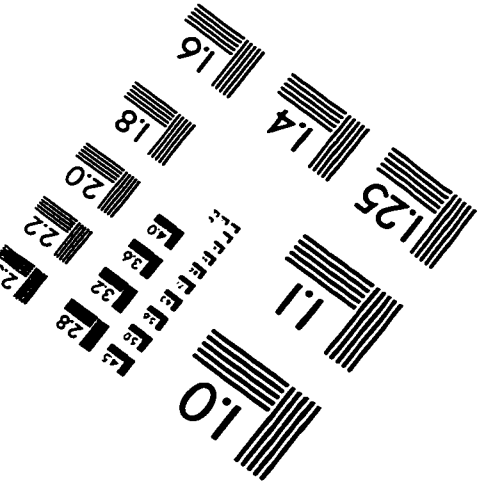
IMAGE EVALUATION
TEST TARGET (QA-3)

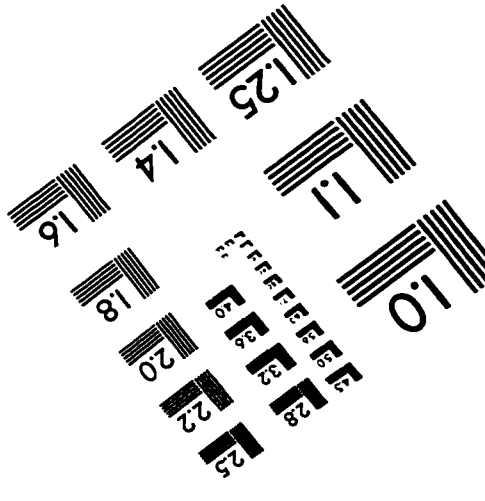












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