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Treatment of 2,4-Dichlorophenol using Soybean Peroxidase

By

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A Dissertation Submitted to the School of Graduate Studies in
Partial Fulfillment of the Requirement for the Degree of
Master of Applied Science
in Civil Engineering (Environmental)

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January, 2000

ABSTRACT

In the presence of hydrogen peroxide (H_2O_2), soybean peroxidase (SBP) extracted from the soybean seed coat catalyzes the polymerization of various chlorinated phenols from wastewater. The resulting polymer precipitates from solution and can be removed through a simple sedimentation or filtration process. To date, the majority of the research has focused on horseradish peroxidase (HRP) as the enzyme source. Recently, researchers have discovered that SBP is thermally more stable and can work in a different pH range than HRP. Since the soybean seedcoat is a waste product of the soybean industry, developing a value-added process to utilize the waste would be economical.

Several limitations need to be resolved prior to this enzymatic process becoming a legitimate treatment. For instance, peroxidase enzyme (PE) inactivation is a primary concern in trying to make the process more viable. Researchers have postulated that the PE is inactivated by entrapment into the forming polymers. To prevent such occurrences, high molecular weight substance such as gelatin and polyethylene glycol (PEG) have been added to solution. PEG 8000 provides a significant increase in protection compared to PEG 3350, a standard additive used for HRP.

To date, researchers studying SBP as an alternative to HRP have focused mostly on the treatment of phenol. The effectiveness of SBP to treat chlorinated phenols is presented in this research. Some of the parameters studied include the effects and interactions of: temperature, reaction time, pH, SBP concentration, PEG molecular weight, PEG concentration, type of substrate, substrate concentration, mode of substrate addition, mode of SBP addition and mode of H_2O_2 addition. SBP was found to be a suitable alternative to HRP offer similar results and in some cases, improved the results.

ACKNOWLEDGMENTS

I would like to this opportunity to express my gratitude to both my supervisors, Dr Kennedy and Dr Warith, for their insightfulness, guidance and unselfish help throughout my research endeavors. I would like to thank them for the advice they provided me, the suggestions they offered and help they gave me in attaining my goals.

A very grateful thank-you to Francisco Aposaga for being very helpful and always willing to lend a hand in and around the laboratory. A special thank-you goes to my colleagues C. Savard and A. Lukasheh for their help throughout my graduate studies. I would like to thank all the professors and support staff of the Department of Civil Engineering and the University of Ottawa, for making my time as an undergraduate and a graduate a very enjoyable and memorable time.

I dedicate this thesis as a symbol of the support and love which I received and cherished from my whole family. I would especially like to express my sincerest gratitude to my wife, Monika whose encouragement and love inspired me and continues to inspire me every day.

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ABBREVIATIONS

hydrogen peroxide (H_2O_2)

soybean peroxidase (SBP)

polyethylene glycol (PEG)

Enzyme Classification (E.C.)

oxidation-reduction (redox)

veratryl alcohol (VA)

plug-flow (PFR)

continuous-flow stirred (CFSTR)

hydraulic retention times (HRT)

Fenton's Reagent (FR)

Advanced Oxidation Processes (AOP)

ozonation (O_3)

ultraviolet (UV)

ultrasound (US)

initial HRP concentration (HRP_0)

dehalogenation number (DN)

2-Chlorophenol (2-CP)

3-Chlorophenol (3-CP)

4-Chlorophenol (4-CP)

2,4-Dichlorophenol (2,4-DCP)

2,4,5-Trichlorophenol (2,4,5-TCP)

pentachlorophenol (PCP)

dibasic sodium phosphate (DSP)

Tris(hydroxymethyl)aminomethane (Tris)

High Pressure Liquid Chromatography (HPLC)

wastewater (WW)

Chemical Kinetics:

A	1st Substrate Concentration
B	2nd Substrate Concentration
P	Reaction Product

Enzyme Kinetics:

E	Enzyme Concentration
S	Substrate Concentration
P	Reaction Product
ES	Enzyme-Substrate Complex
V_o	Intial reaction Rate (velocity)
V_{max}	Maximum Reaction Rate
K_M	Michaelis-Menton Constant

Peroxidase Enzyme Kinetics:

C_I	1st Reaction state of peroxidase enzyme
C_{II}	2nd Reaction state of peroxidase enzyme
C_{III}	3rd Reaction state of peroxidase enzyme
$S^{\bullet}$	Oxidized Substrate Radical
$R^{+\bullet}$	Organic Cation Radical
E_o	Concentration of initial PE;
E_a	Concentration of active PE, considers C_{II} and C_{III} together
E_{iii}	Concentration of C_{III}
E_{inact}	Concentration of permanently inactivated PE
$[AH_2]$	Concentration of aromatic compound
$[H_2O_2$	Concentration of Hydrogen Peroxide
k values	are the rate constants associated with the equations
Fe	Iron
Fe(III)	Ferric Iron
Fe(IV)=O	Oxyferryl Iron

Turnovers Calculations:

C_S	Concentration of Substrate
C_E	Concentration of Enzyme
MW_S	Molecular Weight of Substrate
MW_E	Molecular Weight of Enzyme

CHAPTER ONE

INTRODUCTION

Peroxidase enzymes (PE) are found in mold, bacteria, microorganisms, plants, and animals (Dunford and Stillman, 1976). In the presence of hydrogen peroxide (H_2O_2), acting as an electron acceptor, PE catalyze the oxidative polymerization of phenols, amines and other aromatics to insoluble oligomers. These insoluble oligomers can then be removed through a simple sedimentation or filtration system (Klibanov *et al.*, 1980). To date, the majority of the research has focused on using horseradish peroxidase (HRP) as the PE source.

The use of enzymes for wastewater treatment applications was first proposed in the 1930's. However, experiments using PE to remove toxic phenols and anilines from wastewater were not performed until 1976 by Munnecke and in 1980 by Klibanov *et al.*, (Aitken, 1993). Using HRP, Klibanov *et al.*, (1980) removed up to 30 different phenols and amines from wastewater at efficiencies of up to 99%. Since then, many other researchers have continued to explore the possibilities of using PE in the treatment of wastewater. Increasing regulations on specific compounds is resulting in a growing emphasis to target specific pollutants in air, water, and soil. Many of these compounds are classified as biologically recalcitrant and hazardous and have to be controlled in the environment. Compounds in this group include halogenated aromatics such as chlorinated phenols. There is also a growing trend for methods that destroy or modify pollutants so that they are removed and controlled rather than methods that transfer the pollutants from one phase to another (Aitken, 1993). These trends in waste treatment favour the specificity provided by PE treatment.

HRP has been studied for many years and the success of HRP for the purpose of the removal of aromatic compounds from wastewater is well documented. Recently, soybean peroxidase (SBP) has shown similar success in treating phenols when compared to the results using HRP. More importantly, SBP is obtained from the soybean seed coat, which is a waste-product of the soybean processing industry. According to the Ontario Soybean Growers'

Marketing Board (OSGMB, 1999), there are over 25,000 soybean growers in Ontario alone. With over 2,200,000 acres devoted to growing soybeans in Ontario, developing a process to incorporate the use of SBP technology should be seriously considered for future research and development in order to convert a waste to a value added product.

1.1 Objectives

The general objective of this study is to examine the effectiveness of SBP in treating wastewater contaminated with 2,4-dichlorophenol (2,4-DCP) under a variety of environmental conditions. These environmental conditions include but are not limited to the following:

- length of time required for reaction to go to completion;
- effects of temperature;
- optimum pH range for treating 2,4-DCP with various SBP concentrations;
- determination of relationship between removal rate and initial 2,4-DCP concentration;
- optimum pH range for treating 2,4-DCP with SBP in the presence of the additive polyethylene glycol (PEG);
- increased protection provided by higher molecular weight PEG;
- PEG 8000 concentrations that provides optimum protection;
- minimum SBP concentration required in the presence of PEG at various pH;
- applicability of SBP to treat other chlorinated phenols;
- ability to reuse PE; and
- the effects of various modes of PE and H_2O_2 addition.

This study will determine if SBP is less efficient, as efficient or even more efficient than the commonly used HRP. The effect of a PEG with a molecular weight higher than the standard PEG 3350 will be also be analyzed in various conditions. As well, the experiments will be designed not only to examine SBP but to optimize the various characteristics of the process.

1.2 Experimental Considerations to Meet Objectives

To meet these objectives, a variety of experiments will be conducted using SBP while using previous experimental results with HRP as benchmarks. Since the objectives include determining the optimum of various characteristics, experiments were selected on an ongoing basis based on each set of results obtained. The experiments will be divided into three steps. The first step includes performing trial runs with HRP to ensure that the experimental methods that will be followed are successful. The results from the trial runs are presented in Appendix A. The second step will be performed using SBP to treat 2,4-DCP under a variety of environmental conditions. Results and observations from these tests are presented in Chapter 3. The third step will be to optimize the treatment process in the presence of the protective additive PEG and the results and observations are presented in Chapter 4. This allows for continuous observations and analysis of the results while allowing the flexibility to design the next set of experiments based on previous results.

The experimental data will be analyzed using graphical methods. Mathematical equations will be fit to trends in data and used to interpolate/extrapolate optimum conditions. Results will be described in terms of turnover numbers, a term used to describe the amount of moles of substrate treated per mole of enzyme consumed.

CHAPTER TWO

LITERATURE REVIEW

On the basis of the objectives of this study, the literature review focuses on: (1) peroxidase enzymes and their characteristics; (2) the experimental environment; and (3) reaction products and observations.

2.1 The Basics of Enzymes

Enzymes are specialized proteins used to catalyze biological reactions. Enzymes can catalyze complex sequences of reactions that would require days, weeks, or months in the chemical laboratory. Although enzymes are proteins, and thus relatively fragile molecules, they bring about their extraordinary effects in dilute aqueous solution at various pH and temperature, in sharp contrast to the rather extreme conditions often required to accelerate chemical reactions in the organic laboratory (Lehninger *et al.*, 1993). This advantage makes the PE process attractive. Instead of requiring extreme conditions, enzymes such as PE can be used under moderate conditions to remove specific target organic compounds. In addition, the PE can proceed under a variety of conditions such as various pH and temperature as will be described in the following sections.

2.1.1 Enzyme Nomenclature and Classification

Enzymes have been traditionally named by taking the name of the substrate that they react with and then adding the suffix "-ase" onto it. For example, lactase is an enzyme that reacts with lactose (Hardy, 1998). This approach to naming enzymes was not always practical and several enzymes were given uninformative names such as pepsin and catalase. Therefore, an international enzyme commission developed the Enzyme Classification (E.C.) system to properly identify enzymes. The E.C. system divides enzymes into six major classes and several sets of subclasses. The six major classes; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases are classified according to the type of reaction they catalyze. Oxidoreductase enzymes catalyze an oxidation-reduction (redox) reaction. The E.C. system

assigns numerical values to each of these classes and subclasses. For example, the E.C. name for peroxidase is E.C.1.11.1.7. The first digit (1) of the enzyme classification number represents the class name oxidoreductase. The second digit (11) represents the substrate that the enzyme acts upon (peroxide). The next digit (1) is not defined and the (7) is representative for peroxidase. (Lehninger *et al.*, 1993). Note: Peroxidase (E.C. 1.11.1.7) includes all animal, fungal, plant and bacterial peroxidases therefore the source is usually specified (i.e. horseradish or soybean).

PE are separated into three classes (Welinder, 1992; Banci, 1997). Class I includes the intracellular prokaryotic peroxidase, such as yeast cytochrome c peroxidase (CCP), ascorbate peroxidase (AP), and bacterial catalase-peroxidases. Class II are extracellular fungal peroxidase which include the PE involved in the degradation of lignin: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Class III are secretory plant peroxidase (HRP and SBP) which have multiple tissue-specific functions, one of them being oxidation of toxic compounds.

2.1.2 Peroxidase Enzyme Sources

There is an extensive list of peroxidase sources (Dunford and Stillman, 1976). A few plant sources include horseradish roots, potatoes, white radish, and soybean hulls (Klibanov *et al.*, 1983; Nicell *et al.*, 1992; and McEldoon and Dordick, 1996). Others have mentioned turnips, spinach, and fig tree sap as possible peroxidase sources. Peroxidase polymerization of phenols in plants is considered as the cement like material that prevents plant pathogens from invading plant cells (Kim and Yoo, 1996). Peroxidase from *Arthromyces rasmous* has been shown to treat phenol in solution and shown similar results to HRP (Ibrahim *et al.*, 1997). Peroxidase from carrot hairy root cell culture also exhibits behaviour similar to HRP (Kim and Yoo, 1996). However, this discussion is limited to two plants which have shown promising results, horseradish and soybean.

PE extracted from horseradish plants (HRP) was first discovered in 1937 and was called compound II or HRP-II (Dunford and Stillman, 1976). Since its use in the field of wastewater treatment by Klibanov *et al.*, (1980), researchers have continued to study HRP. The pure extract form of HRP (Sigma product P8000) is a red-brown powder with a molecular weight of 44,000

and is soluble in a 0.1 M potassium buffer, pH 6.0 at 10 mg/ml. Some HRP extracts have several isoenzymes, which may have varying properties (Sigma Data Sheets, 1998).

SBP is extracted from the soybean's hull (also called seed coat), which is a waste-product of the food processing industry. Photos 2.1 , 2.2 and 2.3 depict the soybean and its seedcoat in various growth stages. In Canada, the area of farm land that is being devoted to producing soybeans is continually increasing. Figure 2.1 shows that the area of land devoted to soybean crops was almost 2.5 million acres. In the late '90s there were over 25,000 producers of soybeans in Ontario alone (OSGMB, 1999). SBP may have an advantage over HRP in that unlike HRP there are no isoenzymes associated with it. Isolated SBP powder is goldish, brown in colour. SBP is more reactive in acidic conditions than HRP (McEldoon *et al.*, 1995). McEldoon *et al.*, (1995) performed some veratryl alcohol (VA) oxidation experiments and discovered the optimum pH of SBP to be 2.4 with a working range of 1.5 to 4 (for VA oxidation), a range where HRP does not perform well. (Klibanov *et al.*, 1983). They also examined the oxidation of phenols such as guaiacol and p-cresol and found an optimum of 5.0. Nicell and Wright (1997), compared the rate of substrate utilization for HRP and SBP. Their model indicated that SBP tends to form more compound III (see Enzyme Kinetics section) and is catalytically slower than HRP during oxidation of phenol. Conflicting reports of the "better" PE proves that more research is required to determine what makes one enzyme more suitable than another for a given application.

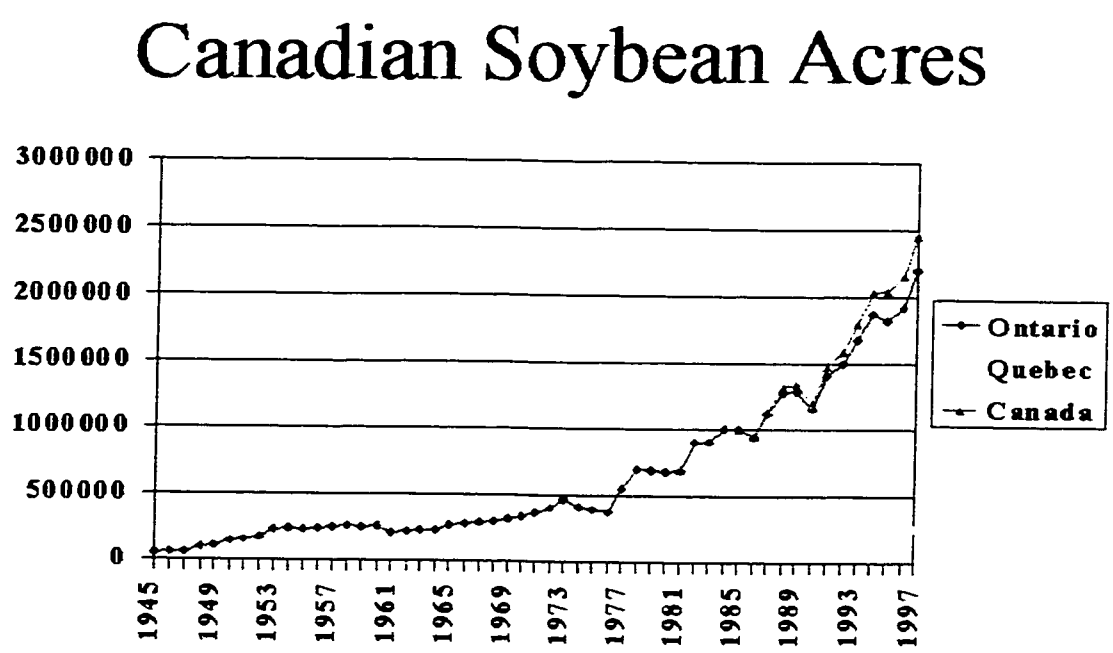


Figure 2.1: Canadian farm land devoted to soybean crops from 1945 to 1997. Source: Ontario Soybean Marketing Board, 1998

Photo 2.1: Picture of a soybean plant.
Source: Iowa State University of Science and Tech.
Cooperative Extension Service, 1994

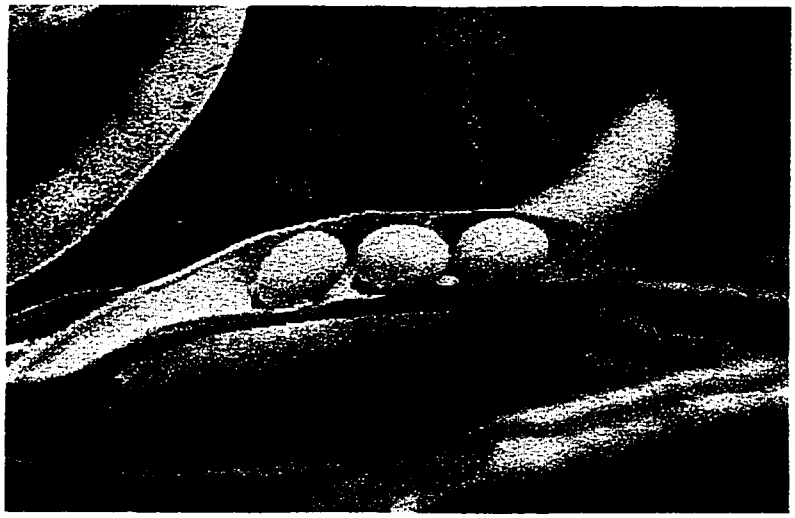


Photo 2.2: Picture of an opened soybean pod with three beans in the white stage surrounded by the seedcoat.
Source: Internet, 1998

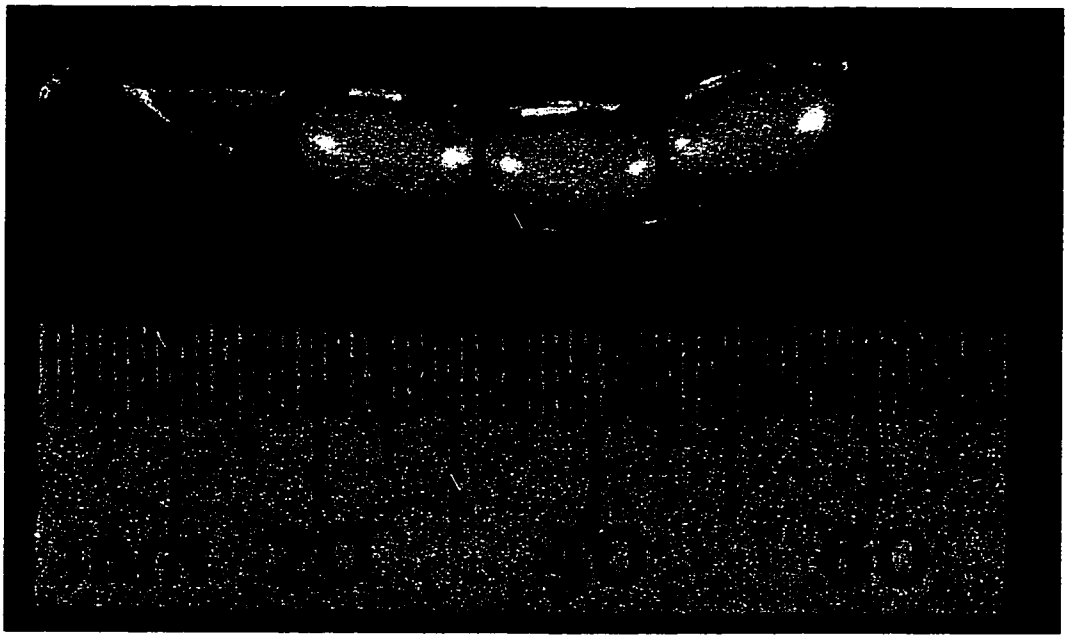


Photo 2.3: Picture of an opened soybean pod with three beans in the green stage surrounded by the seedcoat.
Source: Iowa State University of Science and Tech. Cooperative Extension Service, 1994

2.1.3 Enzyme Structure and Active Site

The proteins that make up an enzyme are generally divided into two major classes depending on their composition: simple and conjugated. Simple proteins are made of amino acids alone whereas conjugated proteins are composed of simple proteins and other organic or inorganic components. The non-amino portion of the conjugated protein is called the prosthetic group. Conjugated proteins are classified with respect to this prosthetic group (Lehninger *et al.*, 1993). All class III PE contain the prosthetic group heme or ferriprotoporphyrin IX (Dunford and Stillman, 1976). HRP, catalase, cytochrome c and hemoglobin all contain this heme group and are classified as hemoproteins. The iron ion contained within this prosthetic group is at rest in the +3 oxidation state (Banci, 1997). This property is essential to the PE kinetics discussed later. Conjugated proteins can also be classified based on the chemical nature of the prosthetic group (Lehninger *et al.*, 1993). Enzymes with a large percentage of carbohydrate associated with their structure are given the prefix "glyco". Both the SBP and HRP are plant glycohemoproteins. The carbohydrate content of HRP is approximately 18.4%. It is postulated that the carbohydrate portion associated with this PE has a significant role in increasing enzyme stablility under elevated temperatures (Dunford and Stillman, 1976). A higher carbohydrate content in SBP could be the reason why SBP has been demonstrated to be more stable at elevated temperatures than HRP (McEldoon and Dordick, 1996).

X-rays diffraction is used to reveal the enzyme structure. However, to date, only a few PE have been examined using x-ray diffraction (Banci, 1997). PE have complicated structures, unique characteristics and specific physical properties. The structural properties of the protein matrix around the prosthetic group (heme for peroxidase) can strongly determine specificity of the enzyme and the nature of the substrates which can be oxidized (Banci, 1997).

Enzymes are typically very large proteins, yet only a small portion of their structure is involved in the reaction. This area is called the active site. The active site is composed of two parts, the catalytic site and the binding site. The catalytic site is where the reaction actually occurs. The binding site is the area that holds the substrate in place. Enzymes use weak non-covalent bonds to hold the substrate in place. The shape of the active site complements the substrate and therefore determines the specificity of the enzyme (Hardy, 1998). Some enzymes

have nearly absolute specificity for a given substrate and will not attack even closely related molecules, whereas others will attack a whole class of molecules sharing a common denominator of structure but at widely different rates (Lehninger *et al.*, 1993).

The following excerpts are from two comprehensive reviews of the structural properties of peroxidase enzymes by Banci, (1997) and Dunford and Stillman (1976).

> *"Class III PE have molecular weights ranging from 35,000 to 100,000. They are divided into two different structural domains, enveloping the heme moiety. The structures of peroxidase are constituted by 10-11 alpha-helices, linked by loops and turns, while beta-structures are essentially absent or are a minor component. Cystene bridges are present in fungal and plant peroxidase (Class II & III). Cystene residues in the proteins form disulphide bridges, which provide only a mild degree of rigidity to the protein. Fungal and plant peroxidase contain two calcium ions whose binding sites have been located in the crystal structure. These calcium ions could have a structural role and could be relevant for maintaining the structure of the active site because of their location and coordination within the PE. It has been found in the Class III plant peroxidase HRP that the removal of the calcium ions significantly reduces enzymatic activity and in particular the rates of reduction of Compound II (Compound II later explained in "Peroxidase Enzyme Kinetics" section). Fungal and plant peroxidases are both glycosylated on the protein surface, (which provides more thermal stability as expressed earlier). Tyrosine is absent in fungal peroxidase (with one exception), making the reduction potential of this class of enzyme higher than in plant peroxidase. PE do not require a major structural change to adapt to different substrates. The distal cavity is the site of interaction with PE and is characterized by two different amino acids, which produce a hydrophilic pocket."*

Several conclusions can be drawn for HRP and SBP with regards to their structures. It is quite possible that the structure of the HRP and the SBP has a mild rigidity allowing it to flex more easily and thereby allowing a larger variety of substrate to enter their active site. This would explain the relative lack of specificity that has been exhibited in experiments using HRP.

Also as mentioned, a high carbohydrate contents protect the enzymes from extreme temperatures and may be the reason for the higher thermal stability exhibited by SBP.

2.1.4 Enzyme Kinetics

Prior to developing a concept for enzyme kinetics, it is necessary to cover several aspects of chemical kinetics. Chemical reactions are most commonly classified on a kinetic basis by reaction order. There are several different types of reactions orders; first, second, pseudo-first, third, zero and mixed orders. First order reactions proceed at a rate exactly proportional to the concentration of one reactant. The following equation 2.1 applies;

$$A \rightarrow P \quad where: \quad -\frac{d[A]}{dt} = k[A] \qquad \text{Equation 2.1}$$

where the product of the reaction, [P] a concentration, is directly proportional to the concentration of [A]. The term -d[A]/dt is the rate at which the concentration [A] decreases. The proportionality constant k relates the rate and concentration at a given temperature. In a second order reaction, the rate is proportional to either the product of the concentrations of the two reactants or to the power of a single reactant. The following equation 2.2 applies to a second order reaction (for two reactants):

$$A + B \rightarrow P \quad where: \quad -\frac{d[A]}{dt} = -\frac{d[B]}{dt} = k[A][B] \qquad \text{Equation 2.2}$$

where the rate of reaction is determined by the product of A and B concentrations. Note that sometimes the concentrations of [A] and [B] can vary greatly and the reaction imitates first order kinetics. This situation is known as a pseudo-first-order reaction. The third order kinetic reaction rate is proportional to three concentrations and occurs very rarely. Finally, the zero order reaction reacts independently of the concentrations involved in the reaction. Many catalyzed reactions are zero order with respect to the reactants. In some cases, the rate depends on the catalyst (enzyme) concentration or some other factor other than the concentrations of the reactants undergoing reaction. Often reactions are neither pure first or second order and are considered mixed order reactions. In order to proceed from reactants to products, the reaction must overcome the energy

of activation. The rate that a chemical reaction proceeds can be accelerated by a rise in temperature or through the addition of a catalyst such as an enzyme. A catalyst accelerates the chemical reactions by lowering the energy of activation. Therefore, less energy is required for the reaction to proceed as is illustrated in Figure 2.2.

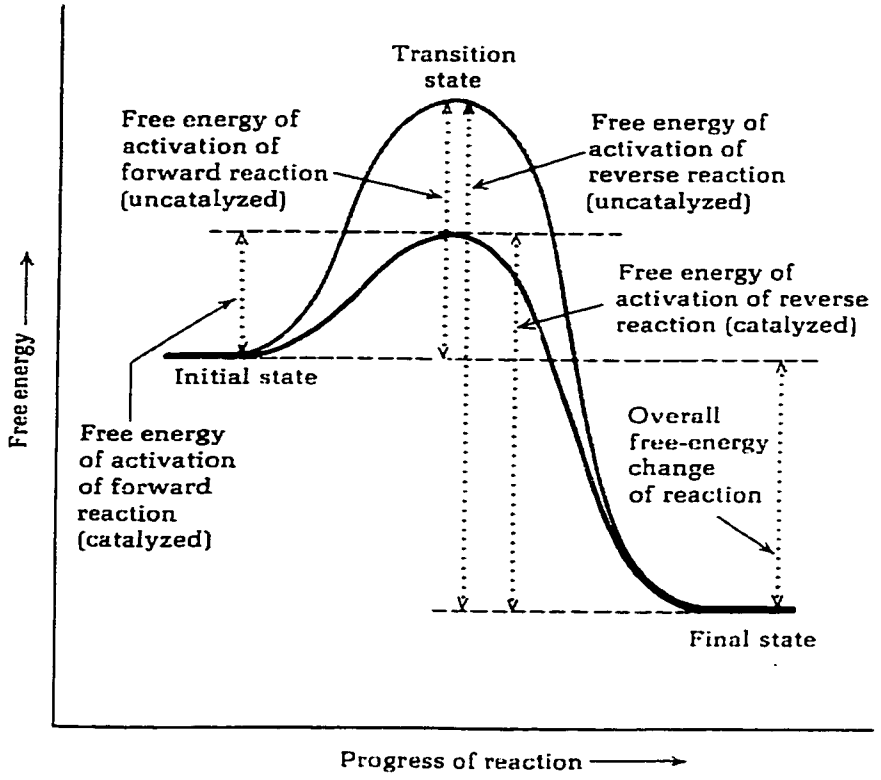


Figure 2.2: Energy diagram for a chemical reaction, uncatalyzed and catalyzed. Source: Lehninger *et al.* (1993)

Reactions catalyzed by enzymes generally follow the principles of chemical kinetics; however, these enzymatic reactions are unique in that they reach "saturation with their substrate". Without a catalyst, a reaction rate will increase with an increase in substrate. When a catalyst (enzyme) is present, the enzymes combine transiently with substrate and lower the energy of activation of the reaction. A lower energy of activation accelerates the chemical reaction. Initially, the relationship between the substrate concentration and the reaction rate increases proportionally, reflecting first order kinetics. However, with an increase in substrate concentration, the reaction rate begins to increase at a lesser rate, reflecting mixed order kinetics.

With a further increase in substrate, the reaction rate becomes independent of the increasing substrate and asymptotically reaches a constant rate. The resulting relationship reflects zero order kinetics and at this point, the enzyme is saturated with its substrate.

A general theory of enzyme kinetics and inhibition is the Michaelis-Menton theory which assumes that the enzyme, E, first combines with substrate, S, to form the enzyme-substrate complex, ES; the latter breaks down in a second step to form free enzyme and the product P (Lehninger *et al.*, 1993). These reactions are considered reversible as shown in equations 2.3a and 2.3b :

$$E + S \leftrightarrow ES \qquad \text{Equation 2.3a}$$

$$ES \leftrightarrow E + P \qquad \text{Equation2.3b}$$

The Michaelis-Menton rate equation for reactions catalyzed by enzymes having only one substrate is derived based on the acceptance of the above equations. The Michaelis-Menton equation (2.3c) is as follows:

$$v_o = \frac{(V_{\max}[S])}{(K_M + [S])} \qquad \text{Equation 2.3c}$$

V_0 is the initial reaction rate (velocity), V_{max} is the maximum reaction rate, S is the initial substrate concentration and K_M is the Michaelis-Menton constant. V_{max} is equal to k +2[ET], where ET is the total enzyme concentration at saturation. Furthermore, the Michaelis-Menton constant K_M is equal to the substrate concentration at which the initial reaction velocity is half the maximum (Lehninger *et al.*, 1993). It is noted that the previous equations express the kinetics for a simple, idealized one-substrate reaction. Many one-substrate reactions involve two or three complexes, as will be shown with the PE system.

2.1.5 Peroxidase Enzyme Kinetics

PE use H_2O_2 to promote the one-electron oxidation of chemicals to free radicals. This occurs through the following steps. Firstly, the heme iron (Fe) of the prosthetic group is in the

ferric state (+3) at resting state. H_2O_2 oxidizes the ferric enzyme (heme[Fe(III)(px)) by two electrons to form what is known as Compound I (C_I) and water (Equation 2.4). C_I contains an oxyferryl (Fe(IV)=O) center and an organic cation radical ($R^{+\bullet}$). The substrate is then oxidized by one electron to a substrate radical (S*) and C_I is reduced by one electron to Compound II (C_{II}), where the organic cation is reduced to its resting state (Equation 2.5). Further oxidation of another substrate molecule (S) by C_{II} returns the enzyme to its ferric Fe(III) resting state (Equation 2.6). This oxidation process occurs through simple electron transfer not binding (Banci, 1997, Barr & Aust, 1994). The substrate radicals that form combine together to produce the polymers that fall out of solution. The reaction proceeds as follows:

$$\mathbf{heme[Fe(III)]_{(PX)} + H_2O_2 \longrightarrow heme[O = Fe(IV) - R^{+\bullet}]_{(Compound\ I)} + H_2O} \quad \text{Equation 2.4}$$

$$\mathbf{heme[O = Fe(IV) - R^{+\bullet}]_{(Compound\ I)} + S \longrightarrow heme[O = Fe(IV)]_{(Compound\ II)} + S^{*}} \quad \text{Equation 2.5}$$

$$\mathbf{heme[O = Fe(IV)]_{(Compound\ II)} + S \longrightarrow heme[Fe(III)]_{(PX)} + S^{*}} \quad \text{Equation 2.6}$$

Source: Banci (1997)

2.1.5.1 Enzyme Activity and Inactivation

HRP and SBP enzyme activity is stated as the number of units per mg of solid extract. One unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C (Sigma, 1998). HRP activities range from 40 to 330 units/mg prior to the addition of any additives and can retain its full activity for at least 2 years when stored at -20°C. SBP has a range between 50-150 units/mg. Mechanisms, which cause an enzyme to become "inactive", are equally as important as the activity of an enzyme and have been summarized. All proteins, including PE, are susceptible to becoming denatured in aqueous solution. Thermal stability can be improved by simply increasing the enzyme concentration or by adding to the reaction mixture other high molecular weight species, such as serum albumin, gelatin, polyalcohols or polyethylene glycol (PEG) or by immobilizing the enzyme (Aitken, 1993).

Enzymes that contain a non-covalently bound prosthetic group such as the heme in the PE are susceptible to inactivation. Heme dissociation in hemoproteins has been observed and may be a function of the pH (Aitken, 1993). HRP has been shown to function within a pH range of 4-10. SBP has demonstrated effective activity at a slightly different pH range of 2.5 to 9.0. When possible, buffering a solution to a desired pH will help avoid these issues.

A phase transfer of the PE involves the absorption of the enzyme to a polymeric oxidation product that is formed (entrapment) in the enzymatic process as is described with the oxidation of aromatics by HRP (Aitken, 1993). Again, the use of an additive that readily binds to the free radicals that are formed in the PE reaction (i.e. sacrotid compound such as PEG) and if possible, repels the enzyme itself can significantly reduce the rate of phase transfer. The use of PEG as a protective additive has been demonstrated using HRP and SBP (Nakamato and Machida, 1992; Wu *et al.*, 1993; Kinsley, 1998).

Excessive amounts of H_2O_2 have shown PE to exhibit a mechanism based (suicide) inactivation. In the absence of the usual electron donors (substrate), the PE/H_2O_2 system behaves as a catalase system where H_2O_2 acts either as an oxidant or as a reductant. Firstly, the reaction of the native enzyme with H_2O_2 yields C_I, with a rate constant $k_1 = 2x10^7\ M^{-1}*S^{-1}$ as shown in Figure 2.3. Following the formation of C_I, one of three pathways is possible; two catalytic pathways and one inactivation pathway. The first catalytic pathway (a) is described as a weak catalase process. A catalase (enzymatic) process is defined as the decomposition of H_2O_2 to oxygen and water by way of enzyme kinetics (Dunford and Stillman, 1976). In the second catalytic pathway (b), the excess H_2O_2 is used as a reductant instead of an oxidant and C_{II} is reduced to C_{III}. The catalytic pathway of the reduction of C_I by H_2O_2 can be carried out through either:

(a) a two-electron transference, yielding O_2 (catalase activity of peroxidase)
with a second order rate constant $k_2 = 5x10^2\ M^{-1}*S^{-1}$.; and

(b) a one-electron transference, with C_{II} formation and yielding superoxide anion

The formation of Compound III (C_{III}) from the reaction of C_{II} with H_2O_2 proceeds at a second order rate estimated as $k_5 = 25\ M^{-1}*S^{-1}$. In addition, C_{III} is known to decompose spontaneously to native peroxidase and the specific rate constant for this first-order reaction is $k_7 = 2.2x10^3\ S^{-1}$. Therefore, C_{III} is considered catalytically inactive because it does not react with a substrate. Also, because of C_{III}'s relatively slow rate of returning to the native enzyme, an accumulation of C_{III} can occur resulting in a loss in catalytic efficiency (Ibrahim *et al.*, 1997). The third pathway, the inactivation pathway, also occurs when excess H_2O_2 is present. The peroxidase is converted into an inactive verdohaemoprotein called P-670 at a rate of k_i (see figure 2.3). Therefore, when the

H_2O_2/reductant substrate (i.e. chlorophenol) ratio is high, based on the kinetic model and the given rate constants, H_2O_2 behaves as a typical suicide substrate of peroxidase leading off towards an inactivation pathway E_i (see figure 2.3). The system could not achieve steady-state and would evolve towards total enzyme inactivation. However, if this ratio is low, then both substances compete for C_I. In this case, the H_2O_2 loses out to a reductant substrate that has a higher rate constant. C_{II} is also reduced to the native enzyme by the same substrate. The catalytic cycle avoids both the formation of C_{III} and the enzyme inactivation pathway and follows the kinetics described in equations 2.1-2.3 as shown in Figure 2.3 (Arnao *et al.*, 1990).

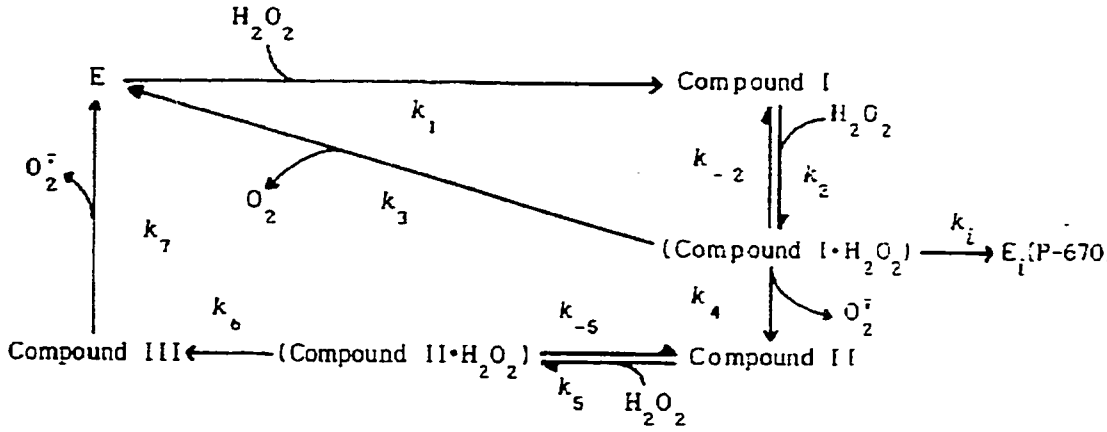


Figure 2.3: Catalytic pathways of peroxidase in the presence of excess H_2O_2
Source: Arnao *et al.*, 1990

Another mechanism of inactivation is irreversible inhibition. This is caused by an inhibitor that binds to the enzyme's active site, thereby preventing it from performing its oxidation process. This issue is important when considering using this PE process in mixed waste streams since there is the possibility that the mixed stream contains these inhibitors. Nicell and Wright (1997) used model parameters to compare the rate of substrate utilization for different peroxidases as well as their susceptibility to C_{III} formation. They listed three kinetic parameters to consider when comparing different peroxidases;

I) the rate of substrate utilization;

II) the rate of inhibition of peroxidase by H_2O_2; and

III) the rate of permanent inactivation of the enzyme.

Quantification of these kinetic parameters will aid in the understanding of the dynamics of the enzymatic treatment process and in the selection and design of a suitable reactor system. The empirical model developed by Nicell and Wright (1997) reflects the proportionality between the rate of formation of the colored products of the assay reaction and the active PE concentration.

2.1.5.2 Peroxidase Enzyme Kinetic Model

Buchanan and Nicell (1999), Buchanan *et al.*, (1998), Buchanan and Nicell (1998), Buchanan and Nicell (1997), Nicell and Wright (1997) and Nicell *et al.*, (1993) presented a series of publications which develop a model for the removal of aromatic substances from aqueous solutions catalyzed by peroxidase. The model is based on parameters obtained using spectrophotometric measuring techniques. Nicell *et al.*, (1993) first developed a kinetic model to predict the removal of 4-chlorophenol by HRP with respect to time in a batch reactor. While the model was suitable for 4-chlorophenol, it was not necessarily applicable to other substrates. Nicell indicated two shortcomings of his model; firstly, the model was based on the assumption that a fixed number of substrate molecules were removed per enzyme molecule inactivated and secondly, there was an over prediction of C_{III} formation. Nicell and Wright (1997) enhanced the model by modeling the inhibition of hydrogen peroxide on the peroxidase activity. The model successfully described the dependence of SBP and HRP activity over the full range of H_2O_2 and peroxidase concentrations studied. Buchanan and Nicell (1997) improved on the model. They considered that a steady-state model could not accurately predict time-dependent concentrations of substrates and PE in a reaction environment. Equations were developed and the result was a pseudo steady-state model represented by three differential equations and one mass balance which were solved by using the 4^{th} order Runge-Kutta method to simultaneously solve the set of equations. The model was calibrated and validated with several batch reactor tests. The final model is capable of predicting the removal of phenol within the range of 0.5-6 mM from a batch reactor and accounts for interactions with free radical formation and polymer entrapment. Buchanan and Nicell (1998) furthered the model by determining the kinetics of peroxidase interactions in the presence of a protective additive. Buchanan *et al.*, (1998) applied the model to both plug-flow (PFR) and continuous-flow stirred (CFSTR) reactors for phenol removal at

a pH of 7 and 25°C with and without an additive (PEG) present. Their work suggested the rate of PE inactivation in the presence of PEG is lower in a CFSTR than in a PFR for equal phenol removals. Simulations also indicated that for short hydraulic retention times (HRT), considerably more PE would be required by a CFSTR than by a PFR for equal phenol removals. The opposite was found to be true for a longer HRT. They also noted that more valuable enzymes would be wasted in a CFSTR than a PFR without a method to recover the enzyme. The latest version of their working model is a simplified kinetic model represented by the following equations (2.7 to 2.11):

$$[H_2O_2] = [H_2O_2]_0 - ([AH_2]_0 - [AH_2]) \qquad \text{Equation 2.7}$$

$$E_a = \frac{E_0 - E_{inact} - [AH_2]}{\frac{2k_a[AH_2]}{k_1[H_2O_2]} + 1} \qquad \text{Equation 2.8}$$

$$\frac{dE_{inact}}{dt} = k_r(E_0 - E_{inact})\sqrt{2k_a E_a [AH_2]} - k_e\left(\frac{d[AH_2]}{dt}\right) \qquad \text{Equation 2.9}$$

$$\left(\frac{d[AH_2]}{dt}\right) = -2k_a E_a [AH_2] \qquad \text{Equation 2.10}$$

$$\frac{dE_{iii}}{dt} = k_b[H_2O_2]E_a - k_c E_{iii} \qquad \text{Equation 2.11}$$

where E_o = concentration of initial PE;

E_a = concentration of active PE, considers C_{II} and C_{III} together

E_{iii} = concentration of C_{III}

E_{inact} = concentration of permanently inactivated PE

$[AH_2]$ = concentration of aromatic compound

$[H_2O_2]$ = concentration of H_2O_2

and the k values are the rate constants associated with the equations (Kinsley, 1998).

Several other researchers, Wu *et al.*, (1993) for example, have optimized certain aspects of the enzymatic process. The results of such analysis are empirical equations strictly based on experimental data and best fit . It is noted that such equations simplify the process and combine many assumptions. Wu *et al.*, (1993) developed two equations, one that represents the minimum amount of HRP required to treat phenol based on phenol concentration at optimum conditions (Equation 2.12) and an equation to determine the minimum PEG required to protect the HRP in the process (Equation 2.13). The equations are as follows:

$$[HRP]_{min} = 0.0435 + 0.0048C_{ph} + 0.0031\ C^2_{ph} \qquad \text{Equation 2.12}$$

$$[PEG]_{min} = 0.0065 + 0.024C_{ph} \qquad \text{Equation 2.13}$$

where C_{ph} is the concentration of the phenol in solution.

2.1.6 Other Peroxidase Enzyme Applications

Plant peroxidase enzymes may be used in a variety of different applications. PE extracted from horseradish plants are already used in "kits" to diagnose viral, bacterial, and parasitic diseases in the medical field (Raley, 1997). They are also used as indicators for food processing and as catalysts for phenolic resin synthesis (McEldoon and Dordick, 1996). Aitken (1993) proposes a list of five applications for the use of enzyme technology to:

1) pre-treat wastewater prior to biological treatment to remove the toxic and hazardous components;
2) remove specific contaminants from aqueous solutions when biological treatment is not feasible such as in ground water contamination; use enzymatic treatment of infrequently generated waste or isolated contamination;
3) use enzyme treatment as a post biological or polishing treatment; and
4) incorporate the plant enzymatic treatment procedure for low volume, high concentration wastewater.

The task of selecting an enzymatic process for the treatment of a waste presents a new set of rules to take into consideration. As with any process selection, safety, efficiency, and cost must be considered. Aitken (1993) also presents certain criteria that must be considered prior to the implementation of a peroxidase application. The following is a summary of those criteria:

- the products of the reaction must be less toxic, more biodegradable or otherwise

more amendable to subsequent treatment than the parent compound;

- the selectivity of target compounds must be verified in laboratory investigations;
- the enzyme must exhibit a reasonable amount of native activity under typical treatment conditions;
- the reactors for enzymatic processes must be relatively simple;
- the enzyme must be relatively stable under the reaction conditions; and
- the enzyme(s) of interest must be available commercially.

2.1.7 Potential Advantages of PE Treatment

The use of PE to remove phenols from wastewater has many advantages over conventional methods such as solvent extraction, microbial degradation, chemical oxidation and adsorption on activated carbon. These advantages include broad substrate specificity, effectiveness over a range of pH, temperatures and substrate concentrations (Klibanov *et al.*, 1983). The specificity of the enzyme process prevents undesirable or unnecessary reactions, which increase reactant consumption and therefore increase cost of operations. Enzymes combine the advantages of selectivity, simplicity, reliability, and predictability of conventional chemical treatment systems (Aitken, 1993). Enzymes can work in conditions that would normally be toxic to microorganisms. Use of enzyme treatment could result in a reduction in consumption of oxidants and a reduction in sludge volume. The enzyme process can also provide a more defined system, which leads to simpler process control (Bewtra *et al.*, 1995). A PE-based treatment could operate under milder, less corrosive conditions; operate in a catalytic manner, operate on trace level organic compounds and on organics not removed by existing chemical/physical process; and reduce the concentrations of adsorbent materials, such as charcoal, for disposal (Nicell *et al.*, 1992).

2.1.8 Potential Disadvantages of Enzyme Treatment

The primary drawback to using PE for industrial purposes is the amount of enzyme required to make a process viable. PE inactivation is postulated to be due to the formation of the phenolic polymers that potentially entrap the PE, thereby removing them from solution and deactivating them. Cooper and Nicell (1996) published a preliminary cost analysis comparing the use of purified HRP, crude HRP, and Iron or Fenton's Reagent (FR). Their results concluded

that the lowest cost estimate for the catalyzed process ($49.70/m^3 U.S., using crude extract and PEG) was still significantly higher than the cost of the Fenton process estimated at $8.10/m^3. Research is currently underway to determine methods of reducing PE inactivation by the addition of additives such as gelatin and polyethylene glycol (PEG) (Nakamoto and Machida, 1992). Conditions such as high organic waste concentrations may also prohibit the use of a strictly enzymatic approach. Conventional biological treatment is often the preferred choice for the treatment of medium to high concentrations of organic matter. Another condition that may not warrant using PE treatment, would be when a variety of pollutants is present in the waste (Aitken, 1993). These two examples show how the PE treatment processcould be used as a pre or post treatment to target specific contaminants. Also, previous studies have shown that the PE process does work in mixed streams and can even remove other non-target compounds in the precipitating polymers (Klibanov *et al.*, 1980).

2.1.9 Other Wastewater Treatment Methods

Examples of other methods used to remove phenol from wastewater include extraction, adsorption on activated carbon, steam distillation, bacteria and chemical oxidation, electrochemical, and irradiation. While not the focus of this work, because of similarities to the PE process, a brief description of some of these processes is provided. Chemical oxidation falls under a category similar to the peroxidase oxidation process called Advanced Oxidation Processes (AOP). AOP's have become very popular lately and a lot of attention and research is being devoted to them. Interestingly, one AOP known as Fenton's Reagent has many similarities to the PE treatment. Fenton's Reagent occurs when an iron ion Fe^{2-} is oxidized by H_2O_2 to Fe^{3+}. In the process powerful hydroxyl radicals (OH^-) are created with the ability to oxidize many substrates. When the substrate in question is an aromatic, the hydroxyl radical may cleave the benzene ring or may create a free radical. There is a possibility that these free radicals form with other free radicals to form polymeric oligomers just like the PE process. Other forms of AOPs include ozonation (O_3), ultraviolet (UV) and ultrasound (US) treatment and combinations of all four mentioned. The definition of an AOP is an oxidation process that generates hydroxyl radicals in sufficient quantity to affect water treatment (Trapido *et al.*, 1998). The degradation rate of 2,4-Dichlorophenol (2,4-DCP) was examined for various AOPs and prioritized in a list

of fastest to slowest. The fastest to slowest process was found to be as follows: $H_2O_2/Fe^{2+}/UV > H_2O_2/Fe^{2+} > O_3/US > O_3 \geq O_3/UV > UV/H_2O_2 \geq UV$. Photo-Fenton, ozonation and its combination with ultrasound was found to be a promising method of phenol destruction (Trapido *et al.*, 1998). Fenton's Reagent has also been studied for the treatment of various chlorinated phenols (Barbeni *et al.*, 1987).

Another method used to treat chlorinated phenols is bioremediation. Simply stated, bioremediation is the application of bacteria that have the capability to consume and destroy target contaminants. Valo *et al.*, (1990) reported successful treatment of simulated ground water contaminated with chlorinated phenols using bacteria immobilized on a polyurethane carrier. Immobilization is known to increase the tolerance of bacteria against the toxicity of chlorophenols and to allow for a longer storage without loss of activity.

2.2 Peroxidase Enzyme Treatment: Operating Ranges and Conditions

This section breaks down the PE treatment process into several subcategories. Most of the research in PE is still in the feasibility stage, meaning, that the PE treatment process is possible and researchers are attempting to make the process economically feasible for practical purposes. In order to do this, a clear understanding of the specific components of the process is required. With a plethora of information available for each component of the process, an attempt is made to draw out some of the more important issues.

2.2.1 Peroxidase Enzymes

Many kinds of PE (Class I, II, III) belonging to the peroxidase family have been tested for their applicability in treating various components of waste streams (Aitken 1993). As more research is performed in this field a set of benchmarks is required to determine the suitability of the numerous PE available. Nicell and Wright (1997) stated that peroxidase can be compared on the basis of kinetic parameters controlling;

1) the rate of substrate utilization;

2) the rate of inhibition of peroxidase by H_2O_2; and

3) the rate of permanent inactivation of the PE.

Rate constants will be dependent on temperature, pH, substrate properties and additives used. In general, HRP is used as the bench to which allother PE are compared.

2.2.1.1 SBP vs HRP

A comparison of the rate constants between HRP and SBP indicates that they have similar k_1 (rate at which native PE is converted to Compound I) values, but SBP appears to be more than an order of magnitude slower than HRP for the oxidation of phenol (Nicell and Wright, 1997). However, it should be kept in perspective that the catalytic process is still completed in a matter of minutes for both process and that a faster rate constant could just as well lead to faster inactivation. McEldoon *et al.*, (1995) have shown SBP capable of oxidizing veratryl alcohol (VA) in a pH range that HRP cannot (pH 2.4). They reported that soybean peroxidase can be classified as highly reactive and thermally stable compared to HRP. Again, this must be taken into context when considering what a standard pH range will be of a treatment process; pH 2.4 is on the rather extreme end. McEldoon *et al.*, (1996) furthered their claim by stating that SBP is a highly thermostable PE and its melting temperature of 90.5°C is highly atypical of HRP and microbial peroxidase. This could be the most significant finding. This phenomenon is attributed to the fact that SBP retains its heme much more tightly that HRP which therefore could imply that the SBP is more structurally sound and that a tightly bound heme aids in maintaining the protein catalytically active. SBP has also been reported to have a longer shelf life than HRP, which is one of the reasons why researchers are looking to replace HRP in medical diagnostic kits with SBP. Polymers formed in the SBP are also being considered for use in manufacturing computer chips, adhesives, car parts and varnishes. Currently; Rick Vierling of Ohio State University has cloned the peroxidase gene and plans to release a new variety of high peroxidase germ plant (Vierling, 1997).

Dec and Bollag (1994a,b) compared the dosages of HRP and H_2O_2 and their effect on the removal of 5.2 mM concentration of 2,4-DCP from an aqueous solution buffered at a pH of 5. Figure 2.4 illustrates the results obtained showing the effect of the HRP activity and the H_2O_2 concentration in the transformation of 2,4-DCP (5.2 mM) in a wastewater (pH = 5.0). It was determined that the optimal conditions for HRP and H_2O_2 dosages were 9.5 units/ml and 5.3 mM, respectively.

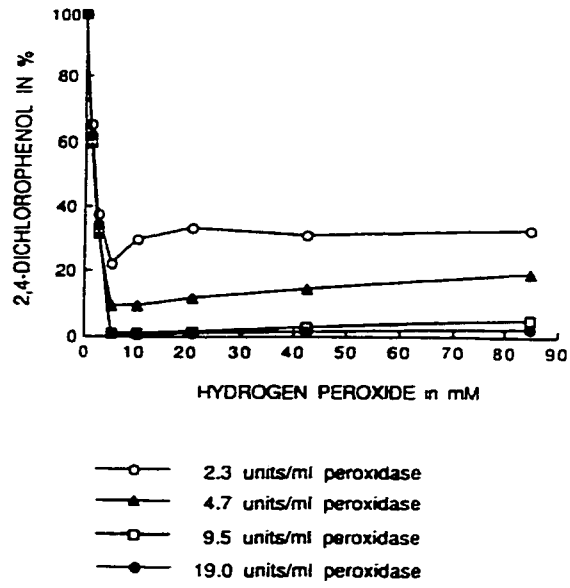


Figure 2.4: Effect of HRP and H_2O_2 concentration in the transformation of 2,4-DCP. Source: Dec and Bollag (1994a,b)

2.2.1.2 Crude/Purified PE

Peroxidase enzymes that have been isolated in pure form from their parent organisms are often preferred over intact organisms containing the enzyme because the purified enzymes act with greater specificity, their potency can be better standardized, they are easier to handle and store and the PE concentration is not dependent on growth rates and ecological conditions (Nicell *et al.*, 1993). Pure HRP or SBP may be purchased through a chemical supplier such as Sigma Chemicals with known activity.

The majority of the research reported using PE utilize purified PE purchased from chemical suppliers. However, several researchers have used crude PE extracted from other plant sources. Alberti and Klibanov (1982) showed that the HRP performance is independent of enzyme purity. However, evidence exists that indicates a lose of HRP activity occurs as the PE is purified. Tatsumi *et al.*, (1996) prepared crude HRP by grating 1 kilogram of horseradish and mixing it with 1 liter of water. The PE protein was precipitated by adding 5 liters of ethanol. The precipitate was then dissolved in 50 ml of water. This solution was desalted and concentrated

by ultrafiltration. Freeze drying produced 233 mg of purified PE powder. The purified activity was 72% of the first water extract (crude enzyme) and the peroxidase activity was 2.5 units/mg. Cooper and Nicell (1996) demonstrated that crude HRP achieved better total phenol removal than the pure HRP for the same PE concentration up to the point of maximum removal. It was suggested that the crude PE might be protected from inactivation due to the proteinaceous matter within the horseradish's root. Dec and Bollag (1994a,b) compared crude potato and white radish PE extracts to the purified enzyme. Crude potato peroxidase from continued to exhibit a significant detoxification potential when applied to wastewater polluted with chlorophenols. This report is an indication that PE isolation might not be necessary and that plant material might constitute inexpensive enzymatic agents for decontamination processes.

2.2.2 Turnovers

A method of describing the efficiency of the enzymatic process is by comparing turnovers. The catalytic lifetime of an enzyme is known as a turnover. "Turnovers" are defined as the number of substrate molecules precipitated from solution per molecule of enzyme consumed and is a measure of how many times the enzyme carries out its catalytic process before being inactivated (Nicell *et al.*, 1993). Figure 2.5 indicates that for HRP, the number catalytic turnovers is a function of pH. Turnovers can be calculated from a proportionality constant and the amount of substrate removed. The proportionality constant is a function of the substrate and the enzyme molecular weights and concentrations. To calculate turnovers for 2,4-DCP use equations 2.14-2.18 (assuming $MW_{SBP} = 44000$ which is HRP's molecular weight):

$$Turnovers = \frac{(Moles_S)}{(Moles_E)} = \frac{\left(\frac{C_S}{MW_S}\right)}{\left(\frac{C_E}{MW_E}\right)} \qquad \text{Equation2.14}$$

$$Turnovers = \frac{\left(\frac{Cs(mg/l)}{163g/mole}\right)}{\left(\frac{C_{SBP}}{44000g/mole}\right)} = 269.939\left(\frac{Cs}{C_{SBP}}\right) \qquad \text{Equation 2.15}$$

$$where \quad C_{SBP} = \left(\frac{Mass_{SBP}}{Activity_{SBP}}\right)\left(\frac{Unit_{SBP}}{ml}\right)\left(\frac{1000ml}{L}\right) \qquad \text{Equation 2.16}$$

$$\therefore C_{SBP} = \left(\frac{360mg}{25000units}\right)\left(\frac{Unit_{SBP}}{ml}\right)\left(\frac{1000ml}{L}\right) = 14.4\left(\frac{Unit_{SBP}}{ml}\right) \qquad \text{Equation 2.17}$$

$$\therefore Turnovers = 18.75\frac{(C_S(mg/l))}{\left(\frac{Unit_{SBP}}{ml}\right)} \qquad \text{Equation2.18}$$

where: C_S = the concentration of the substrate (mg/l)

C_E = the concentration of the enzyme (unit/ml), for SBP $C_E = C_{SBP}$

MW_S = the molecular weight of the substrate

MW_E = the molecular weight of the enzyme, for SBP $MW_E = MW_{SBP}$

$Mass_{SBP}/Activity_{SBP}$ provided by the enzyme producer

Therefore for SBP = 0.01 units/ml and 2,4-DCP = 100 mg/l removed, the maximum number of turnovers that can be achieved is = 187,500. The following table (2.1) provides an example illustrating how increasing the number of turnovers can decrease the cost of removal for 4-methylphenol. The calculations are based on data provided in Figure 2.5.

Table 2.1: Calculation of cost to treat water contaminated with 4-methylphenol.

(Based on Sigma 1998 data, Canadian currency, no tax)

4-methylphenol					
SBP Mass= 360 mg and **SBP Activity** = 25,000 units					
SBP Molecular Weight =44,000 g/mole					
Reactor Volume = 10,000 litres					
SBP Cost/unit =0.00886 $/unit (Sigma, 1998)					
Turnover	**(Unit/l)**	**Volume (l)**	**Units of SBP**	**Cost/unit**	**Total Cost**
10000	187.5	10000	1,874,574	$ 0.00886	$ 16,608.73
40000	46.87	10000	468,650	$ 0.00886	$ 4,152.24

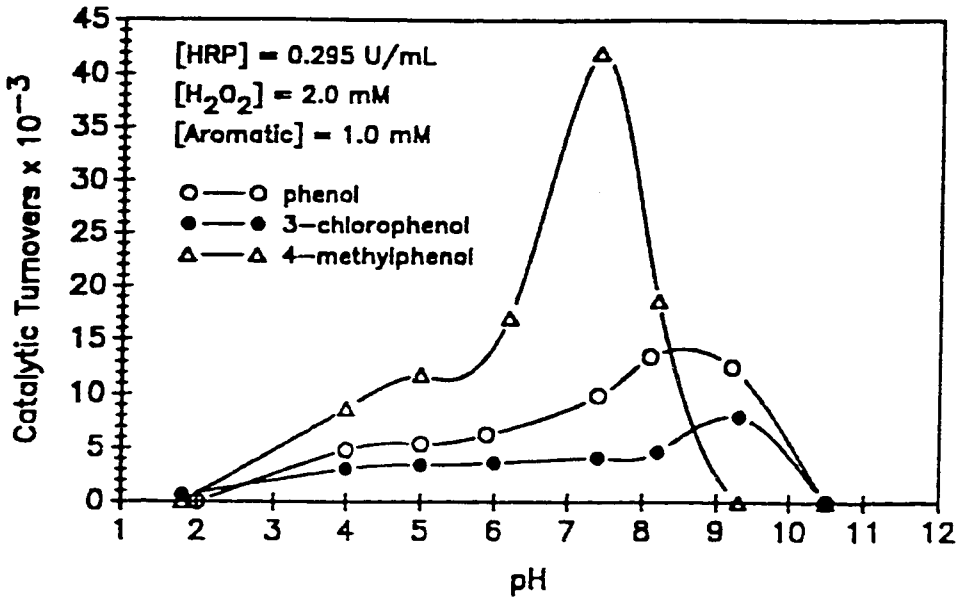


Figure 2.5: Catalytic turnovers accomplished in a batch reactor as a function of pH.

Source: Nicell *et al.*, 1992

2.2.3 Enzyme Enhancement and Genetic Engineering

Methods to increase the PE activity/yield include growth optimization to genetic engineering. Tests have been performed to increase the peroxidase activity within the carrot root via yeast added to the growth stage as a supplement to the cell. This method yielded promising results by increasing the activity from 5.0 units/g to 17.5 units/g without being toxic to the carrot cell (Kim and Yoo, 1996).

2.2.4 Application of PE Systems

results by increasing the activity from 5.0 units/g to 17.5 units/g without being toxic to the carrot cell (Kim and Yoo, 1996).

2.2.4 Application of PE Systems

PE has been used to treat a variety of pollutants or waste-streams which include the following: aromatic amines; bromophenols; chloromethylphenols; chlorophenols; methoxyphenols; methylphenols; phenol; bleach plant effluent; coal conversion effluent; and cotton mill effluent (Aitken, 1993). Klibanov *et al.*, (1980) used HRP to remove over 30 different phenols and aromatic amines (with varying efficiency) from water. It should also be noted that various concentrations of contaminant compounds have been tested for PE treatment. Both high and low concentrations of phenols have been tested. Wu *et al.*, (1993) tested initial phenol concentrations in the range of 1-10 mM (0.1-1.0 g/l) whereas Maloney *et al.*, (1984) effectively treated 2,4-DCP in a "drinking water" range of 0.0025 to 240 mg/l. The enzymatic process has shown to be effective in both high and low substrate concentrations. Ultimately, the efficiency of any PE system can be measured in terms of turnover (the number of contaminant moles treated per mole of enzyme consumed) as previously discussed. The PE system was also shown to indirectly treat water contaminated with PCB. Alone in solution, the PE did not treat PCB; however, when PCB was mixed into a stream containing a compound that is treated by PE such as phenol, the PCB precipitated to a certain extent due to entrapment in the forming polymers (Klibanov *et al.*, 1983). Therefore, a by-product of the PE system is the treatment of compounds that not catalyzed by the process alone.

2.2.5 Electron Donor: Hydrogen Peroxide (H_2O_2)

H_2O_2 is an essential element to the PE process. Several researchers have examined the relationship between the substrate and the donor concentration. Nicell *et al.*, (1992) described a one to one molar relation between the H_2O_2 and phenol as the optimum condition for eight phenolic compounds studied. Concentrations of H_2O_2 higher than 1 mM did not increase the efficiency of removal when using 1 mM of o-chlorophenol (Klibanov *et al.*, 1980). Nicell *et al.*, (1992) achieved 98% removal of 1 mM phenol from solution with 1.2 U/ml in 3 hours using 1 mM of H_2O_2. When the H_2O_2 was increased to 2 mM, over 12 hours was required to achieve the same removal. This difference in time was attribued to the formation of C_{III} (Arnao *et al.*, 1990).

In addition, the rate of color formation is significantly affected by the addition of extra H_2O_2 added to the assay. This coloration is a product of the reaction. Decrease in the rate of color formation was attributed to the inhibitory affect of H_2O_2 on peroxidase (Nicell and Wright, 1997). Wu *et al.*, (1994) observed the effect of batch and semi-batch additions of H_2O_2 on the removal of phenol in concentrations between 1-10 mmol/l. They concluded that the ratio of max H_2O_2 concentration to initial HRP concentration (HRP_o) controlled the removal rate of phenol in the presence of PEG. The optimum ratio $(H_2O_2)_{max}/(HRP_o)$ was reported to be between 10 and 25 μmol H_2O_2/unit HRP.

One of the major concerns related to the inactivation of the PE, is the formation of C_{III} and P-670 as described in the section 2.1.5. C_{III} , the catalytically inactive form of PE and P-670, the permanently inactivated form of PE, are formed by the presence of excessive H_2O_2. Therefore, appropriate control over the amount of peroxide used is necessary to prevent the formation of Compound III. Klibanov *et al.*, (1983) used a molar ratio of 2 mM H_2O_2 to 1.1 mM Phenol. Nicell *et al.*, (1992) showed how a 2:1 ratio increased the time required for a phenol reaction to go to 98% removal. They determined that a reaction that goes to 98% in three hours using a 1:1 ratio takes up to 12 hours to go to completion for a 2:1 ratio. No additional removal was observed when peroxide was present in excess or when the peroxide was added to the reaction mixture gradually. However, Nakamoto and Machida (1992) reported contradicting results and stated that phenol polymerization should be accomplished under controlled H_2O_2 addition, because the rate of H_2O_2 addition influenced phenol removal efficiencies. Wu *et al.*, (1993) also characterized the affects of H_2O_2 addition in an attempt to optimize reaction conditions. Their experiments concluded:

1) The ratio of $(H_2O_2)_{max}$ to $(HRP)_o$ has been found to be a crucial parameter for the phenol removal efficiency. The optimum range of this ratio is between 10 and 25 μmol/unit.
2) Correlations have been established to predict the values of $(H_2O_2)_{max}/(HRP)_o$ for any giving phenol concentration with different H_2O_2 addition modes over the phenol concentration range of 1 to 10 mmol/L.
3) For economic considerations, a H_2O_2 addition mode should be selected which makes a compromise between having the fastest reaction rate and having the minimum number

of aliquots additions.

2.2.6 Temperature

Experiments using phenols and HRP indicated that no loss of activity occurs between 5 and 35°C and that HRP is susceptible to rapid inactivation at temperatures above 45°C (Nicell *et al.*, 1992). Figure 2.6 shows that HRP is capable of catalyzing the polymerization reaction at 65°C. However, reactions should be carried out at temperature below 35°C to prevent significant thermal inactivation (Bewtra *et al.*, 1995). Nicell *et al.*, (1992) reported that optimal catalytic efficiency is be achieved at 5°C. This is an ideal temperature for a ground water treatment approach. SBP has a melting temperature of 90.5°C (McEldoon and Dordick, 1996). This is significant such that, SBP unlike HRP is more thermally stable and can be used at elevated temperatures. As previously mentioned, this characteristic is likely attributed to the fact that SBP holds on to its heme prosthetic group more strongly than HRP.

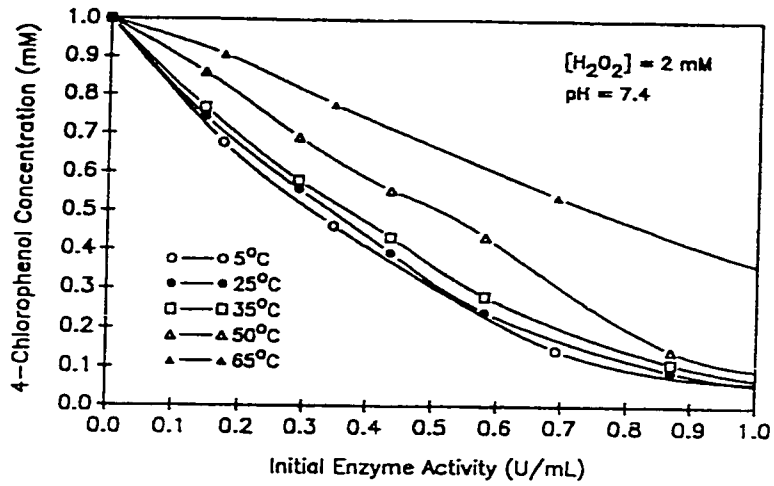


Figure 2.6: Removal of 4-chlorophenol as a function of temperature in a batch reactor Source: Bewtra *et al.*, (1995)

2.2.7 Reaction Kinetics

Wu *et al.*'s 1993 research with HRP allowed the reaction to proceed for 14 hours to go to completion. Other researchers have shown that the reaction is almost instantaneous and that up to 90% of a substrate can be removed in a matter of minutes. For instance, Dec and Bollag (1994a,b) demonstrated that maximum removal could be achieved in 15 minutes with the

majority of the removal occurring in the first 2 minutes. Kinetically speaking, the PE reaction proceeds at an extremely fast rate and samples analyzed with High Performance Liquid Chromatography (HPLC) can verify this. However, analytical methods using spectrophotometric techniques require the formation of color products to determine level of treatment. The formation of colour may not proceed as quickly as the reaction and a lag time may be required prior to analysis to determine the true extent of treatment. Formation precipitate also proceeds at a slower rate than the catalytic reaction. Allowing more time for particulate to form and precipitate out of solution is important when considering the method of filtration, sedimentation or binding. Figure 2.7 shows the transformation with time of 2,4-dichlorophenol (5.2 mM) by HRP (9.5 units/ml) and H_2O_2 (5.3 mM) in a 2,4-D (pesticide) wastewater at a pH of 5 and a temperature of 20°C. Figure 2.8 illustrates the difference in kinetics between peroxidase, lacase and tyrosinase. The graph shows that PE, in comparison to other enzymes, is extremely fast and effective reaching 100% removal of the 2,4-DCP in solution under 5 hours whereas the other PE reactions were incomplete at 25 hours. The HRT of the process is also a very important consideration when determining the size and setup of a reactor system. The PE process is fast and efficient regarding this concern.

Klibanov *et al.*, (1980) indicated that an increase in reaction time can offset the reduction in removal efficiency at lower PE concentrations. The same removal efficiency (>99.8%) was achieved for 1 unit/l of peroxidase over 3 hours and for 0.25 units/l over 24 hours. Wu *et al.*, (1993) also demonstrated that an increase in PE concentration decreased the reaction time significantly. Nicell *et al.* (1993) also working with HRP demonstrated that most of the 4-chlorophenol in a solution was removed in the first minute of reaction and that the removal rate slowed right down to a point where the reaction was considered finished after 3 hours. Cooper and Nicell (1996) also reported that a range of HRP (0.5-16 mM), reactions conducted in batch reactors were complete in significantly less than 3 hours. Recent experiments performed using SBP have shown similar results, in that, treatment of phenol was complete within the 3 hour time frame allotted for the reaction (Kinsley, 1998).

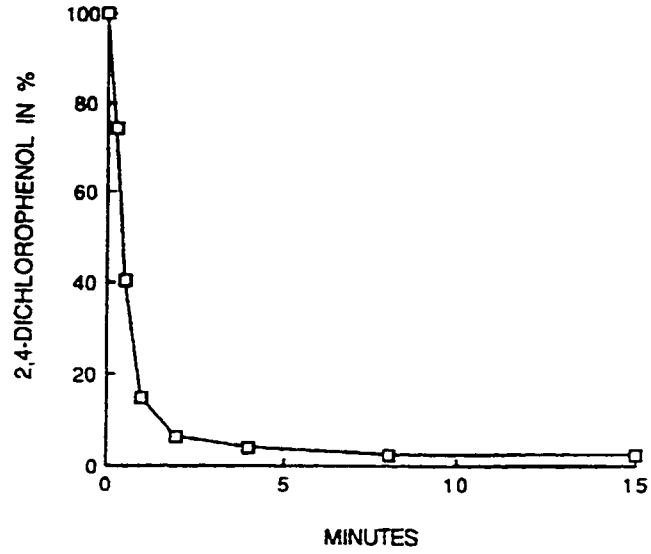


Figure 2.7: Transformation time of 5.2 mM 2,4-DCP by HRP in a 2,4-D wastewater. Source: Dec and Bollag (1994a,b)

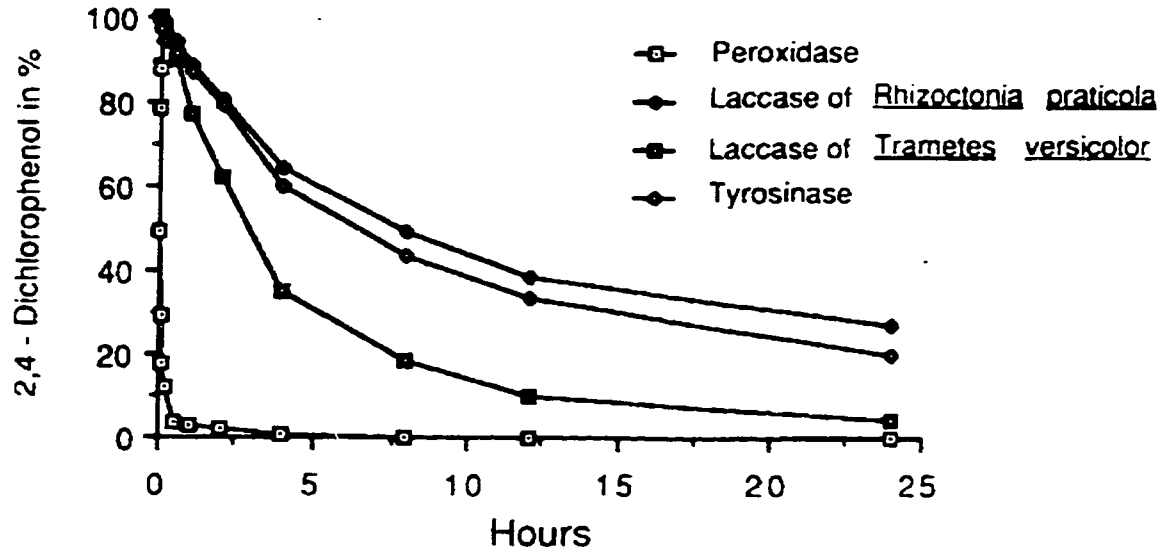


Figure 2.8: Effect of time of incubation on the transformation of 2,4-DCP by various enzymes. (HRP concentration was 0.05 units/ml at 25°C and pH 6.6 and 0.1 mM H_2O_2) Source: Dec and Bollag (1990)

At this point, it is worth noting that discrepancies in results reported regarding reaction rates could be directly attributed to the method of analysis. Samples analyzed using a colorimetric assay do not reflect the actual concentrations in solution at a given time because the rate of colour formation is not necessarily equal to the catalytic rate of the reaction (Kennedy, 1999). Researchers using more accurate techniques such as HPLC for analysis (Dec and Bollag, 1994a,b for example) have reported reaction rates and removal efficiencies in a matter of minutes from initialization with H_2O_2 whereas others using colorimetric methods have reported longer lengths of times. When carrying out a feasibility study to determine the size of a reactor and hydraulic retention time (HRT) required based on the reaction kinetics, the method of obtaining experimental data must be taken into consideration.

2.2.8 Effective pH Ranges

The pH of a mixture is an important environmental condition for the PE process. According to Wu *et al.* (1993), the operating pH range for phenol removal using HRP is 6 to 9 with an optimum around 8.5. When the additive PEG was in the solution, the optimum pH shifted to 8.0. Their studies illustrated that additives in solution can effectively change the optimum operating conditions. Similar studies by Bewtra *et al.* (1995) reported the operating pH range for phenol removal using HRP to be 4 to 10 with an optimum pH of 8.0 (see Figure 2.9). The reason for conflicting results with respect to Wu *et al.* (1993) is incomplete tests as seen in Figure 2.9. No tests were performed at pH 8.5 or 10.0 which would substantiate their conflicting claims. Nicell *et al.* (1992) determined the operating pH range of HRP to be 4 to 10 with a better removal within the range of 6 to 9 for eight different phenolics studied. Klibanov *et al.* (1983), reported that the optimum pH for removal of phenol from industrial aqueous effluent to be 9.0 with a working range between 3 to 12.

Dec and Bollag (1994a,b), determined that the working pH range for HRP in treating 2,4-DCP in a 2,4-D (insecticide) wastewater was between 3 and 10 (see Figure 2.10). At pH 2, there was very little removal of 2,4-DCP and at pH 11 there was no removal. In previous experiments, Dec and Bollag (1990) reported that the substrate being catalyzed had an effect on the optimum pH of the reaction (see Figures 2.11 and 2.12). They compared the effective pH for four different enzymes when treating 4-chlorophenol (4-CP) and 2,4-DCP. In Figure 2.11, the optimum pH for

the treatment of 4-CP with HRP was approximately 8.3 while for the treatment of 2,4-DCP in Figure 2.12, the optimum pH was approximately 6.5. A possible explanation for the different optimum pH for different substrate lies within the structure of the enzyme. A change in pH could potentially change the folding of the PE structure resulting in various active site configurations. Certain configurations would have more specificity towards particular substrate.

In Figure 2.10, the source of the 2,4-DCP mixture is a 2,4-D wastewater and in Figure 2.12 the source is a synthetic mixture prepared in the lab with no other compounds present. Differences in results may be explained by the interaction of other compounds present in the mixture. Therefore, it can be stated that not only does the target compound have an effect on optimum pH, but so do other compounds present in the mixture.

Results from reactions with other enzymes (lacase and tyrosinase) are presented for comparison in Figures 2.11 and 2.12. The results indicate that further to the above mentioned conditons, the optimum pH when treating the same substrate is also specific to the enzyme being used. For this reason, it is possible that peroxidase from different sources (for example SBP) may have different optimum pH as well.

McEldoon *et al.* (1995) found that SBP efficiently catalyzed the oxidation of veratryl alcohol at a pH of 2.4. They also discovered that SBP retains more then 50% of its intrinsic activity at a pH 3 unlike HRP. Their results tend to indicate that SBP is more resistant to acidic condition and holds onto the heme more tightly than HRP does. Information regarding the effects of various pH on the SBP treatment process is limited. SBP studies to date have use the pH optimum based on previous HRP results (Kinsley, 1998). However, results from McEldoon *et al.*, (1995) suggested the SBP functions in a lower pH range than HRP. Based on these findings, more information is required to determine the effective pH range for SBP in treating various substrate.

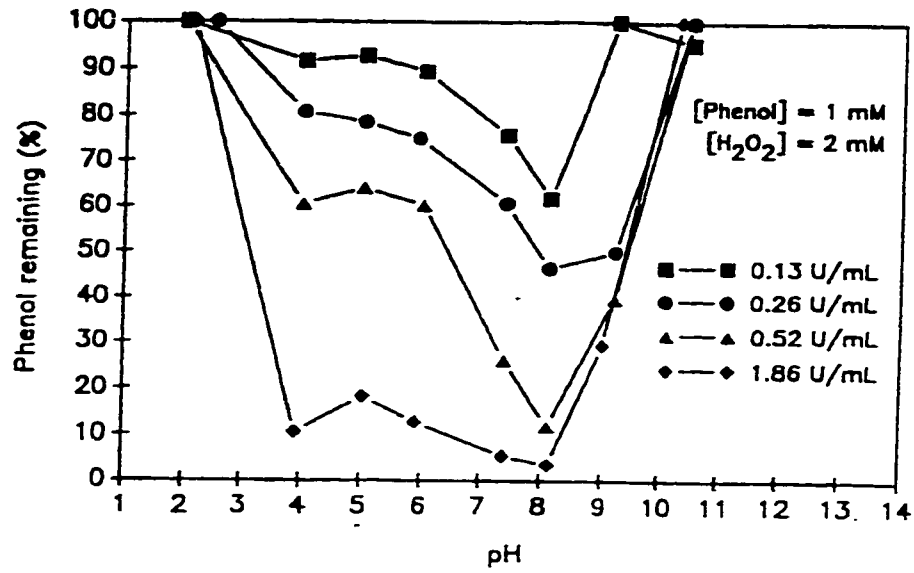


Figure 2.9: Effect of pH on the transformation of phenol by HRP.
Source: Bewtra *et al.* (1995)

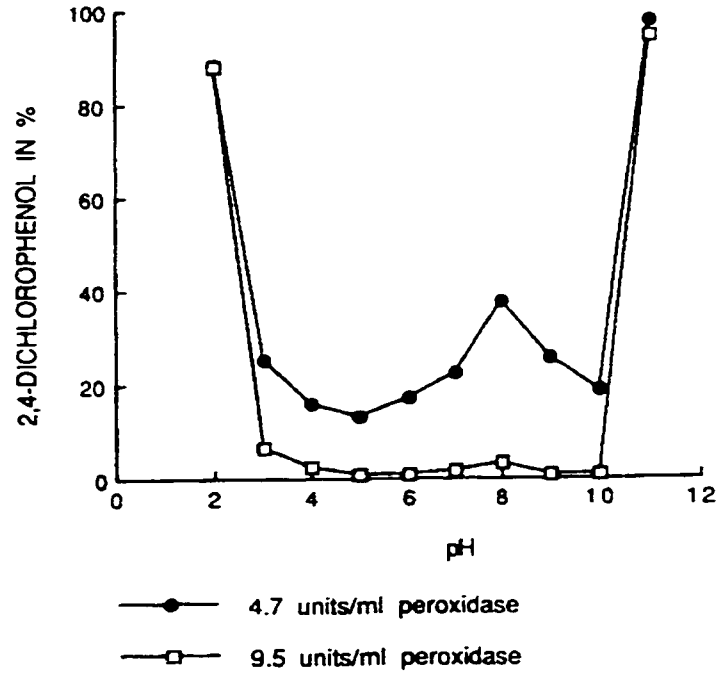


Figure 2.10: Effect of pH on the transformation of 2,4-dichlorophenol (5.2 mM) by HRP in 2,4-D wastewater.
Source: Dec and Bollag (1994a,b)

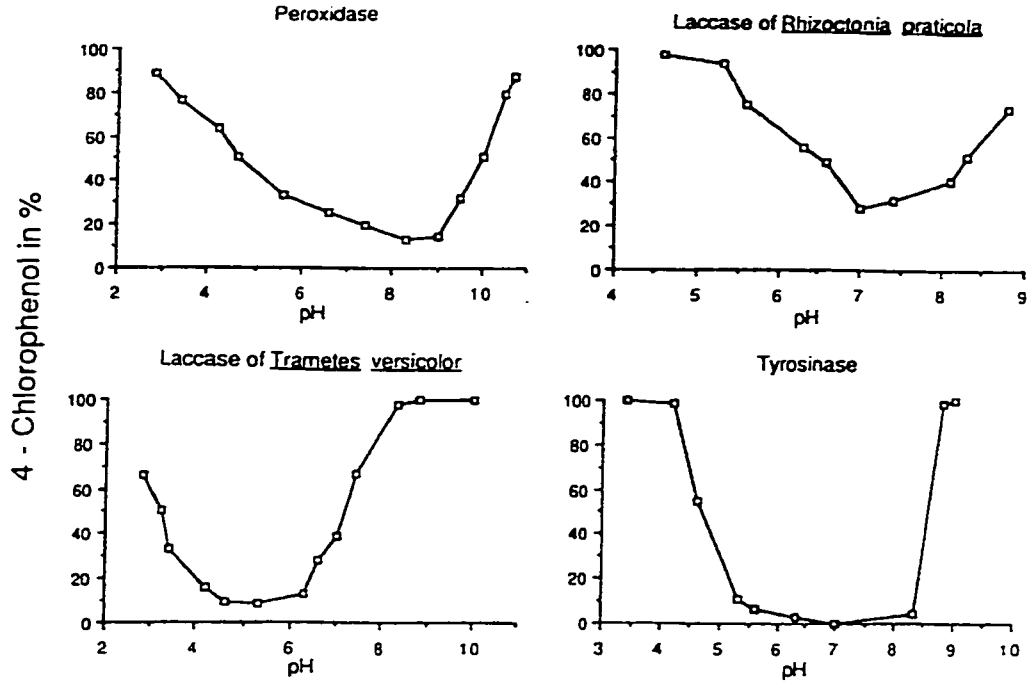


Figure 2.11: Effect of pH on the transformation of 4-chlorophenol by various enzymes.
Source: Dec and Bollag (1990)

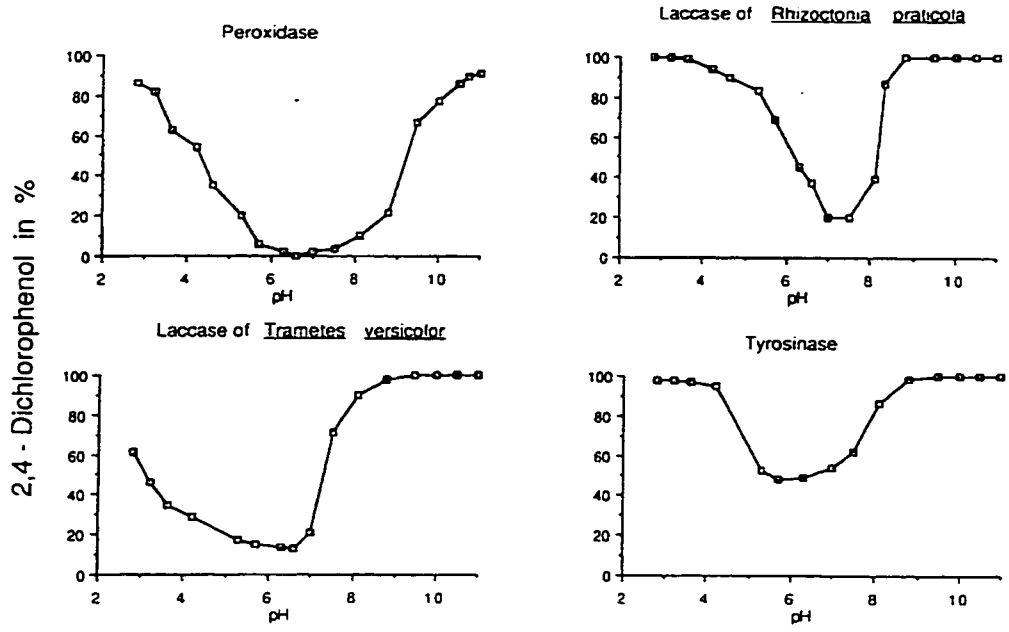


Figure 2.12: Effect of pH on the transformation of 2,4-dichlorophenol by HRP and other enzymes.
Source: Dec and Bollag (1990)

2.2.9 Buffers

Buffer solutions used to control the pH can vary. For example, Nakamoto and Machida (1992) used a buffer solution which has a buffer action between pH 4-10. Alternatively, Wu *et al.*, (1993), prepared several buffer solutions with various buffer capacities (see Table 2.2).

Table 2.2: Buffer solutions and their pH ranges.

Buffer Name	pH range
Acetic acid - sodium acetate	4 to 5
Sodium Phosphate	6 to 8
Boric Acid - Borax	8.0 to 8.5
Sodium Carbonate-bicarbonate	9 to 10

Source: Wu et al., (1993)

Nakamoto and Machida (1992) reported borate to have a protective effect on HRP activity but warned against the use of it as a buffer for wastewater because of its insecticidal properties. Wu *et al.* (1993) also reported that borate is a protective reagent for HRP activity and that less PE concentration was needed to obtain 95% removal of a 1 mM phenol when borate was present. It is important to consider the actions of the buffer reagents with the PE activity. They also reported that borate is not a practical additive because high concentrations were required to provide sufficient protection to increase PE activity leaving a high residual borate concentration in solution after the reaction. Buffers capable of providing an PE with protection from inactivation will ultimately affect the results and their influence on the PE reaction must be considered when analyzing test results.

2.2.10 Scale up and Mode of Operation

The cost and the concentration of PE necessitates the use of small reactor volumes for laboratory experiments. For example, Dec and Bollag (1994a,b) determined that the treatment for a 5.2 mM 2,4-DCP concentration without using an additive, the optimal HRP concentration is 9.5 unit/ml . Using a 1 l reactor, 9500 units would be required for treatment of 2,4-DCP at this given concentration. Using Type II HRP, supplied by Sigma (Canada), results in a total enzyme cost for a single run equal to $17.80/l (Sigma, 1998). Therefore, most experiments are performed in small reactor volumes so that costs are minimized. Wu *et al.*, (1993) used 30 ml batch reactors

at room temperature. Dec and Bollag (1994a,b) used 5 ml samples to determine optimum operating conditions and than proceeded to test a limited number of 1 l samples. Nakamoto and Machida (1992) performed their experiments in 50 ml reactors. The effect of scale up on the PE activity and performance can only be speculated at this time.

2.2.11 Mode of Enzyme Addition

Mode of reactor operation is a very important aspect of the enzymatic process. Klibanov *et al.* (1983) observed that removal efficiency of batch reactions was higher when the PE was added incrementally rather than all at once. Table 2.3 presents the results Nicell *et al.* (1992) obtained comparing the catalytic turnovers in batch reactors to the turnovers in CSTRs for phenol and 4-CP. They reported similar results to Klibanov *et al.* (1983) when comparing batch, semi-batch and continuous flow reactors. Their results indicate that the catalytic turnovers achieved in a continuous stirred tank reactor (CSTR) are higher than those observed in both batch reactors and semi batch when sufficient retention time is provided.

The method of applying the PE (batch, semi-batch or continuous) and the method of catalyzing the PE are important. Ideally, the longer an enzyme is in contact with the target substance and harsh conditions such as extreme pH, the greater the chance of loss of PE efficiency. Most researchers mix the PE in solution with the contaminant before H_2O_2 is added to initiate the reaction. This method provides an incubation period under potentially bad conditions (McEldoon *et al.* 1995). Experimental methods should address how PE and H_2O_2 are added to the process as this can influence final results.

Table 2.3: Catalytic turnovers in Batch and Continuous Stirred Tank Reactors. Source: Nicell *et al.*, (1992)

Aromatic substrate	Reactor type	Retention time (minutes)	Catalytic turnovers
Phenol	Batch	> 180 (complete)	8 500
	CSTR	9.8	5 600
		20.6	7 200
		31.5	8 200
		126.	10 900
4–Chlorophenol	Batch	> 180 (complete)	9 300
	CSTR	8.1	8 600
		17.9	9 400
		28.2	10 100
		126.	16 000

$[Aromatic]_0 = 1.0$ mM $[H_2O_2]_0 = 1.0$ mM $[HRP]_0 = 0.4$ U/mL pH = 7.0

2.2.12 Mixing

Klibanov *et al.*, (1983) reported that PE reactions proceeded with or without mixing. The removal efficiency was independent of whether the mixture was stirred, shaken, or left undisturbed. However, mixing the sample was preferred and Teflon coated magnetic stirrers were often the choice for most researchers. HRP enzymes are hemoproteins containing an iron ion. Dunford and Stillman (1996) discussed the potential magnetic susceptibility of certain PE. Furthermore, Tatsumi *et al.* (1996) performed experiments using HRP immobilized on magnetic particles. Therefore, it may be appropriate to question the extent of the magnetic properties of the Teflon coated stirrers and their affect on the process. Potentially, the PE may become immobilized on the stirrers. Agitations methods using shakers instead of magnetic stirrers would provide suitable mixing yet prevent any misinterpretation of results due to unexpected immobilization.

2.2.13 Chemical Enhancers

The process of using PE to treat a waste stream containing a specific contaminant has been demonstrated in relatively small reactor volumes as previously mentioned. However, the economies of scale indicate that the process is not viable at this moment because of the cost of

the process, primarily due to the cost of the PE. Inactivation of the PE by entrapment within the polymers that are formed and precipitated increase the PE requirement of the process. In order to reduce the cost, PE entrapment within the polymers may be prevented by several methods. Reaction efficiency can be improved by adding other higher molecular weight species (waste products would be ideal), such as serum albumin, gelatin, polyalcohols or polyethylene glycol (PEG) to the mixture to protect the PE. Nakamoto and Machida (1992) reportedly studied milk casein, bovine serum albumin and polyvinyl alcohol but did not publish the results.

2.2.13.1 PEG

Nakamoto and Machida (1992) reported that the amount of HRP required was reduced by 200 fold by using additives, such as gelatin and PEG, when working with phenol concentrations of 10 to 30 g/l. The presence of hydrophilic additives such as PEG that have a greater affinity than HRP for hydroxyl groups on the growing polymers, prevent the PE from being entrapped. In essence, the phenoxy radicals created in the process attach to these additives instead of attaching to the active site of the PE, allowing for a more efficient treatment process. PEG is a nontoxic organic compound that has ben declared fit for human consumption, however it does increase the organic content which results in higher BOD and COD levels in the final effluent (Harris, 1992). PEG is available in a variety of sizes with increasing molecular weight (MW). Nakamoto and Machida (1992) reported that PEG with a MW greater than 1000 g/mol is most effective for enhancing HRP activity. PEG is identified by its molecular weight, therefore; a PEG with a molecular weight of 3350 g/mole is described as PEG 3350.

Table 2.4: Effect of PEG average molecular weight on peroxidase activity in supernatant.

PEG average molecular weight	% Residual peroxidase activity
200	1.5
300	1.3
400	1.3
600	62
1000	92
7500	97
20,000	97

Source: Nakamoto and Machida (1992)

Table 2.4 presents the effect of PEG molecular weights on HRP activity. Wu *et al.*, (1993) demonstrated a reduction in the HRP requirement of 40 and 75 fold for treatment of 1.0 mM and 10.0 mM of phenol respectfully with a PEG concentration of 0.1 to 1.0 g/l. They confirmed Nakamoto and Machida's work (1992) which indicated the best PEG concentration to use was 4 g/l as shown in Figure 2.13. They also noted that an increase in PEG concentration did not increase the reaction time of the process. However, they used colorimetric methods to obtain reaction kinetics information. Formation of colour by-products may take longer to form in a solution containing PEG than in a solution without PEG. Factors such as this must be taken into account when reporting such reaction rate data.

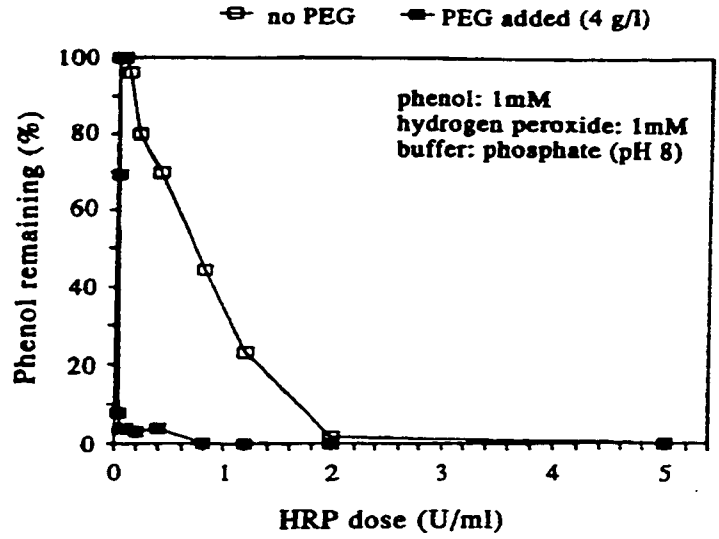


Figure 2.13: Effect of HRP dose on phenol removal in the presence of PEG. Source: Wu *et al.*, (1993)

Cooper and Nicell (1996) reported on the problems of using PEG 3350 as an additive when treating complex wastewater. They tested the effectiveness of PEG 3350 as an additive in foundry wastewater versus synthetic foundry wastewater mixture. They reported that the PEG interacted with other organics in the complex foundry wastewater, thereby limiting the effectiveness of PEG 3350 to protect the HRP from inactivation when compared to the effectiveness without PEG present.

2.2.13.2 Gelatin

Using gelatin as an additive for PE wastewater treatment is limited since gelatin contains 18% nitrogen by weight and its effluent is undesirable for the environment (Cooper and Nicell, 1996). However, Nakamoto and Machida (1992) reported that gelatin may be the most suitable additive for actual applications because it hardly leaves any residual in treated water and they also reported that gelatin was less pH dependent the PEG as shown in Figure 2.14. However, their comparison of gelatin was with PEG 1000 which is the least effective MW for protecting PE. The PEG requirement can be optimized by varying the PEG MW and concentration, whereas gelatin is limited to change in concentration. It is possible that increasing the PEG MW can

decrease the actual amount of PEG required.

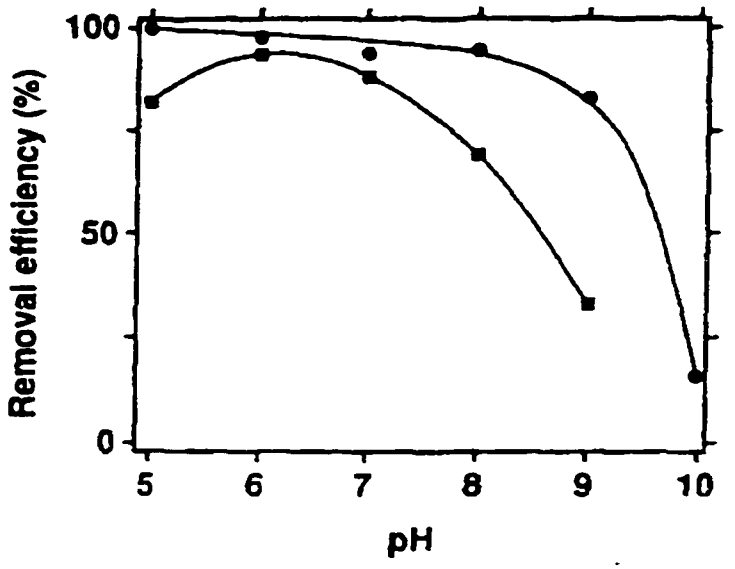


Figure 2.14: Phenol Removal efficiency dependance on pH. Reactions were carried out in GTA buffer containing 10 g/l phenol and 10 mg/l peroxidase. Gelatin (circles) and PEG 1000 (squares) were added at a concentration of 4 g/l.
Source: Nakamoto and Machida 1992

2.2.13.3 Other

Additives may also be used to speed up the sedimentation process once the precipitate is formed. The addition of a coagulating substance, alum, was performed by Cooper and Nicell (1996) to help the sedimentation process. Iron catalyzed oxidation, also known as Fenton's Reagent was investigated using ferrous ammonium sulphate spiked PE mixtures. The presence of iron did not contribute to the removal of phenol nor the consumption of H_2O_2. Tests revealed that even with other organics and trace amounts of iron, 99% of the phenols was removed.

Borate, used mainly to buffer solutions to a desired pH, has potential for increasing the efficiency of the reaction (discussed previously). However, it is undesirable to use borate based on environmental concerns because of the compound's insecticidal properties.

To control the process in time-dependent studies, catalase enzyme can be used to halt the PE activity by using all the H_2O_2. Catalase has the ability to rapidly convert H_2O_2 to oxygen and water. Without any H_2O_2 present in solution, the HRP oxidation process cannot continue.

2.2.14 Immobilized Enzymes

Several researchers have used enzyme immobilization techniques to prevent PE from being trapped and inactivated in the insoluble polymer that forms. There are several methods by which immobilization may be performed: physical adsorption, covalent bonding, crosslinking, inclusion, and encapsulation. Siddique *et al.*, (1993) compared the HRP activity bound on cellulose filter paper, nylon balls, and nylon tubing in the removal of 4-chlorophenol from solution and found greater than 80% removal efficiency for all three matrices. However, they did not present any control results. The data presented are lacking and inconclusive in terms of revealing an increase or decrease in activity when compared to tests when there was no immobilization technique attempted. Tatsumi *et al.*, (1996) showed that HRP immobilized by physical adsorption on magnetite retained 100% of activity whereas using a cross-linking method immobilized only 18% of the HRP and retained only 52% of the HRP activity. However, there was a concern that PE activity may be due to either a bound enzyme or a desorbed enzyme so overnight tests were performed in pure water and showed that no peroxidase was desorbed. They also performed a test in a phosphate solution and reported that 44% of HRP was desorbed back into solution. They continue their discussion without reiterating (or referring) to the fact that their experiments were performed at pH 7 using a phosphate buffer. They also reported that crude peroxidase prepared from horseradish could be immobilized by simple adsorption and thereby potentially removing the need for the PE to be purified. Results of using soluble HRP and immobilized HRP to treat various chlorinated phenols are presented in Table 2.5. With immobilization, the chlorophenols were removed close to 100% from solution. Whereas without immobilization, the results varied from 97% removal for 2,4,6-TCP to only 36% for 2,4,5-TCP. However, the amount of HRP used for all tests remained constant at 0.2 units/ml and this removed 97% of 2,4,6-TCP without immobilization. Therefore, increased removal efficiency was not identified because the HRP was not limiting. Figure 2.15 compares the results of phenol removal with various HRP immobilization techniques. Without using an immobilization technique, only 50% percent of the phenol in solution was removed compared to 75 and 100% for crosslinking and adsorption methods respectively at pH 7 (using phosphate buffer). They also reported that coloured products (formed during the reaction) were removed when phenol was

treated by immobilized HRP. Physical adsorption of peroxidase on magnetite particles offers the advantage of easily separating the precipitate through the use of magnetic devices. Other advantages of using magnetite include removal of colored products, total organic carbons (90%), and absorbable organic halogens (90%) (Tatsumi *et al.*, 1996).

Table 2.5: Removal of chlorophenols from model wastewater by soluble and immobilized HRP.

Chlorophenol	Initial concentration (μM)	Soluble peroxidase (%)	Immobilized peroxidase (%)
p-chlorophenol	200	58	100
2,4-dichlorophenol	200	82	100
2,4,5-trichlorophenol	100	36	99
2,4,6-trichlorophenol	100	97	98
2,3,4,6-tetrachlorophenol	20	81	99
pentachlorophenol	10	55	97

Source: Tatsumi et al, 1996

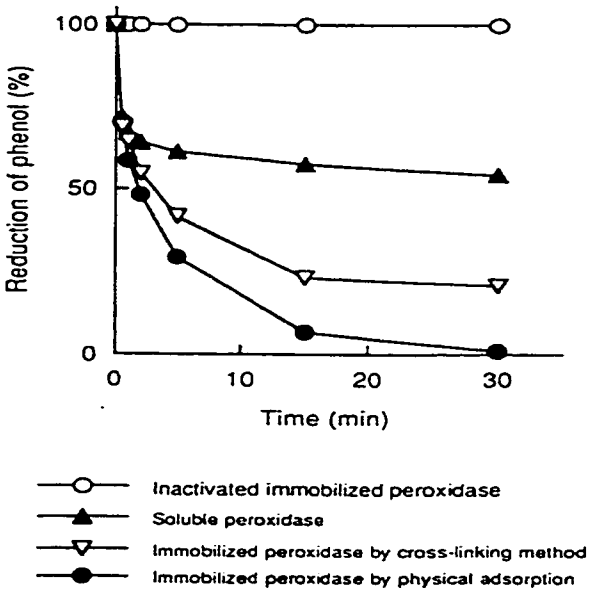


Figure 2.15: Reduction of phenol by soluble and immobilized HRP in the presence of H_2O_2. Source: Tatsumi *et al.*, 1996

2.2.15 Mix Streams vs. Pure Streams

Some of the first PE work performed with complex mixtures examined the treatment of a coal production wastewater which consisted of phenols, amines, and PCBs (Klibanov *et al.*, 1983). Klibanov demonstrated that pollutants such as PCB's that are unreactive with peroxidase can be precipitated if the waste contains other pollutants that are readily removed by the PE system. Cooper and Nicell (1996) examined a foundry wastewater containing 3.5 mM of total phenols (330 mg/l as phenol). They determined that the amount of HRP required to treat the foundry wastewater was in the same range as for treatment of simple synthetic foundry wastewater with the same phenol concentration (no organic compounds or iron in the matrix). This shows that the PE treatment is suitable in even complex streams with multiple and variable compounds and suspended solids (SS) are present.

One of the advantages of the enzymatic process is the environmental condition under which enzymes can operate. SS in foundry wastewater neither enhanced nor detracted from the enzymatic process. Therefore, it was concluded that the SS concentration has no effect (Cooper and Nicell 1996). However, it has been discussed previously that wastewater pretreatment with different additives can greatly improve the overall efficiency of the PE treatment process.

2.3 Reaction Products and Observations

PE catalyzes the oxidation of a variety of organic and some inorganic compounds by H_2O_2 and other peroxides. Oxidation of an electron donating substrate by H_2O_2 involves the overall transfer of two electrons, but most reactions catalyzed by HRP and other peroxidase occur in sequential one electron steps. A single electron oxidation of phenol results in the formation of a phenoxy radicals which may diffuse from the PE active site and undergo a variety of post-enzymatic reactions in aqueous solution. Products from such reactions include quinones resulting from the loss of another electron from the phenoxy radical and coupling products resulting from the reaction of two phenoxy radicals, leading to the formation of dimers and higher molecular weight oligomers (Aitken *et al.*, 1993).

2.3.1 Polymerization

As previously explained, the PE oxidized substrate forms insoluble polymers that precipitate out of solution. The polymers that form have been suggested for use in other applications. Minard *et al.*, (1981) studied the lower molecular weight reactive intermediates formed initially by oxidation and by oxidative coupling of 2,4-DCP with a phenol oxidase from the fungus *Rhizoctonia practicola.* Using a mass spectrometric analysis, oligomers up to the pentamer were identified including two dimeric quinones. Dec and Bollag (1990) determined that the precipitates formed during the polymerization of 2,4-DCP constituted a mixture of oligomers with average molecular weights of up to 800. Dordick *et al.* (1986) tested the properties of larger polymers formed from the reaction of HRP and various phenols. To overcome low substrate solubilities and product molecular weights in water, aqueous organic solutions were used. Water mixed with water-miscible solvents up to 95% were tested. The solvents included dioxane, acetone, dimethylformamide, and methyl formate. The resulting polymers had molecular weights that varied from 400 Daltons (D) to 26,000 D. This method enzymatic treatment of chlorinated phenols in organic solution was proposed as an alternative to producing phenolic resins with formaldehyde. The properties of the phenolic polymers formed using PE suggested that these polymers may be useful as thermoresistant resins containing no formaldehyde or as conductive polymers.

2.3.2 Colourization

Treatment of chlorinated phenols using the PE system produces colour which, in some cases, may be undesirable. Addition of 1 mM of H_2O_2 and 1 unit/ml of HRP to a solution containing 0.1 g/l o-chlorophenol (o-CP) turned the solution dark brown, followed by a gradual production and separation of a brown precipitate. Removal of this precipitate using centrifugation resulted in a colorless solution. 99.8% o-CP removal efficiency was obtained within 3 hours (Klibanov *et al.* 1980). Alum and charcoal have been used to remove the colour that forms through this process. However, alum should be used only when the enzymatic reaction is complete since the PE is immobilized on the alum and removed once the alum is filtered (Ibrahim *et al.*, 1997).

2.3.3 Dehalogenation

Dehalogenation of chloride ions was observed in the transformation of several chlorophenols using HRP, laccase, and an inorganic catalyst birnessite (Dec and Bollag, 1994a,b) . It was determined that the rate of dehalogenation was correlated with that of substrate removal. The dehalogenation number (DN) of a process is equated by determining the percentage of substrate transformed divided by the amount of chloride ions released into solution and by the number of chlorine atoms on the benzene ring. The substrates which they studied included 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, and 2,4,5-trichlorophenol (see Table 2.6). The highest transformation percentage attained was 94% for 2,4-DCP. The percentage of chloride ions released in the process was 24%. This resulted in a dehalogenation number of 2.0. The results were obtained by using 5 ml volume with 9 mM of substrate incubated at 25°C at pH 5 with 4 units/ml HRP and 9 mM H_2O_2.

Table 2.6: Percent transformation (%Tr) and dehalogenation (% Cl) of chlorophenols by an oxidoreductive enzyme (HRP) and the respective dehalogenation numbers (DN):

Chlorophenol	% Tr	% Cl	DN
2-Chlorophenol (2-CP)	14.0	2.3	6.1
3-Chlorophenol (3-CP)	10.0	0.2	50.0
4-Chlorophenol (4-CP)	20.0	6.9	2.9
2,4-Dichlorophenol (2,4-DCP)	94.0	24.0	2.0
2,4,5-Trichlorophenol (2,4,5-TCP)	83.5	12.5	2.2

Source: Dec and Bollag, 1994a,b

Figure 2.16 illustrates the hypothesized free radicals that form during the oxidation of 2,4-DCP. As shown, there are 10 possible combinations. Seven of these combinations involve free radicals with a chlorine atom attached to the aromatic carbon carrying the unpaired electron. The coupling of these radicals should result in the release of the chloride ion Three of these seven combinations involve coupling between two radicals that have chlorine atoms attached to a carbon with the lone electron. In this case, the release of both chlorines can be expected. The

remaining three combinations involve coupling between radicals that do not have chlorine atoms attached to the carbon-with the unpaired electron, and these reactions should not produce chloride ions. In summary, 10 chloride ions were released from 20 molecules which were transformed. The DN for this model is 20/10 = 2, which is general agreement with the results obtained in Figure 2.7 (Dec and Bollag, 1994a,b). Though this model describes the early stages of the enzymatic process, it does not characterize the later stages of polymerization. A mathematical expression (Equation 2.19) was developed to calculate the number of substrate molecules (M) involved in the formation of oligomers up to the pentamer.

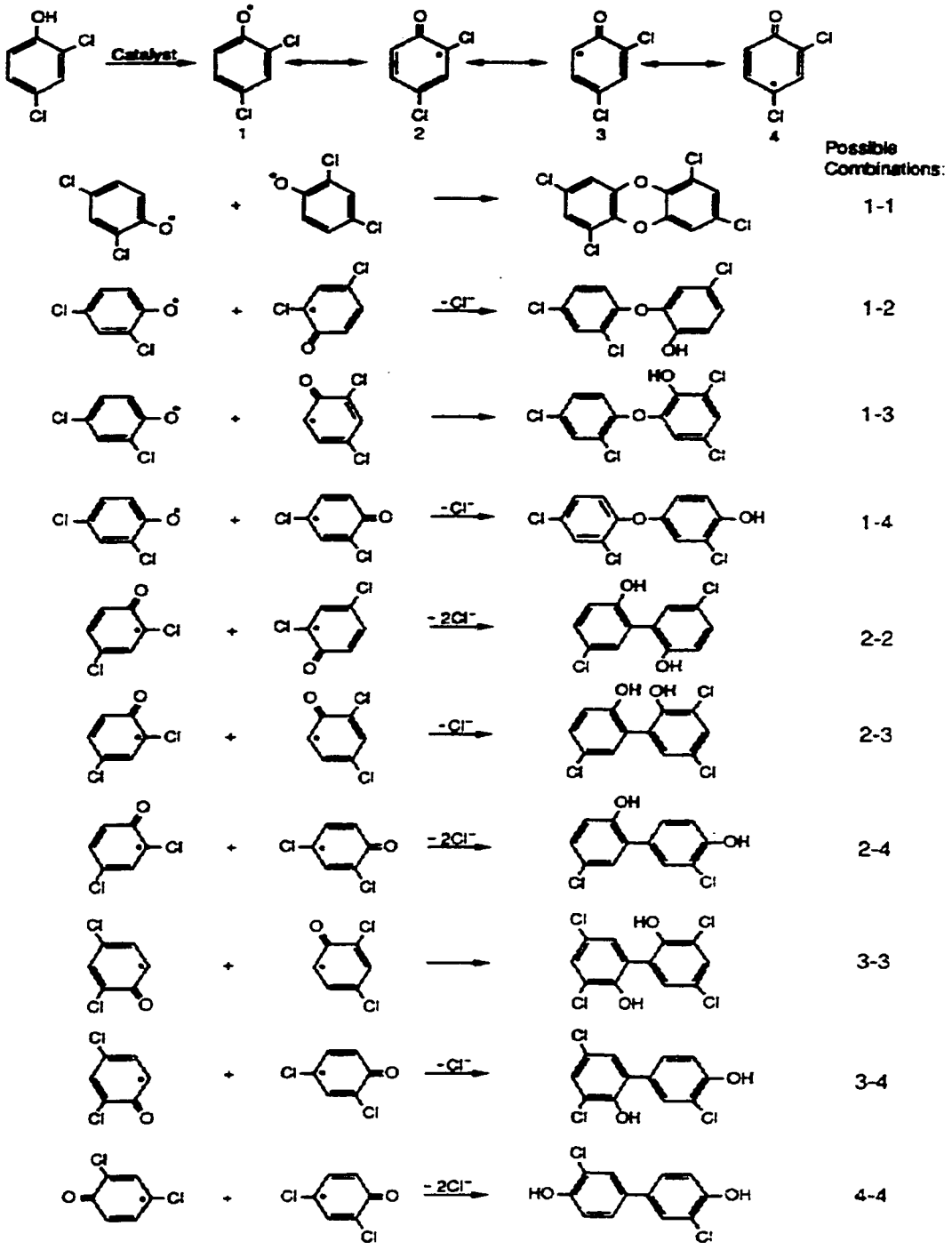


Figure 2.16: Proposed coupling reactions between free radicals generated during the catalytic oxidation of 2,4-dichlorophenol.
Source: Dec and Bollag, 1994a,b

$$M = \sum_{n=1}^{n=4} (n+1)NR^{n-1} \qquad \text{Equation 2.19}$$

$$C = \sum_{n=1}^{n=4} R^{n-1}d + 2(n-1)NR^{n-2} + \tfrac{1}{6}(n-1)(n-2)(n-3)(8NR - 2NR^2) \qquad \text{Equation 2.20}$$

$$C = \sum_{n=1}^{n=4} R^{n+1}d + (n-1)NR^{n-2} + \tfrac{1}{6}(n-1)(n-2)(n-3)(3NR - NR^2) \qquad \text{Equation 2.21}$$

The variable "n" is the oligomerization order (i.e n = 1 for dimerization), "N" is the number of dimerization reactions, and R is the number of radicals. Likewise, an equation to calculate the number of chloride ions (C) released from M molecules was developed for chlorophenols with four radicals (Equation 2.20). For chlorophenols with three radicals, Equation 2.21 was developed. The variable "d" is the number of chlorines lost during dimerization. When the DN value was calculated for 2,4-DCP up to the pentamer, it was discovered that the DN value was equal to 2 (the same for dimerization) and therefore the data from table 2.6 is in agreement for pentamerization.

2.3.4 Potential Toxicity

Microtox™ can be used to measure the acute toxicity of raw samples and treated product mixtures. Microtox™ measures the toxicity in terms of EC_{50} values. The EC_{50} value represents the effective concentration of contaminant which 50% light attenuation occurs in the Microtox™ assay. As the EC_{50} value of a compound or mixture decreases, the toxicity of the compound or mixture increases. Preliminary results indicate that out of four target compounds tested, o-cresol, p-cresol, 2-chlorophenol, and 4-chlorophenol, all but p-cresol resulted in an increased in EC_{50} as a result of enzymatic oxidation (Heck *et al.*, 1992). Other researchers have stated that the oligomers formed in the PE process are potentially less toxic. Table 2.7 presents published findings concerning the toxicity of PE pentachlorophenol (PCP) treatment (Aitken *et al.*, 1994). They indicated that for PCP, pH can have a significant effect on the final toxicity of the reaction products. The results indicate that for PCP, a lower pH of 4 significantly reduces the toxicity

compared to pH 7.

Table 2.7: EC_{50} values (% by volume) of reaction product mixtures and blanks:

Compound	Blank: pH 4	Blank: pH 7	HRP: pH 4	HRP: pH 7
4-Chlorophenol	5.0	7.0	3.7	4.4
p-Cresol	7.1	8.5	Not determined	270
2-Chlorophenol	59	96	Not determined	5.1
o-Cresol	210	91	Not determined	2.1
Phenol	Not determined	200	Not significant	15
Pentachlorophenol	4.6	7.9	69	9.1

Source: Aitken *et al*, 1993

2.4 Recommendations

Cooper and Nicell (1996) recommend the following research prior to the enzymatic process being applied at the full scale:

1) assess the toxicity of the by-products of the reaction;

2) conduct leachate studies and explore disposal options for the solid by-products of the reaction;

3) design and optimize continuous flow treatment systems;

4) determine how the process should be integrated in series with other treatment processes; and

5) increase the database of types of wastewater which can be treated using peroxidase.

2.5 Conclusion

In view of the research presented, it is evident that the technology in question is still in the "feasibility study" stage. While researchers are experimenting with various types of enzyme source materials, the predominant source studied is horseradish. Other sources such as soybeans have been proposed and studied as an alternative to HRP. When considering a feasibility study of a new enzyme source, many variables must be considered from substrate and pH to temperature and additives. The literature review has provided a basis for setting up an experimental process as will be described in the following chapter.

CHAPTER THREE

Soybean Peroxidase Treatment of Chlorinated Phenols

Note: Chapters 3 and 4 are written to be standalone papers which were submitted for publication in a journal. An abstract is provided at the beginning and a conclusion is provided at the end of each paper discussing the results. A summary conclusion is provided in chapter 5.

3.1 Abstract

Peroxidase Enzymes (PE) extracted from various plants are used to remediate aqueous solutions contaminated with chlorinated aromatics. In the presence of hydrogen peroxide (H2O2), PE catalyzes the oxidation of various substrates to free radicals, which then combine to form heavier than water polymers that precipitate out of solution. The precipitate can then be removed using a simple filtration or sedimentation process. This study characterizes the treatment of 2,4-dichlorophenol (2,4-DCP) in solution using soybean peroxidase (SBP) as a catalytic oxidizer. The effects of pH, SBP concentration, additive addition and initial 2,4-DCP concentration are reported. Optimum pH for removal of 2,4-DCP without an additive was found to be pH 8.2. Using the protective additive, polyethylene glycol 3350 (PEG 3350) increased the effectiveness of SBP by a factor of 10. A new pH optimum of 6.0 was also found when SBP was used with PEG-3350. Empirical equations are developed to show the relationships that exist between the examined parameters and 2,4-DCP removal. Treatment of 2,4-DCP using horseradish peroxidase (HRP) was also compared with SBP treatment.

Key words - soybean peroxidase enzyme, 2,4-dichlorophenol, polyethylene glycol, bioremediation.

3.2 Introduction

To date, the majority of the experiments performed have used horseradish for the source of PE. Horseradish peroxidase (HRP), as it is known, has successfully been used in the treatment of wastewater contaminated with phenols, cresol, chlorinated phenols and other compounds. However, in an effort to study the characteristics of the peroxidase enzyme (PE) process and to test the validly of other PE sources, researchers are studying PE from various sources (Aitken, 1993). Recently, peroxidase extracted from the soybean seedcoat has been suggested as an alternative to HRP (Nicell and Wright, 1997). One economical advantage of using soybean peroxidase (SBP) is that the enzyme is derived from the plant's seedcoat which is a byproduct of the soybean processing industry. The use of PE treatment is still in the feasibility stage. Researchers are continually enhancing the removal efficiency. One such method of increasing efficiency includes the use of protective additives. Entrapment of the PE into the forming polymers has been reported as the reason for loss of enzymatic activity. To prevent such entrapment, high molecular weight additives have been used to bind with the forming polymers and prevent the PE from becoming entrapped. One such additive, polyethylene glycol (PEG), has successfully increased the removal efficiency of the process. Nakamoto and Machida (1992) reported on the effects of using PEG with increasing molecular weight for the treatment of phenol using HRP. They reported that PEG with average molecular weight less than 400 was ineffective in suppressing peroxidase activity loss. They also indicated that, PEG with molecular weight greater than 7500 was no more effective than PEG 7500 (average molecular weight, 7500). To that, several researchers have arbitrarily selected PEG 3350 as the optimum molecular weight for added PE protection (Wu *et al.*, 1993). PEG 3350 will be used in this study for comparison to the results of other researchers..

3.3 Materials and Methods

Type II Horseradish Peroxidase (HRP) and Soybean Peroxidase (SBP) were both purchased from Sigma Chemicals in the amounts of 5000 units and 25,000 units respectively and were stored at 0°C until required. The HRP had a Reinheitszahl (RZ) value of 2.0 and the SBP had a RZ value of 1.3. The RZ value is a measure of heme content in the enzyme and not enzymatic activity (Sigma, 1998). The activity of the HRP enzyme was 150 purpurogallin units/mg solid. The activity of the SBP enzyme was 70 purpurogallin units/mg solid. Catalase enzyme from bovine from Sigma-Aldrich Canada was used to consume any remaining H_2O_2 in solution. The catalase had an activity of 21,000 units/mg solid where one unit of catalase enzyme will decompose 1.0 μmole of H_2O_2 per minute at pH 7.0 and 25°C. 2,4-DCP and polyethylene glycol (PEG) with an average molecular weight of 3350 g/mole was also purchased from Sigma Chemicals and stored at room temperature.

3.3.1 Preparation of Chlorinated Phenol Stock Solutions

A primary stock solution containing 1000 mg/l of 2,4-DCP was prepared using the following method. A scintillation vial was tared (VT) for weighing the 2,4-dichlorophenol. In a fume hood, while wearing a respirator and gloves, approximately 0.2 g of chlorinated phenol was measured into the scintillation vial and reweighed (Vs). Following this, a 150 ml beaker (BT) containing a magnetic stirbar was preweighed. The contents of the vial were then rinsed out with two 1 ml washings of 1.0 M NaOH into the beaker. 60 ml of Milli-Q water was added to the beaker. The beaker was then placed on a magnetic stirrer until chlorinated phenol dissolved into solution. Heat was required to aid the dissolution on several occasions. The dissolution would occur over several hours. The final weight of the beaker was measured and recorded as BS. The final solution was transferred to a 125 ml glass bottled, covered with parafilm, and stored at 4°C. Calculation of the stock concentration is as follows:

[CP]mg/l = [VS(g) - VT(g)]/[BS(g)-BT(g)]*1000(mg/g)*1000(ml/l)

(Note: 1 cm^3 water weighs 1 g at 25°C and 1ml = 1 cm^3)

Secondary, diluted and buffered stock solutions containing 125 mg/l of 2,4-DCP were prepared at specific pH's. Solutions with a pH between 9.0 and 7.2 were buffered using a Tris/HCl buffer and solutions with a pH between 7.0 and 2.6 were prepared using dibasic sodium

phosphate (DSP)/citric acid buffer. The following methods were followed to prepare the buffered solutions.

3.3.2 Preparation of Buffer Solutions

3.3.2.1 Tris(hydroxymethyl)aminomethane (Tris) Buffer

Two solutions were required to prepare secondary 2,4-DCP stock solutions buffered with Tris. The first solution, "A" was prepared by dissolving 24.2 g of Tris in Mill-Q water and diluting to 1000 ml resulting in a concentration of 0.2 M. Solution "B" was prepared by diluting 1.66 ml concentrated HCl to 100 ml resulting in a concentration of 0.2 M. The secondary diluted 2,4-DCP stocks were prepared by mixing 50 ml of solution A, "x" ml of B and 25 ml of the 1000 mg/l 2,4-DCP stock solution and then diluting the mixture to 200 ml in a volumetric flask. The 2,4-DCP concentration in these secondary stocks was 125 mg/l. Table 3.1 lists the "x" values in ml required to obtain certain pH's.

Table 3.1: Tris(hydroxymethyl)aminomethane (Tris) buffer preparation:

"x" (ml) of Solution B	pH
0	9.0
26.8	8.0
44.2	7.2

3.3.2.2 Citrate-Phosphate Buffer

Similarly, two solutions were required to prepare the secondary 2,4-DCP stocks buffered with the citrate-phosphate buffer. Solution, "C" was prepared by dissolving 19.21 g of citric acid into Milli-Q water and diluting to 1000 ml resulting in a concentration of 0.1 M. Solution "D" was prepared by dissolving 28.4 g of anhydrous, dibasic sodium phosphate powder in Milli-Q water and diluting to 1000 ml resulting in a concentration of 0.2 M. The secondary diluted 2,4-DCP stocks were prepared by mixing "y" ml of solution C, "z" ml of D, the required amount of the 2,4-DCP stock to bring the final concentration to 125 mg/l and diluting the mixture to a total of 200 ml. Table 3.2 lists the amounts required to obtain certain pH's.

Table 3.2: Citrate-phosphate buffer preparation:

"y" (ml) of Solution C	"z"(ml) of Solution D	pH
35.8	64.2	6.0
48.6	51.4	5.0
61.4	38.6	4.0
79.6	20.4	3.0

3.3.3 Preparation of Enzyme Stock Solution

Solutions containing 5,000 units of HRP per 100 ml and 25,000 units of SBP per 500 ml were prepared by washing out the vial containing the peroxidase enzymes with Milli-Q into 100 ml and 500 ml volumetric flasks. These stock solutions containing 50 units/ml of peroxidase were kept at 4°C to reduce any deactivation of the enzymes and were only removed to make necessary dilutions for experiments.

3.3.3.1 Secondary and Tertiary Enzyme Stock Solutions

In order to reduce the primary stock of 50 units/ml to concentrations such as 0.001 units/ml in the final 25 ml reactor sample, secondary and tertiary dilutions of the original stock were required. These solutions were prepared on the day of experimentation according to the desired final concentrations.

3.3.4 Preparation of PEG Stock Solution

A primary stock solution of PEG was prepared by dissolving 12.5 g of PEG 3350 in Milli-Q water and diluting to 250 ml. This 50 mg/ml concentration of PEG would be diluted to the desired amounts in the secondary and tertiary enzyme stock solutions. Consideration of the desired concentration in the final 25 ml reactor vessel would predetermine the amount of PEG solution mixed in with the enzyme solutions.

3.3.5 Preparation of Reactor Vessels for Testing

Experiments were performed in 30 ml flat bottom clear glass vials with Teflon caps. Plastic vials were avoided in order to prevent any adsorption of the chlorinated phenols to the walls. The total volume of the final solution was 25 ml. The final reaction solutions were comprised of two parts:

i) 20 ml of the buffered, secondary 2,4-DCP solution

ii) 5 ml of secondary or tertiary enzyme stock solution (with or without PEG)

H_2O_2 (Sigma Chemicals) was added to the 25 ml solution in the amount equivalent to a 1:1 molar ratio with the substrate in order to activate the enzyme. A 10µl Hamilton micro-syringe was necessary to dispense the amount of H_2O_2 required. This method to prepare the final reaction mixtures provided the best versatility when testing various pH, substrate concentrations, enzyme concentrations, and PEG concentrations.

The 30 ml reactors were placed on top of a Junior Orbitor (see Photo 3.1) shaker with a box surrounding it to prevent light from decomposing the H_2O_2. Once the reaction was initiated with the H_2O_2, the shaker was set to 50 rpm. It has been determined by several researchers and verified by this researcher that three hours provides an ample amount of time for the peroxidase enzyme reaction to be completed. Following the three hours (for experiments going to completion), the reaction was halted by the addition of catalase enzyme in the necessary amounts to decompose the H_2O_2. The reason for this is to prevent any residual enzyme to react beyond the allotted time frame. Following the addition of the catalase, if suspended particles were visible in solution, the mixtures would be centrifuged at 4°C for 15 minutes at 500 rpm. Following this, a 1 ml sample was drawn from the solution and filtered through a 2 µm millipore filter into a vial ready for High Pressure Liquid Chromatography (HPLC) analysis.

3.3.6 Analyzing Mixtures for Substrate using HPLC

The final solutions were analyzed for residual chlorinated phenols using a Hewlett Packard (model 1090) HPLC fitted with a Hypersil-ODS reverse C18 column able to detect chlorinated phenols (See Photo 3.2). The diode array detector was set at a wavelength of 280 nm at an oven temperature 40°C and the flowrate of the mobile phase was set at 0.3 ml/min. For the mobile phase, an isocratic mixture of HPLC grade methanol (60%) and 0.05 M sodium acetate (40%) at pH 4.7 was applied. The solvents used were HPLC grade methanol and sodium acetate filtered using a 0.2 µm filter. The solvents were degassed prior to use in order to prevent bubbles from entering the column. The HPLC was recalibrated every 20 samples using standard solutions. The calibration curves for the various phenols remained relatively constant throughout the experimental phase. There is an experimental error associated with the HPLC that was taken

into consideration. In order to avoid interpreting background noise in the chromatogram as contaminant, a minimum area of 10 was selected which conveys 0.4 mg/l on the 2,4-DCP calibration scale. Therefore, complete removal of 2,4-DCP is never guaranteed, however in cases where no peak was detected, it is assumed that the amount removed is between 99.6 to 100% based on initial concentration of 2,4-DCP used in tests.

3.3.7 Experimental Conditions

Experimental conditions which were used throughout the experiments (unless noted) are given in Table 3.3. In general, 2,4-DCP and SBP were used for the majority of the tests as substrate and enzyme respectively. However, one experiment tested the effects of SBP treatment of various other chlorinated phenols compounds. Direct contact with light was always limited by covering the shaker with a cardboard box. This reduced the chance of the H_2O_2 being decomposed by UV rays. Previous work indicated that 3 hours was sufficient time to allow the reaction to go to completion (Cooper and Nicell, 1996). A time dependent test verified these results. The temperatures of the mixtures were in equilibrium with the room at a temperature of 22°C. H_2O_2 was applied to the mixture in a 1:1 molar equivalent ratio with the substrate. This ratio is reported to be the optimum dose ratio (Nicell *et al.*, 1992). Less H_2O_2 would be a limiting condition whereas, excessive H_2O_2 concentrations lead to the deactivation of the enzymes due to the formation of Compound III, an enzymatic state in which the enzyme does not recover and is considered deactivated (Arnao *et al.*, 1990).

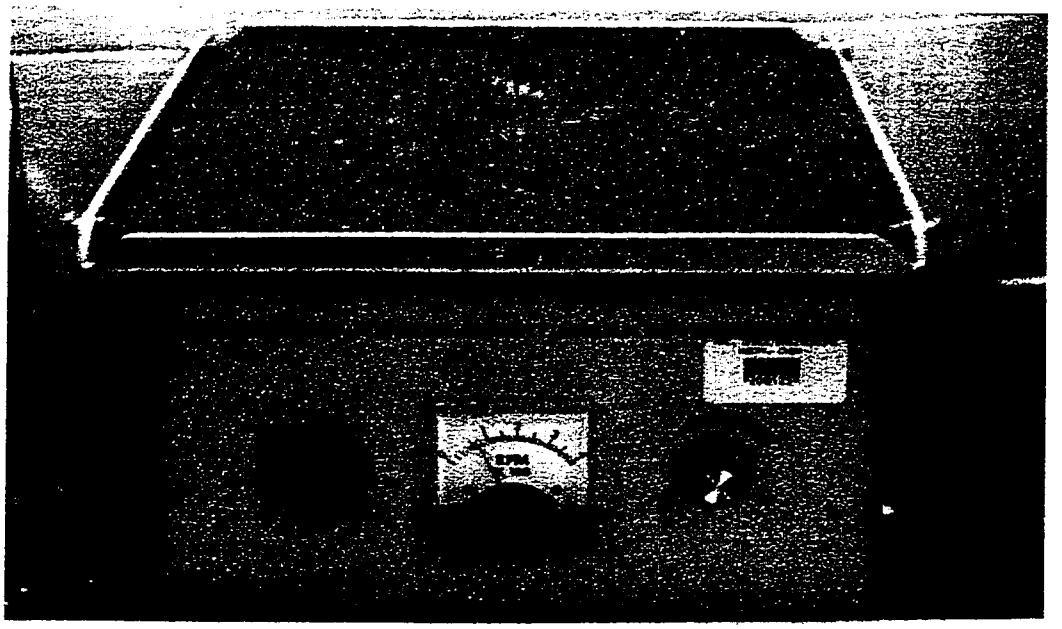


Photo 3.1: Experimental equipment - Junior orbit shaker.

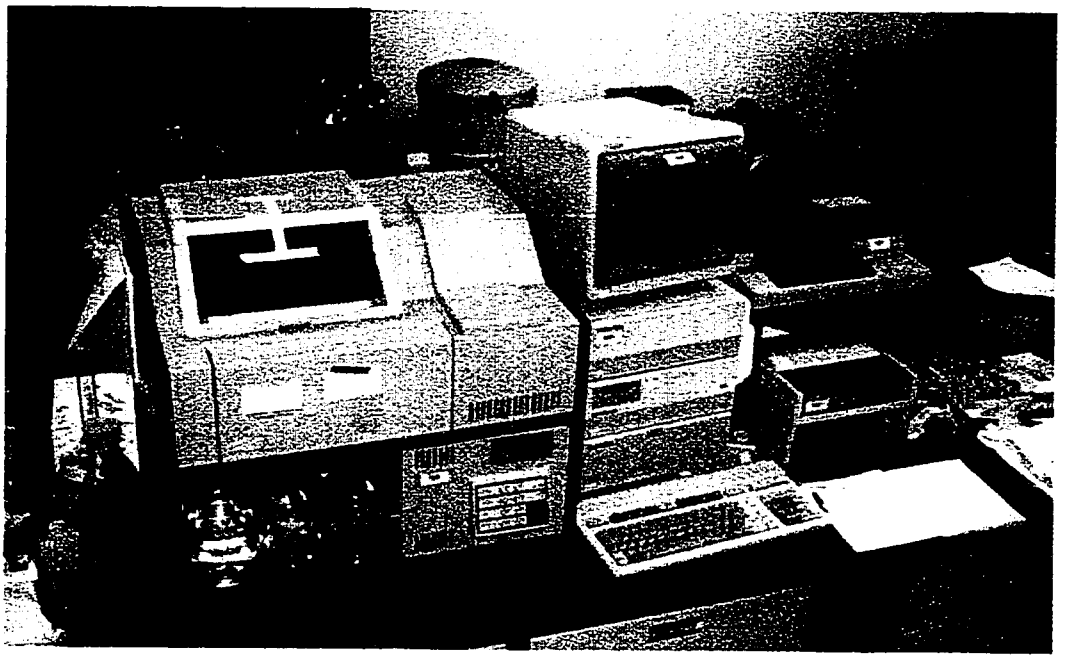


Photo 3.2: Experimental equipment - High Pressure Liquid Chromatography unit.

Table 3.3: Table of experimental conditions:

Potential Experimental Variable	Experimental Conditions used unless otherwise stated	Range of Variable tested during various experiments
2,4-Dichlorophenol	100 mg/l	150 - 500 mg/l
Soybean Peroxidase	0.01 units/ml	0.0005 - 10.0 units/ml
Polyethylene Glycol 3350	Varies	0.002 - 1.2 mg/l
Time	3 hours	5 minutes - 72 hours
Temperature	22°C	4°C & 22°C
H_2O_2	0.613mM	Held Constant
Tris Buffer	pH 8.2	pH 7.2 - 9.2
Citrate-Phosphate Buffer	pH 6.2	pH 2.5 - 7.2
Reactor Set-up	Batch	Batch
Light	Prevented from direct sunlight	Held Constant
Agitation/Mixing	50 rpm shaker	Held Constant
Volume	25 ml	Held Constant
Centrifuged/Filtered	Several mixtures centrifuged, always filtered for HPLC	Held Constant

3.4 Observations and Results

3.4.1 Time and Temperature of Reaction Results

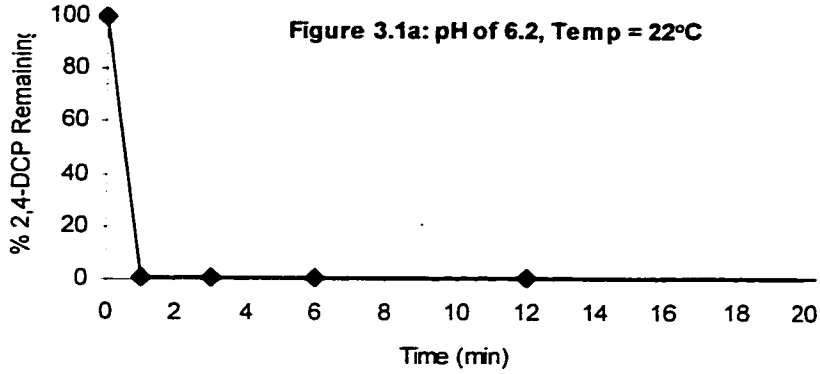
Figures 3.1a-3.1b indicate the time required for 1 unit/ml of SBP to react with and remove 100 mg/l 2,4-DCP from solution with a pH of either 6.2 or 7.2. Inoculating the reaction mixture with a concentrated dose of catalase enzyme at the desired time intervals halted the enzymatic reaction. In approximately 1 min at 22°C, less than 0.4 mg/l of 2,4-DCP remained in solution when tested immediately with the HPLC method. A method of describing PE efficiency is the number of turnovers (TO), which equals the moles of substrate removed per mole of enzyme consumed. Assuming SBP has a molecular weight similar to HRP (44,000), the maximum number of turnovers that can be achieved in this experiment using 1 unit/ml SBP to treat 100 mg/l 2,4-DCP is 1875. Assuming the reactions took exactly one minute to complete, a conservative turnover rate calculated for both pH test (6.2 and 7.2) was 31.2 TO/second (Figure 3.1a and 31.b). Optimizing the SBP concentrating will significantly increase this value. Similar temperature dependent tests were set up in a walk-in cooler with a constant temperature set at 4°C. Prior to adding the H_2O_2 to initiate the reaction, the mixture was given time to reach equilibrium with the surrounding temperature. After 20 minutes, removal was only 95% and it wasn't until approximately 3 hours that 99.6% removal was reached. The corresponding TO rate is 1.48 TO/second after 20 minutes and 0.17 TO/second after 180 minutes. These tests describe the effect that temperature can have on the PE treatment process. Although the final number of turnovers is the same, the rate at which it was reached is significantly lower at lower temperatures. This is very significant when determining the hydraulic retention time (HRT) of a treatment process and for determining the size of reactors required. It is also important if considering the application for groundwater treatment which typically has a colder temperature. The effects of slowing down the kinetics of the enzyme may also play a significant role in process efficiency. There is a possibility that the rate of reaction may either increase or decrease in the potential formation of the inactive form of the enzyme. Further investigation into the kinetics may be desired. The solutions exhibited a visual discolouration upon activation of the peroxidase (See Photo 3.3). Within seconds of injecting the H_2O_2, a cloud forms near the center of the mixture. With time, this cloud becomes more pronounced and the colour of the solution

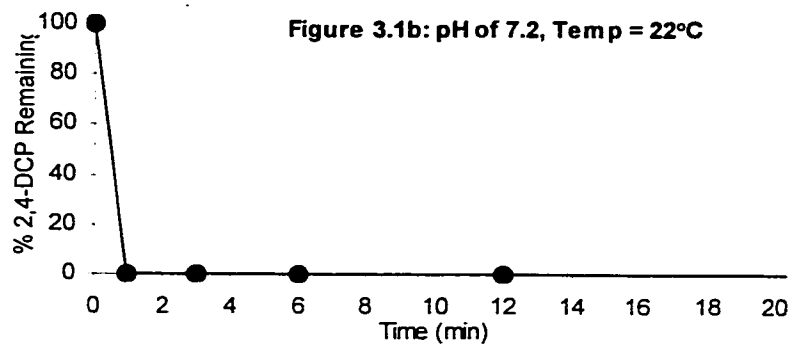
changes. The color of the solution is dependent on the pH, as will be discussed later. For experiments that were run for a longer period of time, visual observations of the reactions revealed a cloud that formed and would densify to the point where visible particles would form at approximately 1 hour. After 3 hours, these particulate would settle to the bottom of the vessel (See Photo 3.4). The time frame of 3 hours is also important in that 100% removal may be reported in the first few minutes of the reaction but a lag time is required for the particulate to form, precipitate out of solution and settle to the bottom of the reactor. These particulates are believed to be responsible for entrapping the active enzyme within its structure thereby rendering the process inactive (Nakamoto and Machida, 1992). Polymer characterization tests performed by Dordick *et al.*, (1986) have unveiled a potential market for the byproduct of this process. Tests have shown that dechlorination also occurs to some extent (Dec and Bollag, 1994a,b). In a trial experiment performed (Appendix A) in an unbuffered solution, a drop in pH was observed, which is a possible indication that chlorine ions are being released into solution.

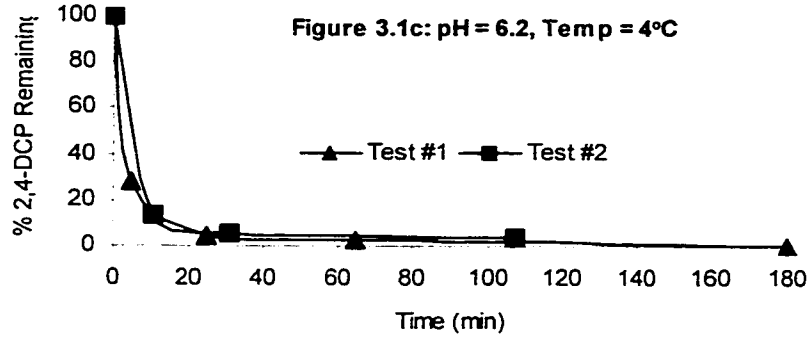
These time dependent tests provided information that served as a benchmark for future test development in this study. This test indicated that up to 100% removal of 2,4-DCP from aqueous solution is achieved at pH 6.2 and 7.2 at 22°C using a minimum 1.0 unit/ml of SBP.

3.4.2 Influence of pH and SBP Enzyme Concentration

The following experimental results focus on the use of SBP as an alternative to HRP. The effect of pH on the removal of 2,4-DCP using SBP was determined in a variety of experiments. The goal of the first set of SBP experiments was to determine the optimum and the sub-optimum pH ranges in which 2,4-DCP can be removed from aqueous solution and to determine the effective range of enzyme concentration without an additive (PEG) present in solution. Previous researchers have determined the optimum pH for removing 2,4-DCP using HRP to be 6.5 with a working range of 3 to 10 (Dec and Bollag, 1990).







Figures 3.1a-3.1c: Effects of time and temperature on the removal of 100 mg/l of 2,4-DCP using 1 unit/ml SBP in a 25 ml batch reactor.

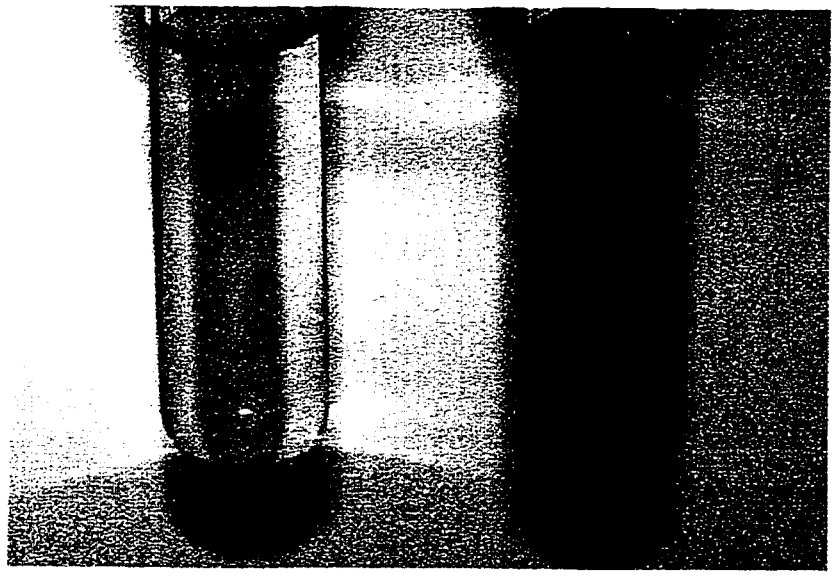


Photo 3.3: Tube on the left, contains no H_2O_2 but does contain 100 mg/l 2,4-DCP and 1 unit/ml SBP. The tube on the right contains 100 mg/l 2,4-DCP, 1 unit/ml SBP and was activated by H_2O_2 at pH 6.2. This photo was taken approx. 1 hour into rxn. Note: visible particulate has formed in tube yet has not settled as shown in Photo 3.4.

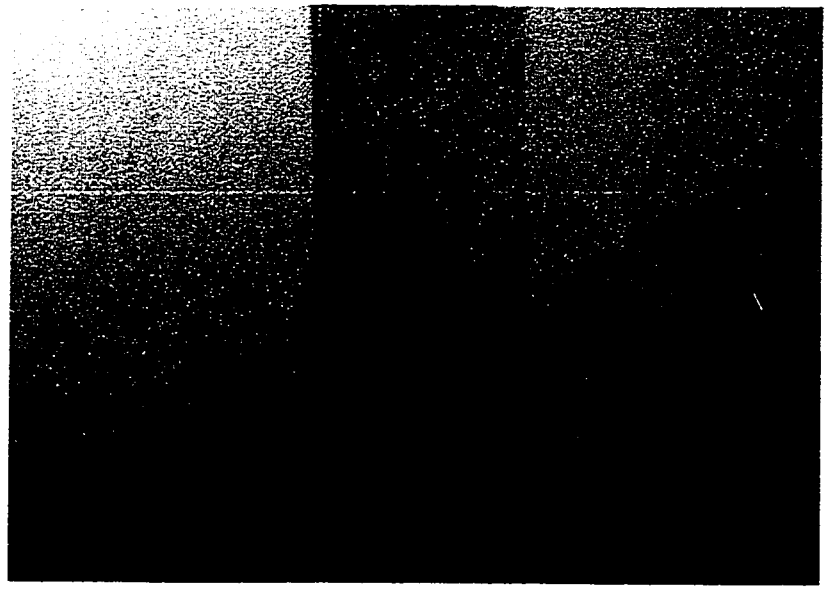
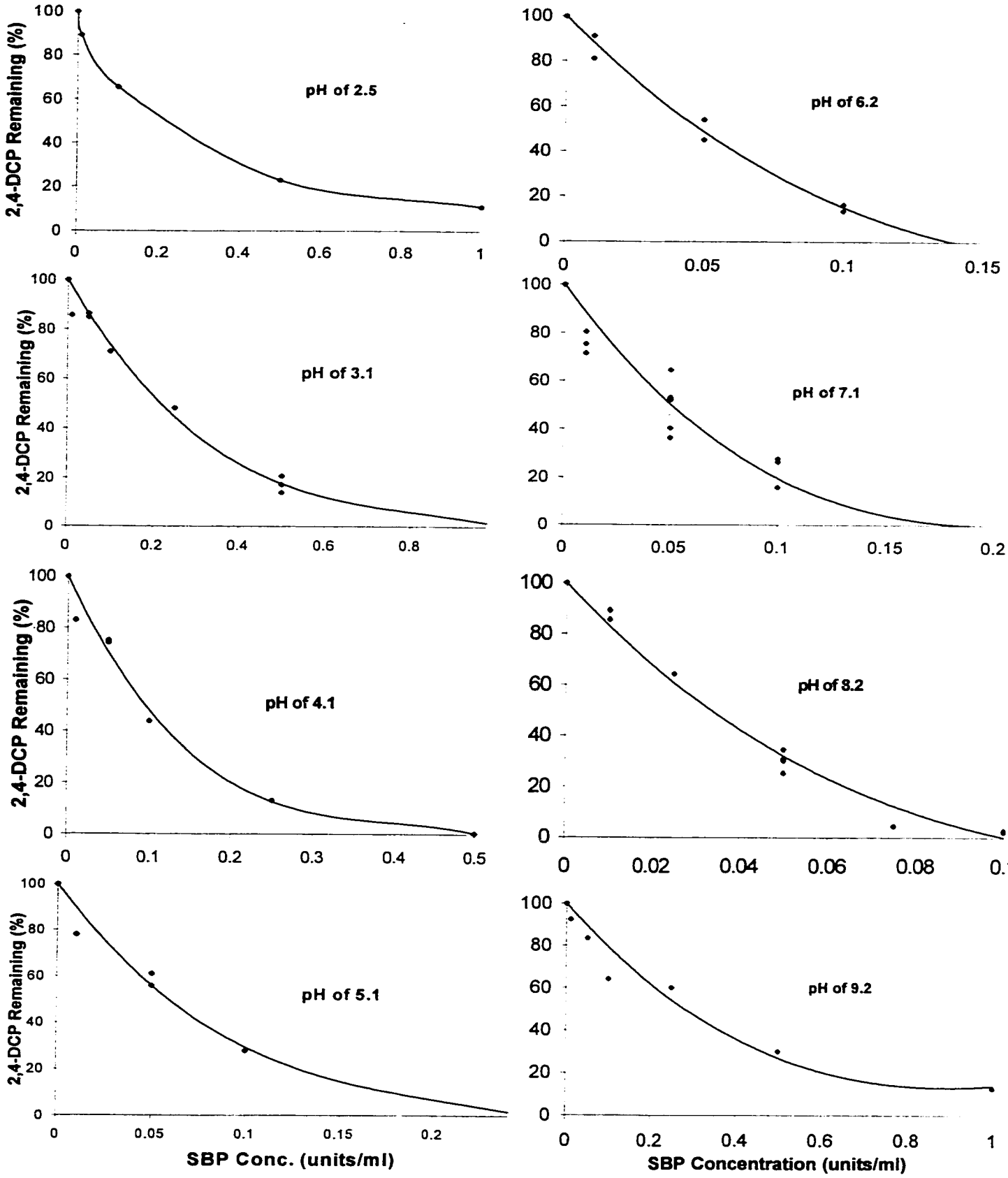


Photo 3.4: The effect of time on the same reaction shown in photo 3.3. Photo is taken at approximately 3 hours. Particulate has had time to coagulate and settle at the bottom of tube. Note: discolouration also lessens with time.

In this study, over 100 tests were performed for pHs ranging from 2.5 to 9.4 and for SBP concentrations between 0.01 and 9 units/ml. The raw data from these experiments are presented in Figures 3.2a - 3.2h. A 3rd degree polynomial was fit to each set of data between the pH range of 3.1 to 9.2. The following equations in Table 3.4 were determined from the polynomials fit to the data. "Y" represents the % 2,4-DCP remaining and "X" represents the amount of SBP in units/ml. The square of the correlation coefficient R^2 is given for each best fir curve. The R^2 values range from 0.90 to 0.99 which are relatively close to 1 therefore indicating that the empirical equations represent the data points fairly accurately. A family of curves was produced from equations 3.1 to 3.7 and is plotted in Figure 3.3. Also, a 3-dimensional surface plot of the data, using a 3rd degree polynomial, is presented in Appendix A page 130a. The effect of SBP concentration, as discussed earlier, is clear and the maximum amount of SBP that is required to remove up to 100 % of the 2,4-DCP for each pH can be determined.

Table 3.4: List of equations derived from Figures 2b-2h

pH	Y =	X =	R^2	Equ.
3.1	$-127.91(X)^3 + 323.38(X)^2 - 294.38(X) + 100$	$0 > X < 1.00$	0.99	Eq 3.1
4.1	$-1331.9(X)^3 + 1599.9(X)^2 - 666.09(X) + 100$	$0 > X < 0.5$	0.99	Eq 3.2
5.1	$-7213.2(X)^3 + 4587(X)^2 - 1094.5(X) + 100$	$0 > X < 0.25$	0.97	Eq 3.3
6.2	$-2671(X)^3 + 3930.7(X)^2 - 1214.2(X) + 100$	$0 > X < 0.14$	0.99	Eq 3.4
7.2	$-5020.5(X)^3 + 4521.9(X)^2 - 1205.4(X) + 100$	$0 > X < 0.20$	0.90	Eq 3.5
8.2	$-15932(X)^3 + 9577.9(X)^2 - 1796.8(X) + 100$	$0 > X < 0.10$	0.99	Eq 3.6
9.2	$-35.865(X)^3 + 173.85(X)^2 - 224.09(X) + 100$	$0 > X < 1.00$	0.96	Eq 3.7



Figures 3.2a-3.2h (pH 2.5-9.2). Effects of increasing SBP concentration for various pH in a 5 ml batch reactor at 22°C (without an additive).

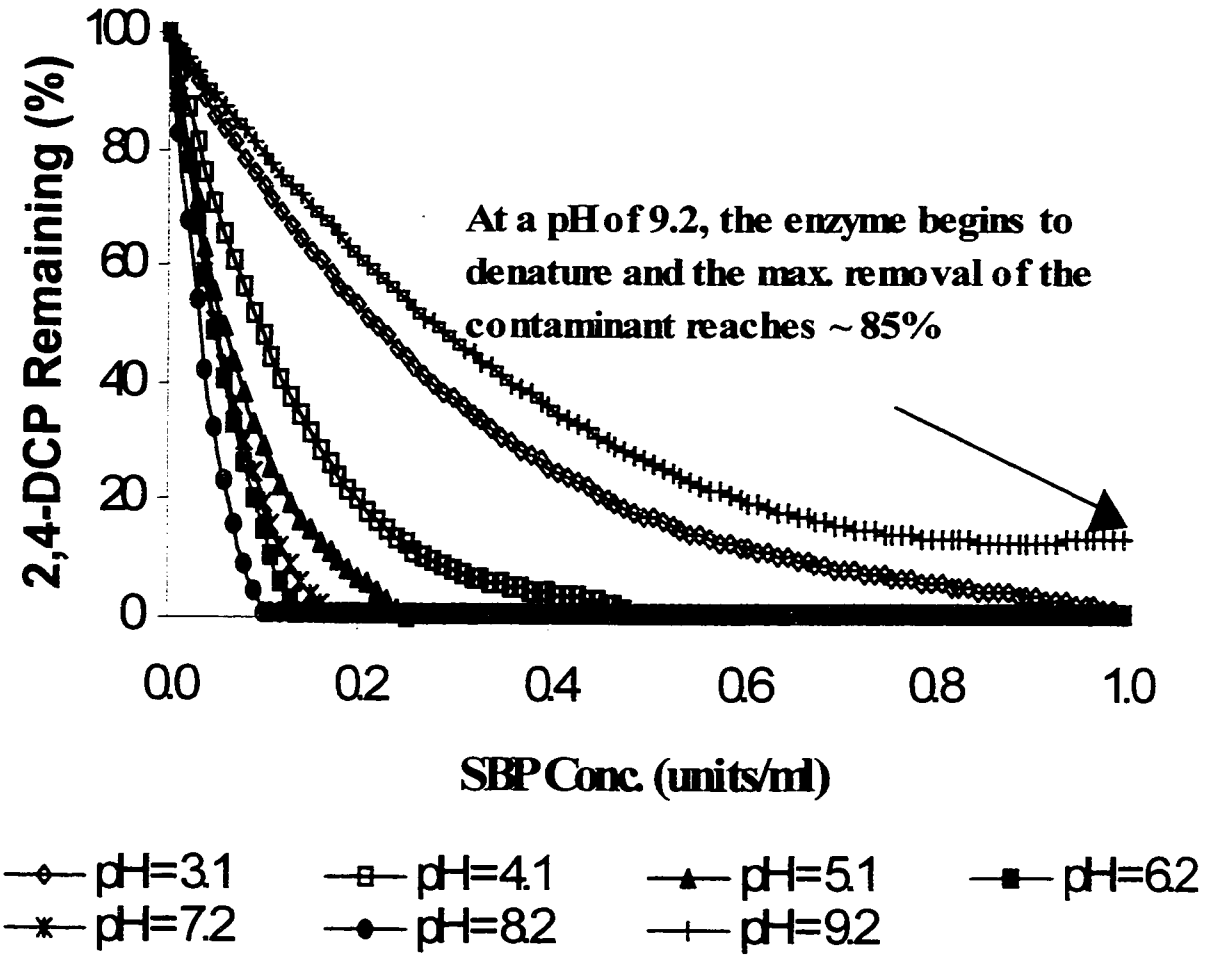


Figure 3.3: Effects of SBP concentration on the removal of 100 mg/l 2,4-DCP in a batch reactor at 22°C for various pH (3.1-9.2).

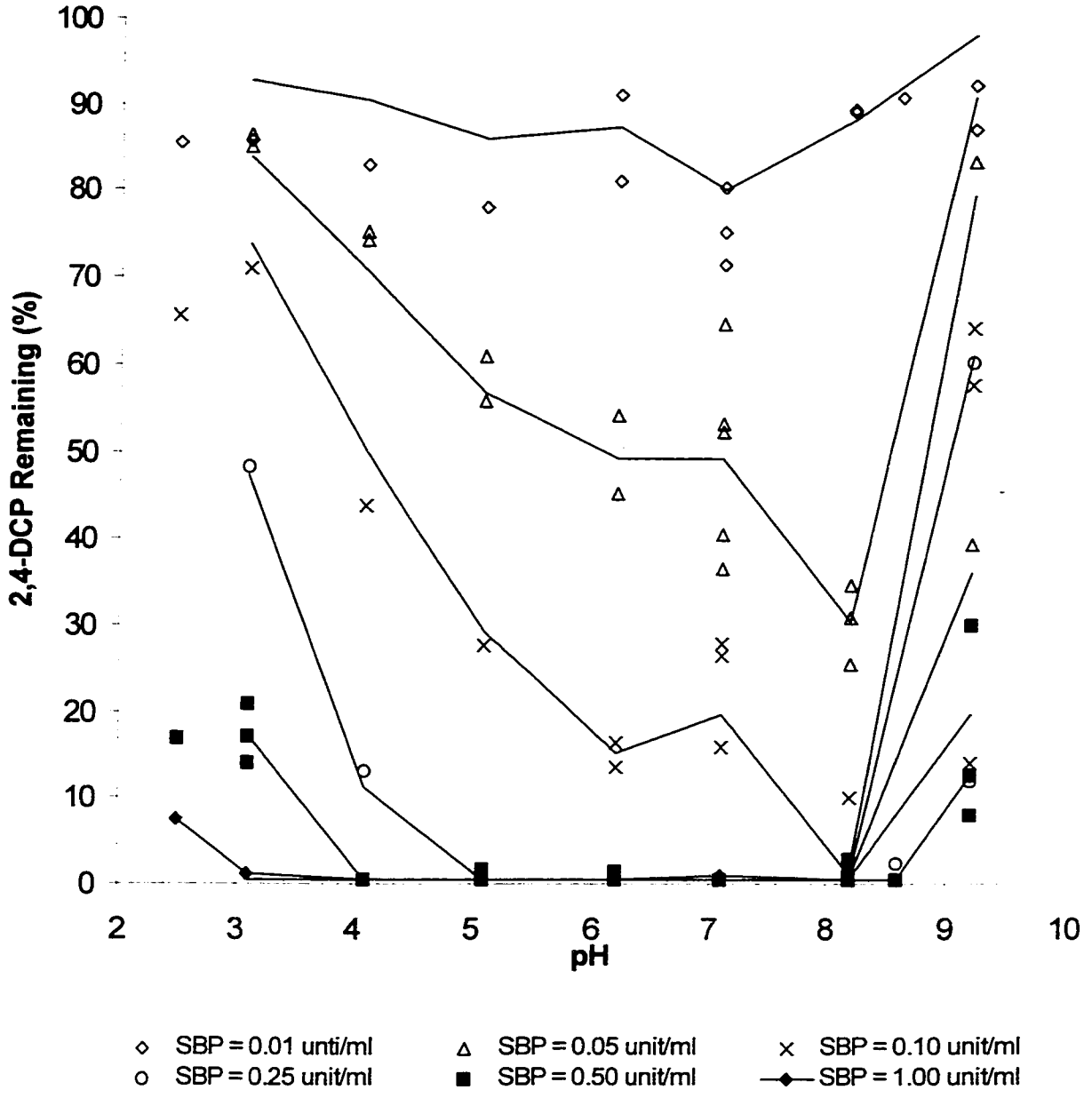


Figure 3.4a: Effects of pH on the removal of 100 mg/l 2,4 DCP in a batch reactor at 22°C for various SBP concentrations (0-1.0 units/ml).

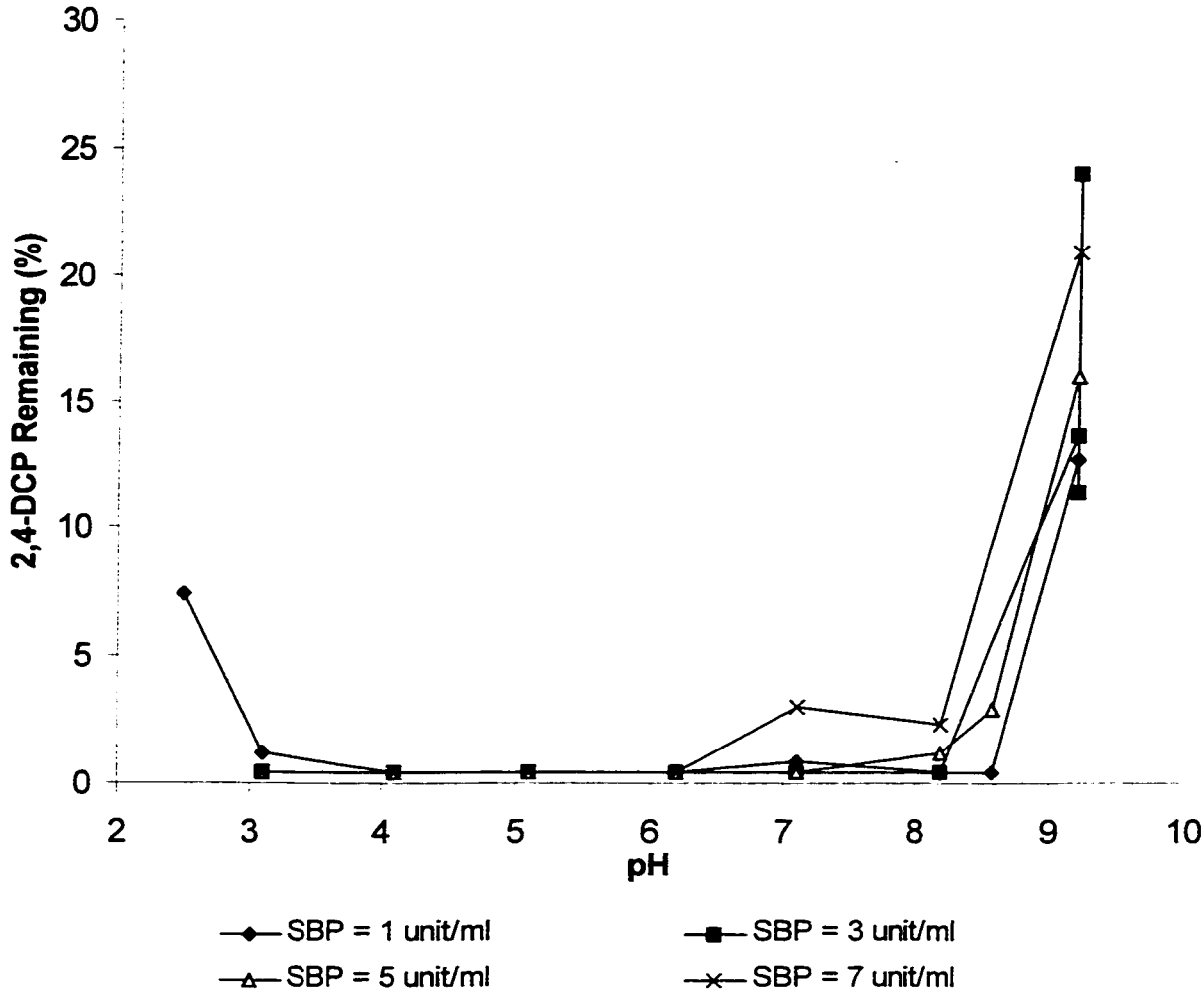


Figure 3.4b: Comparing the effects of pH on the removal of 100 mg/l 2,4 DCP in a batch reactor at 22°C for various SBP concentrations (1.0-7.0 units/ml).

Replacing the X-axis of Figure 3.3 for pH produces Figures 3.4a and 3.4b with the % 2,4-DCP remaining vs. pH. These graphs compare the data obtained from the best fit polynomials (lines) to the raw data points. They accurately reflect the effect of pH on the reaction, however; there was no clear relationship that could fit all the data. A similar graph was produced for the removal of phenol by HRP where the pH range which HRP is reported to have catalytic ability is from 4 to 10 (Bewtra *et al.* 1995). The results obtained in this experiment indicate that SBP can function in more extreme acidic conditions than HRP. As shown in Figure 3.4a, even at pH of 2.5, 1 unit/ml of SBP removed nearly 90% of the 2,4-DCP. Several other important observations relating to the effects of pH can be made from Figure 3.4a. The optimum pH for SBP treatment of 2,4-DCP is approximately 8.2. Interstingly, this is quite different than the results Dec and Bollag (1990) obtained for HRP (optimum pH ~ 6.5). For pH just slighter greater than 8.2, the reaction is severely affected at low SBP concentrations. As shown in Figure 3.4b, 85% removal can be achieved at pH 9.4 but much more SBP is required to do so. It is believed that the peroxidase enzyme is susceptible to major denaturing in even slightly basic solutions. As the pH decreases and becomes more acidic several things occur. Firstly there is a gradual loss of efficiency compared to the basic conditions. Therefore, it is assumed that the enzyme is denaturing but not as quickly as in a basic solution. The second observation is a "bump" which occurs in the curves at pH 7 (See Figure 3.4a). Several lower SBP concentration tests were repeated up to four times at a pHs of 6.1, 7.1, and 8.2 (which included using a different buffer) to try and confirm results. However, more tests performed confirmed that the "bump" was significant. Similar "bumps" have been observed by other researchers investigating the effects of pH on HRP (Bewtra *et al.*, 1995; Dec and Bollag, 1994a,b). It is quite possible that this bump occurs because of interactions of the buffer solutions or maybe it is simply a matter of enzyme kinetics.

Table 3.5 summarizes the results obtained in Figures 3.4a and 3.4b. For various removal rates, the minimum amount of SBP required for a given pH range is provided. At the optimum pH of 8.2 the minimum SBP required to achieve 100% removal of 2,4-DCP is 0.10 units/ml. This removal efficiency is equivalent to 18,746 turnovers. Using the same SBP concentration (0.01 unit/ml) at pH 3.1, only 30% removal is achieved which is equivalent to 5,624 turnovers.

At a pH of 3.1, the minimum SBP required to remove 100% 2,4-DCP is 1.0 units/ml which is equivalent to 1,875 turnovers. Using 0.01 units/ml, at pH 7.1, 20% removal is equivalent to 37,492 turnovers out of a possible 187,458 (i.e. 100% removal in a batch reactor). Since turnovers are inversely proportional to SBP concentration, a decrease in SBP concentration by a factor of ten results in an increase of turnovers by a factor of ten assuming the same removal efficiency. Therefore, it is clear that even though pH has a significant effect on the removal efficiency, if a lower enzyme concentration can be optimized for 100% removal, the increase in efficiency will be much greater than by just adjusting pH.

Table 3.5: Effective pH range for certain removal rates using various concentration of SBP:

% Removal of 100 mg/l 2,4-DCP	SBP Concentration (units/ml)	Effective pH Range
100	1.00	3.1 to 8.6
100	0.50	4.1 to 8.6
100	0.25	5.1 to 8.6
100	0.10	8.2 only
70	0.05	8.2 only
20	0.01	7.1

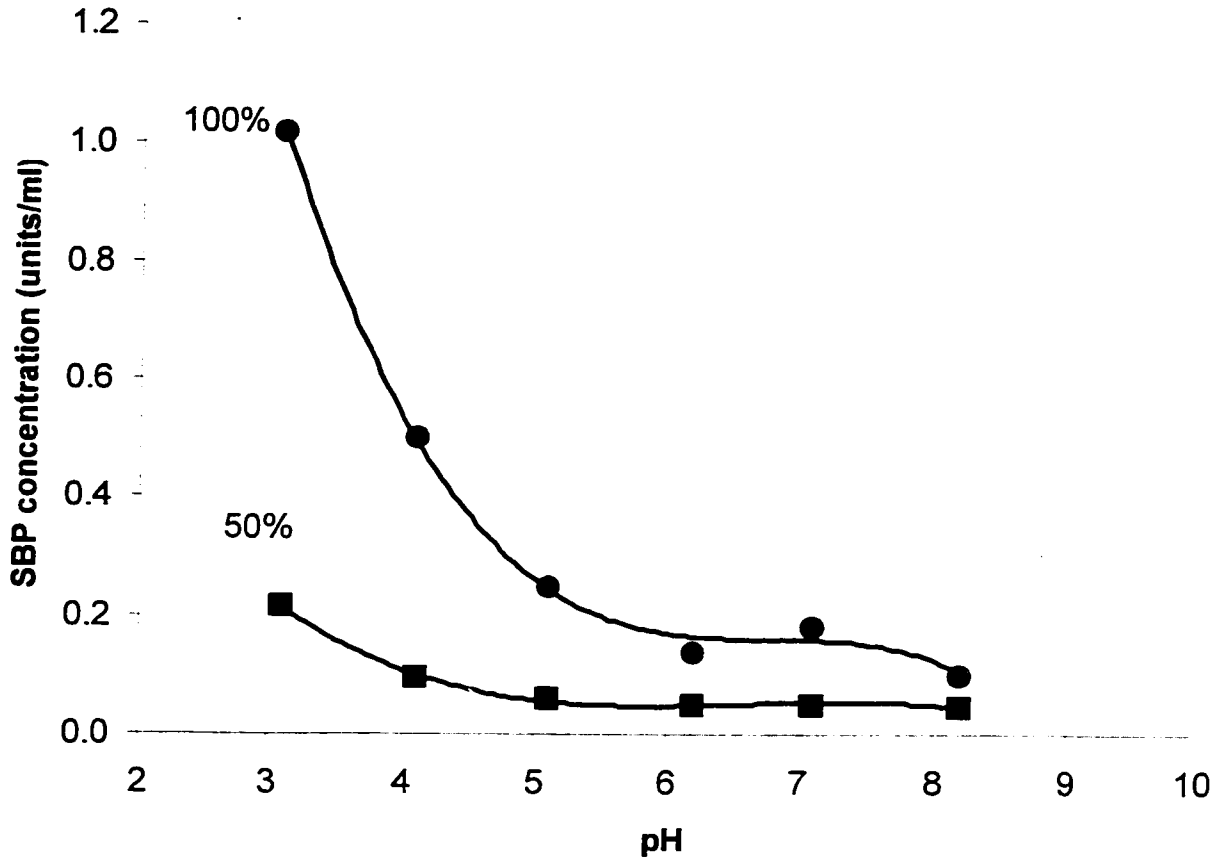


Figure 3.5: Minimum SBP concentration required (without an additive) to remove 50% and 100% of 100 mg/l 2,4-DCP based on results shown in Figure 3.3.

Table 3.6: Minimum SBP required to achieve 50% and 100% removal:

pH	SBP (units/ml) required to remove 100% of 100 mg/l 2,4-DCP	SBP (units/ml) required to remove 50% of 100 mg/l 2,4-DCP
3.1	1.02	0.215
4.1	0.50	0.095
5.1	0.25	0.060
6.2	0.14	0.049
7.2	0.18	0.051
8.2	0.10	0.045

Figure 3.5 and Table 3.6 show the minimum amount of SBP required to remove up to 50% and 100% of 100 mg/l of 2,4-DCP for various pH. Equations were fit through both sets of data and the resulting equations were determined:

For 100% removal: $\mathbf{SBP_{min} = -0.0173pH^3 + 0.35pH^2 - 2.38pH + 5.55}$ **Equation 3.8**

For 50 % removal: $\mathbf{SBP_{min} = -0.0045pH^3 + 0.09pH^2 - 0.57pH + 1.27}$ **Equation 3.9**

where: SBP_{min} = the minumum amount of SBP required

pH = pH of the mixture.

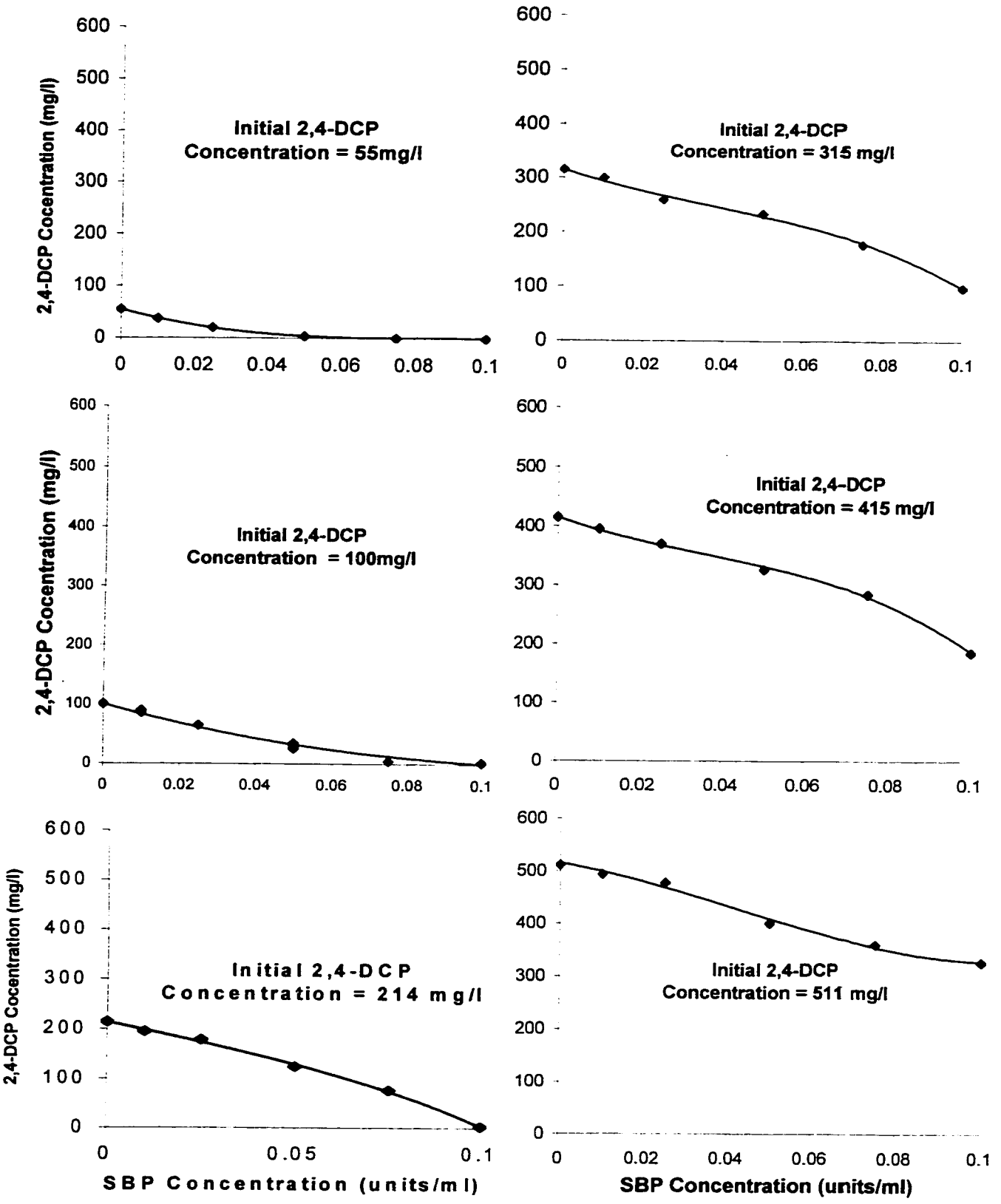
Firstly, when comparing the 100 % removal line, it is observed that for a pH of 3.1 and pH 4.1, 10 and 5 times the amount of SBP required than what is required at pH 8.2. A second observation is the amount of SBP required to remove 100% is 5 times more than what is required to remove half the amount or 50% for pH 3.1 and 4.1. At pH of 8.2, a little more than twice the amount of SBP is required to remove 100%. From these results we can also hypothesize that as the pH approaches it's optimum value (pH 8.2), the relation between enzyme and amount of 2,4-DCP removed approaches linearity.

SBP turnovers are calculated on the assumption that SBP has a molecular weight similar to HRP at 44,000. Dec and Bollag (1994a,b) published results indicating that during the removal 9.0 mM (1467 mg/l) of 2,4-DCP using 4 units/ml of HRP, they achieved 94.0% transformation at pH 5. This translates into 0.34 mg of 2,4-DCP per unit of HRP or 6462 turnovers. For

comparison, using Equation 3.3, 100 mg/l of 2,4-DCP can be transformed with 0.25 units/ml of SBP at pH 5.1. This translates into 7,500 turnovers or roughly 14% more moles transformed than was reported with HRP by Dec and Bollag (1994a,b) at the same pH.

3.4.3 Varying Initial 2,4-DCP Concentration and SBP Enzyme Concentration

The results obtained from the previous experiments were used to determine the conditions used in following experiments. Without an additive present, the optimum pH for the SBP reaction to removal 2,4-DCP was determined to be approximately 8.2. When using this pH for testing purposes, it is imperative that the mixture has a good buffer to avoid the solution from becoming even slightly more basic. A slight shift to the basic side drastically affects the mechanism of the enzymatic process as shown in Figures 3.4a & 3.4b. Figures 3.6a-3.6f present the results from a series of experiments performed using various initial 2,4-DCP concentrations ranging from 55 mg/l to 511 mg/l. The tests were performed using a constant pH of 8.2. The experiments helped determine the amount of 2,4-DCP that could be removed per unit of enzyme in a batch solution. From the previous experiment, the minimum amount of SBP required at pH 8.2 for 100% removal is 0.01 units/ml. Therefore testing SBP concentrations less than 0.01 units/ml will provide informative results because 2,4-DCP removal should not reach 100%. A range of SBP concentration between 0 and 0.1 units/ml were selected for this experiment.



Figures 3.6a-3.6f: Effects of increasing SBP concentration for various initial 2,4-DCP concentrations in a 25 ml batch reactor at pH 8.2 and 22°C.

Table 3.7: Equations to determine 2,4-DCP removed for certain SBP concentrations:

Initial 2,4-DCP (mg/l)	2,4-DCP =	SBP =	R^2	Eq.
55	$-88309(SBP)^3 + 22857(SBP)^2 - 1948.4(SBP) + 55$	0>SBP<0.1	1.00	3.10
100	$-15932(SBP)^3 + 9577.9(SBP)^2 - 1796.8(SBP) + 100$	0>SBP<0.1	0.99	3.11
214	$-61333(SBP)^3 + 1272.4(SBP)^2 - 1618.9(SBP) + 214$	0>SBP<0.1	1.00	3.12
315	$-233736(SBP)^3 + 25812(SBP)^2 - 2418.8(SBP) + 315$	0>X<0.1	1.00	3.13
415	$-284787(SBP)^3 + 30476(SBP)^2 - 2483(SBP) + 415$	0>X<0.1	1.00	3.14
511	$204206(SBP)^3 - 25691(SBP)^2 - 1351(SBP) + 515$	0>X<0.1	0.99	3.15

A 3rd degree polynomial was fit to each of the six raw data sets in Figures 3.6a to 3.6f as shown in Table 3.7. Each equations represents the % 2,4-DCP remaining for the given amount of SBP in units/ml. These six equations and the raw data points of which they represent were plotted on a single chart shown in Figure 3.6g. An apparent trend in the data appears. The fitted curves approach linearity and for each specific enzyme concentration, approx. the same amount of 2,4-DCP was removed no matter what the initial concentration of 2,4-DCP. The slopes of each data point (final - intial 2,4-DCP & excluding those that reached 0.0 mg/l) were determined on per litre basis and plotted in Figure 3.7. The Y axis represents the slopes determined for each point in mg/units. The X axis represents the enzyme concentration in units/ml. The mean of these data points was determined for these points to be 1.77 mg 2,4-DCP/unit with a variance of 0.09 mg 2,4-DCP/unit. 1.77 mg 2,4-DCP/unit would then be equal to 33,180 turnovers. From these slopes a general equation 3.16 representing the amount of SBP required to treat a given concentration of 2,4-DCP at pH 8.2:

SBP (units) = 2,4-DCP (mg)/1.7 **Equation 3.16**

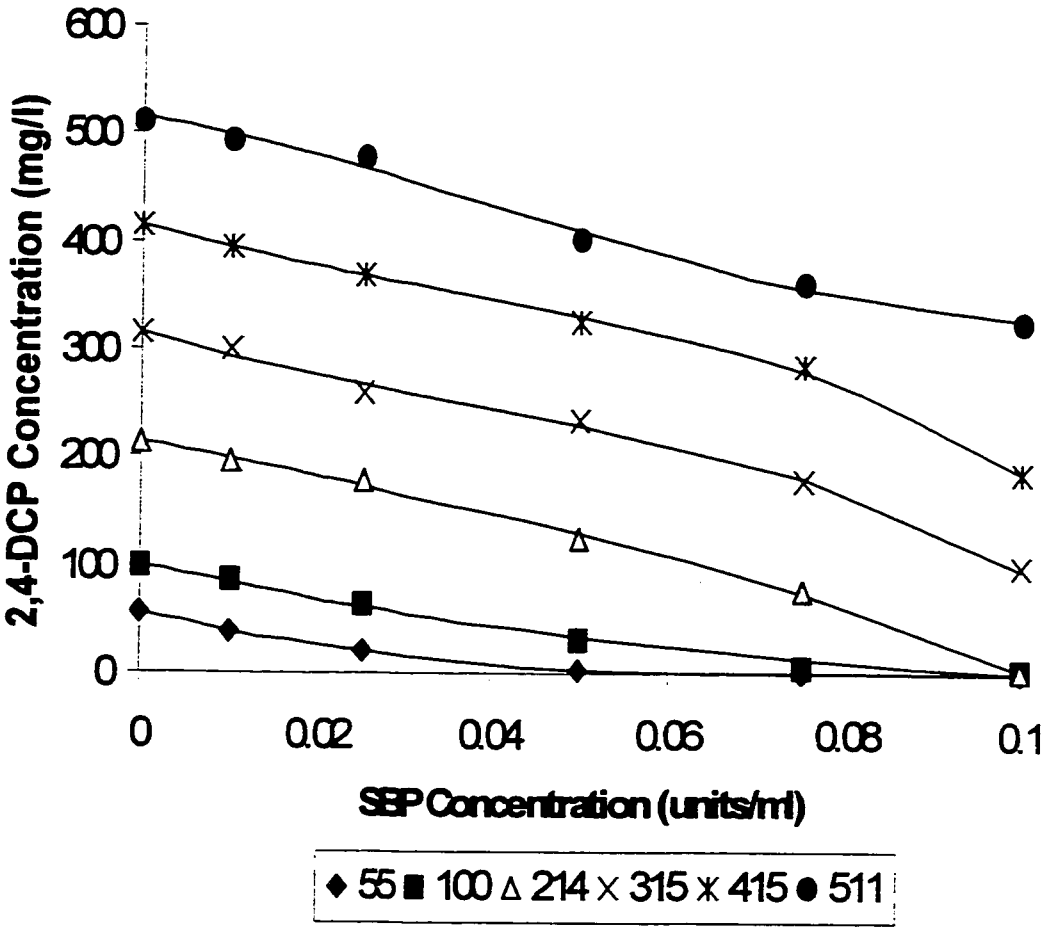


Figure 3.6g: Figures 3.6a-3.6h combined to illustrate the relation between effective 2,4-DCP removal at various initial concentrations for various SBP concentrations.

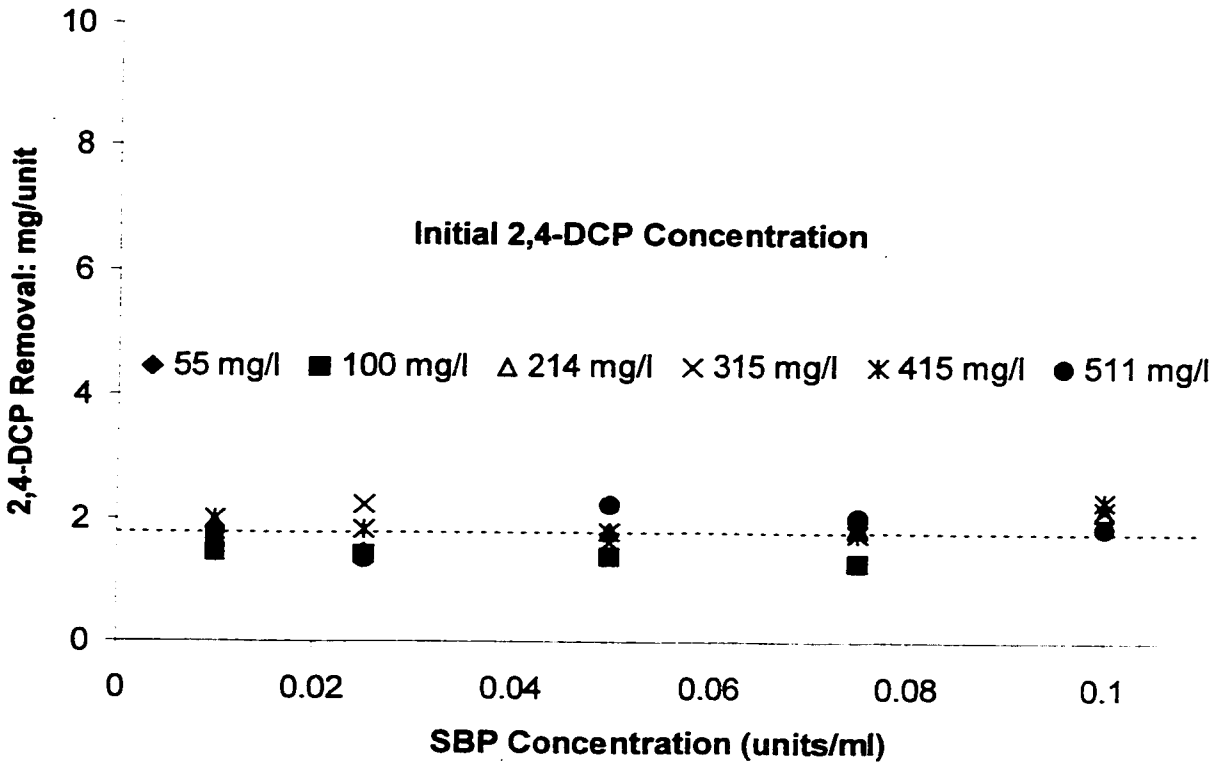
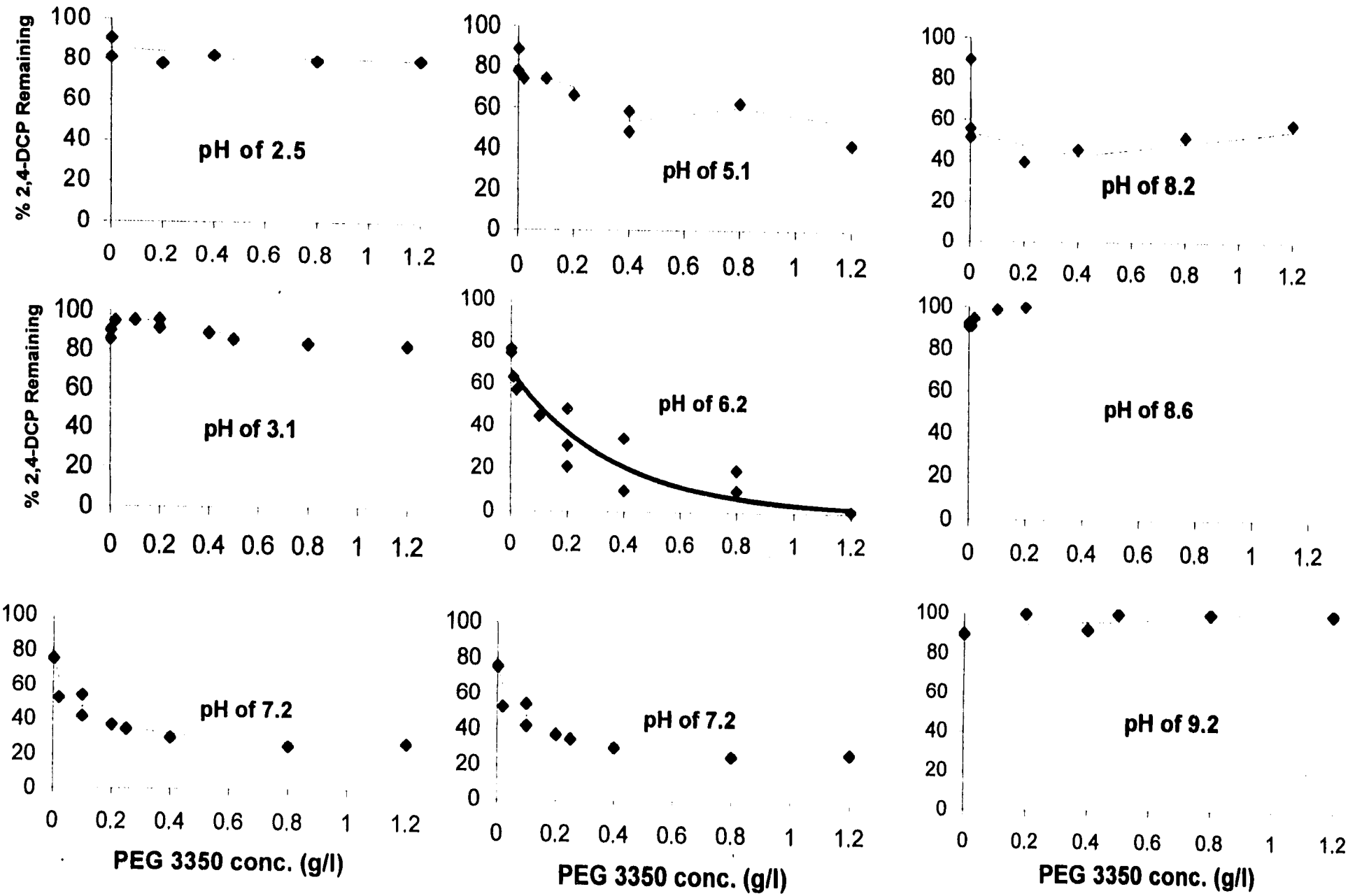


Figure 3.7: 2,4-DCP removed in mg per unit of SBP used in a 1 litre batch reactor. (pH 8.2 and a temperature of 22°C)

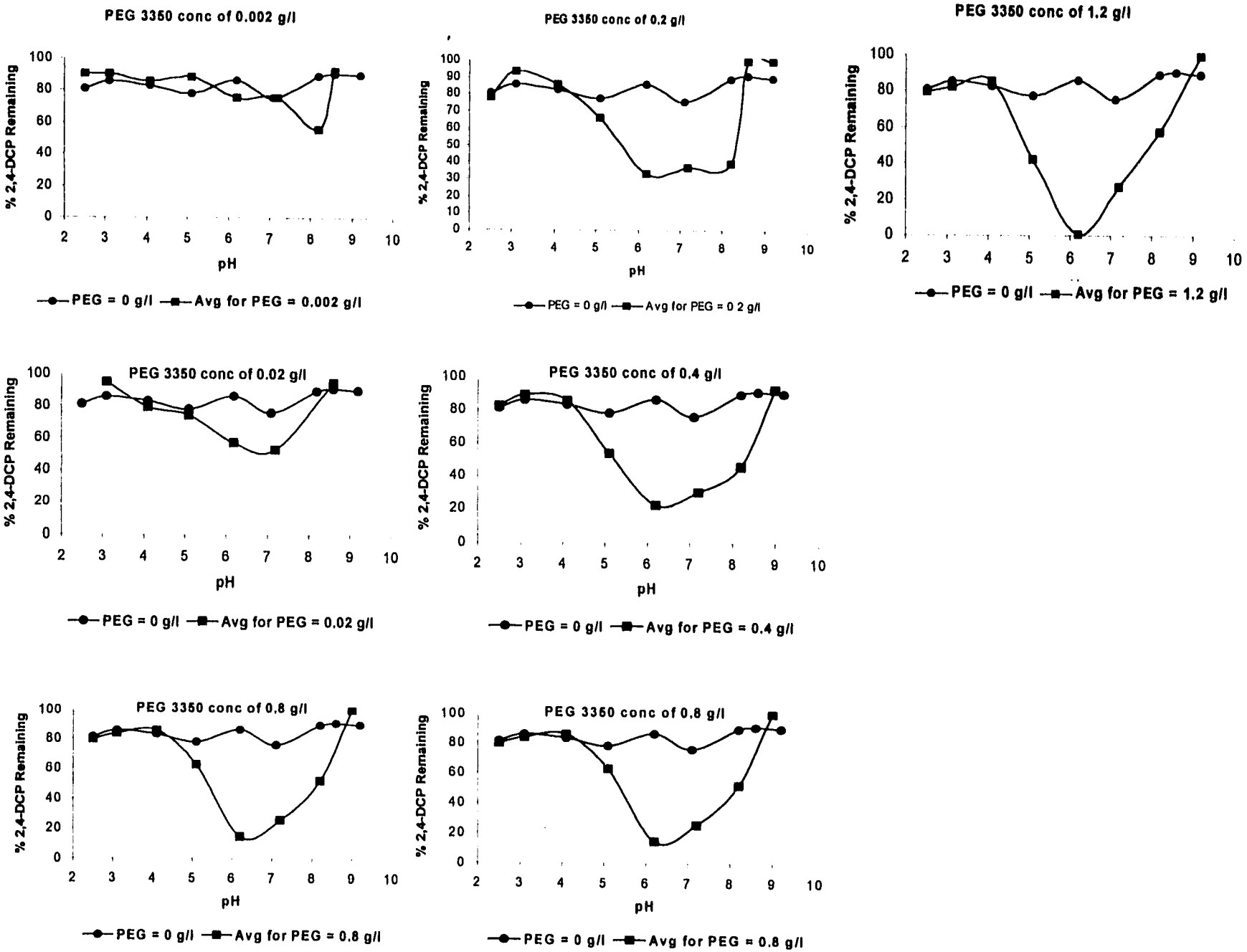
3.4.4 Protective Additive PEG 3350 and the Influence of pH

The last experiment helps characterize the enzymatic reaction using various concentrations of the protective additive polyethylene glycol (PEG)with molecular weight 3350 (PEG 3350). The process kinetics with regards to enzyme activity protection afforded by the PEG is not fully defined. It is believed that, when dissolved into solution with the substrate, the PEG offers the free radicals that form a binding site to attach to rather than having attach to another free radical.

Figure 3.4a indicated that 0.01 units/ml of SBP could only remove 10% - 15% of 100 mg/l pf 2,4-DCP at any pH. This enzyme concentration was selected for further study and served as a benchmark for using PEG as a protective additive. The optimum pH for removal in the presence of PEG 3350 for SBP was determined in Figures 3.8a-3.8i. Addition of PEG resulted in little to no improvement (and even worse removal at very low and low pH) for pH 2.5, 3.1, 4.1, 8.6, and 9.2. Marked improvement was observed between pH 5.1 and 8.2. However, the optimum pH that removed close to 100% with a PEG concentration of 1.2 g/l was pH 6.2. Figures 3.9a-3.9g shows that with an increasing PEG concentration, the removal of 2,4-DCP increases for certain pH values. Unlike the pH tests without PEG, the pH 7 "bump" (Figure 3.4a) is not evident in these results with an additive present. The removal efficiency with PEG 3350 is optimum at pH 6.2 and gradually decreases in both directions. Equations were fit to the data using 2 variables, however a 3rd degree polynomial was fit to all the data to provide the representative contour plot in Figure 3.10. The contour plot reveals how increasing the concentration of PEG 3350 can improve the 2,4-DCP removal rate efficiency especially around the optimum pH of 6.2. The number of turnovers achieved for pH 6.2 without a protective additive equaled approximately 37492. Using 1.2 g/l of PEG 3350, the turnovers increased by a factor of five to 187458 (the max. for this SBP conc. in batch).



Figures 3.8a-3.8i: Illustrate the effects of increasing the concentration of the additive PEG 3350 on the removal of 100 mg/l 2,4-DCP using 0.01 units/ml SBP for various pH in a 25 ml batch reactor at room temperature.



Figures 3.9a-3.9g: Illustrates the effects of pH on the removal of 100 mg/l 2,4-DCP using 0.01 units/ml SBP for various concentrations of the additive PEG 3350 in a 25 ml batch reactor at room temperature.

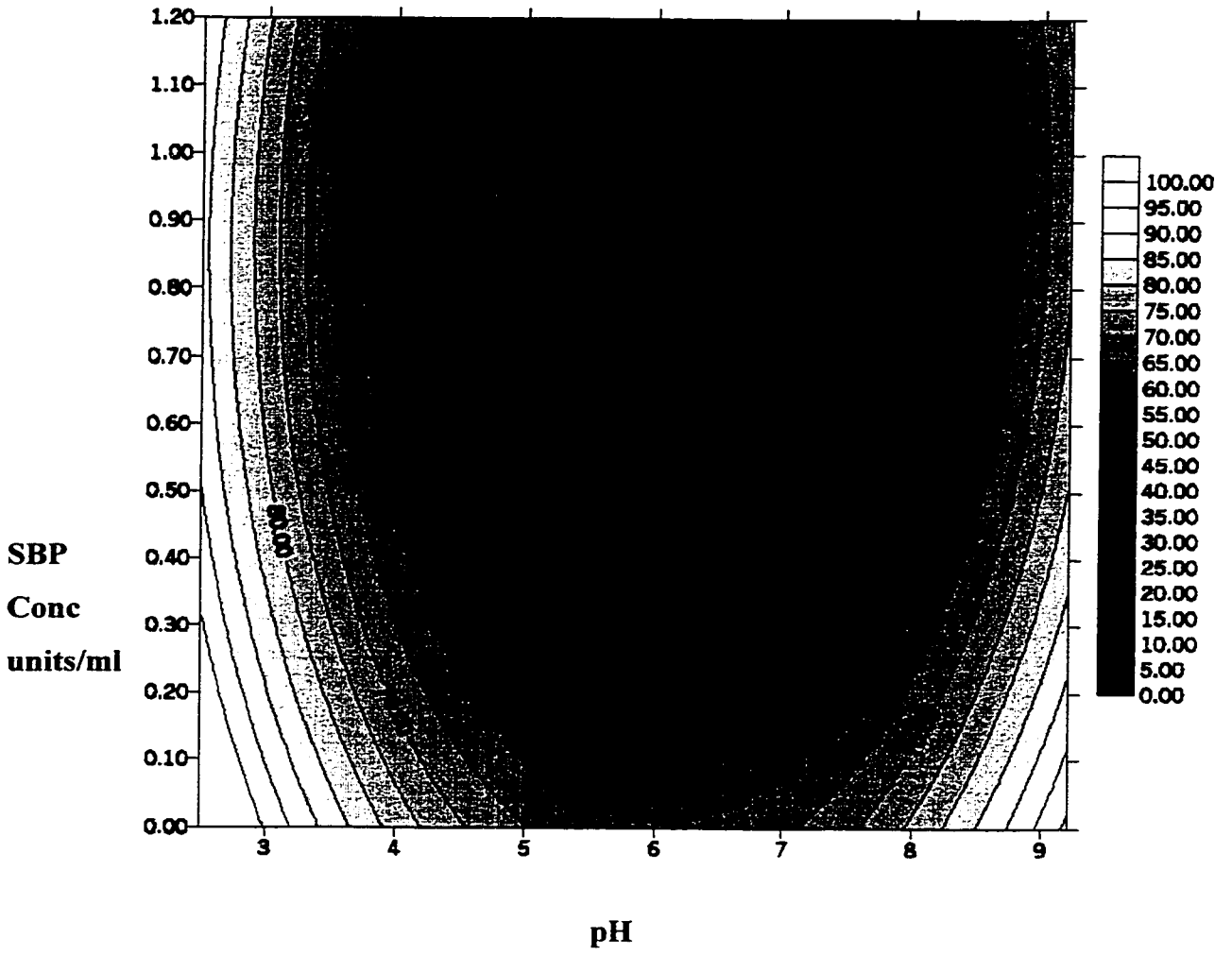


Figure 3.10: Contour representation of Figures 9a-9g. Legend indicates % remaining of 100 mg/l 2,4-DCP in a 25 ml batch reactor at room temperature.

3.5 Conclusion

Several conclusions can be made from these test results. Firstly, time and temperature are important factors to be considered when appliying SBP for treatment of 2,4-DCP. . The colder the temperature of the mixture the slower the rate of reaction. The rate at which the reaction proceeded to remove 100% 2,4-DCP at room temperature compared to 4oC was 180 times faster. However, for the evaluation of SBP for treatment of 2,4-DCP, at the temperatures tested, 3 hours is sufficient time to allow the reaction to occur and for the particulate to form and settle. SBP is a viable alternative to HRP which serves as a good benchmark to compare the two enzymes. The optimum pH for SBP treatment of 2,4-DCP without an additive was determined to be approximately 8.2. At the optimum pH of 8.2 (without an additive), the removal efficiency of various concentrations of 2,4-DCP is linear within the SBP utilization range of 0.001 to 0.1 activity units and can be summarized by stating 1.77 mg of 2,4-DCP is removed per unit of SBP activity. In the presence of the additive PEG- 3350, the optimum pH shifts to pH 6.0 and the removal efficiency increases by factor of 10 compared to no PEG addition.

The use of turnovers (TO) to describe removal efficiency provides a benchmark for comparison. Using the same SBP concentration (0.01 units/ml) with no additive at different pHs increased the number of TO from 5624 at pH 3.1 to 18746 at pH 8.2 (a factor of 3.3) using. Finally, the use of 1.2 g/l PEG-3350 allowed for an increase in TO by a factor of 10 to 187458 at pH 6.2 and SBP of 0.01 units/ml.

3.6 Recommendations for Future Research

Based on the results obtained in this study, recommendations for future research studies which could improve the SBP process are as follows:

1) Investigate the use of higher molecular weight protective additives.

2) Determine the effectiveness of SBP in the treatment of other chlorinated phenols.

3) Apply the SBP process on a larger scale study.

4) Determine the suitability of the PE process to remediate contaminated soil.

5) Characterize the by products formed in the PE process.

CHAPTER FOUR

Using Various Molecular Weights Polyethylene Glycol as a Protective Additive to Soybean Peroxidase for the Treatment of Chlorinated Phenol in Wastewater.

4.1 Abstract

The successful use of soybean peroxidase (SBP) enzymes to treat wastewater contaminated with a halogenated aromatic has been established in Chapter Three. My previous results indicated that SBP is as effective and in certain cases more effective than horseradish peroxidase (HRP) in treating wastewater contaminated with 2,4-dichlorophenol (2,4-DCP). The following section presents the results obtained when using purified SBP, to treat various concentrations of 2,4-DCP and other chlorinated phenols in the presence of polyethylene glycol (PEG) of various molecular weights. The experiments were performed to determine the level of influence of pH, enzyme concentration, initial 2,4-DCP concentration, PEG concentration and varying PEG molecular weight. An experiment was performed to determine the applicability of using SBP to treat other chlorinated phenols including 2-chlorophenol (2-CP), 4-chlorophenol (4-CP) and 2,4,6-trichlorophenol (2,4,6-TCP). Reactor configuration has also been identified as a crucial parameter to the peroxidase enzyme (PE) treatment process. The final experiments were performed under optimum conditions to examine the effects the various methods of applying the substrate dose, the enzyme dose and the H_2O_2 dose has on the final removal efficiency.

Key words - soybean peroxidase enzyme, chlorinated phenol, polyethylene glycol.

4.2 Introduction

In the presence of H_2O_2, PE can catalyze the oxidation of a wide variety of aromatic compounds in aqueous solution. The resulting aromatic free radicals that form combine with each other and precipitate out of solution. Early research experiments focused on the treatment process using horseradish peroxidase (HRP) (Klibanov *et al.*, 1980). Recently however, a promising new alternative to HRP has been undergoing rigorous testing to determine its suitability and its place in this treatment field. Soybean peroxidase, extracted from the seedcoat of the soybean has been shown to be thermally stable and capable of reacting in acidic conditions (McEldoon and Dordick, 1996). These promising characteristics distinguish SBP from other types of PE.

Another recent advancement in the PE treatment field is the use of protective additives which help prolong the catalytic ability of the enzyme. It is hypothesized that the particulate that forms and precipitates out of solution in this reaction, entraps the PE and thereby renders it inactive. High molecular weight additives such as polyethylene glycol (PEG) can be used to suppress the entrapment process. It has been reported that PEG with a molecular weight less than 1000 is ineffective at protecting HRP when treating phenol and that PEG with a molecular weight of 7500 is more efficient than PEG 1000 (Nakamoto and Machida, 1992). Other researchers have continued this research using PEG with a molecular weight of 3350 (PEG 3350) (Wu *et al.*, 1993; Ibrahim *et al.*, 1997). However, there is no supporting documentation that suggests PEG 3350 is more suitable than PEG 7500. A recent study has shown that SBP in the presence of PEG with higher molecular weights can achieve better phenol removal efficiency (Kinsley, 1998). This study will report on the effectiveness of using various molecular weight PEG for the protection of SBP when treating chlorinated phenols.

The effect of pH on HRP catalyzing phenol in the presence of PEG has been documented (Bewtra et al., 1995; Dec and Bollag, 1994a,b). The effect of pH on HRP catalyzing different chlorinated phenols without an additive present has also been reported and the results indicate that the optimum pH of the reaction is not only dependent on the enzyme in question but it is also dependent on the substrate involved (Dec and Bollag, 1990). Recent studies using SBP have also accepted the optimum pH conditions determined for HRP as being optimum for SBP

(Kinsley, 1998). While this may certainly be the case, few (if any) studies have been performed to support these assumptions. The previous chapter in this study examined the effect of various pH on the SBP treatment of 2,4-DCP in the presence of PEG 3350. In this chapter, the effect of a range of acidic pH will be presented for a higher molecular weight PEG 8000 when protecting SBP's treatment of 2,4-DCP.

An experiment was performed using SBP to treat various chlorinated phenols including 2-chlorophenol (2-CP), 4-chlorophenol (4-CP) and 2,4,6-trichlorophenol (2,4,6-TCP). The test revealed that SBP is capable of treating a range of contaminants similar to HRP.

The effects of reactor set up have been described for HRP (Nicell *et al.*, 1993) but information is limited for SBP. By controlling the amount of enzyme and/or H_2O_2 available to the reaction, there is the possibility of limiting the amount of PE inactivation that may occur. Tests were performed to determine the effects of adding the SBP and/or the H_2O_2 in a semi-batch mode. Turnovers are used to express the amount of moles of a compound treated per mole of enzyme. Using turnovers to describe the results provides a benchmark to which other feasibility studies can be measured. A discussion on turnovers is presented at the end of the results.

4.3 Materials and Methods

The materials and methods described in Chapter 3 were used and followed for Chapter 4. 2-CP, 4-CP and 2,4,6-TCP were purchased from Sigma chemicals and prepared in the manner described for 2,4-DCP. All experiments in Chapter 4 were performed at room temperature of 22°C. Any procedures not previously explained will be presented in the appropriate section.

4.4 Observations and Results

4.4.1 Influence of PEG Molecular Weight, PEG Concentration and 2,4-DCP Concentration

An experiment was performed to determine the effects of using various molecular weights of a PEG for the SBP process treating 2,4-DCP. Similar work has been performed with HRP on phenol (Nakamoto and Machida, 1992; Wu *et al.*, 1993). The results with HRP indicated that only molecular weights higher than 1000 were effective at protecting the enzymatic reaction. Experimental conditions determined in Chapter 3 were used in Chapter 4. A pH of 6.2 was used as the optimum pH as previously determined in Chapter 3. The temperature of the reaction mixtures was 22°C. The reactor volume remained at 25 ml. The

reactions were allowed to continue for three hours at which time a 0.5 ml dose of concentrated catalase solution was added to the mixture to remove any remaining H_2O_2. A SBP concentration of 0.01 units/ml was selected from the results of previous experiments in Chapter 3. At a concentration of 0.01 units/ml, SBP exhibited activity but only removed approximately 10 to 15% of the initial 2,4-DCP. The variables studied in this experiment include: initial 2,4-DCP concentrations; PEGs with three different molecular weights (1000, 3350, and 8000) and PEG concentration (0.01 to 2.0 g/l). The data corresponding to this experiment are plotted in Figures 4.1a, 4.1b, and 4.1c. In Figure 4.1a, it is observed that for all concentrations of PEG 1000, there was no improvement in the treatment of the 2,4-DCP when compared to the control test containing only the SBP enzyme. The tests using PEG 3350 in Figure 4.1b showed a marked improvement over using the enzyme alone. The highest concentration of PEG 3350 used (2 g/l) achieved >95% removal for all three initial concentrations of 2,4-DCP. In the final test using PEG 8000, the results indicte a significant increase in the removal where >95% removal was achieved for every concentration of PEG tested, including the 0.1 g/l concentration (Figure 4.1c). These encouraging results indicate that not only can a greater level of removal be achieved using PEG 8000 but that it can be done using a lower concentration of PEG 8000. This is important when considering the enzyme effects of residual PEG in water. Firstly, as the molecular weight of PEG increases, it becomes more recalcitrant and the residual PEG becomes more difficult to remove from the water. This is an important consideration if using very high molecular weight PEG. Therefore, there is a tradeoff between the beneficial effects of high molecular weight PEG on PE reactions and the negative effects of residual PEG in the environment. Ideally, we would prefer using a minimum amount of high molecular weight PEG, yet achieve high PE removal efficiency. PEG 8000 seems to fit this criteria compared to lower molecular weight PEGs. It might also be worth investigating even higher molecular weight PEG if even lower concentrations will be needed to achieve similar or better results that with PEG 8000. Kinsley (1998) reported on the effects of increasing molecular weight PEG on the removal of phenol using SBP. His data shows that based on total phenol removal, PEG 35000 was the optimum molecular weight for the process. However, the overall effectiveness of the higher molecular weight PEG must be compared relative to the other results. When using 0.2 units/ml SBP to treat

1 mM phenol in the presence of 100 mg/l PEG, PEG 3350 removed approx. 50%, PEG 8000 removed approx. 84% and PEG 35000 removed approx. 98%. Therefore by doubling the molecular weight from 3350 to 8000, the removal efficiency was increased by 34%. By increasing the molecular weight by a factor of ten to 35000 (4.4 times higher than 8000), the removal increased by 48% which is only 14% more than PEG 8000. Therefore, based on the previous criteria, by increasing the molecular weight to 35000 and in return achieving only 14% better removal efficiency, the increase may not be justified in terms of increased difficulty in treating residual PEG. Kinsley also studied the residual PEG in solution and determined that COD increases rapidly with increasing PEG and that optimum PEG doses should be used in order to minimize residual PEG.

Photos 4.1 and 4.2 show some of the reaction products observed when using SBP to treat 2,4-DCP both with and without the presence of PEG. Colour formation was also related to the pH of the solution. At lower pH the colour of the mixture after reacting tended to be a more yellow colour, while closer to pH 6 and 8 the colour of the solution was pinkish orange.

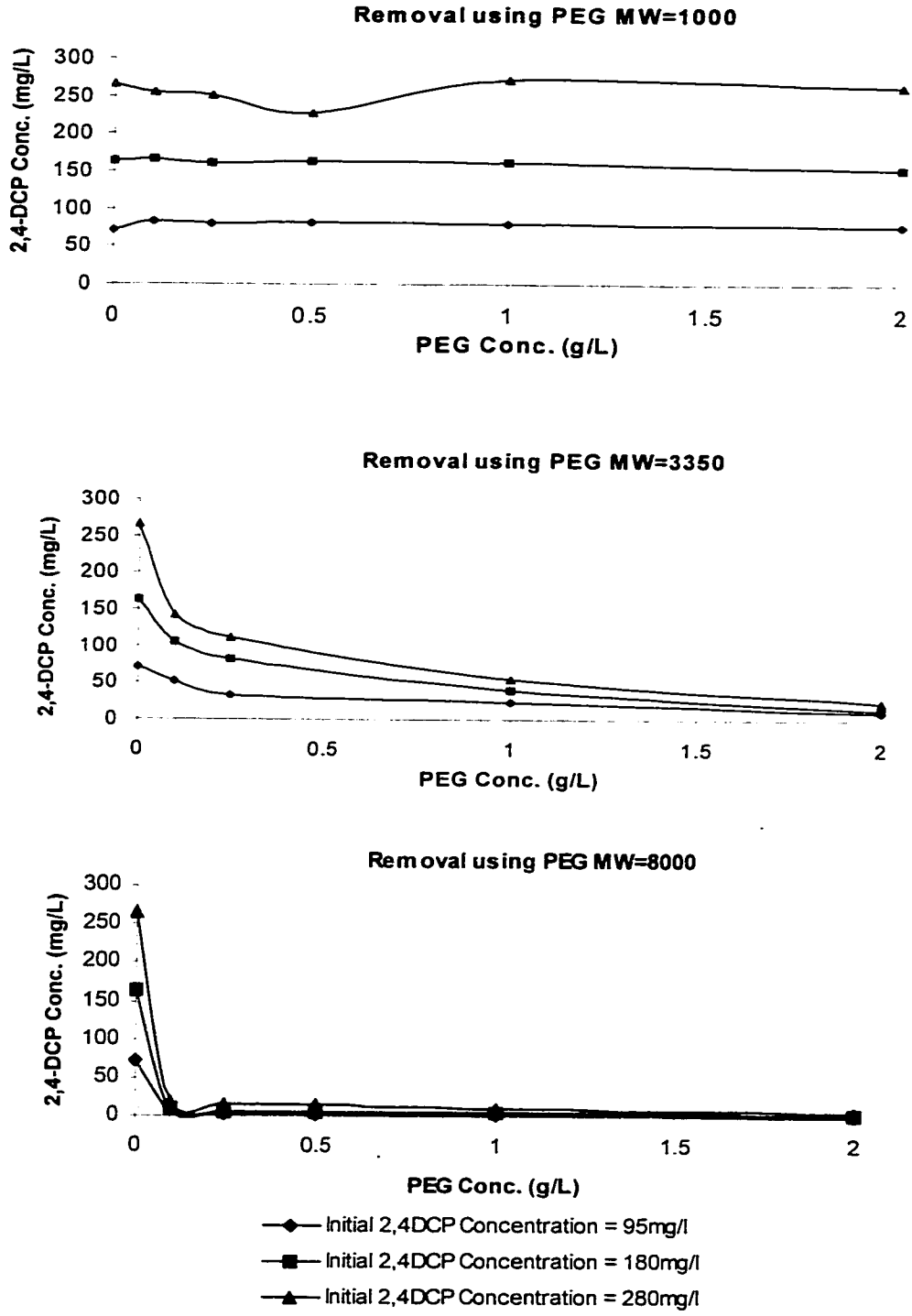


Figure 4.1a-4.1c: Removal of various initial concentrations of 2,4-DCP using 0.01 units/ml SBP with increasing PEG concentration. (PEG 1000, PEG 3350 and PEG 8000)

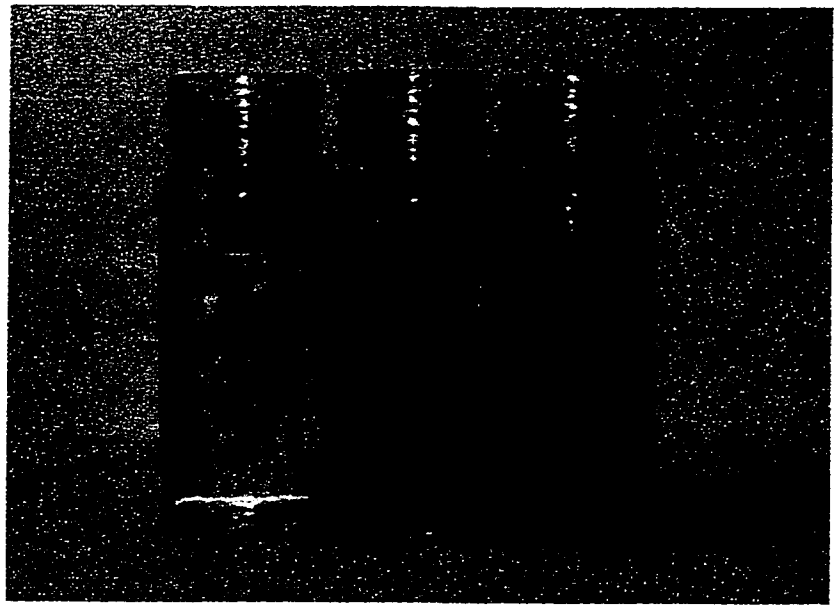


Photo 4.1: Each vial contains 100 mg/l 2,4-DCP buffered at pH 6.2 and 0.01 units/ml SBP activated by H_2O_2. The vial on the left contains 1 g/l PEG 1000, in the middle 1 g/l PEG 3350 and on the right 1 g/l PEG 8000.

Photo 4.2: Effect of increasing SBP concentration from 0, 0.25, 0.5, 0.75, to 1.0 units/ml. (left to right) Each solution of 100 mg/l 2,4-DCP contained no additive but were buffered to pH 6.2

4.4.2 Influence of SBP, PEG 8000 and 2,4-DCP Concentrations.

From the previous results, it was determined that a SBP concentration of 0.01 units/ml can successfully treat up to 300 mg/l of 2,4-DCP using various concentrations of PEG 8000 (Figure 4.2a and 4.2b). Two separate tests were carried out to determine the level of treatment that could be obtained by decreasing the concentration of SBP below 0.01 units/ml while using various concentrations of PEG 8000. Both tests were carried out under similar environments. The solutions were buffered to a pH of 6.2 and maintained at 22°C. The first set of tests performed used an initial 2,4-DCP concentration of 100 mg/l. Three SBP concentrations, 0.0005, 0.001 and 0.005 units/ml were tested using 4 different concentrations (0, 0.2, 1.0 and 2.0 g/l) of PEG 8000. When no PEG was added to the reaction, there was no removal of 2,4-DCP. Table 4.1 summarizes the results obtained from Figure 4.2a and Table 4.2 summarizes the results obtained from Figure 4.2b. When PEG 8000 was used, there was a significant increase in removal for the three SBP concentrations. Without any PEG, the removal was negligible. The maximum number of turnovers that can be achieved using 0.01 units/ml (in batch) assuming 100% removal of 100 and 300 mg/l 2,4-DCP is equal to 187,458 and 562,373 respectively. However, decreasing SBP concentrations used, increases the maximum number of turnovers proportionally. For example, using 0.0005 units SBP/ml and various concentrations of PEG 8000, up to 30% removal was obtained. This translates into a 1,124,746 turnovers; however, the removal efficiency of 30 % is relatively low. Using 0.005 units/ml SBP up to 95% removal of 2,4-DCP was achieved. Not only was this a good removal efficiency, the number of turnovers this translates into is 356,170. Likewise, when treating 300 mg/l 2,4-DCP with 0.001 units/ml, 35 % removal was attained for a turnover number equivalent to 1,968,305. Using 0.005 units/ml, up to 85% removal was achieved equal to 956,034 turnovers. These encouraging removal efficiencies and turnover numbers indicate that the SBP treatment process can be optimized by controlling the SBP concentration, the PEG concentration and the substrate concentration. These results also reflect the idea that semi-batch addition of very small amounts of enzyme could increase the turnover number and increase removal efficiency by controlling the amount of SBP in the reaction at any time and thereby reducing the chance of enzyme inactivation. Also, at the higher SBP concentrations, the percent removal using 2.0 g/l of PEG is approximately 10%

greater than the removal using 0.2 g/l. Similar results were obtained using 300 mg/l 2,4-DCP, therefore indicating that a tenfold increase in PEG 8000 concentration results only in a slight increase in removal efficiency.

Table 4.1: Ratio of 2,4-DCP (mg) removed per unit of SBP. (Initial 2,4-DCP = 100 mg/l and PEG 8000 ranges in concentrations from 0.2, 1.0 and 2.0 g/l): (See Figure 4.2a)

SBP Enzyme Concentration	Amount 2,4-DCP Removed	Percentage of 2,4-DCP Removed	Ratio 2,4-DCP mg removed per unit of SBP
0.0005 units/ml	20 to 30 mg/l	20 to 30 %	40 to 60 mg/unit
0.001 units/ml	30 to 40 mg/l	30 to 40 %	30 to 40 mg/unit
0.005 units/ml	85 to 95 mg/l	85 to 95 %	17 to 19 mg/unit

Table 4.2: Ratio of 2,4-DCP (mg) removed per unit of SBP. (Initial 2,4-DCP = 300 mg/l and PEG 8000 ranges in concentrations of 0.2, 1.0 and 2.0 g/l): (See Figure 4.2b)

SBP Enzyme Concentration	Amount 2,4-DCP Removed	Percentage of 2,4-DCP Removed	Ratio mg 2,4-DCP removed per unit of SBP
0.001 units/ml	75 to 105 mg/l	25 to 35 %	75 to 105 mg/unit
0.005 units/ml	210 to 255 mg/l	70 to 85 %	42 to 51 mg/unit

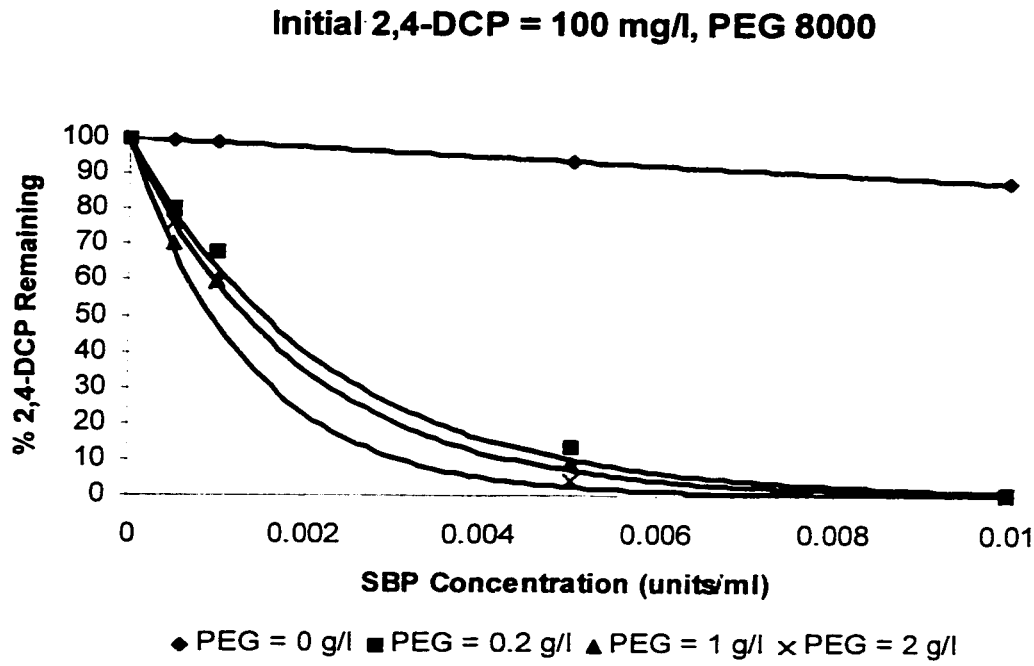
Equations were fit to the experimental data as shown in Figures 4.2a and 4.2b. The equations representing these curves (Equations 4.1 through 4.6) are presented in Tables 4.3 and 4.4. The correlation coefficients indicate excellent fit since they approach unity. These equations are provided to show the mathematical relationships that exist and how SBP concentration is a direct function of removal in the presence of PEG 8000.

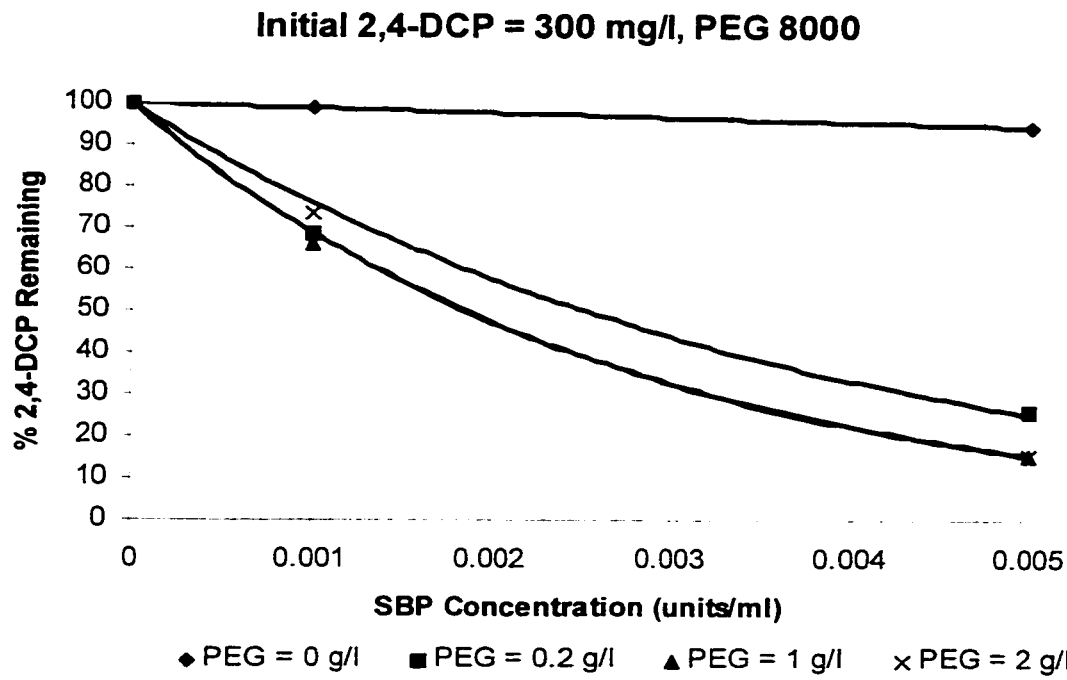
Table 4.3: 2,4-DCP remaining as a function of SBP concentration for various PEG 8000 concentration for initial 2,4-DCP concentration equal to 100 mg/l:

PEG 8000	% 2,4-DCP Remaining	SBP Range units/ml	R^2	Equation
2.0 g/l	$\%_{2,4\text{-DCP}} = 100e^{-749.9[SBP]}$	0 > [SBP] > 0.01	0.99	4.1
1.0 g/l	$\%_{2,4\text{-DCP}} = 100e^{-530.8[SBP]}$	0 > [SBP] > 0.01	1.00	4.2
0.2 g/l	$\%_{2,4\text{-DCP}} = 100e^{-459.6[SBP]}$	0 > [SBP] > 0.01	0.99	4.3

Table 4.4: 2,4-DCP remaining as a function of SBP concentration for various PEG 8000 concentration for initial 2,4-DCP concentration equal to 300 mg/l:

PEG 8000	% 2,4-DCP Remaining	SBP Range units/ml	R^2	Equation
2.0 g/l	$\%_{2,4\text{-DCP}} = 100e^{-275.4[SBP]}$	0 > [SBP] > 0.0005	0.99	4.4
1.0 g/l	$\%_{2,4\text{-DCP}} = 100e^{-375.1[SBP]}$	0 > [SBP] > 0.0005	1.00	4.5
0.2 g/l	$\%_{2,4\text{-DCP}} = 100e^{-373[SBP]}$	0 > [SBP] > 0.0005	1.00	4.6

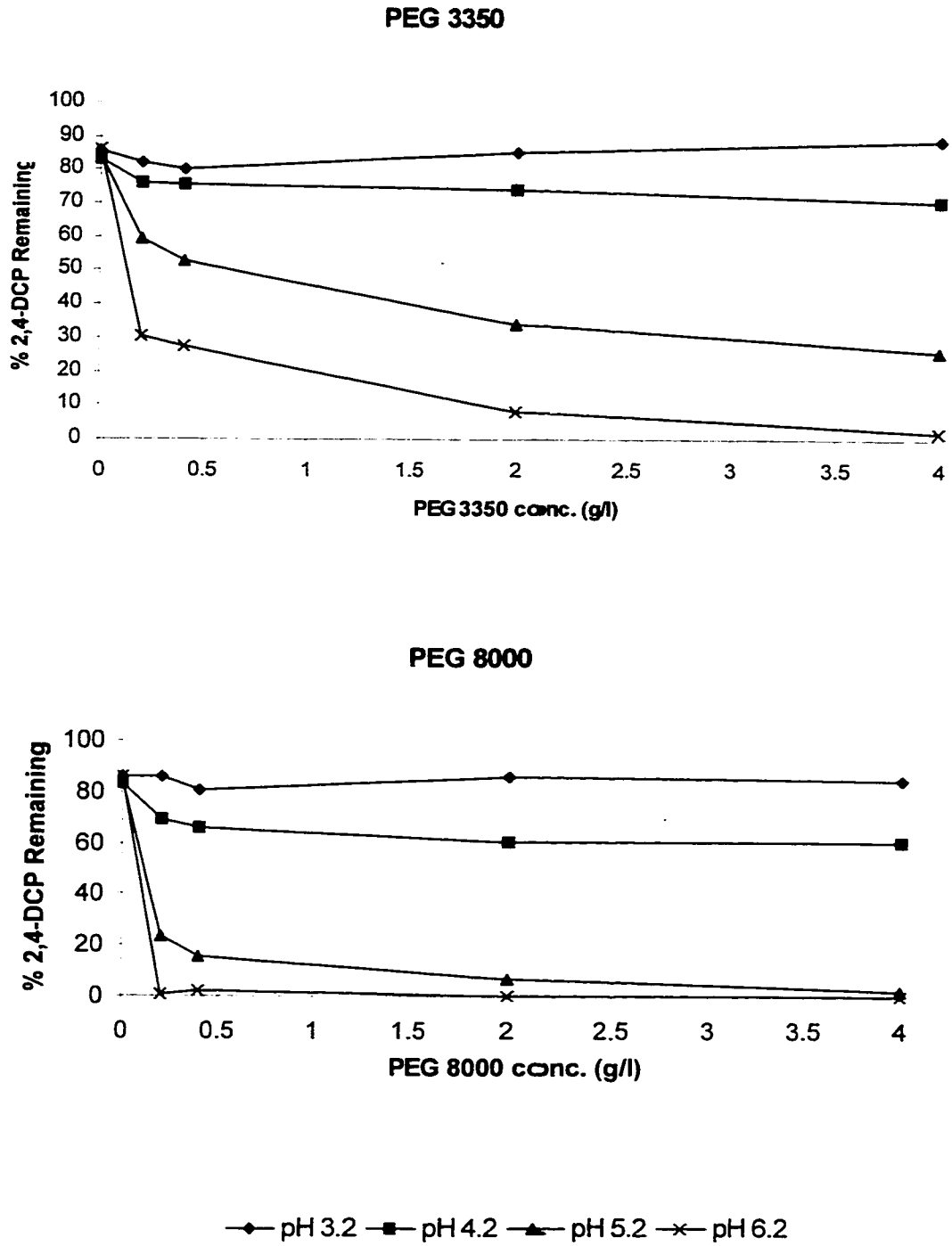




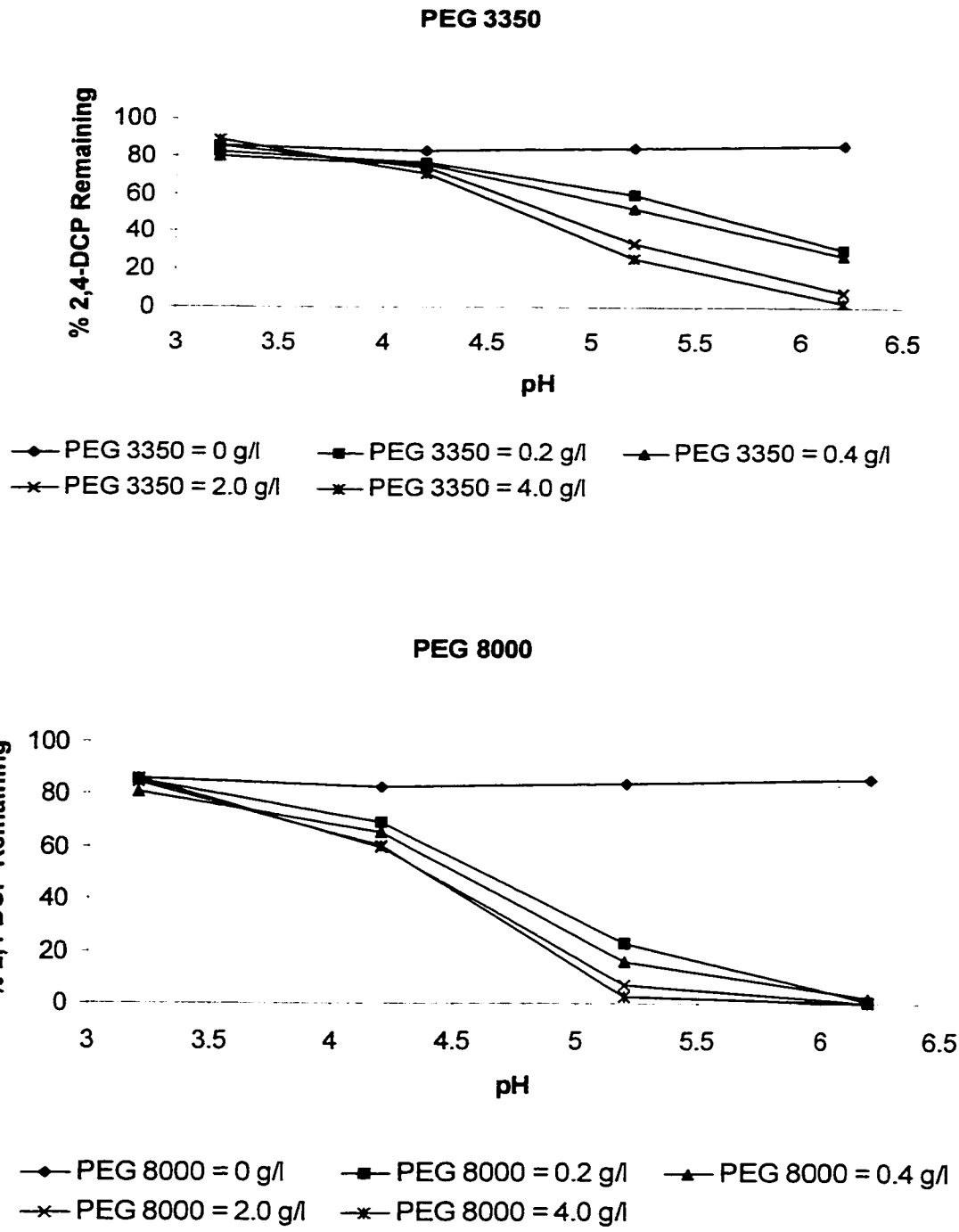
Figures 4.2a & 4.2b: Percent 2,4-DCP remaining versus increasing SBP concentration for various concentrations of PEG 8000. The tests were performed using two initial concentrations of 2,4-DCP 100 and 300 mg/l.

4.4.3 Influence of PEG Molecular Weight, PEG Concentration and pH

The following observations are from experiments investigating the relationships between PEG molecular weight, PEG concentration and pH. The SBP concentration used was constant at 0.01 units/ml. The reaction was run at room temperature in 25 ml reactors as previously described and was stopped after 3 hours with a large dose of catalase. The first set of tests was run using PEG 3350 in concentrations ranging from 0 to 4.0 g/l. The initial 2,4-DCP concentration was 100 mg/l and the H_2O_2 dose was added in a 1:1 molar ratio with the 2,4-DCP. Each set of PEG concentrations was tested at four different pH (3.2, 4.2, 5.2 and 6.2). Identical tests were repeated using PEG 8000 instead of PEG 3350. The results shown in Figures 4.3a, 4.3b, 4.4a and 4.4b indicate that 2,4-DCP removal in the presence of PEG is dependent on both PEG concentration and pH. For PEG 8000 at a pH of 4.2 (See Figure 4.4b), the addition of increasing concentrations of PEG improves the removal by 10 to 20% over the control without PEG. At a pH of 5.2, a significant increase in the removal of 2,4-DCP is observed when PEG 8000 is added. The amount of 2,4-DCP removed for PEG 3350 is 23 to 60% more than the control without PEG, whereas the removal for PEG 8000 is between 65 to 84% more that the control without PEG using the same SBP concentration. Lastly, using pH 6.2, PEG 3350 increased the removal from 14% (control) to 70 ~ 99% (for 0.2 ~ 4.0 g/l PEG 3350). Additionally, PEG 8000 proved to be more efficient at producing removals that were greater than 99% for all PEG 8000 concentrations tested. For comparison, the number of turnovers using 0.2 g/l PEG 3350 at pH 6.2 increased from 22,244 (control) to 131,220 (a factor of 5.9). Using 0.2 g/l PEG 8000 at pH 6.2, the number of turnovers increased from 22,244 (control) to 185,583 (a factor of 8.3).



Figures 4.3a & 4.3b: Percentage of 100 mg/l 2,4-DCP remaining versus increasing PEG concentration at various acidic pH conditions for PEG 3350 and PEG 8000.



Figures 4.4a to 4.4b: Percentage of 100 mg/l 2,4-DCP remaining versus increasing pH for various PEG 3350 and PEG 8000 concentrations.

4.4.4 Influence of SBP Concentration, PEG 8000 Concentration and pH

This series of tests were at three different pHs (3.2, 5.2 and 6.2), using various concentrations of PEG 8000 (0, 0.2, 1.0 and 2.0 g/l) and various SBP concentrations less than 0.01 units/ml. The experiments were performed in 25 ml reactors at 22°C. Direct light was prevented, H_2O_2 was added in one dose in a 1:1 molar ratio with the initial 100 mg/l of 2,4-DCP concentration tested and mixing was performed at 50 rpm on a Junior Orbitor shaker.

The data obtained from the series of tests is plotted in Figures 4.5a to 4.5c. Figure 4.5a presents the data obtained at pH of 3.2. The improved removal of 2,4-DCP compared to the control (no PEG) shows that PEG 8000 can be effective even in acidic conditions. Though the increase in removal is not as good as under optimum pH conditions, it does require 25% less SBP (0.75 units/ml) with PEG 8000 to achieve 100 % removal of the 2,4-DCP than it does without PEG (1.0 units/ml). Also, to achieve 100% removal with PEG 8000 present, 0.75 units/ml of SBP is required. Using this same SBP concentration with no PEG, only 90% removal is achieved. It is important to note that the same 2,4-DCP removal efficiencies (at pH 3.2) were observed when adding 0.2 or 2.0 g/l of PEG 8000. The excess PEG remaining from the 2.0 g/l used could be discharged to the environment which would have a negative effect. Therefore using the least amount of PEG 8000 as possible is most desirable.

At pH 5.2 (Figure 4.5b), a significant increase in removal efficiency occurs in the presence of PEG 8000. In the presence of PEG 8000, only 0.025 units/ml of SBP is required to achieve 100% removal. Ten times that amount of SBP (0.25 units/ml) is required to attain 100% removal without the protection (or enhancement) of PEG. For comparison, using 0.025 units/ml SBP without any PEG present, removed only 60% (compared to 100% with PEG 8000).

At pH 6.2 (Figure 4.5c), results similar to pH 5.2 are obtained but at lower enzyme concentrations. Fourteen times as much SBP is required without PEG and the difference in the removal efficiency for the given PEG concentration range is negligible. What is significant is the level of removal that is achieved for both conditions at SBP concentration of 0.01 units/ml. With 2 g/l of PEG 8000 present, the removal of 2,4-DCP is 100% while without PEG, there is only an 11% removal of 2,4-DCP. The effects of pH in the presence of PEG 8000 on the 2,4-DCP removal efficiency can be shown by comparing the SBP concentrations required to attain

100% removal of 2,4-DCP. In the presence of PEG 8000, 0.75 units/ml SBP is required at pH 3.1 while 0.01 units/ml SBP is required at pH 6.2. This represents a seventy-five fold increase in amount of enzyme required at lower pH.

Figure 4.6 also illustrates the affect that PEG 8000 has at different pH. The number of turnovers for each condition was calculated and plotted in Figure 4.6. The maximum number of turnovers achieved at pH 3.2 using PEG 8000 was 2,500 while the maximum number of turnovers achieved at pH 6.2 using PEG 8000 was 187,458. Therefore, seventy-five times more turnovers were achieved simply by optimizing the pH condition.

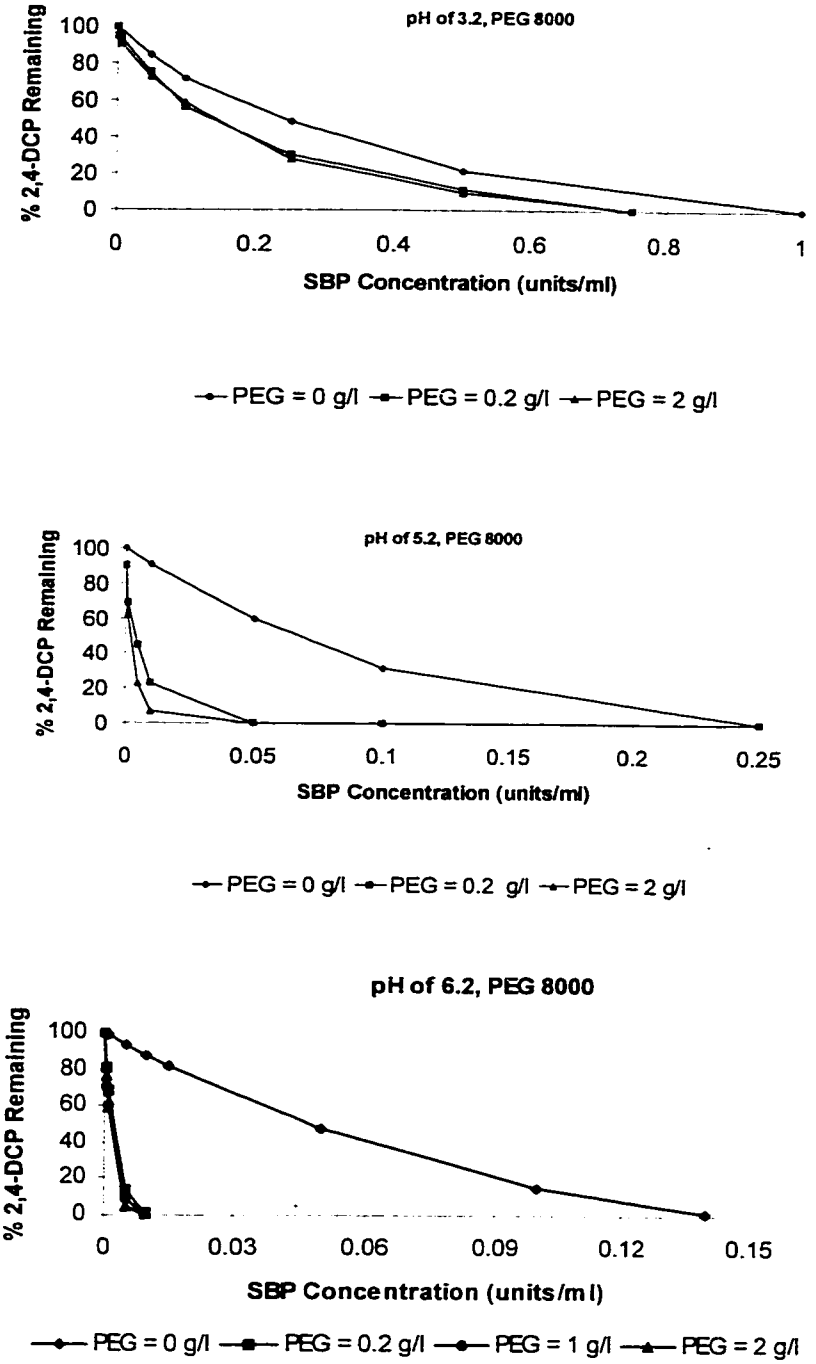


Figure 4.5a to 4.5c: Percentage of 100 mg/l of 2,4-DCP remaining versus increasing SBP Concentration for various PEG 8000 concentrations. The tests were performed at three different pH conditions 3.2, 5.2 and 6.2.

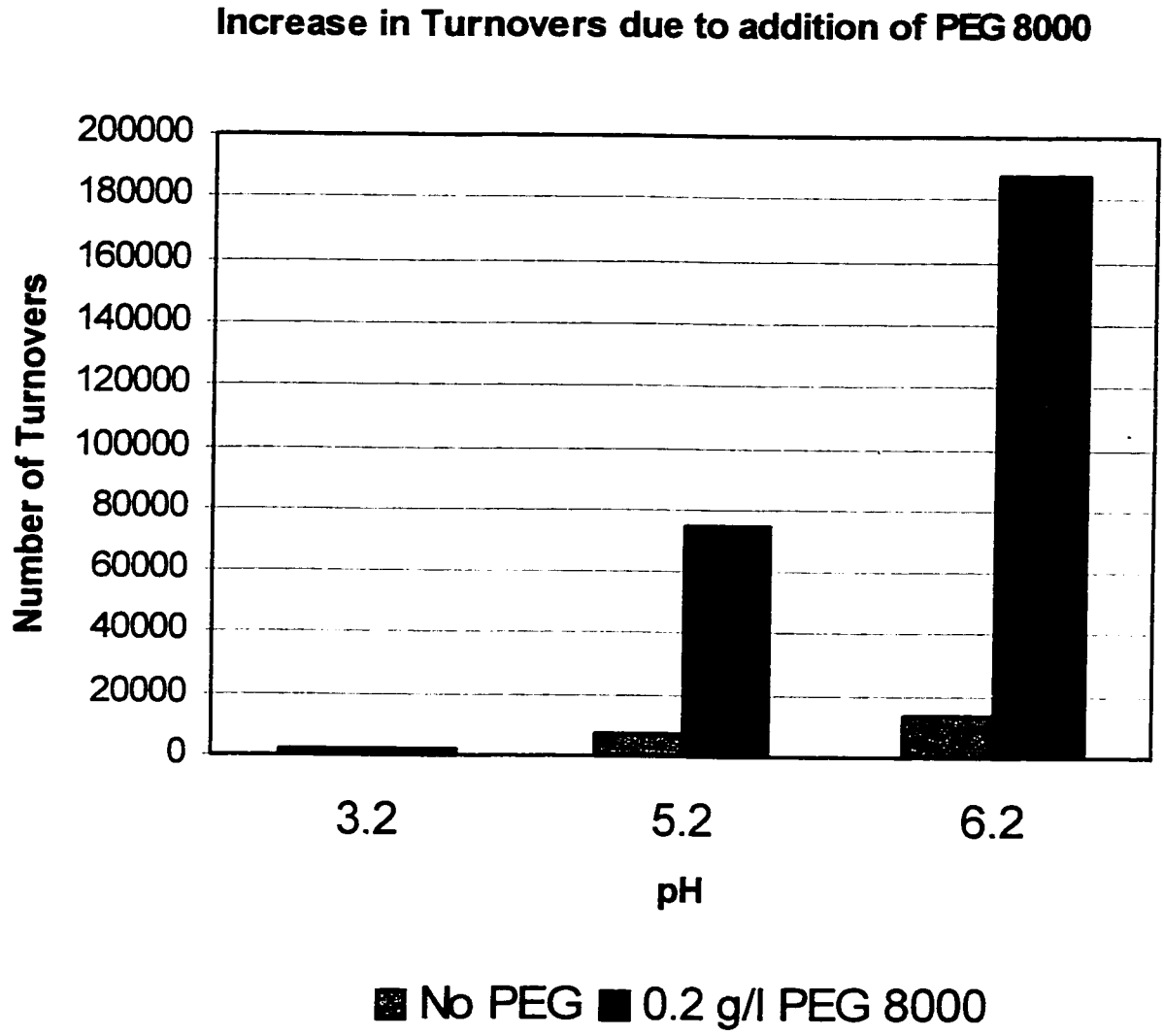


Figure 4.6: Turnovers calculated at 100% removal of 100 mg/l 2,4-DCP with and without PEG 8000 for three different pH (3.2, 5.2 & 6.2).

4.4.5 Influence of SBP Concentration, PEG Concentration and Chlorinated Phenol

These tests were performed to compare the removal efficiency of SBP on two mono-chlorinated phenols 2-chlorophenol (2-CP) and 4-chlorophenol (2-CP) in the presence of PEG 8000. The experiments were performed at pH 6.2. However, as noted earlier, the pH of the solution can affect the specificity of the PE which results in various optimum pH for different substrate.

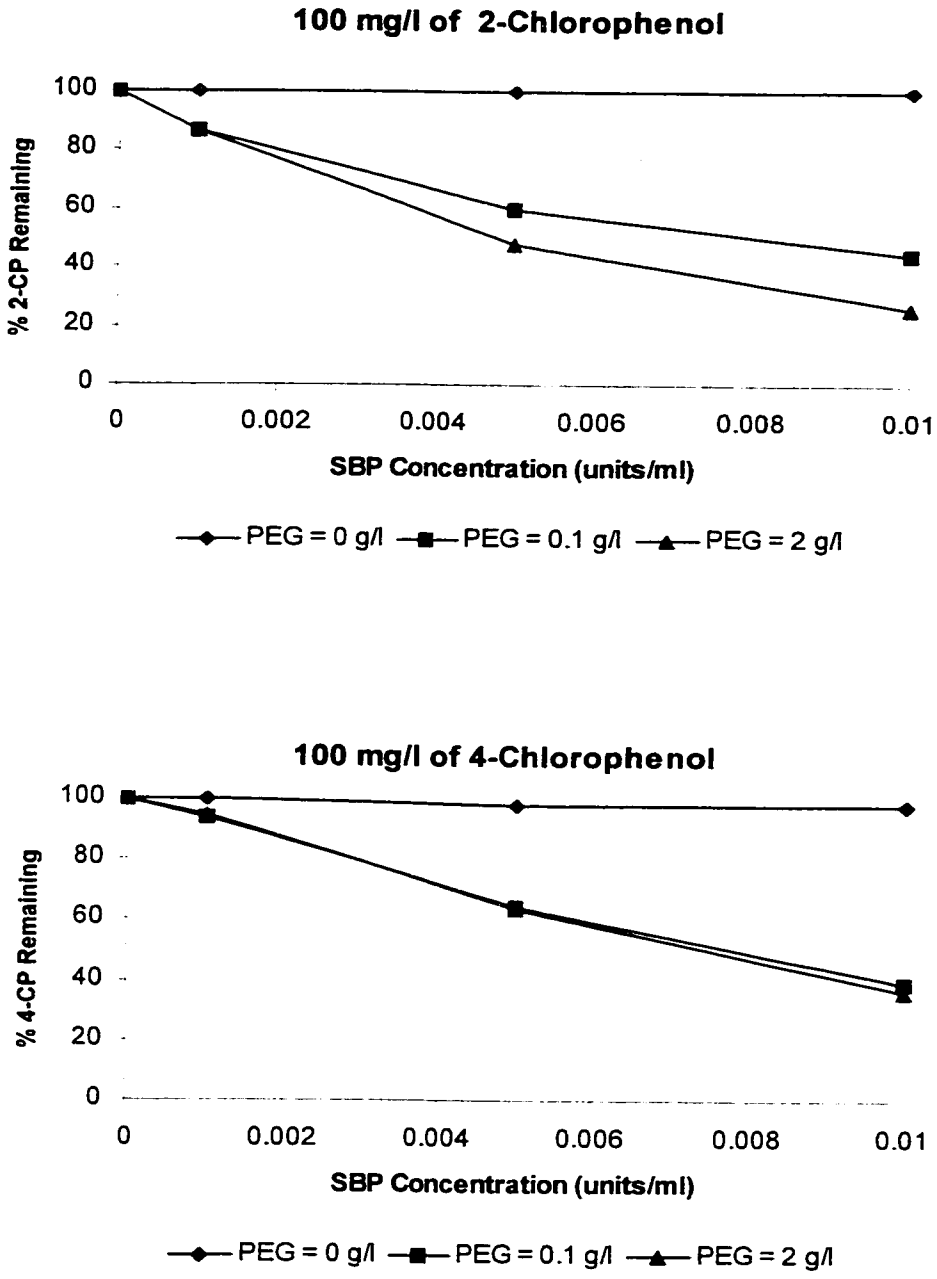
The first test showed that PEG 8000 significantly increased the removal of 100 mg/l 2-CP (Figure 4.7a). No removal was achieved for the 0.01 units/ml SBP when PEG was not present in solution. Using 0.01 units/ml of SBP, up to 70% removal was achieved with 2 g/l PEG 8000 and up to 55% removal was achieved with 0.1 g/l PEG 8000 present. Upon initiating the reaction with H_2O_2, a yellowish, brown colour formed. Visible particulate formed in all samples, however, a large amount of particulate formed in the solution containing PEG with 0.01 units/ml SBP.

Using 100 mg/l 4-CP, 62 and 64% removal of the 4-CP was achieved using 0.1 and 2.0 g/l of PEG 8000 respectively (Figure 4.7b). The solutions formed a murky grey colour when PEG was used. Without PEG, there was no change in colour and there was no detectable removal of 4-CP.

From these results for 2-CP and 4-CP, the exact role that PEG 8000 played in the reaction is unclear. Ideally, if the enzymes were to be trapped in forming polymers, there would have to be a removal of substrate detected. This was not necessarily the case. It is possible that the experimental procedure of preparing the SBP and PEG mixtures prior to mixing them with the substrate affected the results. Solutions of SBP and PEG were sometimes prepared in batch prior to performing the tests and had a long incubation period. It may be possible that the SBP was modified by the PEG. Further exploration of this occurrence on a molecular level could provide the necessary information to make a substantial conclusion.

Some initial tests were also performed on 2,4,6-trichlorophenol (2,4,6-TCP). Up to 65% of 70 mg/l of 2,4,6-TCP was removed from solution using 0.01 units/ml SBP with and without PEG 8000 at pH 7.2. Upon initiation of the reaction, a pinkish, purple colour formed with very little to no visible particulate. A mixture of chlorinated phenols containing 96 mg/l 2-CP, 3 mg/l

4-CP, 83 mg/l 2,4-DCP and 74 mg/l TCP was prepared at pH 7.2 (Figure 4.8). The mixture was prepared to observe the removal of various chlorinated phenols using 0.01 units/ml SBP in the presence of PEG 8000. Without PEG, virtually no removal was observed for any of the substrates and the solution remained clear. When PEG 8000 was present in the enzyme dose, a large amount of visible precipitate formed and the mixture turned brown in colour. The results in general indicate that 2 g/l was the optimum PEG 8000 concentration. Using 2 g/l PEG 8000, 92% 2,4-DCP, 78% 2-CP, 62% 2,4,6-TCP and 16% 4-CP were removed from solution. Both 0.1 and 4.0 g/l of PEG 8000 resulted in less efficient removal of the substrate. This previous experiment has indicated the multi-specific efficiency of the SBP enzyme to remove multiple substrates in the same mixture. However, more work is required to see if preferential polymerization occurs for various substrate provided in the same mixture. This is part of a future study with of colleague.



Figures 4.7a and 4.7b (Top and Bottom): Percentage remaining of chlorinated phenol versus increasing enzyme concentration for various PEG 8000 concentrations. Tests were performed using 100 mg/l 2-CP, 100 mg/l 4-CP and 100 mg/l 2,4-DCP at pH 6.2.

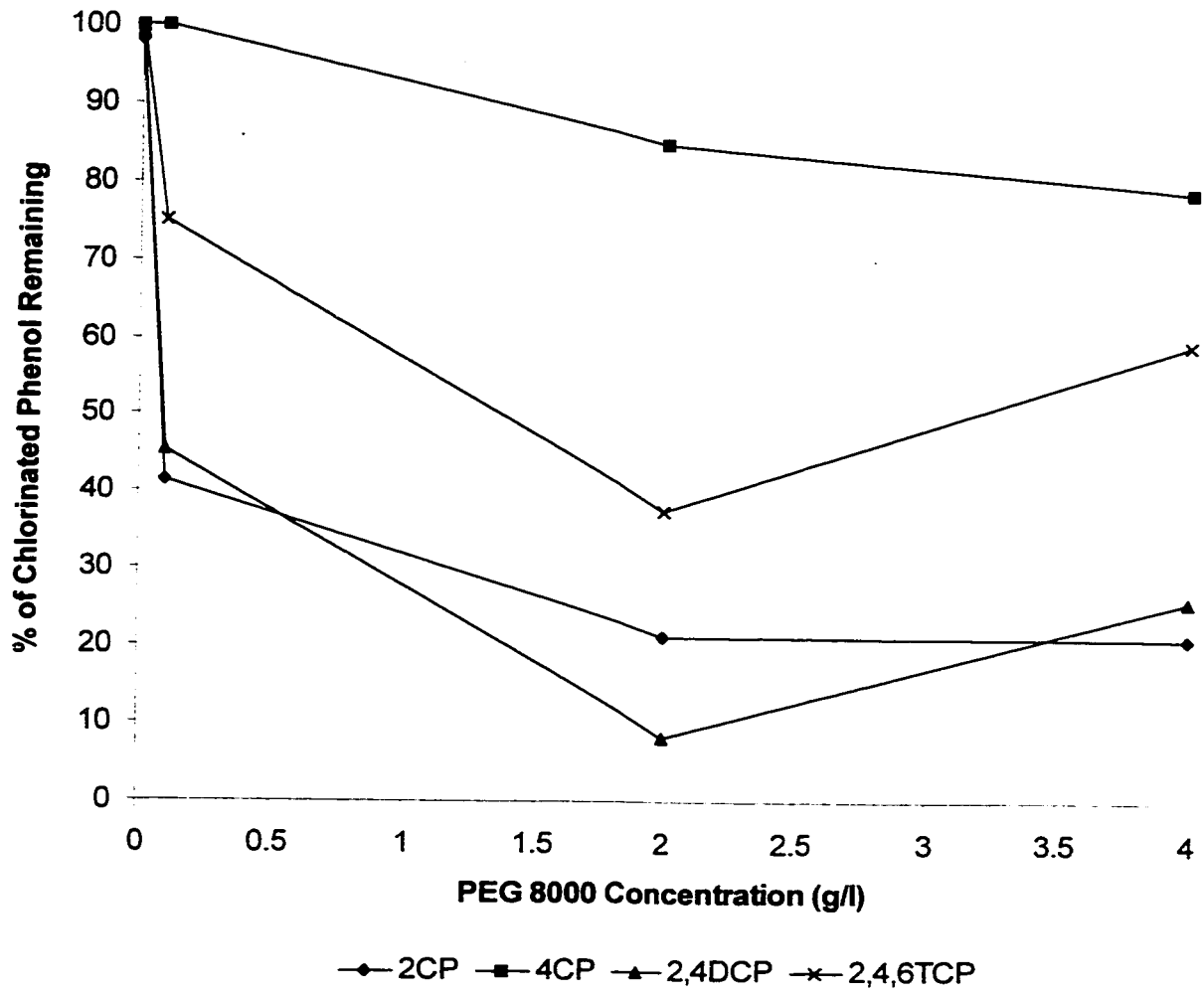


Figure 4.8: Removal of various chlorinated phenols from a single mixture in the presence of increasing concentrations of PEG 8000 using 0.01 units/ml SBP at pH 6.2

4.4.6 Reuse of Enzymes by Applying Additional Substrate

An experiment was performed to determine if an enzyme in solution can be reused when additional substrate is added to the mixture at one hour intervals. The experiments were performed at pH 6.2 at room temperature. The initial concentrations were as follows: SBP = 0.01 unit/ml; PEG 8000 = 1.0 g/l; 2,4-DCP = 100 mg/l and H_2O_2 = 1:1 molar ratio with substrate. The same method of mixing a 5 ml solution of SBP and PEG with a 20 ml buffered solution of substrate (2.5 mg) as previously described was followed. However, after one hour, a sample is drawn for testing and an additional 20 ml of substrate (2.5 mg 2,4-DCP) are added to the mixture along with a new dose of H_2O_2. Following another hour, this step is repeated. At three hours, a sample was drawn but no further substrate was added. A final sample was drawn at five hours for testing. This entire process was performed in duplicate (Test #1 and Test #2) and the results are presented in Figure 4.9. The amount of 2,4-DCP in the mixture at any given time is presented in mg. After one hour, Test #1 reached 98% 2,4-DCP removal efficiency and Test #2 reached just over 90% removal efficiency. Another 2.5 mg of 2,4-DCP was added to solution along with an equimolar spike of H_2O_2. At two hours the reaction continued but less efficiently, achieving 45 and 34% removal of the remaining 2,4-DCP in the duplicate tests. After the third addition only 13% and 8% were removed and it was then concluded that all the residual SBP activity was used up.

The cumulative turnovers over time were plotted in Figure 20. Based on the total 2,4-DCP and SBP concentrations used, the theoretical maximum number of turnover is 562,372. The total number of turnovers achieved after five hours for both test was 313000 or 56% of the theoretical maximum After the first injection, 91 and 98% of the theoretical maximum turnovers was attained (based on one dose of 2.5 mg 2,4-DCP). With the addition of more substrate, the enzyme continues to react but at slower rates. Therefore, while the number of turnovers increased from 184,000 to 313,000, the overall efficiency dropped from 98 to 56% removal. Figure 4.10 shows the deviation of the removal efficiency from the theoretical maximum number of turnovers.

Although semi-batch addition does increase the total number of turnovers, it does not seem to be a suitable method for optimizing the process. The decrease in activity can be

explained by the kinetics of the reaction. If substrate is limiting as it is in this case, excessive H_2O_2 may be present in solution and the enzyme may follow the inactivation path to P-670 or Compound III (as described in the literature review). Therefore, reversing the process and limiting the enzyme or H_2O_2 instead of the substrate may be more efficient in optimizing the reaction as will be shown in the next set of results.

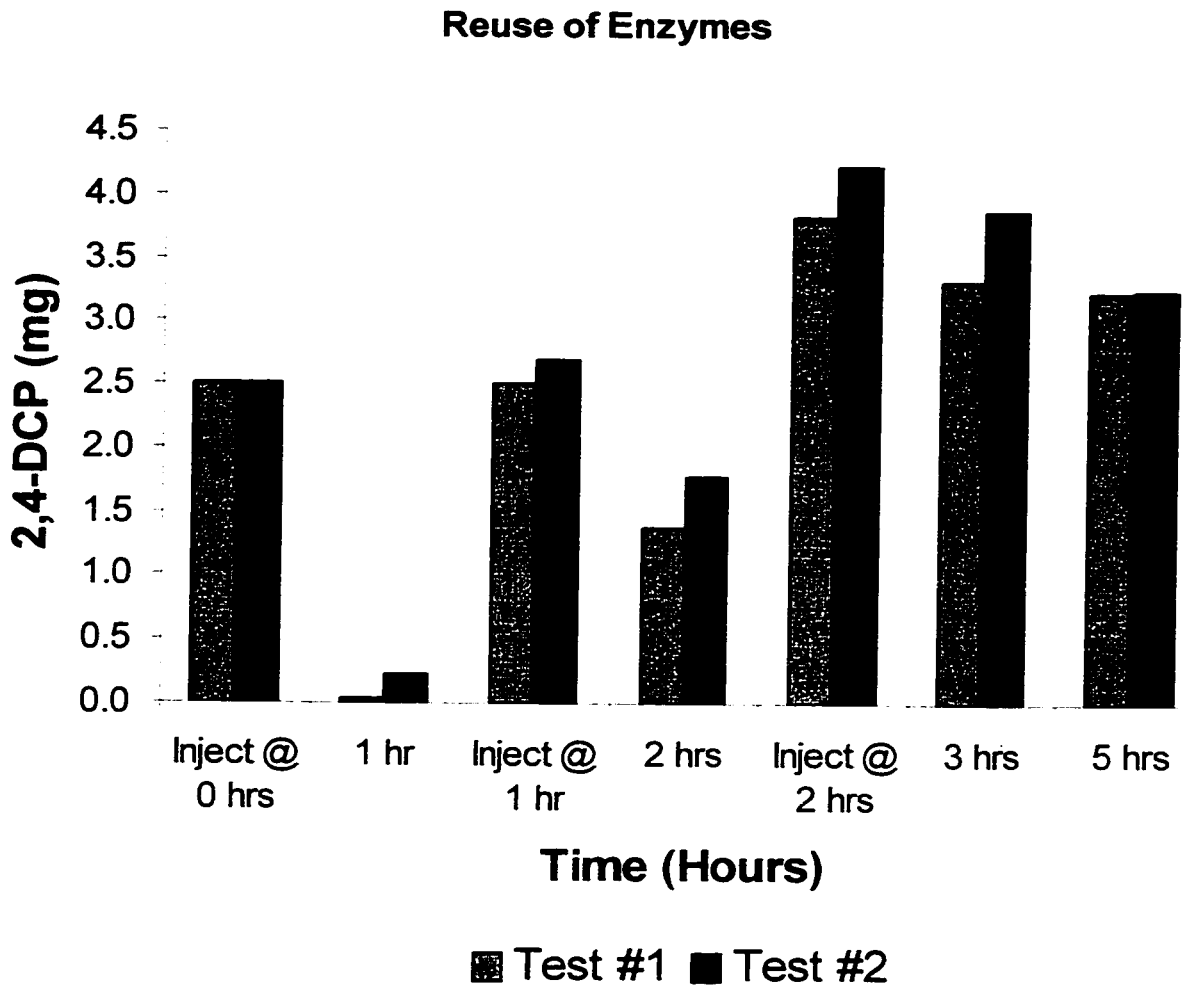


Figure 4.9: 2,4-DCP remaining (in moles) for three equal injections of 2x10-5 moles of 2,4-DCP. The amount of SBP remained constant at 0.25 units (equal to initial dose) and the amount of PEG 8000 remained constant at 25 mg. H_2O_2 was added in three doses in a 1:1 molar ratio to the 2,4-DCP added.

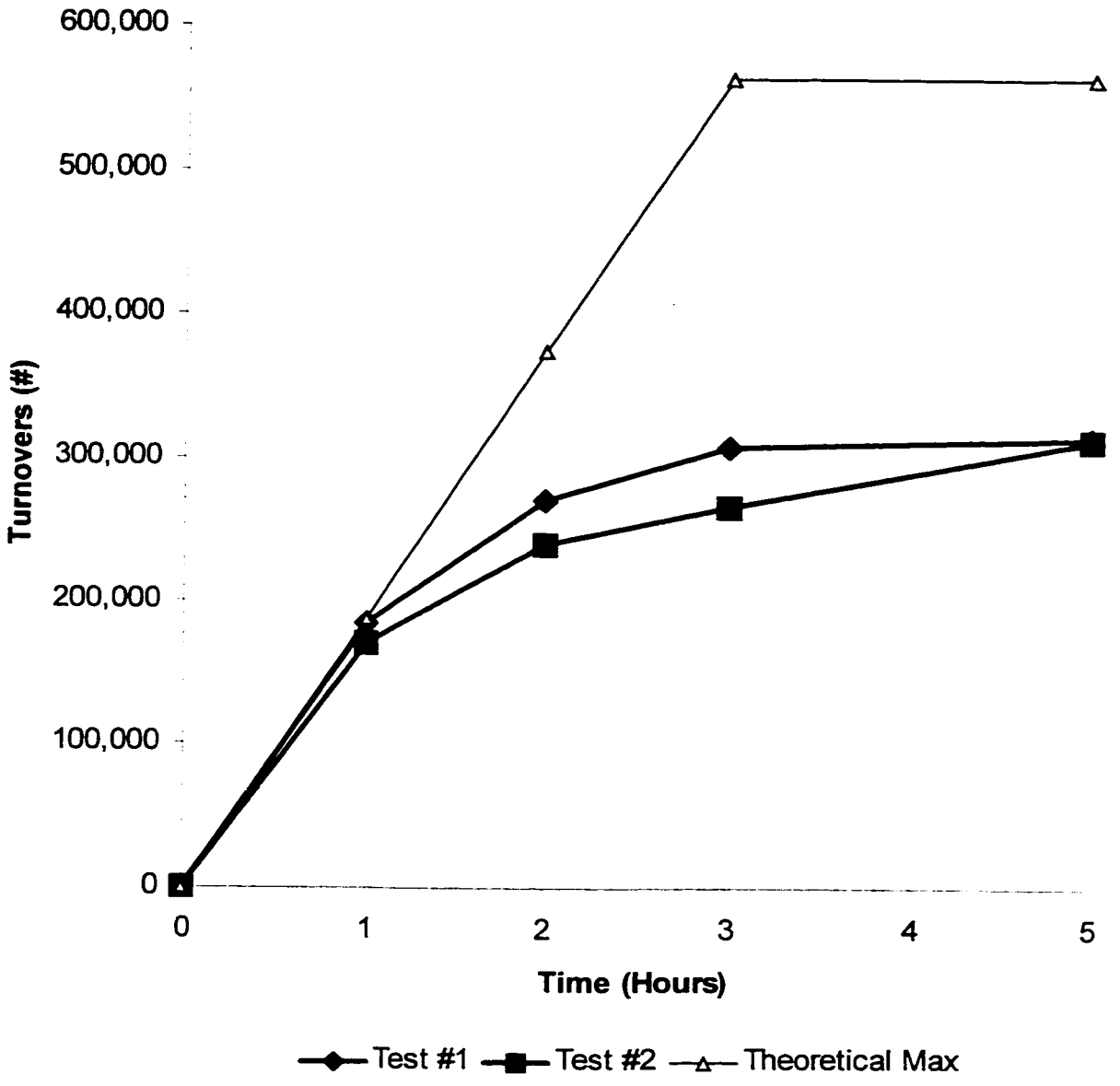


Figure 4.10: Cumulative turnovers calculated at various time intervals indicating the continuing activity of the peroxidase enzyme.

4.4.7 Mode of SBP and H_2O_2 Application

The last set of experiments performed examined the effect of different modes of SBP, PEG and the H_2O_2 addition. The experiment was split into 6 tests and a control. The tests were performed at pH 6.2 and at 22°C and consisted of applying the same total amount of SBP and H_2O_2 in various batch and semi-batch combinations to three different initial 2,4-DCP concentrations 100, 200 and 300 mg/l (Table 4.1).

The first test examined batch additions of SBP and H_2O_2. Similar tests were previously reported in this chapter. Based on these previous results, approximately 40% removal was expected at 100 mg/l 2,4-DCP using 0.0025 units/ml SBP and 0.1 g/l PEG 8000 in batch. Therefore 0.0025 units/ml was selected as a limiting factor in the reaction that could be improved. Table 4.5 shows the various semi-batch addition schemes examined. Results from the tests are presented in Figure 4.11 and Table 4.6. The schemes from the least efficient to most efficient in terms of 2,4-DCP removal, were as follows: #4 (least), #1, #2a, #2b, #3a and #3b (most). The least efficient method #4 used a semi-batch addition of 1/5 the total SBP and a full dose of H_2O_2 every thirty minutes. Ideally, splitting up the SBP dose should improve the reaction efficiency however, it was counteracted by too much H_2O_2. As previously discussed, such conditions create what is called the suicide mechanism. Excessive amounts of H_2O_2 compared to substrate available causes the enzyme to become catalytically inactivated. It is clear that in this case, excessive H_2O_2 is preventing the reaction from achieving the batch test benchmark results.

The remaining tests achieved better results than the control (#1 single batch addition) tests. The results are also presented in Table 4.6 for reference. Test #2a and #2b produced relatively similar results. These results were based on distributing the SBP dose over five equal intervals of time while using a single initial batch dose of H_2O_2 to initiate the reaction The SBP doses in #2a were provided over fifteen minute intervals and the #2b doses were provided over thirty minute intervals. The most effective addition schemes were #3a and #3b which were based on five equal doses of the SBP and H_2O_2 over fifteen minute and thirty minute intervals respectively. Of all the tests, #3b was the most effective. The reason for this is that #3b took advantage of limiting the amount of SBP and H_2O_2 available at each step. This reduces the possible chances of inactivation by excessive H_2O_2 when using a single batch dose. The

removals achieved in #3b were 84%, 76% and 72% for the 100, 200 and 300 mg/l 2,4-DCP concentrations respectively compared to 62%, 52% and 58% for the single batch addition control. In terms of turnovers, the highest number reached in this test was 1,619,634 for the removal of 72% of 300 mg/l of 2,4-DCP. This can be compared to the control which achieved a turnover number of 1,304,705 based on a removal of 58% of 300 mg/l of 2,4-DCP.

Comparing the results of this experiment to the previous experiment (semi-batch substrate addition), it can be seen for this experiment that while turnovers increase, so does the removal efficiency for semi-batch addition of SBP and H_2O_2, which is good. Whereas for the semi-batch addition of substrate, an increase in turnovers resulted in a decrease in overall removal efficiency, which is not so good. Also in this experiment, the difference between the "a" tests (ie #2a and #3a) and the "b" tests (ie #2b and #3b) is the length of time between additions. Generally, the results indicate that a longer period of time between additions "slightly" improves the removal efficiency.

Table 4.5: Various modes of SBP addition and H_2O_2 addition setup:

Time Hrs		#1	#2a	#2b	#3a	#3b	#4
0	No Enzyme	All SBP, All H_2O_2	1/5 SBP, All H_2O_2	1/5 SBP, All H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
0.15			1/5 SBP		1/5 SBP, 1/5 H_2O_2		
0.30			1/5 SBP	1/5 SBP	1/5 SBP, 1/5 H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
0.45			1/5 SBP		1/5 SBP, 1/5 H_2O_2		
1.00			1/5 SBP	1/5 SBP	1/5 SBP, 1/5 H_2O_2	1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
1.15							
1.30				1/5 SBP		1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
1.45							
2.00				1/5 SBP		1/5 SBP, 1/5 H_2O_2	1/5 SBP, All H_2O_2
2.15							
2.30							
2.45							
3.00	Sample	Sample	Sample	Sample	Sample	Sample	Sample

Table 4.6: % 2,4-DCP removal calculated from Figure 4.11:

	% 2,4-DCP Removal		
Test #	100 mg/l	200 mg/l	300 mg/l
1	62	52	58
2a	78	72	60
2b	76	64	62
3a	83	75	70
3b	84	76	72
4	55	47	30

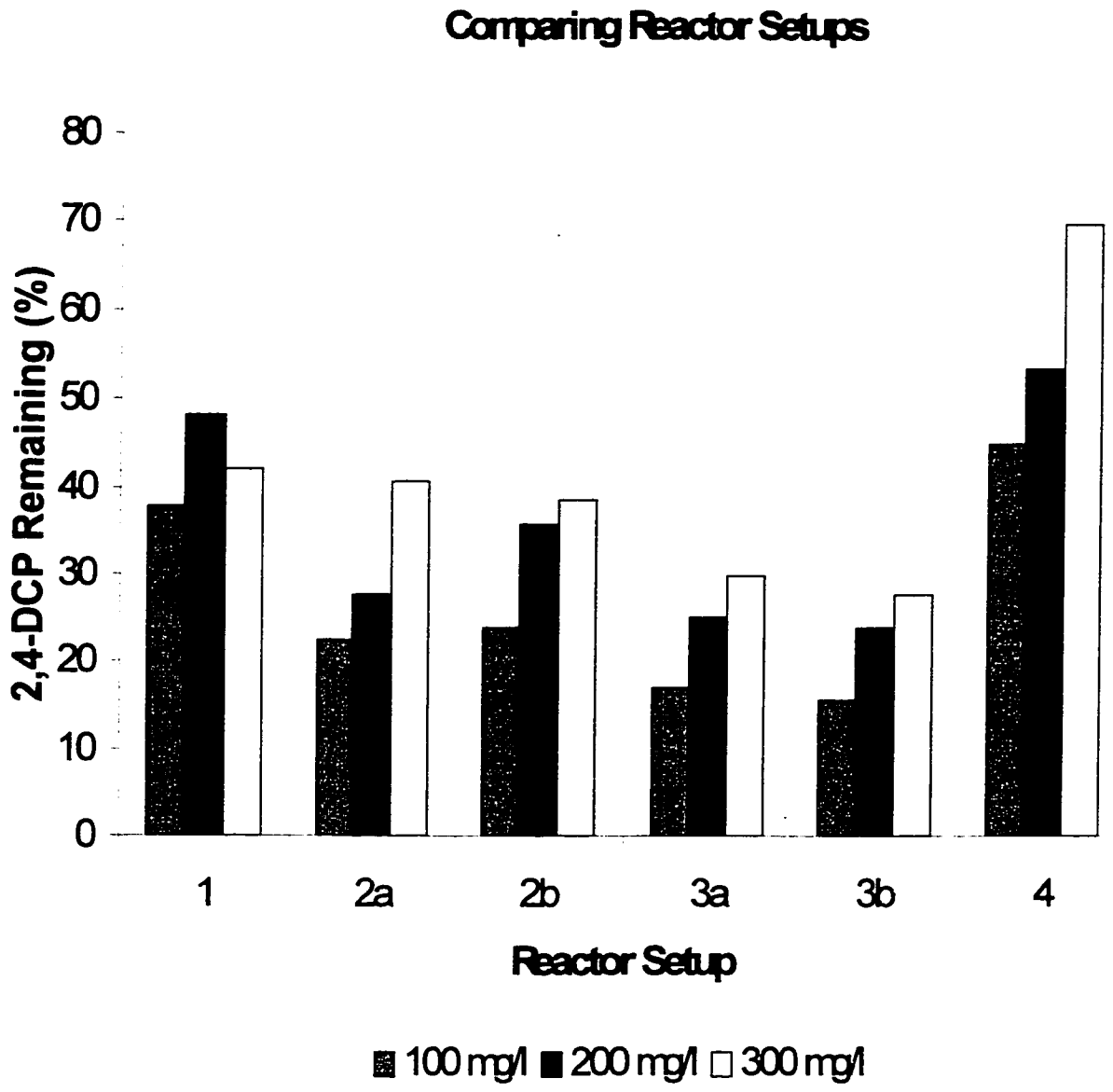


Figure 4.11: Percentage remaining of three initial concentrations of 2,4-DCP (100, 200 and 300 mg/l) for various H_2O_2 and SBP dosage processes. Refer to Table 4.1 which describes the six addition schemes.

4.4.8 Turnovers

In any industrial application, the rate at which an enzyme becomes catalytically inactivated is a critical characteristic in the selection of a feasible treatment process. The catalytic lifetime of an enzyme is known as a turnover. "Turnovers" are defined as the number of substrate molecules precipitated from solution per molecule of enzyme consumed and is a measure of how many times the enzyme carries out its catalytic process before being inactivated (Nicell *et al.*, 1993). Figure 2.5 indicated that for HRP, the number of catalytic turnovers is a function of pH. Turnovers can be calculated from a proportionality constant and the amount of substrate removed. The proportionality constant is a function of the substrate and the enzyme molecular weights and concentrations.

Turnovers are used to express the effectiveness of an enzyme at removing a certain number of moles of substrate. To calculate turnovers for 2,4-DCP use equations 4.1-4.5 (assuming MW_{SBP}= 44000 which is HRP's molecular weight):

$$Turnovers = \frac{(Moles_S)}{(Moles_E)} = \frac{\left(\frac{C_S}{MW_S}\right)}{\left(\frac{C_E}{MW_E}\right)} \quad \text{Equation 4.1}$$

$$Turnovers = \frac{\left(\frac{Cs\,(mg/l)}{163\ g/mole}\right)}{\left(\frac{C_{SBP}}{44000\ g/mole}\right)} = 269.939\left(\frac{Cs}{C_{SBP}}\right) \quad \text{Equation 4.2}$$

$$where \quad C_{SBP} = \left(\frac{Mass_{SBP}}{Activity_{SBP}}\right)\left(\frac{Unit_{SBP}}{ml}\right)\left(\frac{1000\,ml}{l}\right) \quad \text{Equation 4.3}$$

$$\therefore C_{SBP} = \left(\frac{360mg}{25000units}\right)\left(\frac{Unit_{SBP}}{ml}\right)\left(\frac{1000ml}{l}\right) = 14.4\left(\frac{Unit_{SBP}}{ml}\right) \quad \text{Equation 4.4}$$

$$\therefore Turnovers = 18.75 \frac{(C_S(mg/l))}{\left(\frac{Unit_{SBP}}{ml}\right)} \quad \text{Equation 4.5}$$

where: C_S = the concentration of the substrate (mg/l)

C_E = the concentration of the enzyme (unit/ml), for SBP $C_E = C_{SBP}$

MW_S = the molecular weight of the substrate

MW_E = the molecular weight of the enzyme, for SBP $MW_E = MW_{SBP}$

Therefore for SBP = 0.01 units/ml and 2,4-DCP = 100 mg/l removed, the maximum number of turnovers that can be achieved is = 187,500. Table 4.7 provides an example illustrating how increasing the number of turnovers can decrease the cost of removal using SBP treatment and assuming $220/25000 units SBP, activity is 25000 per 360 mg and 10000 litres of water with a 2,4-DCP concentration of 100 mg/l has to be treated. (Sigma, 1998).

Table 4.7: Calculation of cost to treat water contaminated with 2,4-DCP.

(Based on Sigma 1998 data, Canadian currency, no tax)

2,4-DCP =100 mg/l					
2,4-DCP Molecular Weight = 163 g/mole					
SBP Mass= 360 mg					
SBP Activity = 25,000 units					
SBP Molecular Weight =44,000 g/mole					
Reactor Volume = 10,000 litres					
SBP Cost/unit =0.00886 $/unit (Sigma, 1998)					
Turnover	**(Unit/l)**	**Volume (l)**	**Units of SBP**	**Cost/unit**	**Total Cost**
1000	1875	10000	18750000	$ 0.00886	$166,100
10000	187.5	10000	1875000	$ 0.00886	$ 16,610
100000	18.75	10000	187500	$ 0.00886	$ 1,661
1000000	1.875	10000	18750	$ 0.00886	$ 166

From Table 4.7, the influence of the SBP concentration on the removal efficiency is very evident. The concentration of SBP is directly proportional to the total cost of the system. In the first experiment of Chapter Three, 1875 turnovers was achieved using 1 unit/ml SBP. In the most recent test we achieved close to 1,600,000 turnovers (optimum pH, PEG and SBP

concentration). In terms of dollars (Table 4.7) on a large scale, by optimizing the conditions, the enzyme cost of the treatment dropped from $166,087 to under $166.09 which is very substantial.

4.5 Conclusion

The use of PEG-8000 as a protective additive for SBP in the treatment of 2,4-DCP was successfully demonstrated. Optimizing the pH of a solution can increase the efficiency of the reaction when using PEG. By using optimum pH conditions, the PEG-8000 concentration and SBP concentration can be optimized coincidentally to increase the turnover potential and minimize the amount of SBP needed for treatment while simultaneously limiting the effect of residual PEG released in the environment. When selecting the molecular weight of PEG, there is a tradeoff between using very high molecular weight PEG which tends to be more recalcitrant versus the increasing effectiveness of protection as the molecular weight increases. When optimizing the SBP strategy, the SBP enzyme should be limiting otherwise SBP will follow the inactivation path to P-670 or Compound III . Providing substrate in a semi-batch method will increase turnovers but will decrease the removal efficiency, most likely because the enzyme becomes inactivated through various suicide mechanisms. Providing the enzyme in semi-batch increases the turnovers and can also increase the removal efficiency. The stoichiometric ratio of apply H_2O_2 in a 1:1 molar ratio to the substrate should be followed, especially when using semi-batch addition methods. Finally, the use of turnovers is a good method to describe and compare different substrate and enzymes reaction results and characteristics.

4.6 Future Recommendations

Future studies using SBP should look at applying the process on a larger scale. They should determine the suitability of the SBP treating various substrates. Other applications of the SBP process should be studied such as the suitability to soil contamination.

CHAPTER FIVE

Conclusion and Future Recommendations

5.1 Conclusion

The results of this research provided an insight into the functionality of soybean peroxidase in treating aqueous solution contaminated with chlorinated phenols. Conclusions were presented in the last section of each paper. The broad specificity to different substrate of SBP and the wide range of conditions that it is capable of working in make SBP a viable alternative to conventional processes. At this moment, the process of using purified or extracted plant enzyme in the treatment process is still in the feasibility stages but it is a matter of time before large-scale feasibility studies are performed. SBP has shown itself to be capable of treating various chlorinated phenols. It has also been shown to work in very acidic conditions. This research also found that higher molecular weights of the PEG additive affords more protection to SBP than it does for HRP. Also for design purposes, it is essential to consider the modes of addition of all components of the process. As was shown in this research, the mode of addition of enzyme can significantly improve final results.

5.2 Recommendations for Future Research

Two major components of the process most be considered for future studies: 1) Reaction product identification and characterization and 2) Cost reduction through condition optimizing. Other recommendations include applying the process in a soil environment in similar conditions as Fenton's Reagent. Enzymes may have access to sites of chemical partitioning in soil which micro-organisms do not have access to such as micropores (Aitken, 1993). Similarities between the enzyme oxidation process to a chemical oxidation process utilizing what is known as Fenton's Reagent ($Fe^{2+} + H_2O_2$) are very encouraging. Similar products are formed in each process. Fenton's Reagent has been applied to both the wastewater and soil environment. Also new technologies such as genetic engineering and mass production of peroxidase enzymes could have a significant effect on improving the PE process. As the trend of using soybeans in new and inventive processes and products, the need to dispose of the "waste seed coat" will continue. Therefore, taking one person's waste to treat another could have no better meaning.

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APPENDIX A - Trial Results

Preliminary tests were performed using HRP to verify the experimental procedure that was followed for testing SBP's ability to treat chlorinated phenols. Initial test conditions were set based on previous work presented by a variety of researchers The first set of experiments using HRP provided necessary information as to whether the appropriate procedure was developed (Figure A1). The first test included six HRP concentrations, 0.5, 1.0, 2.5, 6.0 and 10.0 units/ml, mixed into an unbuffered solution of 100 mg/l 2,4-DCP, spiked with equimolar (to the substrate) amounts of H_2O_2 and allowed to react for 3 hours. Observations from this study provided necessary information required in determining if the experimental procedure was appropriate. Firstly, the reaction vessels utilized were of a plastic nature. A concern that the substrate may "attach" itself to the polymer surface was acknowledged and future tests were performed in glass vials. Secondly, using an equimolar amount of H_2O_2 proved effective as reported in many other research papers. In an effort to limit the amount of variables, this 1:1 molar ratio between H_2O_2 and the substrate was used throughout the experimentation process. The results indicated that there was sufficient time for the reaction to go to completion for all HRP concentrations tested except for 0.5 units/ml (Graph #1). Theoretically, with the addition of a concentrated acid such as HCl to an unbuffered solution, the pH will lower so much that the acidic conditions will denature the enzyme rendering the enzyme inactive. However, peroxidase enzymes can perform in acidic conditions, especially SBP which is more stable that HRP at lower pH. One solution, particularly in the case of peroxidase enzymes is to make the solution basic (ie. pH >10) where the enzyme is very susceptible to denaturing. This method was abandoned for reasons that it would not be effective in buffered solutions. Therefore, for the remaining experiments, any time dependent experiments were deactivated by the addition of a small but effective amount of catalase enzyme.

The second experiment in an unbuffered solution showed that a drop in pH occurred for almost every reaction. A HRP concentration of 10 units/ml was used to remove 100mg/l of 2,4-DCP. A prolonged period of time (>72 hours) was permitted for the reaction. One explanation for the drop in pH, hypothesized by (Dec and Bollag, 1994a,b), is that dechlorination has

occurred and that an increase in chlorine ions in solutions lends to a more acidic condition. Discoloration and precipitate formation provided evidence that a reaction was proceeding as expected.

The final tests performed with HRP examined the effects of an additive, 4 g/l of PEG 3350, mixed into solution. Figures A2 and A3 compare the results obtained for an experiment to remove 100 mg/l of 2,4-DCP with and without an additive in slightly basic conditions (pH 7.1-9.8). The results indicated that the use of the additive actually reduced the removal efficiency of the enzyme. This reduced efficiency was attributed to either a high PEG concentration or to the fact that without any additive, removal was near 100%, therefore any addition of an additive would be interfering we these already satisfactory results. It was decided that in order to test the effects of an additive with SBP, the "working range" concentration of SBP for various pH would have to be determined first.

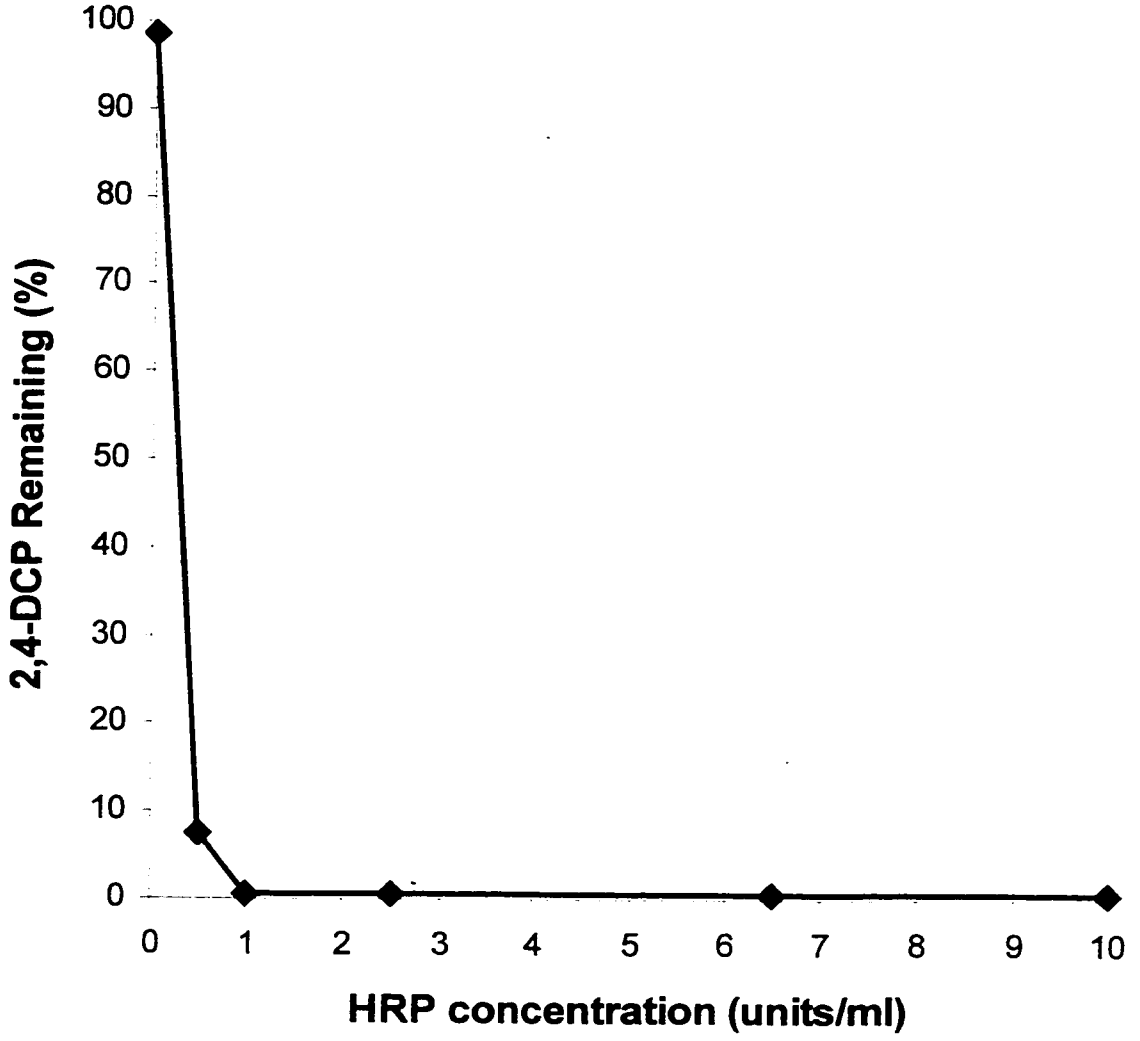


Figure A1: Trial Results - Percentage of 100 mg/l 2,4-DCP remaining as a function of Horseradish Peroxidase (HRP) enzyme concentration in an unbuffered solution at 22°C. (Incubation time > 24hrs)

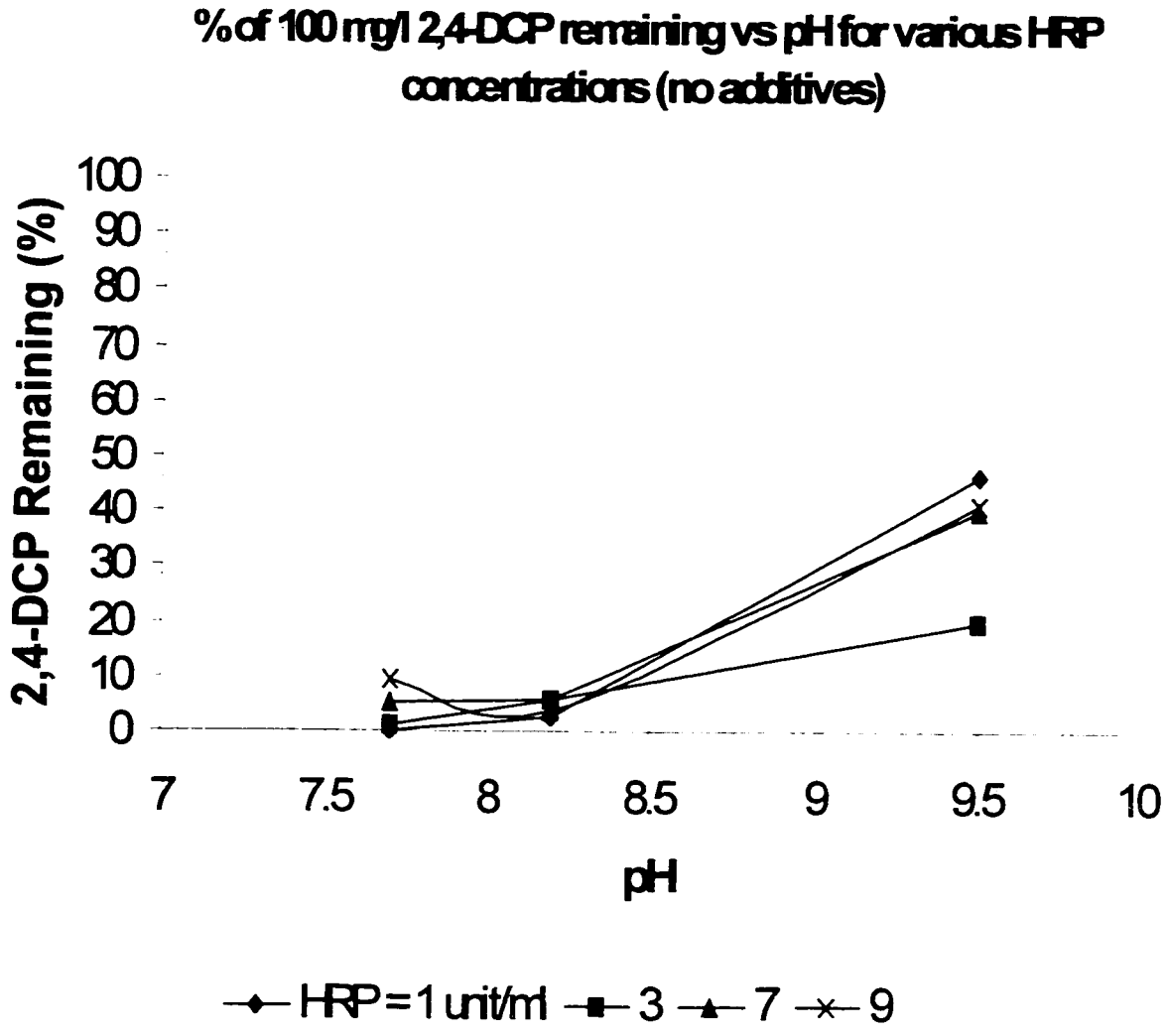


Figure A2: Percentage remaining of 100 mg/l 2,4-DCP versus increasing pH for various concentration of HRP (1, 3, 7, and 9 units/ml) without any additives at 22°C. (Incubation time > 24hrs)

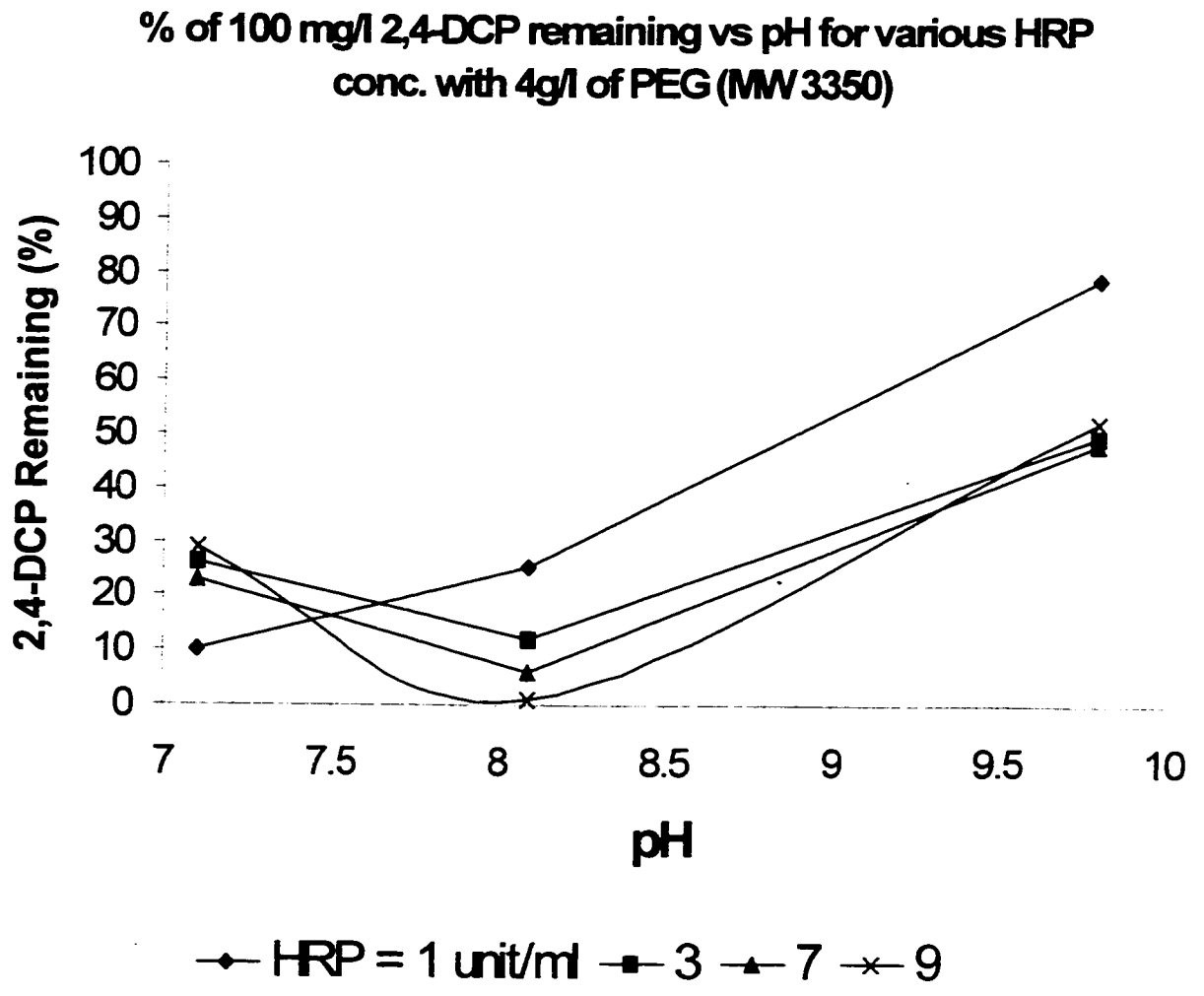
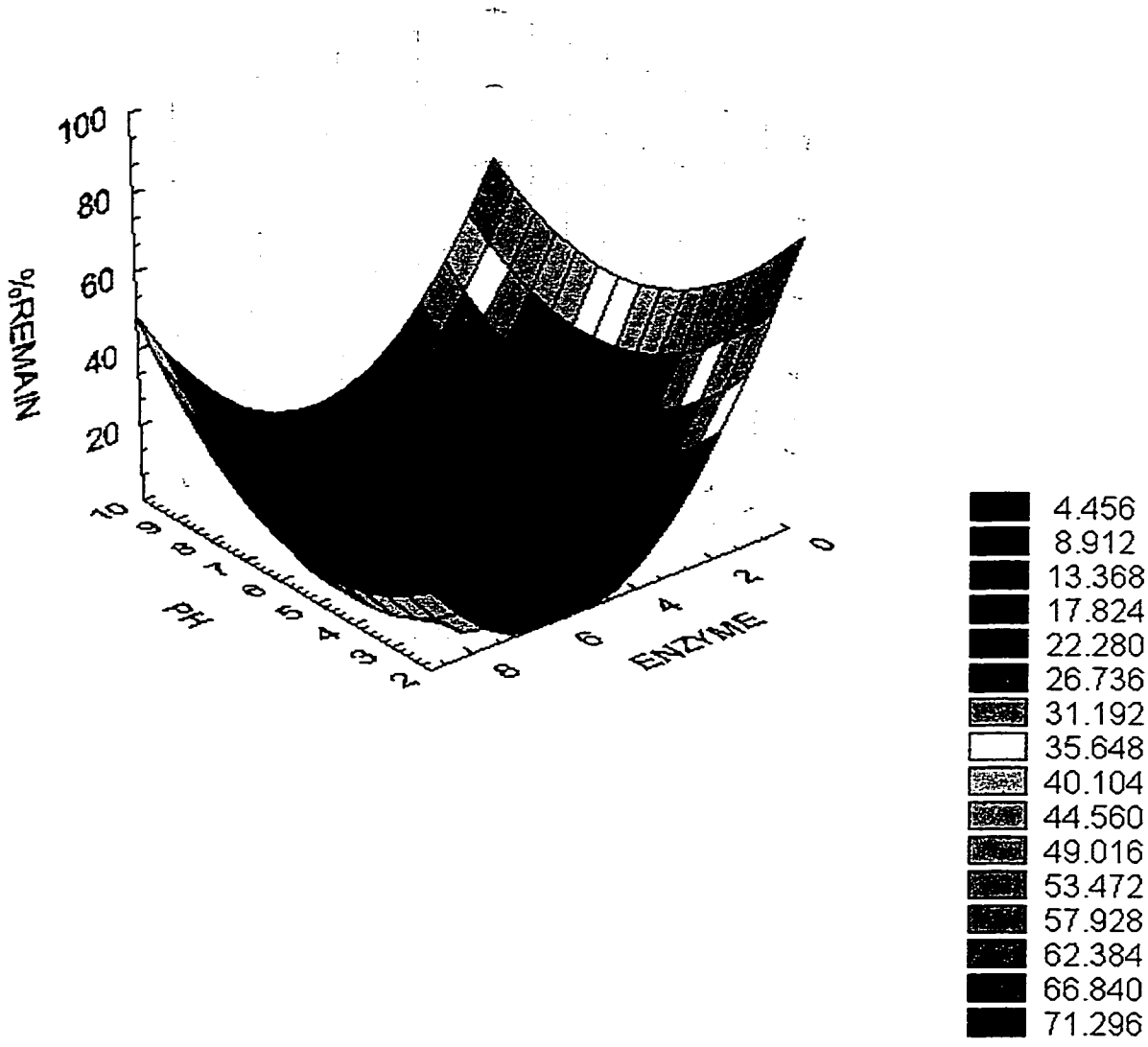


Figure A3: Percentage remaining of 100 mg/l 2,4-DCP versus increasing pH for various concentration of HRP (1, 3, 7, and 9 units/ml) with 4 g/l of PEG 3350 used as a protective additive at 22°C.

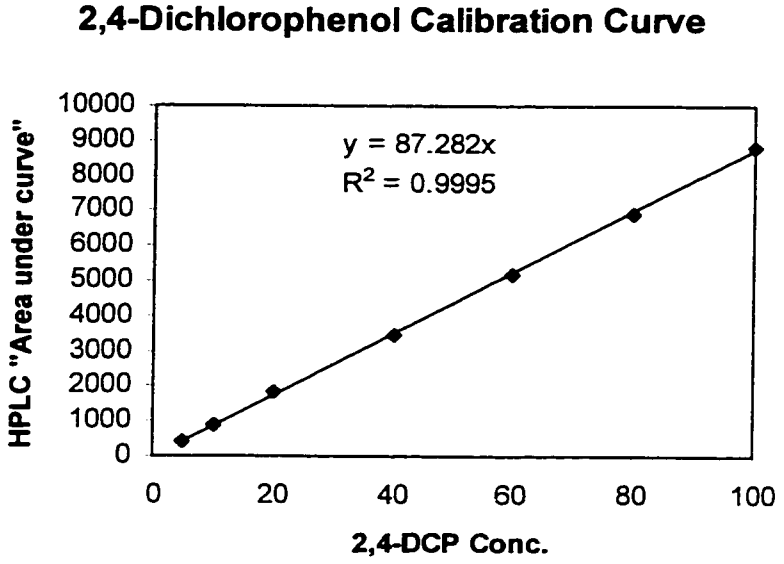
Surface plot comparing effects of SBP concentration and pH on 2,4-DCP removal

z = 108.893-19.503*x-27.684*y+1.466*x*x+0.614*x*y+2.246*y*y



A 3-dimensional, 3-degree polynomial was fitted to the data points used in Figures 3.2a to 3.2f. A plot of the results is presented above. The vertical axis represents % remaining of 100 mg/l 2,4-DCP. On the two horizontal axis the effects of pH and enzyme concentration in units/ml were determined.

APPENDIX B - HPLC Calibration Curves



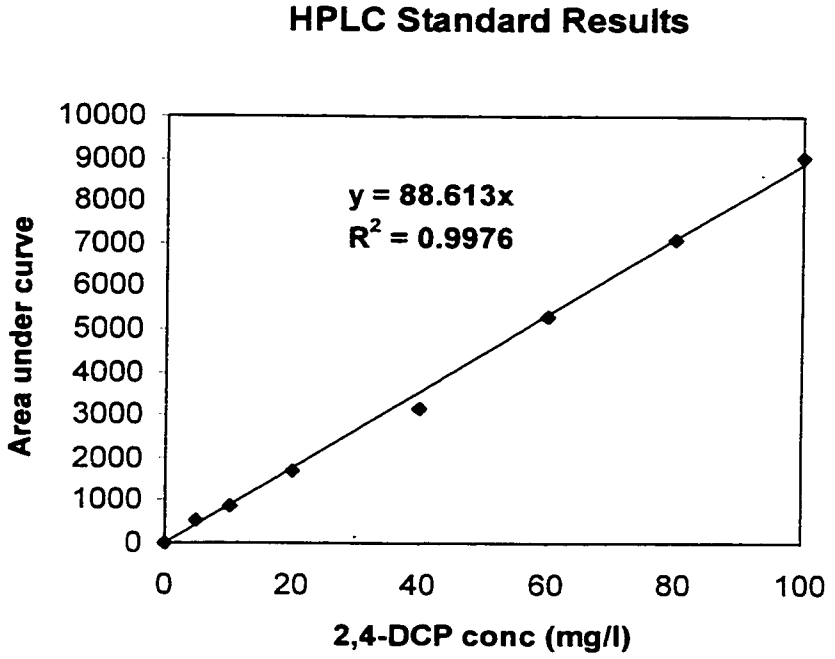


Figure B1 & B2: Examples of calibration curves used by HPLC integrator to determine chlorinated phenol concentrations.

APPENDIX C - HPLC Chromatograms

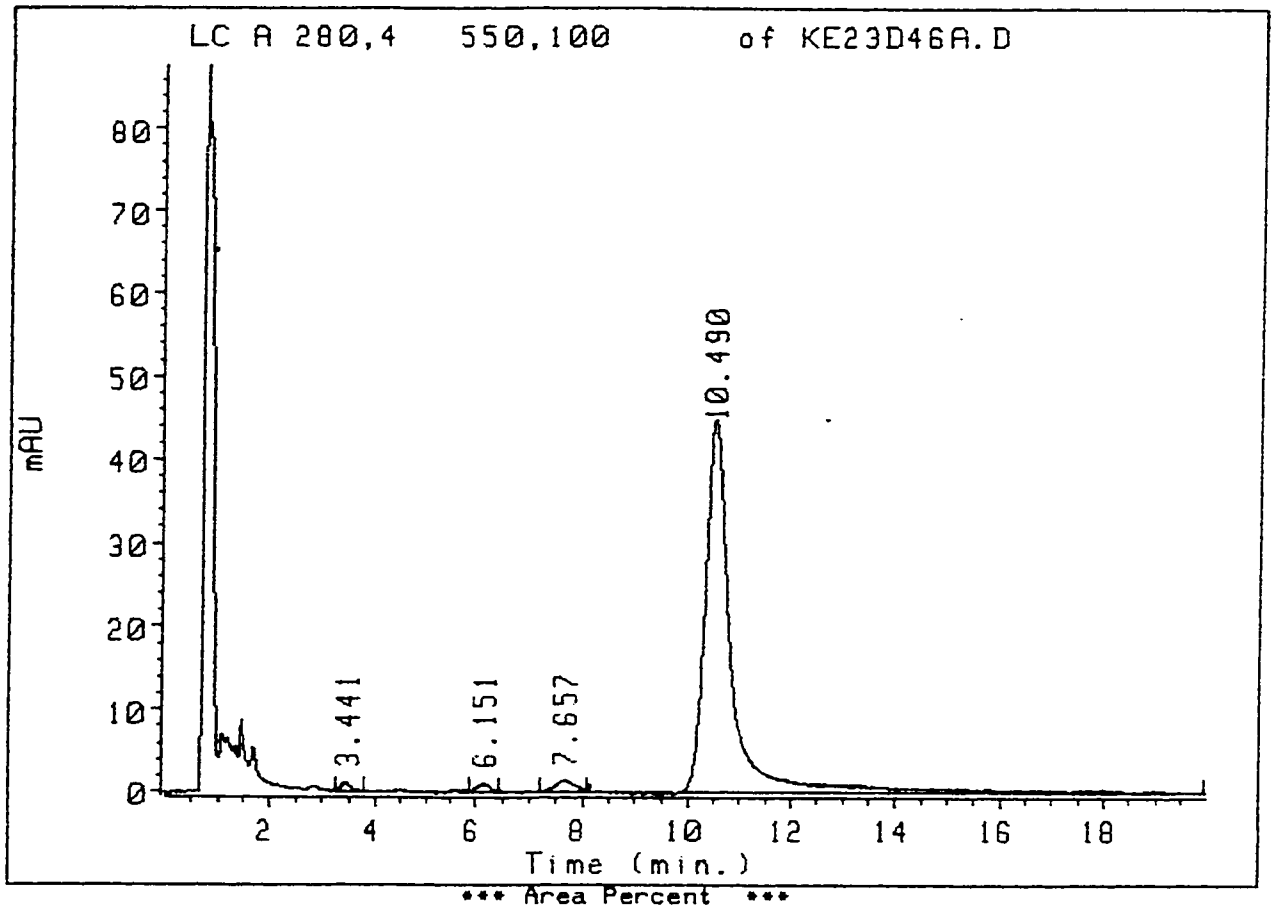


Figure C1: Example HPLC results from experiment testing 0.05 units/ml SBP and 0.2 g/l PEG 8000 to treat 100 mg/l 2,4-DCP at pH 3.1 and temperature 22°C.

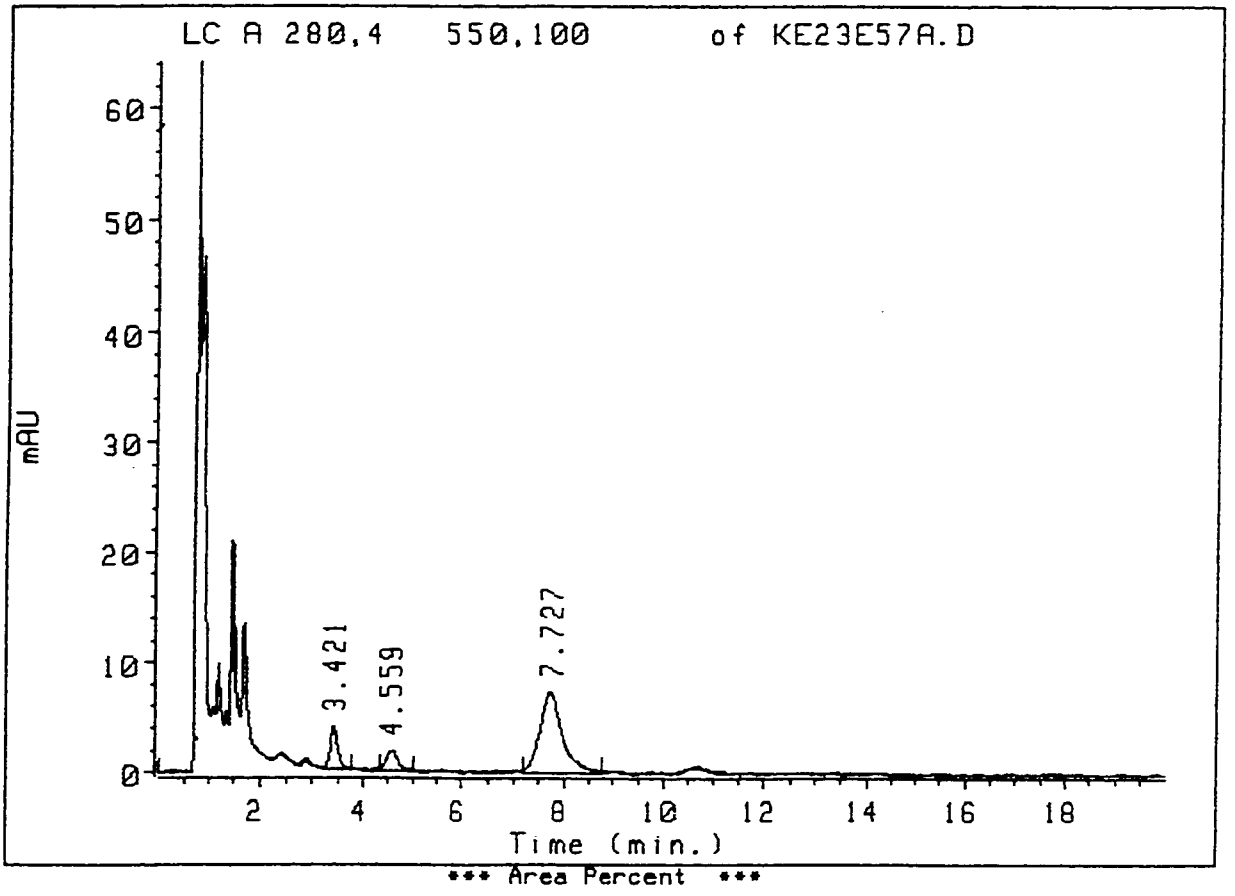


Figure C2: Example HPLC results from experiment testing 0.05 units/ml SBP and 0.2 g/l PEG 8000 to treat 100 mg/l 2,4-DCP at pH 5.1 and temperature 22°C.

APPENDIX D - Numerical Calculations

Table D1: Calculated % remaining of 100 mg/l 2,4-DCP for equations 2.1 to 2.8

Enzyme	3.1	4.1	5.1	6.2	7.2	8.2	9.2	Avg.
0.00	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0.01	97.1	93.5	89.5	88.2	88.4	83.0	97.8	91.1
0.02	94.2	87.3	79.9	77.3	77.7	67.8	95.6	82.8
0.03	91.5	81.4	71.1	67.0	67.8		93.4	75.2
0.04	88.7	75.8	63.1		58.7		91.3	68.2
0.05	86.1	70.5	55.8			32.1	89.2	61.9
0.06	83.5	65.5		40.7		23.2	87.2	56.0
0.07	80.9	60.8	43.4	33.4	36.1	15.7	85.2	50.8
0.08	78.5	56.3	38.1	26.7	29.9	9.4	83.2	46.0
0.09	76.0		33.4	20.6	24.5	4.3	81.2	41.7
0.10	73.7		29.2	15.2	19.7	0.4	79.3	37.9
0.11	71.4	44.3	25.5	10.4	15.4	0.4	77.4	35.0
0.12	69.1	40.8	22.2	6.3	11.8	0.4	75.6	32.3
0.13	66.9	37.5	19.4	2.7	8.7	0.4	73.7	29.9
0.14	64.8	34.5	16.9	0.4	6.1	0.4	71.9	27.8
0.15	62.7	31.6	14.7	0.4	4.0	0.4	70.2	26.3
0.16	60.7	28.9	12.8	0.4	2.3	0.4	68.4	24.8
0.17	58.7	26.5	11.1	0.4	1.1	0.4	66.8	23.5
0.18	56.7	24.2	9.5	0.4	0.4	0.4	65.1	22.4
0.19	54.9	22.1	8.2	0.4	0.4	0.4	63.5	21.4
0.20	53.0	20.1	6.9	0.4	0.4	0.4	61.8	20.4
0.21		18.3	5.6	0.4	0.4	0.4	60.3	19.5
0.22		16.7	4.4	0.4	0.4	0.4	58.7	18.7
0.23	47.8	15.2	3.2	0.4	0.4	0.4	57.2	17.8
0.24	46.2	13.9	1.8	0.4	0.4	0.4	55.7	17.0
0.25	44.6	12.7	0.4	0.4	0.4	0.4	54.3	16.2
0.26	43.1	11.6	0.4	0.4	0.4	0.4	52.9	15.6
0.27	41.6	10.6	0.4	0.4	0.4	0.4	51.5	15.0
0.28	40.1	9.7	0.4	0.4	0.4	0.4		14.5
0.29	38.7	8.9	0.4	0.4	0.4	0.4	48.8	14.0
0.30	37.3	8.2	0.4	0.4	0.4	0.4	47.5	13.5

0.31	36.0	7.6	0.4	0.4	0.4	0.4	46.2	13.1
0.32	34.7	7.0	0.4	0.4	0.4	0.4	44.9	12.6
0.33	33.5	6.6	0.4	0.4	0.4	0.4	43.7	12.2
0.34	32.3	6.1	0.4	0.4	0.4	0.4	42.5	11.8
0.35	31.1	5.8	0.4	0.4	0.4	0.4	41.3	11.4
0.36	30.0	5.4	0.4	0.4	0.4	0.4	40.2	11.0
0.37	28.9	5.1	0.4	0.4	0.4	0.4	39.1	10.7
0.38	27.8	4.8	0.4	0.4	0.4	0.4	38.0	10.3
0.39	26.8	4.6	0.4	0.4	0.4	0.4	36.9	10.0
0.40	25.8	4.3	0.4	0.4	0.4	0.4	35.9	9.7
0.41	24.8	4.1	0.4	0.4	0.4	0.4	34.9	9.3
0.42	23.9	3.8	0.4	0.4	0.4	0.4	33.9	9.0
0.43	23.0	3.5	0.4	0.4	0.4	0.4	32.9	8.7
0.44	22.2	3.2	0.4	0.4	0.4	0.4	32.0	8.4
0.45	21.4	2.9	0.4	0.4	0.4	0.4	31.1	8.1
0.46	20.6	2.5	0.4	0.4	0.4	0.4	30.2	7.8
0.47	19.8	2.1	0.4	0.4	0.4	0.4	29.4	7.5
0.48	19.1	1.6	0.4	0.4	0.4	0.4	28.5	7.3
0.49	18.3	1.1	0.4	0.4	0.4	0.4	27.7	7.0
0.50	17.7	0.4	0.4	0.4	0.4	0.4	26.9	6.7
0.51	17.0	0.4	0.4	0.4	0.4	0.4	26.2	6.5
0.52	16.4	0.4	0.4	0.4	0.4	0.4	25.4	6.3
0.53	15.8	0.4	0.4	0.4	0.4	0.4	24.7	6.1
0.54	15.2	0.4	0.4	0.4	0.4	0.4	24.0	5.9
0.55	14.6	0.4	0.4	0.4	0.4	0.4	23.4	5.7
0.56	14.1	0.4	0.4	0.4	0.4	0.4	22.7	5.5
0.57	13.6	0.4	0.4	0.4	0.4	0.4	22.1	5.4
0.58	13.1	0.4	0.4	0.4	0.4	0.4	21.5	5.2
0.59	12.6	0.4	0.4	0.4	0.4	0.4	20.9	5.1

0.60	12.2	0.4	0.4	0.4	0.4	0.4	20.4	4.9
0.61	11.7	0.4	0.4	0.4	0.4	0.4	19.9	4.8
0.62	11.3	0.4	0.4	0.4	0.4	0.4	19.3	4.7
0.63	10.9	0.4	0.4	0.4	0.4	0.4	18.9	4.5
0.64	10.5	0.4	0.4	0.4	0.4	0.4	18.4	4.4
0.65	10.2	0.4	0.4	0.4	0.4	0.4	17.9	4.3
0.66	9.8	0.4	0.4	0.4	0.4	0.4	17.5	4.2
0.67	9.5	0.4	0.4	0.4	0.4	0.4	17.1	4.1
0.68	9.1	0.4	0.4	0.4	0.4	0.4	16.7	4.0
0.69	8.8	0.4	0.4	0.4	0.4	0.4	16.4	3.9
0.70	8.5	0.4	0.4	0.4	0.4	0.4	16.0	3.8
0.71	8.2	0.4	0.4	0.4	0.4	0.4	15.7	3.7
0.72	7.9	0.4	0.4	0.4	0.4	0.4	15.4	3.6
0.73	7.7	0.4	0.4	0.4	0.4	0.4	15.1	3.5
0.74	7.4	0.4	0.4	0.4	0.4	0.4	14.8	3.5
0.75	7.2	0.4	0.4	0.4	0.4	0.4	14.6	3.4
0.76	6.9	0.4	0.4	0.4	0.4	0.4	14.4	3.3
0.77	6.7	0.4	0.4	0.4	0.4	0.4	14.2	3.3
0.78	6.4	0.4	0.4	0.4	0.4	0.4	14.0	3.2
0.79	6.2	0.4	0.4	0.4	0.4	0.4	13.8	3.1
0.80	6.0	0.4	0.4	0.4	0.4	0.4	13.6	3.1
0.81	5.7	0.4	0.4	0.4	0.4	0.4	13.5	3.0
0.82	5.5	0.4	0.4	0.4	0.4	0.4	13.4	3.0
0.83	5.3	0.4	0.4	0.4	0.4	0.4	13.3	2.9
0.84	5.1	0.4	0.4	0.4	0.4	0.4	13.2	2.9
0.85	4.9	0.4	0.4	0.4	0.4	0.4	13.1	2.9
0.86	4.6	0.4	0.4	0.4	0.4	0.4	13.0	2.8
0.87	4.4	0.4	0.4	0.4	0.4	0.4	13.0	2.8
0.88	4.2	0.4	0.4	0.4	0.4	0.4	13.0	2.7
0.89	4.0	0.4	0.4	0.4	0.4	0.4	13.0	2.7

0.90	3.7	0.4	0.4	0.4	0.4	0.4	13.0	2.7
0.91	3.5	0.4	0.4	0.4	0.4	0.4	13.0	2.6
0.92	3.3	0.4	0.4	0.4	0.4	0.4	13.1	2.6
0.93	3.0	0.4	0.4	0.4	0.4	0.4	13.1	2.6
0.94	2.8	0.4	0.4	0.4	0.4	0.4	13.2	2.6
0.95	2.5	0.4	0.4	0.4	0.4	0.4	13.3	2.5
0.96	2.3	0.4	0.4	0.4	0.4	0.4	13.4	2.5
0.97	2.0	0.4	0.4	0.4	0.4	0.4	13.5	2.5
0.98	1.7	0.4	0.4	0.4	0.4	0.4	13.6	2.5
0.99	1.4	0.4	0.4	0.4	0.4	0.4	13.7	2.4
1.00	1.1	0.4	0.4	0.4	0.4	0.4	13.9	2.4
Avg.	28.8	13.2	8.4	6.2	6.7	4.6	37.1	

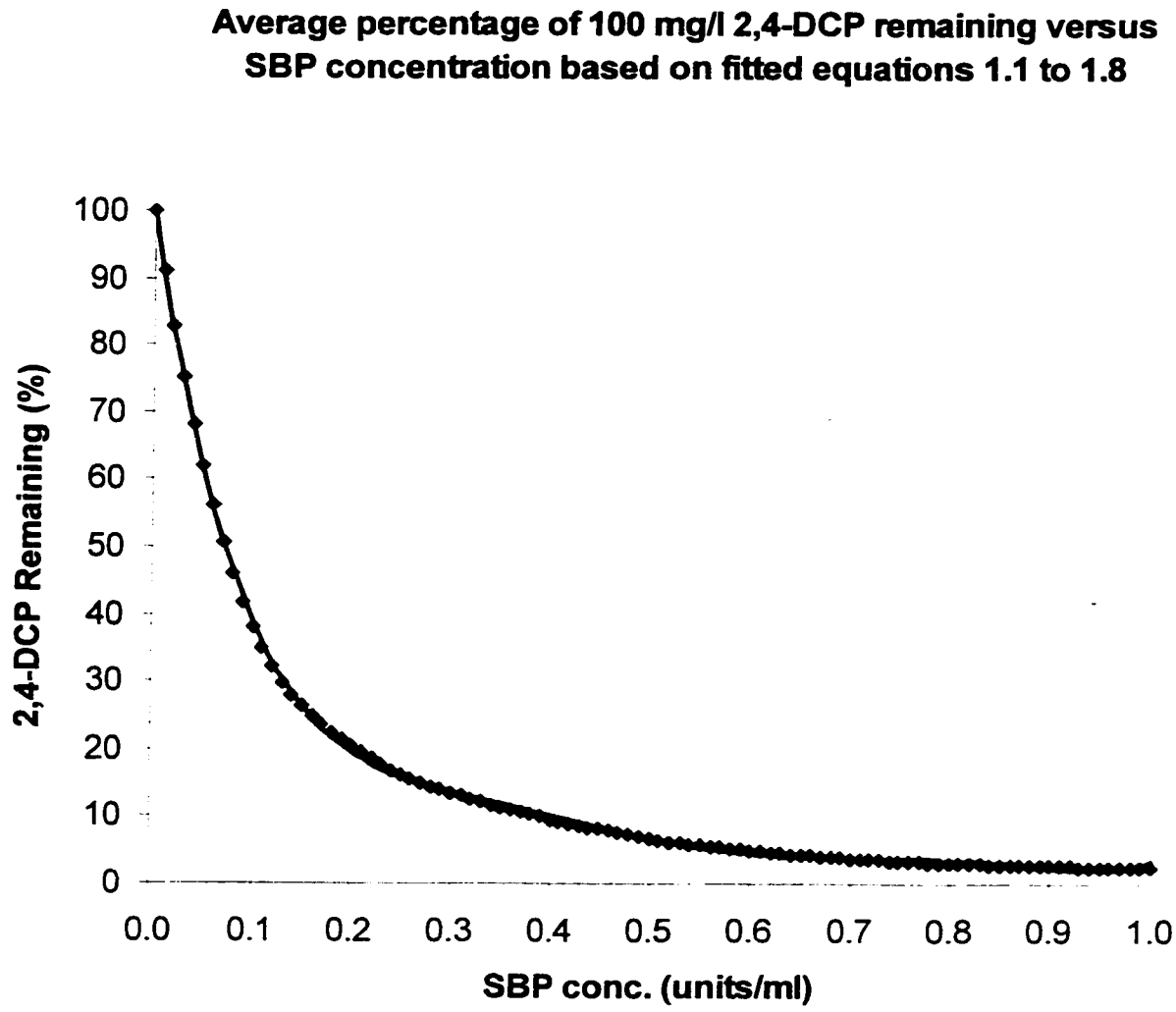


Figure D1: Average removal of 100 mg/l 2,4-DCP as a function of SBP concentration in a batch reactor at 22°C for a pH range of 3.1~ 9.2

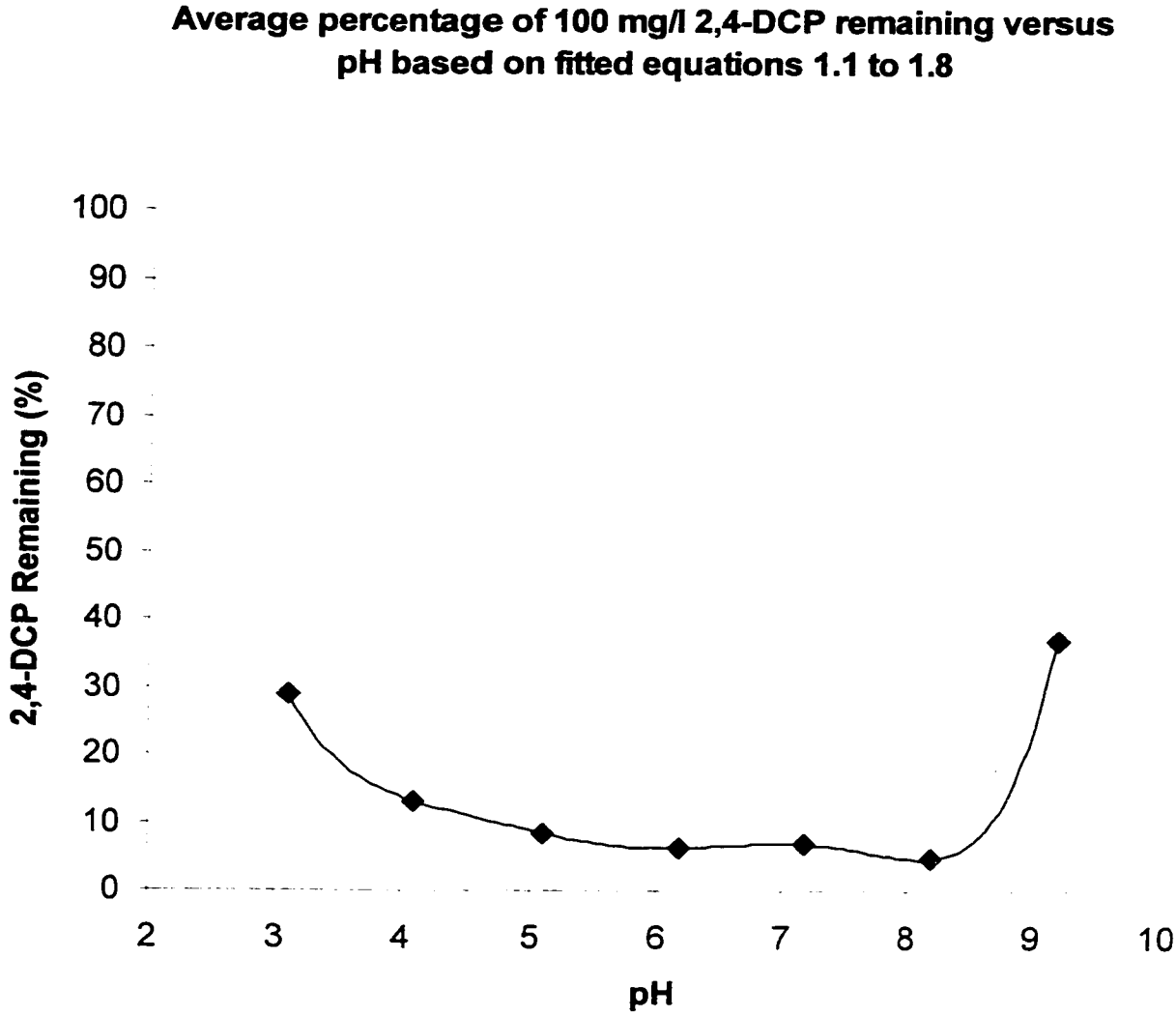


Figure D2: Average removal of 100 mg/l 2,4-DCP as a function of pH in a batch reactor at 22°C for a SBP concentration range of 0 ~ 1 units/mL

APPENDIX E - Raw Data

HRP Test: JA20												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
S1	119.1	0	N/A	N/A	N/A	N/A	9.350	3034	N/A	119.1	N/A	N/A
S2	117.0	0	N/A	N/A	N/A	N/A	9.363	2982	N/A	117.0	N/A	N/A
S3	134.7	0	N/A	N/A	N/A	N/A	9.483	3431	N/A	134.7	N/A	N/A
S4	136.2	0	N/A	N/A	N/A	N/A	9.471	3469	N/A	136.2	N/A	N/A
S5	141.1	0	N/A	N/A	N/A	N/A	9.471	3595	N/A	141.1	N/A	N/A
S6	137.7	0	N/A	N/A	N/A	N/A	9.384	3508	N/A	137.7	N/A	N/A
1	95.3	1	9.8	0.613	3350	4	9.174	1909	N/A	74.9	21.3	381
2	93.6	1	8.1	0.613	3350	4	9.295	599	N/A	23.5	74.9	1315
3	107.7	1	7.1	0.613	3350	4	9.628	283	N/A	11.1	89.7	1811
4	108.9	1	9.5	0.613	N/A	0	9.532	1293	N/A	50.8	53.4	1091
5	112.9	1	8.2	0.613	N/A	0	9.543	71	N/A	2.8	97.5	2064
6	110.2	1	7.7	0.613	N/A	0	0	0	N/A	0.0	100.0	2065
7	95.3	3	9.8	0.613	3350	4	9.684	1208	N/A	47.4	50.2	299
8	93.6	3	8.1	0.613	3350	4	9.788	288	N/A	11.23	88.0	515
9	107.7	3	7.1	0.613	3350	4	9.835	719	N/A	28.04	74.0	498
10	108.9	3	9.5	0.613	N/A	0	9.859	548	N/A	21.37	80.4	547
11	112.9	3	8.2	0.613	N/A	0	9.736	166	N/A	6.47	94.3	665
12	110.2	3	7.7	0.613	N/A	0	8.166	23	N/A	0.90	99.2	683
13	95.3	7	9.8	0.613	3350	4	9.878	1178	N/A	45.94	51.8	132
14	93.6	7	8.1	0.613	3350	4	10.062	1116	N/A	43.52	53.5	134
15	107.7	7	7.1	0.613	3350	4	9.972	637	N/A	24.84	76.9	222
16	108.9	7	9.5	0.613	N/A	0	10.034	1127	N/A	43.95	59.6	174
17	112.9	7	8.2	0.613	N/A	0	10.073	162.7	N/A	6.34	94.4	285
18	110.2	7	7.7	0.613	N/A	0	10.135	1127	N/A	43.95	60.1	177
19	95.3	9	9.8	0.613	3350	4	10.229	1279	N/A	49.88	47.6	95
20	93.6	9	8.1	0.613	3350	4	9.346	24.07	N/A	0.94	99.0	193
21	107.7	9	7.1	0.613	3350	4	10.268	804.6	N/A	31.38	70.9	159
22	108.9	9	9.5	0.613	N/A	0	10.227	1143	N/A	44.58	59.1	134
23	112.9	9	8.2	0.613	N/A	0	10.288	98.23	N/A	3.83	96.6	227
24	110.2	9	7.7	0.613	N/A	0	10.325	269	N/A	10.49	90.5	208

SBP Test: JA28												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
Stock #1	136.4	0	9.2	0	N/A	N/A	8.95	3475	N/A	136.4	N/A	N/A
Stock #2	134.9	0	8.2	0	N/A	N/A	9.05	3436	N/A	134.9	N/A	N/A
Stock #3	134.7	0	7.2	0	N/A	N/A	9.03	3432	N/A	134.7	N/A	N/A
1A	109.1	0.05	9.2	0.613	N/A	N/A	9.00	2320	N/A	91.0	16.6	6778
1B	109.1	0.5	9.2	0.613	N/A	N/A	9.03	397	N/A	15.5	85.8	3509
1C	109.1	1	9.2	0.613	N/A	N/A	9.12	170	N/A	6.6	93.9	1921
1D	109.1	3	9.2	0.613	N/A	N/A	9.00	671	N/A	26.3	75.9	518
1E	109.1	5	9.2	0.613	N/A	N/A	9.06	444	N/A	17.4	84.0	344
1F	109.1	7	9.2	0.613	N/A	N/A	9.08	583	N/A	22.8	79.1	231
1G	109.1	9	9.2	0.613	N/A	N/A	9.11	798	N/A	31.2	71.4	162
2A	107.9	0.05	8.2	0.613	N/A	N/A	9.09	**700**	N/A	27.4	74.6	30165
2B	107.9	0.5	8.2	0.613	N/A	N/A	8.97	25	N/A	0.9	99.1	4010
2C	107.9	1	8.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	2015
2D	107.9	3	8.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	672
2E	107.9	5	8.2	0.613	N/A	N/A	**9.10**	32	N/A	**1.3**	98.8	400
2F	107.9	7	8.2	0.613	N/A	N/A	**9.10**	64	N/A	**2.5**	97.7	282
2G	107.9	9	8.2	0.613	N/A	N/A	9.19	26	N/A	1.0	99.1	223
3A	107.8	0.05	7.2	0.613	N/A	N/A	9.10	1112	N/A	43.6	59.5	24052
3B	107.8	0.5	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	4026
3C	107.8	1	7.2	0.613	N/A	N/A	**9.10**	24	N/A	**1.0**	99.1	2002
3D	107.8	3	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	671
3E	107.8	5	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	403
3F	107.8	7	7.2	0.613	N/A	N/A	9.25	84	N/A	3.2	97.0	280
3G	107.8	9	7.2	0.613	N/A	N/A	**9.25**	**90**	N/A	**3.5**	96.8	217

SBP Test: FE01												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
Stock #1	115.4	0	6.2	0	N/A	N/A	8.90	2941	N/A	115.4	N/A	N/A
Stock #2	117.9	0	5.1	0	N/A	N/A	9.00	3003	N/A	117.9	N/A	N/A
1A	92.3	0.05	6.2	0.613	N/A	N/A	9.07	**300**	N/A	11.7	87.3	30221
1B	92.3	0.5	6.2	0.613	N/A	N/A	9.17	36	N/A	1.3	98.6	3412
1C	92.3	1	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	1724
1D	92.3	3	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	575
1E	92.3	5	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	345
1F	92.3	7	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	246
1G	92.3	9	6.2	0.613	N/A	N/A	9.04	129	N/A	5.0	94.6	182
2A	94.3	0.05	5.1	0.613	N/A	N/A	9.07	1465	N/A	57.5	39.1	13805
2B	94.3	0.5	5.1	0.613	N/A	N/A	9.15	41	N/A	1.6	98.3	3476
2C	94.3	1	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	1760
2D	94.3	3	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	587
2E	94.3	5	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	352
2F	94.3	7	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	251
2G	94.3	9	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	196
3A	93.3	0.05	4.1	0.613	N/A	N/A	9.08	1786	N/A	70.1	24.9	8707
3B	93.3	0.5	4.1	0.613	N/A	N/A	9.15	117	N/A	45.1	51.6	1807
3C	93.3	1	4.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	1742
3D	93.3	3	4.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	581
3E	93.3	5	4.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	348
3F	93.3	7	4.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	249
3G	93.3	9	4.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	194
1H	93.3	0.05	3.1	0.613	N/A	N/A	9.34	893	N/A	35.0	62.5	21862
2H	93.3	0.5	3.1	0.613	N/A	N/A	9.17	332	N/A	13.0	86.1	3011
3H	93.3	1	3.1	0.613	N/A	N/A	9.21	29	N/A	1.1	98.8	1729
4A	93.3	3	3.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	581
4B	93.3	5	3.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	348
4C	93.3	7	3.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	249
4D	93.3	9	3.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	194

SBP Test: FE04a												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
1A	100.0	0.05	3.1	0.613	N/A	N/A	9.13	2201	N/A	86.4	13.6	5111
1B	100.0	0.05	4.1	0.613	N/A	N/A	9.32	1894	N/A	74.3	25.7	9635
1C	100.0	0.05	5.1	0.613	N/A	N/A	9.12	1422	N/A	55.8	44.2	16587
1D	100.0	0.05	6.2	0.613	N/A	N/A	9.16	1155	N/A	45.3	54.7	20517
1E	100.0	0.05	7.2	0.613	N/A	N/A	9.26	930	N/A	36.4	63.6	23832
1F	100.0	0.05	8.2	0.613	N/A	N/A	9.29	789	N/A	30.9	69.1	25892
2A	100.0	0.5	3.1	0.613	N/A	N/A	9.20	439	N/A	17.2	82.8	3105
2B	100.0	0.5	4.1	0.613	N/A	N/A	9.30	11	N/A	0.4	99.6	3734
2C	100.0	0.5	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3734
2D	100.0	0.5	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3734
2E	100.0	0.5	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3734
2F	100.0	0.5	8.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3734

SBP Test: FE04bTime Test at 20C												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Time
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	(min)
4A	100.0	1	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	1
4B	100.0	1	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3
4C	100.0	1	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	6
4D	100.0	1	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	12
3A	100.0	1	6.2	0.613	N/A	N/A	9.33	324	N/A	12.7	87.3	60
3C	100.0	1	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	180
5A	100.0	1	7.2	0.613	N/A	N/A	9.27	12	N/A	0.4	99.6	1
5B	100.0	1	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3
5C	100.0	1	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	6
5D	100.0	1	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	12
3B	100.0	1	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	60
3D	100.0	1	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	180

SBP Test: FE04c											
Solution Name	**CPi (mg/l)**	**Enzyme unit/ml**	**pH**	**H2O2 (mM)**	**PEG MW**	**PEG (g/L)**	**Ret. Time (min)**	**HPLC AREA**	**Peak Height**	**CPf (mg/l)**	**Note: Following are mixtures**
3E	100 2-CP	1	6.2	0.778	N/A	N/A	N/A	<10	N/A	0.4	2CP
3F	100 4-CP	1	6.2	0.778	N/A	N/A	4.06	111	N/A	4.3	4CP
3Gi	Mix*	1	6.2	N/A	N/A	N/A	3.39	75	N/A	2.9	*2CP, 4CP, 2,4-DCP
3Gii	Mix*	1	6.2	N/A	N/A	N/A	4.22	49	N/A	1.9	*2CP, 4CP, 2,4-DCP
3Giii	Mix*	1	6.2	N/A	N/A	N/A	9.46	120	N/A	4.6	*2CP, 4CP, 2,4-DCP
3H	Mix*	1	7.2	N/A	N/A	N/A	N/A	<10	N/A	0.4	*2CP, 4CP, 2,4-DCP

SBP Test: KE01												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
KE01A03	100.0	0.05	3.1	0.613	N/A	N/A	8.97	2166	N/A	85.0	15.0	5640
KE01A04	100.0	0.5	3.1	0.613	N/A	N/A	9.02	529	N/A	20.7	79.3	2972
KE01A05	100.0	0.25	3.1	0.613	N/A	N/A	9.00	1230	N/A	48.2	51.8	3882
KE01A06	100.0	0.1	3.1	0.613	N/A	N/A	8.97	1810	N/A	71.0	29.0	5436
KE01A07	100.0	0.01	3.1	0.613	N/A	N/A	8.98	2183	N/A	85.7	14.3	26871
KE01A08	100.0	0.5	4.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3734
KE01A09	100.0	0.25	4.1	0.613	N/A	N/A	9.05	336	N/A	13.2	86.8	6511
KE01A11	100.0	0.1	4.1	0.613	N/A	N/A	9.01	1118	N/A	43.8	56.2	10529
KE01A12	100.0	0.01	4.1	0.613	N/A	N/A	9.00	2116	N/A	83.0	17.0	31865
KE01A13	100.0	0.25	5.1	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	7469
KE01A14	100.0	0.1	5.1	0.613	N/A	N/A	9.04	706	N/A	27.7	72.3	13551
KE01A15	100.0	0.01	5.1	0.613	N/A	N/A	8.99	1985	N/A	77.9	22.1	41478
KE01A16	100.0	0.25	6.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	7469
KE01A17	100.0	0.1	6.2	0.613	N/A	N/A	9.03	348	N/A	13.6	86.4	16202
KE01A18	100.0	0.01	6.2	0.613	N/A	N/A	8.99	2065	N/A	81.0	19.0	35534
KE01A19	100.0	0.25	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	7469
KE01A23	100.0	0.1	7.2	0.613	N/A	N/A	9.04	409	N/A	16.0	84.0	15750
KE01A24	100.0	0.01	7.2	0.613	N/A	N/A	9.00	1328	N/A	51.9	48.1	90096
KE01A25	100.0	**0.5**	8.2	0.613	N/A	N/A	9.02	76	N/A	2.9	97.1	3640
KE01A26	100.0	**0.25**	8.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	7469
KE01A27	100.0	**0.1**	8.2	0.613	N/A	N/A	9.04	70	N/A	2.7	97.3	18235
KE01A28	100.0	**0.01**	8.2	0.613	N/A	N/A	8.98	2275	N/A	89.2	10.8	20176
KE01A29	100.0	**0.05**	9.2	0.613	N/A	N/A	9.00	1004	N/A	39.4	60.6	22737
KE01A30	100.0	**0.5**	9.2	0.613	N/A	N/A	9.02	320	N/A	12.5	87.5	3279
KE01A31	100.0	**0.25**	9.2	0.613	N/A	N/A	9.00	305	N/A	11.9	88.1	6604
KE01A32	100.0	**0.1**	9.2	0.613	N/A	N/A	9.01	362	N/A	14.1	85.9	16098
KE01A33	100.0	0.01	9.2	0.613	N/A	N/A	8.98	2218	N/A	87.0	13.0	24355

SBP Test: KE05												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
1A	100.0	0.01	6.2	0.613	3350	0.002	8.84	1899	N/A	74.5	25.5	47801
1B	100.0	0.01	6.2	0.613	3350	0.002	8.84	1952	N/A	76.6	23.4	43931
1F	100.0	0.01	8.6	0.613	3350	0.002	8.79	2356	N/A	92.4	7.6	14222
1G	100.0	0.01	8.6	0.613	3350	0.010	8.79	2316	N/A	90.9	9.1	17068
1H	100.0	0.01	8.6	0.613	3350	0.020	8.79	2410	N/A	94.5	5.5	10262
2A	100.0	0.01	8.6	0.613	3350	0.100	8.80	2518	N/A	98.9	1.1	2154
2B	100.0	0.01	8.6	0.613	3350	0.200	8.80	2558	N/A	100.0	0.0	0
2C	100.0	0.01	6.2	0.613	N/A	N/A	8.83	2322	N/A	91.1	8.9	16641
2D	100.0	0.05	6.2	0.613	N/A	N/A	8.85	1381	N/A	54.2	45.8	17187
2E	100.0	0.1	6.2	0.613	N/A	N/A	8.90	416	N/A	16.3	83.7	15690
2F	100.0	0.01	7.2	0.613	N/A	N/A	8.87	1821	N/A	71.5	28.5	53489
2G	100.0	0.05	7.2	0.613	N/A	N/A	8.87	1648	N/A	64.6	35.4	13263
2H	100.0	0.1	7.2	0.613	N/A	N/A	8.92	709	N/A	27.8	72.2	13542
3A	100.0	0.01	8.2	0.613	N/A	N/A	8.87	2277	N/A	89.4	10.6	19938
3B	100.0	0.05	8.2	0.613	N/A	N/A	8.82	884	N/A	34.7	65.3	24499
3C	100.0	0.1	8.2	0.613	N/A	N/A	8.86	58	N/A	2.2	97.8	18333
3D	100.0	0.01	8.6	0.613	N/A	N/A	8.80	2313	N/A	90.8	9.2	17303
3E	100.0	0.25	8.6	0.613	N/A	N/A	8.89	59	N/A	2.3	97.7	7326
3F	100.0	0.5	8.6	0.613	N/A	N/A	8.88	437	N/A	17.1	82.9	3109
3H	100.0	0.01	9.2	0.613	N/A	N/A	8.83	2353	N/A	92.3	7.7	14369

SBP Test: KE06												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
1A	100.0	0.01	6.2	0.613	3350	0.010	8.88	1602	N/A	62.8	37.2	69641
1B	100.0	0.01	6.2	0.613	3350	0.020	8.88	1451	N/A	56.9	43.1	80732
1C	100.0	0.01	6.2	0.613	3350	0.100	8.90	1134	N/A	44.4	55.6	104152
1D	100.0	0.01	6.2	0.613	3350	0.200	8.90	1226	N/A	48.1	51.9	97363
1E	100.0	0.01	3.1	0.613	3350	0.002	8.99	2297	N/A	90.1	9.9	18488
1F	100.0	0.01	4.1	0.613	3350	0.002	8.90	2177	N/A	85.4	14.6	27305
1G	100.0	0.01	5.1	0.613	3350	0.002	8.89	2261	N/A	88.7	11.3	21221
1H	100.0	0.01	7.2	0.613	3350	0.002	8.90	1926	N/A	75.6	24.4	45787
2A	100.0	0.01	8.2	0.613	3350	0.002	8.90	1305	N/A	51.2	48.8	91459
2B	100.0	0.01	3.1	0.613	3350	0.020	8.87	2418	N/A	94.8	5.2	9658
2C	100.0	0.01	3.1	0.613	3350	0.200	8.90	2438	N/A	95.7	4.3	8099
2D	100.0	0.01	4.1	0.613	3350	0.200	8.90	2221	N/A	87.2	12.8	24073
2E	100.0	0.01	5.1	0.613	3350	0.200	8.94	1684	N/A	66.1	33.9	63576
2F	100.0	0.01	7.2	0.613	3350	0.200	8.96	943	N/A	37.0	63.0	118132
2G	100.0	0.01	8.2	0.613	3350	0.200	8.97	1002	N/A	39.3	60.7	113769
2H	100.0	0.01	9.2	0.613	3350	0.200	8.90	2542	N/A	100.0	0.0	0
3A	100.0	0.01	7.2	0.613	N/A	N/A	8.93	2049	N/A	80.4	19.6	36712
3B	100.0	0.01	9.2	0.613	N/A	N/A	8.99	349	N/A	13.6	86.4	161906
3C	100.0	0.01	3.1	0.613	3350	0.500	8.94	2185	N/A	85.7	14.3	26806
3D	100.0	0.01	9.2	0.613	3350	0.500	8.94	2579	N/A	100.0	0.0	0
3E	100.0	0.01	6.2	0.613	3350	0.500	8.97	1316	N/A	51.6	48.4	90707

SBP Test: KE07Note: Temperature - = 4C												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Time
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	(min)
A	100.0	1	6.2	0.613	N/A	N/A	8.98	7715	N/A	28.0	72.0	5
B	100.0	1	6.2	0.613	N/A	N/A	8.99	1220	N/A	4.6	95.4	25
C	100.0	1	6.2	0.613	N/A	N/A	8.97	651	N/A	2.4	97.6	45
D	100.0	1	6.2	0.613	N/A	N/A	8.96	651	N/A	2.4	97.6	65
E	100.0	1	6.2	0.613	N/A	N/A	N/A	< 10	N/A	0.4	99.6	180
F	100.0	1	6.2	0.613	N/A	N/A	8.94	3449	N/A	13.7	86.3	11
G	100.0	1	6.2	0.613	N/A	N/A	8.98	1230	N/A	5.1	94.9	31
H	100.0	1	6.2	0.613	N/A	N/A	9.02	834	N/A	3.3	96.7	107

SBP Test: KE09												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
1A	100.0	0.1	9.2	0.613	N/A	N/A	9.15	1468	N/A	57.5	42.5	7958
1B	100.0	0.25	9.2	0.613	N/A	N/A	9.15	1532	N/A	60.1	39.9	2989
1C	100.0	0.5	9.2	0.613	N/A	N/A	9.18	765	N/A	30.0	70.0	2625
1D	100.0	1	9.2	0.613	N/A	N/A	9.21	324	N/A	12.7	87.3	1637
1E	100.0	3	9.2	0.613	N/A	N/A	9.19	291	N/A	11.4	88.6	554
1F	100.0	0.25	8.6	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	7469
1G	100.0	0.5	8.6	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	3734
1H	100.0	1	8.6	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	1867
2A	100.0	5	8.6	0.613	N/A	N/A	9.24	75	N/A	2.9	97.1	364
2B	100.0	0.01	8.6	0.613	N/A	N/A	9.20	2753	N/A	**100.0**	0.0	0
2C	100.0	0.01	8.2	0.613	N/A	N/A	9.23	407	N/A	15.9	84.1	157611
2D	100.0	0.01	8.2	0.613	N/A	N/A	9.24	N/A	N/A	**48.1**	51.9	97290
2E	100.0	0.01	8.2	0.613	N/A	N/A	9.27	1895	N/A	74.3	25.7	48101
2F	100.0	0.01	7.2	0.613	N/A	N/A	9.23	1919	N/A	75.3	24.7	46360
2G	100.0	0.05	7.2	0.613	N/A	N/A	9.33	1331	N/A	52.2	47.8	17915
2H	100.0	0.05	7.2	0.613	N/A	N/A	9.33	1354	N/A	53.1	46.9	17584
3A	100.0	0.1	7.2	0.613	N/A	N/A	9.27	679	N/A	26.6	73.4	13764
3B	100.0	0.01	8.2	0.613	N/A	N/A	9.24	2273	N/A	89.2	10.8	20265
3C	100.0	0.1	8.2	0.613	N/A	N/A	9.34	260	N/A	10.1	89.9	16849
3D	100.0	0.01	7.2	0.613	3350	0.100	9.33	1064	N/A	41.7	58.3	109246
3E	100.0	0.01	7.2	0.613	3350	0.020	9.32	1340	N/A	52.5	47.5	88993
3F	100.0	0.01	7.2	0.613	3350	0.800	9.30	N/A	N/A	**7.1**	92.9	174148
3G	100.0	0.01	6.2	0.613	3350	0.800	9.40	N/A	N/A	**9.9**	90.1	168899
3H	100.0	0.01	5.1	0.613	3350	0.020	9.31	N/A	N/A	**74.2**	25.8	48364
4A	100.0	0.01	5.1	0.613	3350	0.100	9.38	N/A	N/A	**74.2**	25.8	48364
4B	100.0	0.01	4.1	0.613	3350	0.020	9.32	N/A	N/A	**79.2**	20.8	38991
4C	100.0	0.01	4.1	0.613	3350	0.100	9.38	N/A	N/A	**74.9**	25.1	47052

SBP Test: KE11												
Solution	2,4-DCPi	Enzyme	pH	H2O2	PEG	PEG	Ret. Time	HPLC	Peak	2,4-DCPf	%	Turnovers
Name	(mg/l)	unit/ml		(mM)	MW	(g/L)	(min)	AREA	Height	(mg/l)	Rem.	
Stand. 100	97.5	0	N/A	0	N/A	N/A	9.06	2486	70	97.5	N/A	N/A
Stand. 60	59.6	0	N/A	0	N/A	N/A	9.10	1519	43	59.6	N/A	N/A
Stand. 20	20.2	0	N/A	0	N/A	N/A	9.09	516	14.5	20.2	N/A	N/A
Stand. 10	12.8	0	N/A	0	N/A	N/A	9.12	327	7.3	12.8	N/A	N/A
1A	100.0	0.01	4.1	0.613	3350	0.400	9.08	2221	N/A	87.2	12.8	24071
1B	100.0	0.01	3.1	0.613	3350	1.200	9.07	2104	N/A	82.6	17.4	32687
1C	100.0	0.01	5.1	0.613	3350	0.400	9.11	1237	N/A	48.5	51.5	96592
1D	100.0	0.01	5.1	0.613	3350	0.800	9.14	1594	N/A	62.5	37.5	70295
1E	100.0	0.01	6.2	0.613	3350	0.400	9.17	250	N/A	9.8	90.2	169168
1F	100.0	0.01	6.2	0.613	3350	0.800	9.18	492	N/A	19.3	80.7	151370
1G	100.0	0.01	6.2	0.613	3350	1.200	9.26	23	N/A	0.9	99.1	185818
1H	100.0	0.01	2.5	0.613	3350	0.400	9.17	2092	N/A	82.1	17.9	33635
2A	100.0	0.01	6.2	0.613	3350	0.200	9.25	525	N/A	20.6	79.4	148846
2B	100.0	0.01	6.2	0.613	3350	1.200	9.23	775	N/A	30.4	69.6	130462
2C	100.0	0.01	7.2	0.613	3350	0.400	9.29	121	N/A	4.7	95.3	178720
2D	100.0	0.01	7.2	0.613	3350	1.200	9.27	694	N/A	27.2	72.8	136510
2E	100.0	0.01	8.2	0.613	3350	0.400	9.32	566	N/A	22.2	77.8	145870
2F	100.0	0.01	8.2	0.613	3350	0.800	9.33	1320	N/A	51.8	48.2	90412
2G	100.0	0.01	8.2	0.613	3350	1.200	9.49	1479	N/A	58.0	42.0	78724
2H	100.0	0.01	8.2	0.613	3350	0.002	9.47	1415	N/A	55.5	44.5	83489
3A	100.0	0.01	8.2	0.613	3350	0.200	9.47	N/A	13.8	**19.2**	80.8	151393
3B	100.0	0.01	9.2	0.613	3350	0.400	9.47	2353	N/A	92.3	7.7	14384
3C	100.0	0.01	9.2	0.613	3350	0.800	9.53	2640	N/A	100.0	0.0	0
3D	100.0	0.1	9.2	0.613	N/A	N/A	9.52	1636	N/A	64.2	35.8	6720
3E	100.0	0.5	9.2	0.613	N/A	N/A	9.53	203	N/A	7.9	92.1	3451
3F	100.0	1	9.2	0.613	N/A	N/A	9.62	98	N/A	3.8	96.2	1804
3G	100.0	0.01	3.1	0.613	3350	0.400	9.61	1784	N/A	70.0	30.0	56324
3H	100.0	0.01	3.1	0.613	3350	0.800	9.60	2127	N/A	83.4	16.6	31041
4A	100.0	0.01	2.5	0.613	N/A	N/A	9.77	N/A	58	**80.9**	19.1	35883
4B	100.0	0.1	2.5	0.613	N/A	N/A	9.72	N/A	47	**65.5**	34.5	6463
4C	100.0	0.5	2.5	0.613	N/A	N/A	9.70	N/A	16.5	**23.0**	77.0	2887
4D	100.0	1	2.5	0.613	N/A	N/A	9.77	N/A	8	**11.2**	88.8	1666
5A	100.0	0.01	2.5	0.613	3350	0.002	9.70	N/A	65	**90.6**	9.4	17590
5B	100.0	0.01	2.5	0.613	3350	0.200	9.80	N/A	56	**78.1**	21.9	41110
5C	100.0	0.01	2.5	0.613	3350	0.800	9.77	N/A	57	**79.5**	20.5	38497

SBP Test: KE12&14												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
Stand. 100	100.0	0	8.2	0	N/A	N/A	9.21	2589	72.1	100.0	N/A	N/A
1A	55.5	0	8.2	0	N/A	N/A	9.25	1414	38.8	**55.5**	N/A	N/A
1B	55.5	0.01	8.2	0.340	N/A	N/A	9.27	943	25.8	36.9	33.4	34779
1C	55.5	0.05	8.2	0.340	N/A	N/A	9.27	84	2	3.2	94.2	19596
1D	55.5	0.1	8.2	0.340	N/A	N/A	N/A	<10	0	0.4	99.3	10327
1E	55.5	0.5	8.2	0.340	N/A	N/A	N/A	<10	0	0.4	99.3	2065
1F	55.5	1	8.2	0.340	N/A	N/A	N/A	<10	0	0.4	99.3	1033
1G	55.5	1	8.2	0.340	N/A	N/A	N/A	<10	0	0.4	99.3	1033
1H	55.5	5	8.2	0.340	N/A	N/A	N/A	<10	0	0.4	99.3	207
2A	214.1	0	8.2	0	N/A	N/A	9.13	5455	152	**214.1**	N/A	N/A
2B	214.1	0.01	8.2	1.314	N/A	N/A	9.14	4964	138	194.9	9.0	36116
2C	214.1	0.05	8.2	1.314	N/A	N/A	9.20	3170	89	124.4	41.9	33644
2D	214.1	0.1	8.2	1.314	N/A	N/A	9.32	75	3	3.0	98.6	39584
2E	214.1	0.5	8.2	1.314	N/A	N/A	9.25	31	1	1.2	99.5	7984
2F	214.1	1	8.2	1.314	N/A	N/A	9.27	870	25	34.1	84.1	3375
2G	214.1	5	8.2	1.314	N/A	N/A	9.31	117	4.3	4.5	97.9	786
3A	315.0	0	8.2	0	N/A	N/A	9.07	6930	215	**315.0**	N/A	N/A
3B	315.0	0.01	8.2	1.933	N/A	N/A	9.08	7636	206	299.7	4.9	28661
3C	315.0	0.05	8.2	1.933	N/A	N/A	9.13	5971	159	234.4	25.6	30234
3D	315.0	0.1	8.2	1.933	N/A	N/A	9.27	790	25	31.0	90.2	53242
3E	315.0	0.5	8.2	1.933	N/A	N/A	9.28	397	13	15.5	95.1	11227
3F	315.0	1	8.2	1.933	N/A	N/A	9.33	41	2	1.6	99.5	5875
3G	315.0	5	8.2	1.933	N/A	N/A	9.19	4136	114	162.3	48.5	572
4A	386.6	0	8.2	0	N/A	N/A	9.02	10090	268	386.6	N/A	N/A
4B	386.6	0.01	8.2	2.372	N/A	N/A	9.02	10565	262	377.9	2.2	16223
4C	386.6	0.05	8.2	2.372	N/A	N/A	9.06	7512	226	326.0	15.7	22713
4D	386.6	0.1	8.2	2.372	N/A	N/A	9.24	2904	N/A	114.0	70.5	51094
4E	386.6	0.5	8.2	2.372	N/A	N/A	9.14	5101	N/A	200.2	48.2	6986
4F	386.6	1	8.2	2.372	N/A	N/A	9.13	5161	N/A	202.6	47.6	3449
4G	386.6	5	8.2	2.372	N/A	N/A	9.15	4083	N/A	160.3	58.5	848

5A	100.0	0	6.8	0	N/A	N/A	9.21	2605	N/A	100.0	N/A	N/A
5C	100.0	0.05	6.8	0.613	N/A	N/A	9.29	167	N/A	6.5	93.5	35040
5D	100.0	0.1	6.8	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	18672
5B	100.0	0.25	6.8	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	7469
5E	100.0	0.5	6.8	0.613	N/A	N/A	9.31	274	N/A	10.7	89.3	3347
5F	100.0	1	6.8	0.613	N/A	N/A	9.28	303	N/A	11.9	88.1	1652
5G	100.0	5	6.8	0.613	N/A	N/A	9.29	10	N/A	0.4	99.6	373

SBP Test: KE15&16												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
100mg/l	101.4	0	6.2	0	N/A	N/A	9.24	2584	N/A	101.4	N/A	N/A
60mg/l	60.9	0	6.2	0	N/A	N/A	9.25	1552	N/A	60.9	N/A	N/A
20mg/l	20.5	0	6.2	0	N/A	N/A	9.28	523	N/A	20.5	N/A	N/A
1a	95.5	0	6.2	0	1000	0.000	9.21	2434	N/A	95.5	N/A	N/A
1b	95.5	0.01	6.2	0.613	1000	0.100	9.21	2119	N/A	83.1	13.0	23253
1c	95.5	0.01	6.2	0.613	1000	0.250	9.22	2037	N/A	79.9	16.3	29210
1d	95.5	0.01	6.2	0.613	1000	0.500	9.23	2076	N/A	81.4	14.8	26426
1e	95.5	0.01	6.2	0.613	1000	1.000	9.24	2056	N/A	80.7	15.5	27815
1f	95.5	0.01	6.2	0.613	1000	2.000	9.27	1986	N/A	77.9	18.4	33017
1g	95.5	0.01	6.2	0.613	3350	0.100	9.26	1316	N/A	51.6	45.9	82272
1h	95.5	0.01	6.2	0.613	3350	0.250	9.27	844	N/A	33.0	65.4	117129
2a	95.5	0.01	6.2	0.613	**3350**	**0.500**	9.24	2254	N/A	88.5	7.4	13244
2b	95.5	0.01	6.2	0.613	3350	1.000	9.29	617	N/A	24.2	74.7	133729
2c	95.5	0.01	6.2	0.613	3350	2.000	9.36	257	N/A	10.0	89.5	160241
2d	95.5	0.01	6.2	0.613	8000	0.100	9.35	173	N/A	6.8	92.9	166370
2e	95.5	0.01	6.2	0.613	8000	0.250	9.39	62	N/A	2.4	97.5	174550
2f	95.5	0.01	6.2	0.613	8000	0.500	9.35	61	N/A	2.3	97.6	174744
2g	95.5	0.01	6.2	0.613	8000	1.000	9.41	39	N/A	1.5	98.4	176274
2h	95.5	0.01	6.2	0.613	8000	2.000	9.40	<10	N/A	0.4	99.6	178334
3a	180.2	0	6.2	0	1000	0.000	9.24	N/A	**131**	**180.2**	N/A	N/A
3b	180.2	0.01	6.2	1.227	1000	0.100	9.28	N/A	**126**	**173.3**	3.8	12891
3c	180.2	0.01	6.2	1.227	1000	0.250	9.27	N/A	**123**	**169.2**	6.1	20626
3d	180.2	0.01	6.2	1.227	1000	0.500	9.28	N/A	**122**	**167.8**	6.9	23205
3e	180.2	0.01	6.2	1.227	1000	1.000	9.28	N/A	**124**	**170.5**	5.3	18048
3f	180.2	0.01	6.2	1.227	1000	2.000	9.31	N/A	**117**	**160.9**	10.7	36096
3g	180.2	0.01	6.2	1.227	3350	0.100	9.33	N/A	**81**	**111.4**	38.2	128914
3h	180.2	0.01	6.2	1.227	3350	0.250	9.37	N/A	**64**	**88.0**	51.1	172745
4a	180.2	0.01	6.2	1.227	**3350**	**0.500**	9.30	N/A	**130**	**178.8**	0.8	2578
4b	180.2	0.01	6.2	1.227	**3350**	**1.000**	9.25	N/A	**188**	**258.6**	-43.5	-146962
4c	180.2	0.01	6.2	1.227	3350	2.000	9.47	327	N/A	12.5	93.1	314369
4d	180.2	0.01	6.2	1.227	8000	0.100	9.49	244	N/A	9.5	94.7	319998
4e	180.2	0.01	6.2	1.227	8000	0.250	9.51	139	N/A	5.4	97.0	327669
4f	180.2	0.01	6.2	1.227	8000	0.500	9.49	125	N/A	4.9	97.3	328616
4g	180.2	0.01	6.2	1.227	8000	1.000	9.51	88	N/A	3.4	98.1	331322
4h	180.2	0.01	6.2	1.227	8000	2.000	9.54	50	N/A	1.9	98.9	334114
5a	287.7	0	6.2	0	1000	0.000	9.49	7329	N/A	287.7	N/A	N/A

5b	287.7	0.01	6.2	1.840	1000	0.100	9.34	N/A	**186**	**255.8**	11.1	59674
5c	287.7	0.01	6.2	1.840	1000	0.250	9.36	N/A	**183**	**251.7**	12.5	67409
5d	287.7	0.01	6.2	1.840	1000	0.500	9.35	N/A	**165**	**226.9**	21.1	113818
5e	287.7	0.01	6.2	1.840	1000	1.000	9.36	6839	N/A	268.4	6.7	36073
5f	287.7	0.01	6.2	1.840	1000	2.000	9.36	6686	N/A	262.4	8.8	47315
5g	287.7	0.01	6.2	1.840	3350	0.100	9.43	3626	N/A	142.3	50.5	272451
5h	287.7	0.01	6.2	1.840	3350	0.250	9.46	2851	N/A	111.9	61.1	329438
6a	287.7	0.01	6.2	1.840	3350	0.500	9.43	6457	N/A	253.4	11.9	64143
6b	287.7	0.01	6.2	1.840	3350	1.000	9.54	1410	N/A	55.3	80.8	435521
6c	287.7	0.01	6.2	1.840	3350	2.000	9.60	624	N/A	24.5	91.5	493396
6d	287.7	0.01	6.2	1.840	8000	0.100	9.60	419	N/A	16.4	94.3	508461
6e	287.7	0.01	6.2	1.840	8000	0.250	9.60	374	N/A	14.6	94.9	511777
6f	287.7	0.01	6.2	1.840	8000	0.500	9.63	380	N/A	14.9	94.8	511298
6g	287.7	0.01	6.2	1.840	8000	1.000	9.66	271	N/A	10.6	96.3	519298
6h	287.7	0.01	6.2	1.840	8000	2.000	9.67	118	N/A	4.6	98.4	530596

SBP Test: KE20												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
1-200	214.1	0.025	8.2	1.227	N/A	N/A	9.58	3282	N/A	128.8	39.9	63996
2-200	214.1	0.075	8.2	1.227	N/A	N/A	9.60	1942	N/A	76.2	64.4	34468
3-300	315.0	0.025	8.2	1.840	N/A	N/A	9.45	6612	N/A	259.6	17.6	41540
4-300	315.0	0.075	8.2	1.840	N/A	N/A	9.51	4530	N/A	177.8	43.6	34292
5-400	386.6	0.025	8.2	2.454	N/A	N/A	9.36	9421	N/A	369.9	4.3	12511
6-400	386.6	0.075	8.2	2.454	N/A	N/A	9.41	7236	N/A	284.0	26.5	25634
7-400	386.6	**0.12**	8.2	2.454	N/A	N/A	9.42	6556	N/A	257.3	33.4	20188
8-500	493.5	0	8.2	3.067	N/A	N/A	9.29	12569	N/A	493.5	0.0	N/A
9-500	493.5	0.01	8.2	3.067	N/A	N/A	9.28	13020	N/A	N/A	N/A	N/A
10-500	493.5	0.025	8.2	3.067	N/A	N/A	9.29	N/A	**298**	**477.4**	3.3	12044
11-500	493.5	0.05	8.2	3.067	N/A	N/A	9.36	N/A	**250**	**400.5**	18.8	34852
12-500	493.5	0.075	8.2	3.067	N/A	N/A	9.41	N/A	**205**	**328.4**	33.4	41253
13-500	493.5	0.1	8.2	3.067	N/A	N/A	9.42	N/A	**220**	**352.4**	28.6	26435
14-500	493.5	0.12	8.2	3.067	N/A	N/A	9.42	8702	N/A	341.6	30.8	23730
15-500	493.5	0.13	8.2	3.067	N/A	N/A	9.44	7245	N/A	284.4	42.4	30150
16-100	100.0	**.01+.13**	6.8	0.613	N/A	N/A	9.69	330	N/A	12.9	87.1	11658
17-100	100.0	0.05	6.8	0.613	N/A	N/A	9.65	1195	N/A	46.9	53.1	19924
18-100	100.0	0.1	6.8	0.613	N/A	N/A	9.64	30	N/A	1.1	98.9	18537
20-100	95.5	0.025	7.2	0.613	N/A	N/A	9.63	1667	N/A	65.4	31.5	22591
21-100	95.5	0.075	7.2	0.613	N/A	N/A	9.69	801	N/A	31.4	67.1	16026
22-100	95.5	0.12	7.2	0.613	N/A	N/A	N/A	<10	N/A	0.4	99.6	14862
23-200	180.0	0.025	7.2	1.227	N/A	N/A	9.56	3769	N/A	148.0	17.8	24030
24-200	180.0	0.075	7.2	1.227	N/A	N/A	9.54	4374	N/A	171.7	4.6	2081
25-200	180.0	0.12	7.2	1.227	N/A	N/A	9.61	2074	N/A	81.4	54.8	15404
26-300	287.7	0.025	7.2	1.840	N/A	N/A	9.49	5854	N/A	229.8	20.1	43404
27-300	287.7	0.075	7.2	1.840	N/A	N/A	9.53	N/A	**151**	**207.6**	27.8	20021
28-300	287.7	0.12	7.2	1.840	N/A	N/A	9.56	N/A	**127**	**174.6**	39.3	17666

SBP Test: KE23&24												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
100mg/l	102.4	0	7.2	0	N/A	N/A	9.52	2567	67	102.4	N/A	N/A
60mg/l	60.7	0	7.2	0	N/A	N/A	9.56	1521	41	60.7	N/A	N/A
10mg/l	10.8	0	7.2	0	N/A	N/A	9.61	272	7.5	10.8	N/A	N/A
1a	100.0	0.01	3.1	0.613	3350	0.200	9.57	2065	60	82.4	17.6	33041
1b	100.0	0.01	3.1	0.613	3350	0.400	9.57	2006	60	80.1	19.9	37385
1c	100.0	0.01	3.1	0.613	3350	2.000	9.62	2143	59.5	85.5	14.5	27204
1d	100.0	0.01	3.1	0.613	3350	4.000	9.69	2224	60	88.8	11.2	21085
1e	100.0	0.01	3.1	0.613	8000	0.200	9.69	2145	60	85.6	14.4	26988
1f	100.0	0.01	3.1	0.613	8000	0.400	9.69	2021	59.5	80.7	19.3	36272
1g	100.0	0.01	3.1	0.613	8000	2.000	9.70	2154	60	85.9	14.1	26367
1h	100.0	0.01	3.1	0.613	8000	4.000	9.77	2125	59.5	84.8	15.2	28481
2a	100.0	0.01	4.1	0.613	3350	0.200	9.82	1910	54	76.2	23.8	44594
2b	100.0	0.01	4.1	0.613	3350	0.400	9.82	1890	54	75.4	24.6	46106
2c	100.0	0.01	4.1	0.613	3350	2.000	9.85	1854	51	74.0	26.0	48788
2d	100.0	0.01	4.1	0.613	3350	4.000	9.92	1773	49	70.8	29.2	54823
2e	100.0	0.01	4.1	0.613	8000	0.200	9.92	1733	48	69.1	30.9	57843
2f	100.0	0.01	4.1	0.613	8000	0.400	9.94	1652	47.5	65.9	34.1	63933
2g	100.0	0.01	4.1	0.613	8000	2.000	9.99	1506	41	60.1	39.9	74819
2h	100.0	0.01	4.1	0.613	8000	4.000	10.03	1509	41	60.2	39.8	74585
3a	100.0	0.01	5.1	0.613	3350	0.200	10.04	1494	41	59.6	40.4	75745
3b	100.0	0.01	5.1	0.613	3350	0.400	10.06	1319	36	52.6	47.4	88792
3c	100.0	0.01	5.1	0.613	3350	2.000	10.12	854	26	34.1	65.9	123549
3d	100.0	0.01	5.1	0.613	3350	4.000	10.20	652	18	26.0	74.0	138703
3e	100.0	0.01	5.1	0.613	8000	0.200	10.19	581	16	23.2	76.8	144006
3f	100.0	0.01	5.1	0.613	8000	0.400	10.19	394	12	15.7	84.3	157961
3g	100.0	0.01	5.1	0.613	8000	2.000	10.23	168	5	6.7	93.3	174865
3h	100.0	0.01	5.1	0.613	8000	4.000	10.32	57	3	2.3	97.7	183198
4a	100.0	0.01	6.2	0.613	3350	0.200	10.28	687	22	27.4	72.6	136071
4b	100.0	0.01	6.2	0.613	3350	0.400	10.30	766	21	30.5	69.5	130201
4c	100.0	0.01	6.2	0.613	3350	2.000	10.38	208	6	8.3	91.7	171913
4d	100.0	0.01	6.2	0.613	3350	4.000	10.47	54	3	2.2	97.8	183395
4e	100.0	0.01	6.2	0.613	8000	0.200	10.44	21	2	0.9	99.1	185850

4f	100.0	0.01	6.2	0.613	8000	0.400	10.31	50	1	2.0	98.0	183731
4g	100.0	0.01	6.2	0.613	8000	2.000	N/A	< 10	0	0.0	100.0	187382
4h	100.0	0.01	6.2	0.613	8000	4.000	N/A	< 10	0	0.0	100.0	187382
5a	100.0	0.05	3.1	0.613	8000	0.200	10.49	1706	44	68.1	31.9	11969
5b	100.0	0.1	3.1	0.613	8000	0.200	10.52	1278	36	51.0	49.0	9185
5c	100.0	0.25	3.1	0.613	8000	0.200	10.53	679	20	27.1	72.9	5465
5d	100.0	0.5	3.1	0.613	8000	0.200	10.59	260	7	10.4	89.6	3361
5e	100.0	0.05	3.1	0.613	8000	2.000	10.54	1653	44	66.0	34.0	12762
5f	100.0	0.1	3.1	0.613	8000	2.000	10.55	1333	34	53.2	46.8	8779
5g	100.0	0.25	3.1	0.613	8000	2.000	10.63	631	18	25.2	74.8	5610
5h	100.0	0.5	3.1	0.613	8000	2.000	10.67	432	12	17.3	82.7	3102
6a	100.0	0.05	5.1	0.613	8000	0.200	N/A	< 10	0	0.0	100.0	37476
6b	100.0	0.1	5.1	0.613	8000	0.200	N/A	< 10	0	0.0	100.0	18738
6c	100.0	0.05	5.1	0.613	8000	2.000	N/A	< 10	0	0.0	100.0	37476
6d	100.0	0.1	5.1	0.613	8000	2.000	N/A	< 10	0	0.0	100.0	18738
6e	100.0	N/A	3.1	0.613	N/A	N/A	10.66	2223	59	88.7	11.3	N/A
6f	100.0	N/A	6.2	0.613	N/A	N/A	10.67	2245	59	89.6	10.4	N/A
6g	100.0	0.01	6.2	0.613	8000	0.010	10.74	295	8	11.8	88.2	165391
6h	100.0	0.01	6.2	0.613	8000	0.050	10.73	117	3	4.7	95.3	178693
7a	100.0	0.005	6.2	0.613	8000	0.100	10.73	343	9	13.7	86.3	323553
7b	100.0	0.005	6.2	0.613	8000	1.000	10.74	216	5	8.6	91.4	342572
7c	100.0	0.005	6.2	0.613	8000	2.000	10.79	107	3	4.3	95.7	358892
7d	100.0	0.001	6.2	0.613	8000	0.100	10.73	1700	45	67.8	32.2	602908
7e	100.0	0.001	6.2	0.613	8000	1.000	10.75	1497	40	59.7	40.3	754564
7f	100.0	0.001	6.2	0.613	8000	2.000	10.89	1479	38.3	59.0	41.0	768311
7g	100.0	0.0005	6.2	0.613	8000	0.100	10.94	2009	48.78	80.1	19.9	744463
7h	100.0	0.0005	6.2	0.613	8000	1.000	10.97	1759	46.27	70.2	29.8	1117334
8a	100.0	0.0005	6.2	0.613	8000	2.000	10.97	1905	47.255	76.0	24.0	899783
8b	300.0	0.005	6.2	1.840	8000	0.100	10.96	1918	46.086	76.5	74.5	837862
8c	300.0	0.005	6.2	1.840	8000	1.000	11.03	1150	29.837	45.9	84.7	952734
8d	300.0	0.005	6.2	1.840	8000	2.000	11.06	1138	29.223	45.4	84.9	954457
8e	300.0	0.001	6.2	1.840	8000	0.100	10.92	5099	127.38	203.5	32.2	1809854
8f	300.0	0.001	6.2	1.840	8000	1.000	10.97	4924	95.64	196.5	34.5	1940657
8g	300.0	0.001	6.2	1.840	8000	2.000	11.00	5468	136.16	218.2	27.3	1533986
8h	100.0	0.005	5.1	0.613	8000	0.100	11.13	1123	32.51	44.8	55.2	206861

9a	100.0	0.005	5.1	0.613	8000	1.000	11.17	684	20.37	27.3	72.7	272645
9b	100.0	0.005	5.1	0.613	8000	2.000	11.21	582	72.87	23.2	76.8	287868
9c	100.0	0.001	5.1	0.613	8000	0.100	11.19	1722	97.86	68.7	31.3	586812
9d	100.0	0.001	5.1	0.613	8000	1.000	11.22	1497	93.59	59.7	40.3	755259
9e	100.0	0.001	5.1	0.613	8000	2.000	11.27	1551	42	61.9	38.1	714668
9f	100.0	0.005	3.1	0.613	8000	0.100	11.25	2119	54.61	84.6	15.4	57906
9g	100.0	0.005	3.1	0.613	8000	1.000	11.28	2001	52.82	79.8	20.2	75625
9h	100.0	0.005	3.1	0.613	8000	2.000	11.30	2049	53.632	81.8	18.2	68333
10a	100.0	0.001	3.1	0.613	8000	0.100	11.33	2139	53.423	85.3	14.7	274975
10b	100.0	0.001	3.1	0.613	8000	1.000	11.33	2063	52.816	82.3	17.7	331707
10c	100.0	0.001	3.1	0.613	8000	2.000	11.36	2153	55.237	85.9	14.1	264234

SBP Test: KE23&24: 2 CP												
Solution	**2CPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2CPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
10d	103.3	N/A	6.2	N/A	N/A	N/A	3.67	2791	225.56	103.3	0.0	N/A
10e	103.3	0.01	6.2	0.803	N/A	N/A	3.68	2708	218.42	100.2	3.0	7266
10f	103.3	0.01	6.2	0.803	8000	0.100	3.68	**1196**	96.19	44.2	57.2	140257
10g	103.3	0.01	6.2	0.803	8000	2.000	3.69	**715**	61.9	26.5	74.4	182530
10h	103.3	0.005	6.2	0.803	8000	0.100	3.69	1630	130.16	60.3	41.6	204174
11a	103.3	0.005	6.2	0.803	8000	2.000	3.70	1301	107.82	48.1	53.4	262001
11b	103.3	0.001	6.2	0.803	8000	0.100	3.70	2348	184.75	86.9	15.9	389460
11c	103.3	0.001	6.2	0.803	8000	2.000	3.70	2344	183.42	86.7	16.0	392362

SBP Test: KE23&24: 4 CP												
Solution	**4CPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**4CPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
11d	100.0	N/A	6.2	N/A	8000	N/A	4.81	**2399**	152.14	100.3	-0.3	N/A
11e	100.0	0.01	6.2	0.778	8000	N/A	4.83	**2350**	145.86	98.2	1.8	4207
11f	100.0	0.01	6.2	0.778	8000	0.100	4.83	**933**	63.49	39.0	61.0	145019
11g	100.0	0.01	6.2	0.778	8000	2.000	4.58	867	61.353	36.2	63.8	151567
11h	100.0	0.005	6.2	0.778	8000	0.100	4.56	**1542**	107.37	64.5	35.5	168961
12a	100.0	0.005	6.2	0.778	8000	2.000	4.58	1521	105.69	63.6	36.4	173036
12b	100.0	0.001	6.2	0.778	8000	0.100	4.56	**2250**	153.29	94.1	6.0	141417
12c	100.0	0.001	6.2	0.778	8000	2.000	4.82	2274	139.94	95.0	5.0	118020

SBP Test: KE23&24: 246 TCP												
Solution	**246-TCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**246-TCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
12d	70.0	N/A	6.2	N/A	8000	N/A	22.60	1782	94.92			
12e	70.0	0.01	6.2	0.506	8000	N/A	22.65	822	12.537			
12f	70.0	0.01	6.2	0.506	8000	0.100	23.02	949	13.422			
12g	70.0	0.01	6.2	0.506	8000	2.000	23.05	960	13.684			
12h	70.0	0.005	6.2	0.506	8000	0.100	23.04	1350	18.23			
13a	70.0	0.005	6.2	0.506	8000	2.000	23.10	1239	17.573			
13b	70.0	0.001	6.2	0.506	8000	0.100	23.14	1582	21.624			
13c	70.0	0.001	6.2	0.506	8000	2.000	23.22	1381	21.054			

SBP Test: KE23&24												
Solution	**Mix**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**Mix**	**%**	**Turnovers**
Mix A												
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
14d-2CP	96.3	0.01	6.2	0.749	8000	0.100	3.80	1082	90.88	40.0	58.4	133750
14d-4CP	3.1	0.01	6.2	0.024	8000	0.100	4.48	80	2.85	3.3	-8.5	-617
14d-2,4DCP	82.8	0.01	6.2	0.508	8000	0.100	11.96	946	19.689	37.7	54.5	84577
14d-2,4,6TCP	74.0	0.01	6.2	0.375	8000	0.100	23.24	1000	16.27	55.5	25.0	28600
Mix B												
14e-2CP	96.3	0.01	6.2	0.749	8000	2.000	3.78	554	46.432	20.5	78.7	180169
14e-4CP	3.1	0.01	6.2	0.024	8000	2.000	4.46	62	3.7426	2.6	15.2	1106
14e-2,4DCP	82.8	0.01	6.2	0.508	8000	2.000	11.99	170	8.6004	6.8	91.8	142617
14e-2,4,6TCP	74.0	0.01	6.2	0.375	8000	2.000	23.28	**500**	10.482	27.8	62.5	71544
Mix C												
14f-2CP	96.3	0.01	6.2	0.749	8000	4.000	3.81	547	46.686	20.3	79.0	180730
14f-4CP	3.1	0.01	6.2	0.024	8000	4.000	4.46	58	3.7476	2.4	21.2	1543
14f-2,4DCP	82.8	0.01	6.2	0.508	8000	4.000	12.08	539	8.9381	21.5	74.1	115018
14f-2,4,6TCP	74.0	0.01	6.2	0.375	8000	4.000	23.49	790	16.98	43.8	40.7	46636
Mix D												
14g-2CP	96.3	0.01	6.2	0.749	8000	N/A	3.79	2553	201.3	94.5	1.9	4371
14g-4CP	3.1	0.01	6.2	0.024	8000	N/A	4.92	40	2.57	1.7	45.4	3310
14g-2,4DCP	82.8	0.01	6.2	0.508	8000	N/A	11.98	2058	51.474	82.1	0.9	1396
14g-2,4,6TCP	74.0	0.01	6.2	0.375	8000	N/A	23.33	1330	20.99	73.8	0.2	258
Mix E												
14h-2CP	96.3	N/A	6.2	N/A	N/A	N/A	3.80	2602	206.63	96.3	N/A	N/A
14h-4CP	3.1	N/A	6.2	N/A	N/A	N/A	4.80	73	3.21	3.1	N/A	N/A
14h-2,4DCP	82.8	N/A	6.2	N/A	N/A	N/A	12.00	2076	53.312	82.8	N/A	N/A
14h-2,4,6TCP	74.0	N/A	6.2	N/A	N/A	N/A	23.38	1333	21.097	74.0	N/A	N/A

24DCP300												
15a	296.6	0.001	6.2	1.840	8000	1.000	11.06	7434	155.64	296.6	0.0	49

SBP Test:Semibatch addition of 24DCP												
Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
0~1hr	100.0	0.01	6.2	0.613	8000	4	N/A	N/A	N/A	1.6	98.4	184,458
Add	20ml of 125mg/l											
1~2hr	57.7	0.0055	6.2	0.613	8000	4	N/A	N/A	N/A	31.5	45.4	90,011
Add	20ml of 125mg/l											
2~3hr	61.7	0.0038	6.2	0.613	8000	4	N/A	N/A	N/A	53.5	13.3	39,932
No Addition												
3~5hr	53.5	0.0038	6.2	0.613	8000	4	N/A	N/A	N/A	52.5	1.9	4,933
												319,333

Solution	**2,4-DCPi**	**Enzyme**	**pH**	**H2O2**	**PEG**	**PEG**	**Ret. Time**	**HPLC**	**Peak**	**2,4-DCPf**	**%**	**Turnovers**
Name	**(mg/l)**	**unit/ml**		**(mM)**	**MW**	**(g/L)**	**(min)**	**AREA**	**Height**	**(mg/l)**	**Rem.**	
0~1hr	100.0	0.01	6.2	0.613	8000	4	N/A	N/A	N/A	9.2	90.8	1,702,113
Add	20ml of 125mg/l											
1~2hr	61.8	0.0055	6.2	0.613	8000	4	N/A	N/A	N/A	40.7	34.2	726,397
Add	20ml of 125mg/l											
2~3hr	68.1	0.0038	6.2	0.613	8000	4	N/A	N/A	N/A	62.6	8.1	268,792
No Addition												
3~5hr	62.6	0.0038	6.2	0.613	8000	4	N/A	N/A	N/A	52.5	16.1	498,242
												3,195,545