HORSERADISH PEROXIDASE-CATALYZED REMOVAL OF PHENOLS FROM INDUSTRIAL WASTEWATERS

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ABSTRACT

Horseradish peroxidase (HRP) enzyme catalyzes the oxidation of aqueous phenols by hydrogen peroxide resulting in the formation and precipitation of polymeric products. The technical feasibility of this enzymatic process for the treatment of industrial wastewaters that contain phenols was investigated. Bench-scale experiments were conducted in which the influences of selected components in the waste matrix on treatment efficiency were assessed.

Experiments with synthetic phenol solutions revealed that the toxicity of HRP-treated phenol solutions declines with time and is dependent on the presence of protective additives, the mode of reagent addition, and the presence of wastewater constituents. Also, many phenolic solutions can be completely detoxified by providing an additional dose of hydrogen peroxide after the completion of the enzymatic reaction. Chitosan was found to be an effective additive for reducing the amount of enzyme required to accomplish phenol transformation in synthetic and actual wastewaters.

HRP was able to accomplish the treatment of phenols in the presence of a wide range of concentrations of suspended solids, salts, metals, and other inorganic and organic species that are frequently present in industrial wastewaters. In certain instances, treatment was more effective in a real waste matrix as compared to the treatment of pure solutions of phenol due to the presence of particular waste components. However, sulfide, manganese(II) and low quantities of cyanide negatively impacted upon the enzymatic transformation of phenol.

It was demonstrated that actual industrial wastewaters collected from pulp and paper production and petroleum refining operations could be treated with HRP and H_2O_2 to meet regulatory discharge limits for phenol. The enzymatic process can selectively target phenols, which are a major source of toxicity in the wastes, for treatment. Treatment of real wastewaters may require higher than stoichiometric doses of hydrogen peroxide due to the inherent peroxide demand of reduced wastewater constituents or catalytic decomposition of peroxide. HRP-treatment improved wastewater quality as reflected in a significant toxicity reduction. It can also result in a substantial decrease in the biochemical and chemical oxygen demands.

SOMMAIRE

L'enzyme de peroxydase de raifort (PR) catalyse l'oxydation des phénols aqueux par le peroxyde d'hydrogène ayant pour résultat la formation et la précipitation des produits polymères. La faisabilité technique du processus enzymatique pour le traitement des eaux usagées industrielles qui contiennent des phénols a été étudiée. Des expérimentes en laboratoire ont été réalisés dans lesquelles les influences des composants choisis dans la matrice de rebut sur l'efficacité de traitement ont été évaluées.

Les expérimentes avec les solutions synthétiques de phénol ont indiqué que la toxicité des solutions de phénol, qui avaient être traité avec PR, diminue avec le temps et dépend de la présence des additifs protecteurs, de la façon de l'ajout de réactifs, et de la présence des constituants d'eau usagée. Aussi, beaucoup de solutions phénoliques peuvent être complètement détoxiquées en fournissant une dose supplémentaire de peroxyde d'hydrogène après l'accomplissement de la réaction enzymatique. Chitosan s'est montré un additif pertinent pour réduire la quantité d'enzyme exigée pour accomplir la transformation de phénol dans les eaux usagées synthétiques et réelles.

Le PR a été capable d'accomplir le traitement des phénols en présence d'une large gamme de concentrations des solides en suspension, les sels, des métaux, et d'autres espèces inorganiques et organiques qui sont fréquemment présentes dans les eaux usagées industrielles. Dans certains cas, le traitement était plus pertinent dans une vraie matrice de rebut par rapport au traitement des solutions pures du phénol due à la présence des composants particuliers de perte. Cependant, le sulfure, manganèse(II) et les faibles quantités de cyanure ont négativement effectué sur la transformation enzymatique du phénol.

On l'a démontré qu'avec les eaux usagées industrielles réelles recueilli à partir de la production de pâte et papier et des opérations de raffinage du pétrole pourrait être traité avec PR et H_2O_2 pour rencontrer des limites de normalisation de décharge pour le phénol. Le processus enzymatique peut sélectivement viser les phénols, qui sont une source importante de toxicité dans les pertes, pour le traitement. Le traitement de vraies eaux usagées peut exiger des doses plus hautes que stoechiométriques de peroxyde d'hydrogène dû à la demande inhérente de peroxyde les constituants réduits d'eau usagée ou la décomposition catalytique du peroxyde. Le traitement à PR a amélioré la qualité d'eau usagée par une réduction significative de la toxicité et peut également avoir comme conséquence une diminution substantielle des demandes d'oxygène chimique et biochimique.

Dedicated to my Grandfather Erhard Kloninger

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1. INTRODUCTION

1.1 The Application of Enzymes in Wastewater Treatment

Over the last several decades, public awareness of the adverse impact of pollution on human health and the integrity of ecosystems has led to increasingly stringent standards for the discharge of wastes into the environment. In the field of wastewater treatment, this has motivated the improvement of conventional treatment methods and stimulated the development of novel strategies to reduce the concentration of toxic or biorefractory contaminants in the waste streams. Most treatment methods can broadly be classified as either physical, biological or chemical treatment processes.

Physical treatment involves the separation of pollutants from the waste stream according to their physical properties such as size, density, hydrophobicity or electric charge. Typical examples include filtration, sedimentation, solvent extraction or ion exchange. Chemical treatment methods employ chemical reagents and/or specific reaction conditions in order to accomplish the chemical transformation of the target pollutants into innocuous compounds. Biological treatment methods utilize the metabolic and catabolic processes that take place in living microbial biomass to degrade pollutants or to convert them into innocuous or value-added materials.

In recent years, researchers have focused attention on the development of treatment processes based on the actions of biological catalysts called enzymes. Enzymatic treatment methods fall between the traditional classifications of chemical and biological treatment systems because they involve chemical transformations that are mediated by catalysts that have been isolated from living cells. Enzymes are specific proteins that catalyze the multitude of reactions that take place in every living organism. Like any other catalysts, they reduce the activation energy and thus make reactions possible that otherwise would be too slow to be of practical importance. However, they differ from chemical catalysts in several important aspects (Voet and Voet, 1995):

1) The rates of enzyme catalyzed reactions are at least several orders of magnitude greater than those of the corresponding chemically catalyzed reactions and

typically factors of 10^6 to 10^{12} greater than those of the corresponding non-catalyzed reactions.

2) Enzyme catalyzed reactions occur under relatively mild conditions: such as temperatures below 100°C, atmospheric pressure and nearly neutral pH's. In contrast, efficient chemical catalysis often requires elevated temperatures and pressures as well as extremes of pH.

3) Generally, enzymes have a vastly greater degree of specificity than do chemical catalysts with respect to the identities of their reactants, which are termed "substrates" in enzyme-catalyzed reactions, and their products.

In the field of wastewater treatment, these differences translate into a number of advantages of enzymatic over chemical treatment processes. The high specificity of enzymes for their substrates makes it possible to selectively remove target pollutants without unnecessary or undesirable side reactions, which often consume large amounts of chemical reactants in treatment processes that employ chemical catalysts (Aitken, 1993). Due to their specificity and high reaction rates, enzymes can be used to target specific pollutants, which may be present in trace quantities and therefore may not be effectively removed using conventional treatment strategies (Aitken, 1993). Additionally, the operation at low temperatures and pressures reduces energy requirements (Cheetham, 1995) and avoids the need for costly equipment such as heat exchangers or high-pressure reaction vessels. Also, operation at near neutral pH reduces corrosion and avoids the need for waste neutralization. Finally, most enzymes are inherently non-hazardous; thus residues that may be present in the treated effluent pose minimal risks to the environment and are of low pollution potential.

Enzymatic treatment also offers advantages over biological treatment processes (Nicell *et al.*, 1993). Isolated enzymes are far less complex systems than microbial consortia. This makes them less sensitive to many environmental disturbances that may affect living biomass. For example, enzymes can be used to treat pollutants at high and low concentrations and they are not susceptible to shock loading effects resulting from changes in pollutant concentration that can damage or inhibit the metabolic activity of microbial biomass. Also, many enzymes can act on or in presence of compounds that are toxic to microorganisms and some are able to reliably operate under much wider ranges

of temperature, salinity and pH than most microbial cultures. Enzymes are tailored to efficiently accomplish the transformation of target compounds. Therefore, they are able to selectively remove pollutants that can pass unaltered through biological treatment systems due to their recalcitrant nature. Furthermore, the high reaction rates associated with enzymatic catalysis leads to faster responses to shock loadings and allows for shorter hydraulic retention times resulting in a smaller reactor vessel. In addition, the quantity of solids produced from enzymatic processes is small in comparison with sludge produced during biological treatment as result of microbial growth. Finally, enzymes are easy to handle and store and they can be used without delay since they do not require acclimatization to a particular wastewater stream.

Although enzymatic treatment systems can have many advantages, they cannot replace chemical or biological processes in most wastewater treatment applications. In particular, conventional biological and chemical oxidation processes are superior in instances where a variety of different contaminants need to be treated simultaneously (Aitken, 1993). For example, in most cases, conventional activated sludge treatment is much more appropriate to treat municipal wastewaters when the main objective is to reduce the concentration of inorganic nutrients and a broad range of organic compounds (often expressed as biological or chemical oxygen demand). In contrast, enzymatic processes are more appropriate in applications where selective removal of specific contaminants is required. Aitken (1993) lists the following possible applications for enzyme-based processes:

1) removal of specific toxic or inhibiting pollutants from a complex industrial waste mixture prior to on-site or off-site biological treatment;
2) removal of specific chemicals from dilute mixtures, for which conventional mixed-culture biological treatment might not be feasible (e.g., treatment of contaminated groundwater);
3) polishing of treated wastewaters to meet limitations on specific pollutants or whole effluent toxicity criteria;
4) treatment of wastes generated infrequently or in isolated locations including spill sites or abandoned waste disposal sites;

5) treatment of low volume, high concentration process waters at the point of generation in a manufacturing facility in order to allow for their re-use in the plant, to facilitate recovery of soluble products, or to remove pollutants known to cause problems downstream when mixed with other wastes from the plant.

To date, a number of areas have been identified for potential application of enzymes in waste treatment (Karam and Nicell, 1997). In particular, the treatment of phenols and aromatic amines using oxidative enzymes has recently received much attention.

1.2 Removal of Phenols from Industrial Wastewaters

Phenols are typically present in wastewaters produced by oil refineries, chemical plants, manufacturing facilities for explosives, resins or wood preservatives, coke ovens and pulp and paper plants (Patterson, 1985).

Typical concentrations of phenols in industrial wastewaters range from tens to thousands of milligrams per liter; however, substantially higher or lower levels are also frequently encountered (Patterson, 1985). These reported concentrations are often a result of a standard analytical methodology that measures a group of chemically similar pollutants rather than the single species phenol (i.e., hydroxybenzene). Thus, the collective term *phenols* represents a category of compounds comprising substituted phenols (including cresols and chlorophenols), polyphenols and phenoxyacids (Patterson, 1985).

Regulations on discharge limits for phenols in industrial effluents may vary according to the industry sector, the receiving water body or the amounts of substance released. For example, maximum permissible discharge concentrations into the Baltic Sea have been limited to 0.5 mg/L by the Helsinki Committee (Kamenev *et al.*, 1995). In the United States, permissible discharge limits for foundries range from 0.1 to 5 mg/L depending on the state, the particular facility and the receiving stream (Fuller and Tomlin, 1988).

A variety of conventional treatment methods exist for the removal of phenols from industrial wastewaters. For concentrated phenolic solutions (more than 500 mg/L), recovery through solvent extraction may in some cases be economically attractive (Patterson, 1985). However, in most cases subsequent removal of residual phenol in the

waste stream is required, thereby increasing the overall costs. Very concentrated phenol solutions can also be incinerated (Patterson, 1985).

Wastewaters containing 5 to 500 mg/L of phenols are considered to be of intermediate strength. In the absence of high concentrations of toxic substances, biological treatment is widely employed for these wastes. Activated sludge, aerated lagoons, oxidation ditches, and trickling filters, among others, have been successfully applied (Patterson, 1985). However, these processes frequently exhibit instabilities arising from fluctuations in organic or hydraulic loadings. The biological activity can also be disrupted by a gradual accumulation of toxic compounds (e.g., chromium) in such treatment systems (Patterson, 1985). Additionally, the treatment efficiency can be highly dependent on the concentration of other compounds in the waste stream such as sulfate or sulfides. Also, temperatures below 10°C have been reported to reduce phenol removal efficiencies (Vela and Ralston, 1978).

Another treatment option for dilute and intermediate strength phenolic wastewaters is adsorption to activated carbon. Using this process, phenol concentrations can be reduced to extremely low levels. However, rapid phenol breakthrough in carbon columns has been reported, which can be ascribed to a lack in the specificity of the adsorption, and which results in elevated treatment costs (Patterson, 1985).

Chemical oxidation using oxidants such as permanganate, chlorine, chlorine dioxide, ozone or hydrogen peroxide has also been proposed for the treatment of intermediate and low strength phenolic solutions. Hydrogen peroxide is believed to have several advantages over the other chemical oxidants, including moderate costs and lower risk of the formation of toxic reaction products (Patterson, 1985; Debellefontaine, 1996). The actual oxidizing species are hydroxyl radicals, which form from H_2O_2 upon activation with a metallic catalyst, most often ferrous iron. This mixture of ferrous iron and H_2O_2 is known as Fenton's reagent. Treatment with Fenton's reagent will frequently necessitate the addition of acids since the optimum conversion occurs at pH 3 to 4, with sharp reductions in efficiency occurring at higher and lower pH (Patterson, 1985). Typically, 3 to 4 mg H_2O_2 / mg phenol (8 to 11 mol/mol) are needed for the treatment of pure phenol solutions (Patterson, 1985). (Note that complete oxidation of 1 mole of phenol to CO_2

requires 14 moles of H_2O_2; thus only partial oxidation is achieved with this treatment.) Fenton's process also requires relatively high amounts of iron catalysts (up to 300 mg/L).

In summary, problems associated with conventional treatment methods may result from insufficient reliability, a lack in specificity for the targeted phenols, or the need for high quantities of chemical reagents or harsh reaction conditions. Thus, some industrial systems may encounter difficulties in employing these processes for their specific applications. Therefore, alternative treatment methods based on enzymes are being explored.

Several enzymes belonging to the class of oxidoreductases have been used for the treatment of phenolic contaminants (Karam and Nicell, 1997). Of these, horseradish peroxidase (HRP) is one of the most studied in the relatively new area of enzymatic wastewater treatment. HRP catalyzes the oxidation of a variety of aqueous phenols and aromatic amines with H_2O_2 to their respective radicals. These highly reactive compounds undergo further non-enzymatic reactions leading to the formation of insoluble polymers that can be separated from the solution by sedimentation or filtration (Klibanov *et al.*, 1980). Several advantages make HRP a promising candidate for industrial application. These include:

- its potential application to a broad range of hydroxylated aromatics and aromatic amines including chlorophenols, benzidines, quinolines, naphthols and biphenyls (Alberti and Klibanov, 1982; Klibanov and Morris, 1981; Klibanov, 1982), many of which are recalcitrant or toxic to microorganisms;
- operation under broad ranges of pH (5-9) and temperature (5 to 50°C) (Dec and Bollag, 1990; Nicell *et al.*, 1993);
- application to a wide range of phenol concentrations, with treatment having been demonstrated for initial concentrations ranging from as low as 0.01 mg/L (Maloney *et al.*, 1986) to as high as 9100 mg/L (Nakamoto and Machida, 1992);
- requirement of an equimolar amount of hydrogen peroxide per mol of phenol treated (Nicell *et al.*, 1992), which is substantially less than the amount of peroxide consumed during Fenton's process;

- short reaction times which result in low hydraulic retention times and fast responses to shock loadings (Buchanan, 1996);
- lower sludge production than that of biological treatment methods.

However, before this process can be implemented at the industrial scale, several significant problems must be overcome.

HRP is susceptible to permanent inactivation mainly by reaction products such as free radicals (Ator and Ortiz de Montellano, 1987) and possibly also by polymeric products (Nakamoto and Machida, 1992). This limits the extent of phenol conversion that can be achieved by a given dose of the enzyme catalyst. The catalytic lifetime of the enzyme can be substantially extended in the presence of protective additives such as high molecular weight polyethylene glycol (PEG) (Nakamoto and Machida, 1992). However, there are indications that some of the PEG remains in solution after completion of the phenol removal reaction (Kinsley and Nicell, 2000), which is problematic because of the low biodegradability of this polymer. Also, the presence of PEG enhances the concentration of dissolved and possibly toxic reaction products (Ghioureliotis and Nicell, 1999). These drawbacks may prohibit the use of PEG on an industrial scale. Some studies indicate that chitosan, a natural polysaccharide, could be a possible alternative to PEG due to its biodegradability and potential ability to adsorb toxic reaction products (Sun *et al.*, 1992). More detailed research on the effectiveness and the required quantity of chitosan is needed to evaluate its usefulness.

Concerns have been raised about the nature and toxicity of the reaction products resulting from the HRP-catalyzed phenol polymerization since it was observed that some phenolic solutions can be more toxic after HRP-treatment than before (Aitken *et al.*, 1994; Ghioureliotis and Nicell, 2000). The toxicity can be attributed to the formation of trace amounts of soluble toxic reaction products, while the phenolic polymers seem to be non-toxic (Aitken *et al.*, 1994). Little is currently known about the impact of reaction conditions on the residual toxicity of HRP-treated solutions. Also, previous toxicity investigations were conducted using pure solutions of one or two phenolic compounds and might not adequately reflect the conditions of actual industrial effluents, where a variety of different compounds are usually present.

Enzyme-derived phenolic polymers exhibit slow mineralization kinetics similar to naturally occurring lignins and humic acids (Farrell *et al.*, 1998). Nevertheless, methods for their safe disposal have not yet been investigated adequately.

The effectiveness of the HRP-based process for the treatment of synthetic wastewaters consisting of buffered solutions of selected aromatics has been demonstrated in numerous studies. However, real wastewaters vary widely in many characteristics such as the concentration and composition of phenolic compounds, the pH, the quantity of suspended solids, total and dissolved organics and various other chemical species, such as metals or other inorganic compounds, which may interfere with the enzymatic reaction. Nevertheless, very little information is available about the impacts of common wastewater constituents on the performance of the HRP-based phenol removal process.

Also, only a few studies (Klibanov *et al.*, 1983; Cooper and Nicell, 1996; Rejaee and Nicell, 1994) have dealt with the treatment of actual industrial wastewaters. Additional investigations with real effluents are required in order to gain further understanding of the challenges, limitations and possible advantages associated with the application of the HRP-based process under conditions encountered in an industrial environment. These studies should include detailed investigations on the effect of the wastewater matrix on the efficiency of phenol removal, the required doses of HRP and H_2O_2, the effectiveness of protective additives and the residual toxicity of the treated effluent.

1.3 Objectives and Scope

The primary objective of this research was to evaluate the technical feasibility of the HRP-based process for its application to industrial wastewaters. To this end, bench-scale experiments were carried out to investigate the treatment of synthetic solutions and actual wastes containing aqueous phenols.

The synthetic waste studies were performed in order to investigate methods to reduce enzyme requirements and toxicity levels and to evaluate the influence of various species that are commonly present in waste matrices on enzymatic treatment effectiveness. Specifically, these studies were used to assess:

1) the effectiveness of chitosan in comparison with other additives in reducing the amount of enzyme required to accomplish phenol transformation;

2) the influence of reaction conditions, such as the reaction time or the presence of additives, on the toxicity of HRP-treated phenolic solutions;

3) the influence of wastewater parameters including the concentrations of suspended solids, reducing anions, heavy metals, and other inorganic and organic compounds on treatment effectiveness (e.g., substrate transformation and toxicity reduction) and enzyme and peroxide consumption.

Experiments were conducted with wastewaters collected from a pulp and paper production facility and a petroleum refinery with the goal of evaluating:

1) the extent of phenol removal as a function of the quantities of HRP and H_2O_2 supplied to the wastewater;

2) the ability of the enzymatic treatment process to reduce phenol concentrations to levels that meet common regulatory discharge requirements;

3) the effect of the enzymatic treatment on common wastewater quality parameters including toxicity and chemical (COD) and biochemical (BOD) oxygen demands;

4) the impact of the wastewater matrix on the enzymatic process by comparing the treatment of real and synthetic wastewaters;

5) the effectiveness of protective additives in a real waste matrix;

6) the impact of selected dissolved organic compounds on the treatment efficiency.

2. LITERATURE REVIEW

2.1 Peroxidase Enzymes

All living organisms produce peroxidases. These enzymes are involved in a variety of biosynthesis or degradation processes or in defense against pathogens or oxidative pressure (Henriksen *et al.*, 1998). Most of the known peroxidases belong to the plant peroxidase superfamily. These enzymes are a group of metalloproteins that contain a central heme as a prosthetic group. The plant peroxidase superfamily can be subdivided into three broad classes based on structural divergence. The Class I peroxidases are found intracellulary in plants, yeast and bacteria. To this class belong the cytosolic ascorbate oxidase and the cytochrome c peroxidase. The Class II and III peroxidases are found in fungi and plants and are generally located outside the cell. The Class II peroxidases are represented by examples such as the lignin degrading manganese-peroxidase from the fungus *Phanaerochaete chrysosporium*, while the Class III peroxidases include the widely studied horseradish peroxidase (Henriksen *et al.*, 1998).

Extracellular peroxidases are mainly located in the growing cell walls (Fry, 1986) and they have been implicated in the lignification process that confers structural rigidity to plant cell walls. Thus, the natural substrates of these peroxidases seem to be phenolic lignin precursors, also referred to as monolignols, such as *p*-coumaryl or coniferyl alcohol (Lewis *et al.*, 1999). Evidence also exists that peroxidases act on phenolic molecules bound to cell wall polymers thereby crosslinking adjacent macromolecules (Fry, 1986).

Oxidative enzymes that use H_2O_2 as the electron acceptor are also found in the animal kingdom. Examples include thyroid peroxidase, lactoperoxidase (found in milk), myoloperoxidase, and glutathione peroxidase (Dunford and Stillman, 1976).

2.1.1 Structure of horseradish peroxidase

Shannon *et al.* (1966) isolated seven peroxidase isoenzymes from horseradish roots, which differed in their electrophoretic mobility, their amino acid composition and carbohydrate content. These isoenzymes were subdivided onto two groups; one comprising the acidic isoenzymes (HRP-A1, -A2, -A3), whereas the other fraction

contained the slightly basic isoenzymes, of which isoenzyme C (HRP-C) is the most abundant. Later studies revealed characteristic differences between these two isoenzyme groups in their catalytic activity with various substrates (Kay *et al.*, 1967; Marklund *et al.*, 1974; Hiner *et al.*, 1996). Most commercial preparations contain mainly the isoenzyme C (Dunford, 1991); thus this enzyme molecule will be the main focus of this discussion.

Horseradish peroxidase C is a slightly basic glycoprotein consisting of 308 amino acids. The active site of the native enzyme contains the ferric heme prosthetic group (Dunford, 1991). Eight carbohydrate side chains are attached to the protein via asparagine residues. It was suggested that the carbohydrates contribute to the stability of the enzyme at elevated temperatures (Dunford and Stillman, 1976). Also, two Ca^{2+} ions are tightly bound to each HRP molecule. These ions are required to maintain the structural environment of the heme (Ogawa *et al.*, 1979). The total molecular weight of HRP-C is approximately 42 kDa (Dunford, 1991).

The crystal structure of HRP-C has been solved recently (Gajhede *et al.*, 1997). It revealed that the enzyme molecule consists of 10 helices (A-J). Two domains are clearly defined: the N-terminal domain I (consisting of helices A-D) and the C-terminal domain II (consisting of helices F-J). Helix E links both domains. Binding of the heme occurs in a pocket made of both domains between helix B and the C-terminus of helix F.

The heme prosthetic group of the native enzyme, also referred to as ferriprotoporphyrin IX, contains a Fe^{3+} ion in its center which is coordinated to four pyrrole nitrogens of a near planar porphyrin ring structure (Figure 2.1).

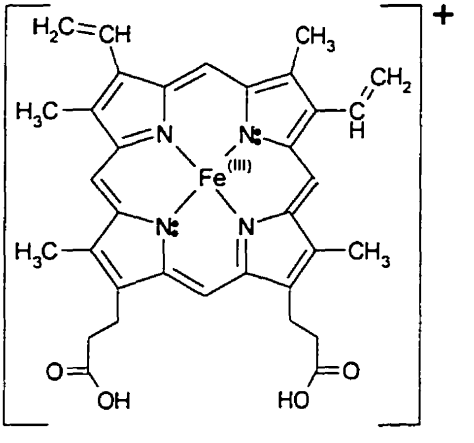


Figure 2.1: Ferriprotoporphyrin IX (heme prosthetic group of HRP).
Adapted from Dunford and Stillman (1976).

The fifth coordination position is occupied by the imidazole side chain of a histidine residue of helix F of the surrounding protein. The sixth coordinate position of the ferric iron is vacant. Note that the formal charge of ferriprotoporphyrin IX is +1, since two of the four heme pyrrole nitrogen atoms bear a negative charge (Dunford, 1991). The porphyrin ring system is aromatic and therefore has an excellent ability to conduct electrons (Dunford and Stillman, 1976).

2.1.2 Catalytic activity

The mechanism of the primary catalytic cycle of HRP is shown in Figure 2.2.

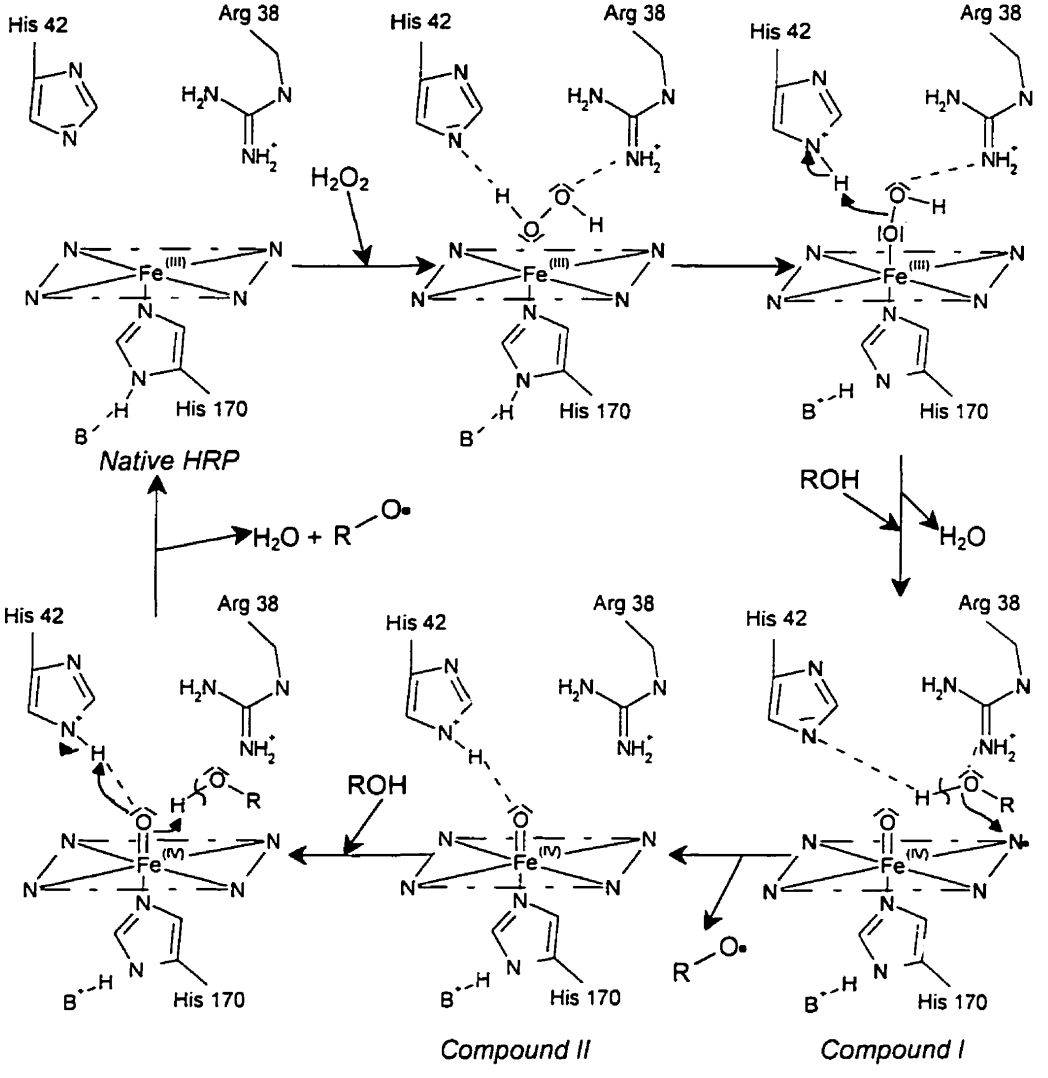


Figure 2.2: Primary catalytic cycle of horseradish peroxidase.

The first step is the oxidation of the native enzyme (also referred to as ferriperoxidase, since it contains ferric Fe(III) iron) with hydrogen peroxide. Initial binding of H_2O_2 occurs at the vacant sixth coordinate position of Fe^{3+} (Dunford, 1991). Upon transfer of a H^+ to a distal histidine (His 42), an iron-peroxide bond is formed, which is followed by heterolytic cleavage of the O-O bond releasing water as the leaving group. This reaction is facilitated by a negative charge on the His 170 and positive charges on the His 42 and Arg 38 amino residues of the enzyme (Dunford, 1991). Following these processes, the ferric iron $[Fe(III)]^{3+}$ is oxidized to the ferryl $[Fe(IV)=O]^{2+}$ group and the porphyrin ring system $(Porph)^{2-}$ into a π-radical intermediate $(Porph^{\cdot})^{-}$. The resulting enzyme form, containing the π-cation radical $(Porph^{\cdot}[Fe(IV)=O])^{+}$ in the active site, is referred to as Compound I (HRP_I) (Dunford, 1991).

The next step is the reduction of Compound I in a one electron-reduction step by a reducing substrate, usually a phenolic molecule (ROH) (Dunford, 1991). Electron transfer occurs from the substrate molecule to the porphyrin ring so that the π-cation radical disappears. Studies with substituted hydrazines indicate that the electron is transferred to the region of the δ-meso carbon and the 8-methyl group of the heme (Ortiz de Montellano, 1987). Simultaneously, a proton is transferred from the phenolic hydroxyl group to the imidazol side chain of His 42, which is assisted by a hydrogen bond between Arg 38 and the phenolic oxygen (Henriksen *et al.*, 1999). The resulting phenolic radical will dissociate from the enzyme leaving the peroxidase in the (Porph[Fe(IV)=O]) form, which is referred to as Compound II (HRP_{II}).

In the final step of the catalytic cycle, another phenolic molecule reduces Compound II in a one electron-reduction step returning the enzyme to its native form (HRP_N). In Compound II, a protonated distal group, most probably His 42, is hydrogen bonded to the ferryl oxygen atom, which is of crucial importance for the catalytic activity of this peroxidase intermediate (Dunford, 1991). Proton and electron transfers from the phenolic substrate occur to the ferryl group simultaneously, reducing iron (IV) to iron (III) and forming water as the leaving group, with the oxygen atom originating from the $[Fe(IV)=O]^{2+}$ (Dunford, 1991). The two protons in the leaving water molecule are derived from the protonated distal group and the reducing substrate. The result is the formation of

another phenolic radical and the return of the peroxidase to its ferric state $(Porph[Fe(III)])^+$ (Dunford, 1991).

The main catalytic cycle can be summarized by the following set of equations:

$$\underset{HRP_N}{(Porph\,[Fe(III)])^+} + H_2O_2 \xrightarrow{k_1} \underset{HRP_I}{(Porph^{\cdot}[Fe(IV)=O])^+} + H_2O \qquad (1.1)$$

$$\underset{HRP_I}{(Porph^{\cdot}[Fe(IV)=O])^+} + R\text{-}H \xrightarrow{k_2} \underset{HRP_{II}}{(Porph\,[Fe(IV)=O])} + R\cdot + H^+ \qquad (1.2)$$

$$\underset{HRP_{II}}{(Porph\,[Fe(IV)=O])} + R\text{-}H + H^+ \xrightarrow{k_3} \underset{HRP_N}{(Porph\,[Fe(III)])^+} + R\cdot + H_2O \qquad (1.3)$$

Typical values for the rate constants k_1, k_2 and k_3 are given in Table 2.1.

Table 2.1: Kinetic constants for horseradish peroxidase reactions at pH 7.0 and 25°C.

CONSTANT	VALUE	UNITS	LITERATURE SOURCE
k_1	$2.0 \cdot 10^7$	$M^{-1}s^{-1}$	Yamazaki and Nakajima (1986)
[a]k_2	$2.76 \cdot 10^6$	$M^{-1}s^{-1}$	Job and Dunford (1976)
[a]k_3	$3.38 \cdot 10^5$	$M^{-1}s^{-1}$	Buchanan and Nicell (1998)
[b]k_4	$1 \cdot 10^3$	$M^{-1}s^{-1}$	Nakajima and Yamazaki (1987)
[c]k_5	1.76	s^{-1}	Arnao *et al.* (1990)
[c]k_6	$7.85 \cdot 10^{-3}$	s^{-1}	Arnao *et al.* (1990)
[c]k_I	$3.92 \cdot 10^{-3}$	s^{-1}	Arnao *et al.* (1990)
k_{eff}	$4.71 \cdot 10^{-2}$	s^{-1}	Buchanan and Nicell (1998)
k_{app}	20.3	$M^{-1}s^{-1}$	Buchanan and Nicell (1998)
[a]k_7	95	$M^{-1}s^{-1}$	Tamura and Yamazaki (1972)

[a] For phenol as the reducing substrate.
[b] Extrapolated from data at 5°C to 20°C using Arrhenius equation and activation energy supplied by Nakajima and Yamazaki (1987).
[c] Values measured at pH 6.3.

Hydrogen peroxide also reacts with Compound I. These reactions were studied in detail by Arnao and co-workers (1990).

The first step is the formation of a complex between Compound I and H_2O_2:

$$\underset{HRP_I}{(Porph^{\cdot}[Fe(IV)=O])^{+}} + H_2O_2 \xrightarrow{k_4} \underset{HRP_I:H_2O_2}{(Porph^{\cdot}[Fe(IV)=O])^{+}:H_2O_2} \qquad (1.4)$$

This complex can dissociate to release molecular oxygen through the following reaction:

$$\underset{HRP_I:H_2O_2}{(Porph^{\cdot}[Fe(IV)=O])^{+}:H_2O_2} \xrightarrow{k_5} \underset{HRP_N}{(Porph\,[Fe(III)])^{+}} + O_2 + H_2O \qquad (1.5)$$

In this reaction peroxide acts as a reducing substrate by being oxidized to oxygen and reducing Compound I back to the native state. Thus, HRP is also able to decompose H_2O_2 into oxygen and water; a reaction that is typically catalyzed by catalase enzymes. Note that the reaction of Compound I with peroxide is approximately 4 orders of magnitude slower than with phenol. Thus, in the presence of a reducing compound, reaction (1.5) is only a minor reaction pathway. In fact, Nicell (1994) estimated that during the oxidation of 1 mM 4-chlorophenol the hydrogen peroxide consumed in the catalase reaction amounts to less than 0.02 % of its initial concentration.

While reaction (1.5) is the main reaction involving the $HRP_I:H_2O_2$ complex, two other minor reaction pathways exist. H_2O_2 can also reduce Compound I in a one-electron reduction step resulting in the formation of Compound II and the release of a superoxide anion radical:

$$\underset{HRP_I:H_2O_2}{(Porph^{\cdot}[Fe(IV)=O])^{+}:H_2O_2} \xrightarrow{k_6} \underset{HRP_{II}}{(Porph\,[Fe(IV)=O])} + {}^{\cdot}O_2^{-} + 2\,H^{+} \qquad (1.6)$$

The complex can also be converted to a permanently inactive form of the enzyme referred to as verdohemoprotein. This compound absorbs light at a peak wavelength of 670 nm and is therefore often referred to as "P-670".

$$HRP_I:H_2O_2 \xrightarrow{k_i} \text{P-670} \qquad (1.7)$$

Reactions (1.6) and (1.7) are very slow compared to reaction (1.5) (see Table 2.1).

Apart from the three enzyme forms involved in the major catalytic cycle - ferriperoxidase, Compound I and Compound II - the active enzyme can also be reversibly

converted into two other stable enzyme forms: namely, Compound III, also referred to as oxyperoxidase, and ferroperoxidase, which contains ferrous iron Fe(II).

Oxyperoxidase can be regarded as a resonance hybrid with contributing structures represented as (Porph[Fe(II)-O_2]) ↔ (Porph[Fe(III)-$^{\cdot}O_2$]) (Dunford, 1991). It is three oxidation states above the ferric enzyme. Oxyperoxidase can be formed through three mechanisms: the binding of an oxygen molecule to ferroperoxidase, the binding of a superoxide radical molecule to the ferric enzyme (HRP_N) or through oxidation of Compound II with H_2O_2 (Dunford, 1991).

Nakajima and Yamazaki (1987) proposed two reaction pathways for the conversion of Compound II to Compound III. In one pathway the ferryl oxygen atom is converted to $OH^{\cdot}$, which departs from the heme. Subsequently, H_2O_2 binds at the sixth coordinate position of iron, thereby forming oxyperoxidase:

$$\underset{HRP_{II}}{\text{(Porph [Fe(IV)=O])}} + H^{+} \longrightarrow \text{(Porph [Fe(IV)])}^{2+} + OH^{\cdot} \qquad (1.8)$$

$$\text{(Porph [Fe(IV)])}^{2+} + H_2O_2 \longrightarrow \underset{HRP_{III}}{\text{(Porph [Fe(III)-}^{\cdot}O_2\text{])}} + 2\,H^{+} \qquad (1.9)$$

The other pathway involves the reduction of Compound II to ferric peroxidase with H_2O_2 as the reductant resulting in the formation of a superoxide anion radical.

$$\underset{HRP_{II}}{\text{(Porph [Fe(IV)=O])}} + H_2O_2 \longrightarrow \underset{HRP_{N}}{\text{(Porph [Fe(III)])}^{+}} + {}^{\cdot}O_2^{-} + H_2O \qquad (1.10)$$

Note the similarity of this reaction with the reduction of Compound I to Compound II by hydrogen peroxide. The superoxide anion radical immediately combines with the ferric enzyme to form oxyperoxidase. This reaction is reversible. Thus, Compound III spontaneously decomposes to the native enzyme releasing a superoxide radical according to:

$$\underset{HRP_{III}}{\text{(Porph [Fe(III)-}^{\cdot}O_2\text{])}} \xrightarrow{k_{eff}} \underset{HRP_{N}}{\text{(Porph [Fe(III)])}^{+}} + {}^{\cdot}O_2^{-} \qquad (1.11)$$

Overall the conversion of Compound II to oxyperoxidase can be expressed as:

$$HRP_{II} + H_2O_2 \xrightarrow{k_{app}} HRP_{III} + H_2O \qquad (1.12)$$

In a way that is similar to Compound I and Compound II, Compound III is reduced in a one electron reduction step by substrates such as phenols to Compound I (Tamura and Yamazaki, 1972).

$$\underset{HRP_{III}}{(\text{Porph}\,[\text{Fe(III)-}\dot{O}_2])} + \text{R-H} + H^+ \xrightarrow{k_7} \underset{HRP_I}{(\text{Porph}^{\cdot}[\text{Fe(IV)=O}])^{+}} + \text{R}\cdot + H_2O \quad (1.13)$$

The reaction rates of reaction (1.13) are orders of magnitude smaller than the corresponding reactions of Compound I and Compound II (see Table 2.1). Thus, reactions of Compound III do not significantly contribute to the conversion of reducing substrates.

Ferrous, Fe(II), peroxidase is formed through reduction of ferric peroxidase. Dithionite ($S_2O_4^{2-}$) or borohydride (BH_4^-) have been used as the reducing agents for this reaction (Wittenberg *et al.*, 1967). Ferrous HRP reacts rapidly with H_2O_2 to Compound II (Noble and Gibson, 1970) and combines with oxygen to form oxyperoxidase (Compound III) (Wittenberg *et al.*, 1967).

2.1.3 Substrates of horseradish peroxidase

Apart from hydrogen peroxide, organic peroxides including alkylhydroperoxides and peracetic acid can also oxidize the ferric enzyme to Compound I (Baek and Van Wart, 1992).

Organic substrates that reduce Compound I and Compound II are mainly aromatic compounds bearing at least one hydroxyl or amino group. A variety of phenols, naphthols, anilines and benzidines belong to this group (Klibanov *et al.*, 1980, Klibanov and Morris, 1981; Josephy *et al.*, 1982). Recently, the oxidation of organic sulfides to the corresponding sulfoxides gained some interest (Baciocchi *et al.*, 1996). In this reaction the sulfide (R_2S) is first oxidized by Compound I to the radical cation ($R_2S^{\cdot}$), which then further reacts with Compound II to the corresponding sulfoxide ($R_2S{=}O$), whereby the oxygen in the sulfoxide molecule originates from the ferryl oxygen of Compound II. Some non-aromatic compounds such as β-diketones are also oxidized to the corresponding radicals (Teixeira *et al.*, 1999).

HRP also oxidizes a number of inorganic compounds including ferrocyanide, iodide, nitrite and hydrogen sulfite (Dunford and Stillman, 1976). Iodide and hydrogen sulfite are

interesting exceptions among the reducing substrates since they reduce Compound I directly in a two electron reduction step to the native enzyme. The reaction of iodide with Compound I can be described as (Dunford and Stillman, 1976):

$$HRP_I + I^- \longrightarrow HRP_N + I^+ \qquad (1.14.)$$

The I^+ ion rapidly reacts with excess iodide to liberate molecular iodine (I_2).

2.1.4 Inhibitors of horseradish peroxidase

Enzyme inhibitors are compounds that slow or prevent the transformation of a target substrate without irreversibly destroying the enzyme's catalytic activity. Classically, three forms of inhibition are distinguished: competitive, non-competitive and uncompetitive (Voet and Voet, 1995). Competitive inhibitors compete with the substrate for binding at the same site on the enzyme. Such an inhibitor usually resembles the substrate, but differs in that it is unreactive. Non-competitive inhibitors bind to a different site, but block the conversion of the substrate to products. Uncompetitive inhibitors are only able to bind to the enzyme-substrate complex, but not to the free enzyme itself.

In an early work, Theorell (1951) reported that peroxidase activity is reversibly inhibited in the presence of 10^{-5} to 10^{-6} M sulfide or cyanide, while fluoride, azide and hydroxylamine inhibit at concentrations of around 10^{-3} M.

Recent studies have revealed that cyanide, azide and fluoride bind in their protonated form to the vacant sixth coordinate position of the ferric heme of the native peroxidase. Thus, these compounds mimic the first step of the reaction of the enzyme with H_2O_2 and inhibit the formation of Compound I (Dunford, 1991). For example, cyanide binds to ferriperoxidase according to:

$$\underset{HRP_N}{(\text{Porph [Fe(III)]})^+} + HCN \longrightarrow \underset{HRP\text{-}CN}{(\text{Porph [Fe(III)-CN]})} + H^+ \qquad (1.15.)$$

The liberated proton is taken up by the distal His 42, which forms a hydrogen bond to the nitrogen of the bound cyanide (Dunford, 1991). The binding rate constant has been determined by Dunford *et al.* (1978) as $1.05 \cdot 10^5$ $M^{-1}s^{-1}$ at pH 7.0 and 25°C. The

dissociation rate constant of HRP-CN is nearly pH independent and in the order of 0.2 s^{-1} (Dunford and Stillman, 1976). This leads to a dissociation constant for HRP-CN of $2 \cdot 10^{-6}$ M.

The binding of fluoride occurs in a similar fashion with a dissociation constant of 10^{-3} M (Dunford and Stillman, 1976).

Guilbault *et al.* (1966) studied the inhibition of HRP by various compounds using homovanillic acid as a flourometric substrate. The enzymatic assay employed by Guilbault *et al.* contained approximately 0.3 mM reducing substrate and 2 mM H_2O_2 in pH 8.5 Tris buffer. The concentrations of some compounds that caused 50% inhibition of HRP activity under the specified conditions are listed in Table 2.2.

Table 2.2: Concentration of substances causing a 50% inhibition of HRP under the assay conditions described by Guilbault *et al.* (1966)

Compound in Assay	**Concentration (M)**
Sulfide (S^{2-})	$5.25 \cdot 10^{-5}$
Hydroxylamine (NH_2OH)	$2.57 \cdot 10^{-4}$
Cyanide (CN^-)	$6.76 \cdot 10^{-4}$
Mn^{2+} (Manganese II)	$5.18 \cdot 10^{-5}$
Co^{2+} (Cobalt II)	$7.59 \cdot 10^{-5}$
Pb^{2+} (Lead II)	$2.57 \cdot 10^{-4}$
Fe^{2+}, Fe^{3+} (Iron II, III)	$3.16 \cdot 10^{-4}$
Cu^{2+} (Copper II)	$6.46 \cdot 10^{-4}$
Cd^{2+} (Cadmium II)	$4.17 \cdot 10^{-3}$
Ni^{2+} (Nickel II)	$5.25 \cdot 10^{-3}$

Typically, the percent inhibition was found to be linearly dependent on the logarithm of the concentration of the inhibitor. Halogen anions, nitrate, sulfate and phosphate as well as several metal ions including Cu^+, Zn^{2+}, Hg^{2+} and Al^{3+} were found to have no effect on enzyme activity. Lobarzewski *et al.* (1990) studied the impact of metal ions on the activity of soluble and immobilized cytoplasmatic cabbage peroxidase using an assay containing 1.5 M guaiacol and 50 mM H_2O_2 in 50 mM phosphate buffer pH 7.0. Among the metals tested, only Hg^{2+} seemed to significantly inhibit soluble peroxidase at

concentrations higher than 0.2 mM, while Cu^{2+} and Mn^{2+} slightly enhanced peroxidase activity. In this study, the metal ions were employed in concentrations ranging from 0.1 to 1 mM; however, the reduced solubility of many metal ions in the presence of phosphate was not mentioned. Another study reports that Cu^{2+} inhibits the activity of moss peroxidase for the oxidation of guaiacol (Bakardjieva *et al.*, 1997). Based on these reports it seems that the effect of metal ions on enzyme activity is dependent on the reaction conditions and the type of enzyme employed.

2.1.5 Stability of horseradish peroxidase

HRP is irreversibly inactivated when incubated in the presence of H_2O_2 through the formation of verdohemoprotein (P_{670}) (Arnao *et al.*, 1990; Baynton *et al.*, 1994), while incubation in the presence of phenolic substrates does not lead to activity loss (Nicell *et al.*, 1993).

HRP possesses an unusually high stability in aqueous solutions. Concentrated solutions can be stored in distilled water at 4°C for at least one month without significant loss in activity (Nicell *et al.*, 1993). However, like most other enzymes, HRP is inactivated during prolonged incubation at elevated temperatures. For example, Nicell *et al.* (1993) report that HRP lost less than 10% of its activity when incubated at or below 35°C for one hour at neutral pH. Incubation at 55°C led to 90% activity loss over one hour, while at 72°C virtually all activity was lost almost immediately. The rate of thermal inactivation appeared to be biphasic, which was attributed to the existence of heat stable and heat resistant isoenzymes in the enzyme preparation used. Tamura and Morita (1975) also observed a biphasic thermal inactivation of an acidic isoenzyme of Japanese-radish peroxidase, but attributed this behavior to a three-step inactivation mechanism involving reversible dissociation of the heme from the protein, irreversible denaturation of the apoenzyme and irreversible modification of the heme. Chattopadhyay and Mazumdar (2000) studied the thermal unfolding of HRP using circular dichroism and fluorescence techniques. Thermal unfolding proceeded in two distinct phases. The first phase, occurring at 35 to 55°C, was associated with a change in the tertiary structure of the heme region. This was followed in the second phase at 50 to 93°C by a release of the heme from the protein cavity and subsequent changes in the secondary structure of the enzyme.

The bound calcium ions and the disulfide bridges contributed significantly to the thermal stability of the enzyme.

Enzymes are only stable within a limited pH range. Outside this range, changes in the charges of the ionizable amino acid residues result in modifications of the tertiary structure of the enzyme molecule and eventually lead to denaturation (Zubay, 1993). However, individual enzymes can widely vary in their stability and activity as a function of pH (Zubay, 1993). As reported by Nicell *et al.* (1993), HRP retained more than 90 % of its activity after incubation for 48 hours at pH between 6 and 9, but the enzyme was severely inactivated outside this pH range. HRP inactivation at acidic pH can at least partly be attributed to unfolding of the protein structure, resulting in heme release as observed by Chattopadhyay and Mazumdar (2000). With decreasing pH, the unfolding of the heme pocket shifted to lower temperatures allowing heme release to take place at room temperature while below pH 4.5. Moreover, according to Boivard *et al.* (1982), several factors affect the rate of activity loss at pH 5.0 and 25°C. The stability of HRP decreased with decreasing concentrations of enzyme, increasing concentrations of buffers, and in the presence of EDTA. The extent of inactivation depended also on the buffer species used. HRP was most stable in citrate buffers and least stable in phosphate buffers. The effect of EDTA can be explained by the fact that free Ca^{2+} readily interchanges with Ca^{2+} bound to HRP C (Haschke and Friedhoff, 1978); thus, upon incubation with EDTA, bound Ca^{2+} is slowly removed from the enzyme, which may lead to enhanced heme release.

2.2 Factors Affecting Peroxidase-based Phenol Removal

2.2.1 Activity loss over the course of the phenol oxidation reaction

HRP is rapidly inactivated when catalyzing the oxidation of aromatic substrates with H_2O_2 (Baynton *et al.*, 1994). Inactivation cannot be attributed to the action of H_2O_2 alone since the reactions leading to the formation of the permanently inactive verdohemoprotein (P-670) are very slow in the presence of a reducing substrate (Nicell, 1994). Rather, it is generally believed that under ambient conditions inactivation by reaction products is the predominant mechanism that limits the amount of substrate that can be transformed by a

given amount of enzyme. Various studies have been performed to elucidate the mechanisms underlying this form of inactivation.

Kapeluich *et al.* (1997) studied HRP inactivation over the course of *p*-iodophenol oxidation. The enhanced chemoluminescence reaction was used to monitor HRP activity on-line. In this reaction, luminol is oxidized by *p*-iodophenol radicals, which are generated via the peroxidase reaction, to a compound that further reacts to a chemoluminescent product. When the authors injected the enzyme into a chemoluminescence reaction mixture the recorded light output rapidly decreased; however, when fresh enzyme was injected the light intensity was completely restored and the rate of subsequent light decay was identical to the initial decay rate. The authors concluded that inactivating species did not accumulate in the reaction mixture and that they were therefore very unstable and reactive. Moreover, using an assay containing the reducing substrate ABTS, it was shown that the addition of oxygen radical scavengers did not reduce the rate of inactivation over the course of *p*-iodophenol oxidation. This indicates that inactivation was not caused by reactive oxygen species such as hydroxyl or superoxide anion radicals, which could possibly form through decomposition of hydrogen peroxide. However, lower rates of inactivation were recorded when *p*-iodophenol was oxidized in the presence of luminol. Furthermore, HRP was almost completely protected from inactivation in the presence of 1 mg/mL of bovine serum albumin (BSA) or 1 mM of the polycation 2,5-ionene. Based on these results the authors conclude that inactivation results from phenoxy radicals attacking the enzyme molecule. When BSA is present the radicals will presumably interact with this protein and thus fewer radicals will attack and inactivate the enzyme. According to this theory the protective role of luminol consists in its ability to reduce phenoxy radicals. In a subsequent study the same group was able to demonstrate binding of *p*-iodophenol reaction products to HRP as well as its suppression in the presence of BSA (Egorov *et al.*, 1998).

Similarly, Ator and Ortiz de Montellano (1987) report that HRP was inactivated during the oxidation of phenylhydrazine due to the formation of phenyl radicals. Inactivation was accompanied by covalent binding of two radiolabeled substrate derivatives to the enzyme molecule. Two modified hemes were isolated from inactivated HRP. One of them contained a phenyl moiety bound to a meso-carbon of the

protoporphyrin ring. The other modified heme resulted from the oxidation of an adjacent methyl group by a phenyl radical. The authors argue that, based on the high regiospecificity of the observed heme transformations, only a small sector of the heme molecule is accessible to interactions with phenyl radicals. Also, since only a fraction of the bound radiolabeled phenyl was recovered along with the modified hemes, covalent modification of the protein globule was probably more important for activity loss than heme modification.

Interaction of proteins with products generated from peroxidase-catalyzed reactions can result in protein crosslinking as demonstrated by Leatham *et al.* (1980). The extent of crosslinking depended on the protein species examined and on the phenolic substrate used, indicating that phenolic radicals or other reactive products differ in their ability to react with proteins and thus presumably to inactivate enzymes. In fact, using *Coprinus cinereus* peroxidase, Aitken and Heck (1998) observed that the turnover capacity, defined as the amount of phenolic substrate removed per mol of peroxidase inactivated, depended highly on the type of phenol oxidized. For a number of monosubstituted phenols the turnover capacity increased as the electronegativity of the substituent on the benzyl ring decreased. Moreover, the turnover capacity correlated relatively well to the Hammett radical ($\sigma\cdot$) values of the *meta* and *para* substituted phenols suggesting that at least at the initial stages of the reaction phenoxy radicals were primarily responsible for enzyme inactivation.

On the other hand, according to Subrahmanyam *et al.* (1990) covalent binding to the protein globule may also involve reactions of *p,p'*-diphenoquinone, a product which forms from a phenolic dimer, with cysteine residues of the protein molecule. These investigators also report that the majority of the protein-bound phenol oxidation products were attached to the enzyme molecule through non-covalent interactions.

Nakamoto and Machida (1992) proposed that HRP could also be inactivated through interaction with phenolic polymers that form spontaneously when high concentrations of phenolic radicals are produced. They observed that the concentration of HRP in the aqueous phase rapidly decreased over the course of phenol oxidation presumably due to attachment of the enzyme to the phenolic polymer that precipitated out of solution. Also, activity assays conducted in the presence and absence of the precipitate revealed that

more than 90 % of the total activity was associated with the phenolic polymer during most stages of the reaction. Thus, most of the polymer-bound enzyme was at least initially still active. The addition of gelatin to the reaction mixture suppressed the attachment of HRP to the polymer and at the same time significantly decreased enzyme inactivation. Based on these observations, the authors suggest a two-step process of activity loss. Initially, HRP molecules adhere to the phenol polymer while retaining their catalytic activity; then they are inactivated presumably because the polymer encloses them and hinders the access of the substrate to the enzyme's active site. In later publications this process was termed "inactivation through entrapment" (Buchanan and Nicell, 1997).

In order to test whether the enzyme could be inactivated purely through the contact with phenolic polymers, Wu *et al.* (1998) isolated the precipitate that had formed during the HRP-catalyzed oxidation of a 2 mM phenol solution. When a certain amount of HRP was incubated in the presence of these precipitates approximately 30 % of the initial activity was lost within one hour. The observed inactivation was relatively slow compared to the rapid activity loss that occurs during phenol turn-over conditions. For example, in the same study, Wu *et al.* (1998) show that 90 % of the enzyme was inactivated within 10 minutes when catalyzing the oxidation of a 0.5 mM phenol solution. Since the concentration of the precipitate employed in the HRP-incubation experiment was not quoted, it is difficult to evaluate the significance of these results. Other investigators suggest that simple adsorption to the polymer is insignificant compared to inactivation through radicals and polymer entrapment occurring during phenol turn-over conditions (Buchanan and Nicell, 1997). Their conclusions are based on kinetic modeling of batch and continuous flow reactions.

Taken together, these studies indicate that reactive products do not immediately inactivate the enzyme in the vicinity of the active site where they are formed. Rather, they diffuse into solution where they can combine with other radicals or interact with amino acids of the HRP molecule, but also with other species present in the reaction mixture. This feature makes it possible to extend the enzyme's catalytic life by the addition of compounds to the reaction mixture that are capable of neutralizing inactivating reaction products.

2.2.2 Impact of the type of phenol

The quantity of phenolic substrate that can be oxidized within a certain time using a given amount of peroxidase will depend on the reactivity of the reducing substrate with Compounds I and II as well as on the capacity of the reaction products formed to inactivate the enzyme under the given conditions. Also, the ability of reaction products to compete with the original substrate for the reduction of HRP may affect the rate and extent of substrate conversion.

Reaction rates between *para* and *meta* substituted phenols and Compounds I and II generally decrease with increasing Hammett constant of the substituent (Job and Dunford, 1976; Dunford and Adeniran, 1986). High Hammett constants are assigned to electron withdrawing substituents, which increase the redox potential of the phenol molecule, thereby rendering it thermodynamically more difficult to oxidize (Job and Dunford, 1976; Diaz *et al.*, 1998). Accordingly, Candeias *et al.* (1997) show that reaction rates increase with increasing thermodynamic driving force for the phenol oxidation reaction. Nitrophenols, which have high redox potentials can therefore not be treated by HRP (Job and Dunford, 1976; Klibanov *et al.*, 1980). However, disubstituted phenols show lower reactivity than expected based on their oxidizability, suggesting that in these cases steric effects may also play a role (Job and Dunford, 1976). Also, anilines are generally less reactive with Compound I than phenols (Job and Dunford, 1976).

At the same time, one-electron oxidation potentials of the phenols influence the homolytic bond dissociation energy of the O-H bond, which is a measure of the stability of the resulting phenoxy radicals (Aitken and Heck, 1998). Phenols that are more difficult to oxidize tend to have higher homolytic bond dissociation energies implying that their radicals exhibit higher reactivities. These types of phenols possess a stronger capacity to inactivate the peroxidase enzyme resulting in low amounts of substrate transformed per enzyme molecule inactivated (Aitken and Heck, 1998).

A number of studies have compared the treatment of different phenols with HRP in batch reactions. As expected, higher quantities of HRP are required to treat phenols bearing electron-withdrawing groups. For example, bromophenols are more difficult to treat than the corresponding chlorophenols (Dec and Bollag, 1990), which on the other hand are more difficult to treat than methylphenols or methoxyphenols (Nicell, *et al.*,

1993; Wu *et al.*, 1993a; Dec and Bollag, 1990). The position of the substituent on the aromatic ring was also shown to have an important impact. For chlorophenols, the amount of HRP required to accomplish a specified degree of treatment increases in the order: *para-* < *ortho-* < *meta-* position resulting in approximately 2.5-fold higher enzyme requirements for the treatment of 3-chlorophenol than for 4-chlorophenol (Nicell *et al.*, 1993; Wu *et al.*, 1993a). Similarly, removal efficiencies of bromophenols decrease in the same order (Dec and Bollag, 1990) suggesting that phenols bearing electron-withdrawing groups in the *meta*-position may be generally the most difficult to treat. On the other hand, among the methylphenols, 2-methylphenol is the most resistant to HRP-treatment followed by the *meta-* and *para* substituted congeners (Dec and Bollag, 1990; Nicell *et al.*, 1993; Wu *et al.*, 1993a). Aitken and Heck (1998) observed the same order for the different methyl-, and chlorophenols when testing their capacity to be transformed by *Coprinus cinereus* peroxidase.

2.2.3 Impact of reaction temperature and pH

Temperature has a two-fold effect on enzymatic reactions. With most enzymes, the rate of substrate conversion as well as the rate of thermal inactivation increases with temperature (Zubay, 1993). This leads to an apparent increase in enzyme activity until a temperature is reached where thermal inactivation becomes predominant. Above this point the apparent activity drops abruptly. Nicell *et al.* (1993) demonstrate that HRP follows this general behavior. The activity of the enzyme increased steadily with temperature up to a maximum at 50°C and than rapidly fell off due to thermal inactivation. Under phenol turn-over conditions, temperature could also affect other factors that have an impact on the rate and extent of phenol conversion, such as the rate of enzyme inactivation by hydrogen peroxide or reaction products. However, only a limited amount of data is available on the impact of reaction temperature on the overall enzymatic phenol removal process.

Dec and Bollag (1990) reported that the degree of 2,4-dichlorophenol conversion in batch reactors was only slightly affected by the reaction temperature in the range of 5 to 55°C. The reactions were carried out for a relatively long time (8 hours) using an amount of HRP that led to a high extent of 2,4-dichlorophenol conversion (approximately 93 % at

25°C). Similarly, when applying long reaction times Nicell *et al.* (1992) observed no significant difference in the amount of 4-chlorophenol removal for high levels of phenol conversion (approximately 90 %) when the reaction temperature was varied from 5 to 50°C. However, at lower enzyme doses and thus lower levels of substrate conversion, less 4-chlorophenol removal was achieved at 50°C compared to the removal at 5 to 35°C. Enzyme requirements increased substantially when the temperature was raised to 65°C, presumably due to thermal inactivation (Nicell *et al.*, 1992).

Nicell *et al.* (1993) studied HRP-catalyzed removal of phenolic compounds as a function of pH. Phenol, all six monochloro and monomethylphenols as well as of 2,4-dichlorophenol were included in this investigation. For all phenolic compounds studied, the enzyme demonstrated some catalytic ability over a pH range of 4 to 10 but only accomplished good removal over the pH range of 6 to 9. No catalytic ability below a pH of 2 and above 10 was apparent. The optimum pH in terms of the minimum amount of HRP required to accomplish conversion was between 8 and 9 for all phenols studied, except for 4-methylphenol, which showed optimum treatment at pH 7. Dec and Bollag (1990) arrived at slightly different results when they investigated the treatment of some chlorophenols. They report that 4-chlorophenol was best removed at pH 8.3 and 2,4-dichlorophenol at pH 6.5. They also observed that that the optimum pH for the treatment with HRP decreased with an increase of the number of chlorine atoms attached to the aromatic ring. The acidity of phenols increases when electron-withdrawing substituents are present on the aromatic ring (Loudon, 1988). This may explain the requirement for an acidic environment for the oxidation of polysubstituted chlorophenols since HRP can only oxidize phenols in their neutral form (see Figure 2.2).

2.2.4 Impact of the peroxide dose

According to the primary catalytic cycle of HRP (equations 1.1 to 1.3.), half a mole of H_2O_2 is needed to oxidize one mole of phenolic substrate. However, significantly higher peroxide requirements were observed under phenol turn-over conditions. For example, Nicell *et al.* (1992) observed a one-to-one stoichiometry between H_2O_2 and eight phenolic compounds in batch reactions when the reactions were limited by H_2O_2. The authors attributed the higher peroxide requirements to the participation of phenolic oligomers in

the catalytic cycle. They also claim that as the phenolic polymer grows in size, the ratio of peroxide consumed to aromatic substrate removed should approach unity as a limit. Similarly, Wu *et al.* (1993a) observed a broad optimum for phenol removal around a molar ratio of 1. Dec and Bollag (1994a) obtained maximum 2,4-dichlorophenol conversion when a molar equivalent of H_2O_2 was supplied to the reaction mixture.

The above mentioned studies also show that increasing the H_2O_2 concentration beyond the amount required to treat the phenolic compounds reduces the phenol removal efficiency in batch reactors. For example, the removal efficiency of 2,4-dichlorophenol was decreased up to a maximum of 10% when the H_2O_2/2,4-dichlorophenol ratio was increased to 16. Similar observations are reported by Wu *et al.* (1993b) and Nicell (1991) and may be partially explained by enzyme inactivation due to the H_2O_2-induced formation of verdohemoprotein (P-670). High doses of hydrogen peroxide also lead to the conversion of a significant fraction of the active enzyme into Compound III. While HRP that is arrested in this form is practically inactive as a catalyst, it is still susceptible to inactivation by reaction products. Thus, in the presence of reaction products, reversible inhibition by H_2O_2 may lead to irreversible inactivation.

2.2.5 Impact of the reactor configuration

Nicell *et al.* (1992) proposed that the degree of enzyme inactivation through free radicals could be reduced by lowering the free radical concentration and the amount of enzyme available for inactivation at any instant. This theory was tested by continuously adding the enzyme to a batch reactor containing peroxide and phenol. This approach resulted in a more than two-fold increase in the number of substrate molecules removed per enzyme molecule inactivated (Nicell *et al.*, 1992). In continuous flow stirred tank reactors (CFSTRs), reactant and enzyme concentrations are reduced to low steady-state levels immediately upon entering the reactor. This leads to low rates of enzyme inactivation and Compound III formation and thus increases the efficiency of enzyme use (Nicell *et al.*, 1992). Conditions leading to reduced enzyme inactivation can also be maintained in plug flow reactors (PFRs) into which the enzyme is fed at multiple ports (i.e., a step feed PFR) (Buchanan *et al.*, 1998).

Enzyme inactivation rates are strongly reduced in the presence of protective additives (Buchanan and Nicell, 1998). In this case, CFSTRs seem to be less advantageous, since a high proportion of the active enzyme will be wasted in the effluent due to the reduced rates of catalysis in this reactor type (Buchanan *et al.* 1998). Based on the results of kinetic modelling, Buchanan *et al.* concluded that the optimum reactor configuration depends upon the presence of protective additives, the available retention time, the initial phenol concentration, and the desired level of phenol removal.

2.2.6 Co-precipitation

Klibanov *et al.* (1980) reported that when two substrates were present in the reaction mixture, the one that was easily removed by HRP enhanced the transformation of the compound that was more difficult to remove. Furthermore, in subsequent studies it was observed that even non-substrates such as naphthalene and azobenzene (Klibanov, 1982) or polychlorinated biphenyls (PCBs) (Klibanov *et al.*, 1983) were removed from the solution in the presence of phenolic substrates. Klibanov *et al.* (1983) suggested two possible mechanisms to be responsible for this phenomenon. First, the free radicals that are produced from the easily removed substrates could react with (i.e., co-polymerize) and co-precipitate other compounds in the reaction mixture. Alternatively, co-precipitation could also occur due to adsorption of difficult to-remove pollutants to the forming polymer. The occurrence of co-precipitation has important implications for the treatment of real wastewaters, which in most cases will contain substrates with different reactivities and also a variety of compounds that are unreactive with HRP.

Research on co-polymerization has focused on the study of the underlying mechanism and on the products that are formed when different substrates are present in the reaction mixture. The latter aspect involves the identification of reaction products and/or their characterization in terms of toxicity or UV absorbance. In particular, a number of studies have focused on reactions of xenobiotic chlorinated phenols in the presence of naturally occurring humic constituents with the aim to elucidate the fate of phenolic pollutants in the aquatic or soil environment (Bollag, 1992). Enhanced removal of these relatively unreactive substrates was observed when the reactions were carried out in the presence of low molecular weight humic precursors such as guaiacol (2-

methoxyphenol), 2,6-dimethoxyphenol (Roper *et al.*, 1995) and several phenolic acids including ferulic, *p*-coumaric and *p*-hydroxybenzoic acids (Morimoto and Tatsumi, 1997). These humic phenolic compounds are good substrates of HRP. Moreover, enhancement of chlorophenol transformation was also observed in the presence of high molecular weight fulvic (Sarkar *et al.*, 1988) and humic acids (Dec and Bollag, 1994b). It seems unlikely that HRP directly oxidizes these polymeric compounds.

Park *et al.* (1999) examined in more detail the effect of various phenolic humic compounds on the transformation of 4-chlorophenol with horseradish peroxidase and other oxidative enzymes. In the case of HRP, the humic constituents studied could be sub-divided into three groups. Many compounds (for example syringaldehyde) enhanced 4-chlorophenol transformation, some had no effect, while others, particularly phenols containing two hydroxylgroups such as catechol or hydroquinone, reduced 4-chlorophenol transformation. The authors argue that the co-substrate has to fulfill two requirements in order to enhance the transformation of a less reactive substrate present in the reaction mixture. Firstly, it needs to possess a high reactivity towards the enzyme. Secondly, the primary oxidation products of both substrate and co-substrate need to be able to combine (couple) with each other. According to this theory catechol and hydroquinone, although fulfilling the first requirement, do not meet the second since they are oxidized to quinones, which the authors claim are unreactive with free radicals generated from 4-chlorophenol. Rather, due to their high reactivity these co-substrates reduce 4-chlorophenol transformation through competition for the enzyme's active site. However, Park *et al.* (1999) do not offer an explanation on how the coupling of substrate and co-substrate oxidation products may enhance the extent of substrate conversion. It may be suggested, following the line of their reasoning, that if the substrate oxidation products possess strong enzyme inactivating capabilities, the process of coupling with free radicals that are generated in large numbers from the reactive co-substrate may prevent these enzyme inactivating species from attacking and inactivating the enzyme molecule.

Importantly, not only the extent but also the rate of substrate oxidation was greatly accelerated in the presence of an enhancing co-substrate. This acceleration was attributed to radical transfer from the co-substrate oxidation product to the substrate molecule (Park

et al., 1999). The radical transfer mechanisms may offer an alternative explanation for the enhancing or inhibiting effect of co-substrates. Highly reactive co-substrates may "activate" substrate molecules by oxidizing them to free radicals, thereafter returning to the enzyme's active site to be again oxidized to radicals. Thus, co-substrates, which are transformed to products that are not capable of effectively activating the substrates, will not enhance but rather inhibit substrate conversion. The mechanism of radical transfer was also used to explain the enhanced removal through oxidative binding of chlorophenols to high molecular weight humic acids (Dec and Bollag, 1994b). The authors propose that chlorophenol radicals oxidize phenolic residues on the high molecular weight humic acid to the corresponding radicals. This allows the subsequent coupling of chlorophenol radicals to the humic material.

Evidence for radical transfer being an important mechanism in co-polymerization can also be drawn from results obtained by Simmons *et al.* (1989) who studied the laccase-catalyzed co-oxidation of guaiacol and 4-chloroaniline. Similar to peroxidase, laccase oxidizes guaiacol to the corresponding free radical but is relatively unreactive with 4-chloroaniline. In the presence of guaiacol, however, 4-chloroaniline is transformed to a higher degree. According to the authors, the structure of some reaction products suggests that the first step leading to their formation was the radical transfer from a guaiacol radical to a 4-chloroaniline molecule. They also suggest that other types of reaction products were formed through direct coupling reactions between the 4-chloroaniline and guaiacol-derived quinone dimers (Simmons *et al.*, 1989).

Interestingly, guaiacol could not enhance the transformation of 4-chloroaniline when the reaction was catalyzed by horseradish peroxidase, even though this enzyme readily oxidizes guaiacol presumably to the same reaction products as the laccase (Simmons *et al.*, 1989). Furthermore, the oxidation of guaiacol was slowed down when 4-chloroaniline was present in the reaction mixture. The difference between the two enzymes suggests that, apart from non-enzymatic reactions involving co-substrate-derived products, other factors such as the susceptibility of the enzyme to be inactivated by substrate-derived reaction products may determine whether a co-substrate will be able to enhance the transformation of a substrate.

The notion that apart from radicals, other longer-lived reaction products may also be involved in the co-polymerization process is supported by results obtained by Roper *et al.* (1995). These authors observed a significant amount of 2,4,5-trichlorophenol removal (72 %) when this compound was incubated with an enzyme-free filtrate containing reaction products generated from the HRP-catalyzed oxidation of 2,6-dimethoxyphenol. 2,4,5-trichlorophenol removal was attributed to a chemical reaction with the oxidation products of the co-substrate.

2.2.7 The use of protective additives

Nakamoto and Machida (1992) report that HRP inactivation over the course of the reaction substantially decreased when the reaction was carried out in the presence of 4 g/L of gelatin. This resulted in a 200-fold reduction in the amount of HRP required to treat a solution containing 10 g/L (~100 mM) phenol. Similar effects were also observed with other proteins, polyvinylalcohol and borate. Also, polyethylene glycol (PEG) (see Figure 2.3) of a molecular weight of or above 1000 Da reduced enzyme inactivation. Borate was considered to be unsuitable for wastewater treatment due to its insecticidal action.

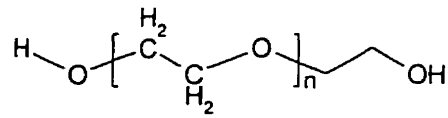


Figure 2.3 : Structure of the polyethylene glycol (PEG) polymer.

Wu *et al.* (1997) compared gelatin and PEG of an average molecular weight of 3350 Da (PEG_{3350}) in terms of their usefulness for the treatment of 1 mM phenolic solutions. Higher amounts of gelatin than of PEG_{3350} were required to achieve maximum protection of the enzyme. Also, the addition of excess gelatin reduced phenol removal and prevented the formation of a precipitate. An excess of PEG_{3500} had no adverse effect on treatment. Furthermore, in contrast to PEG, gelatin consists of approximately 18 % nitrogen by weight, which may adversely increase nitrogen levels in the treated wastewater (Nicell *et al.*, 1995). Another advantage of PEG is its extremely low toxicity (Harris, 1992). Thus, PEG was preferred in further investigations for the use in HRP-catalyzed wastewater treatment.

Nicell *et al.* (1995) report that due to the protective action of PEG_{3350}, about 20 times less HRP was required for 90% treatment of phenol solutions with initial concentrations in the range of 0.5 to 10 mM. Wu *et al.* (1993b) found that PEG_{3350} was more effective at higher initial phenol concentrations, with 40- and 75-fold reductions in enzyme requirements for 95% treatment for initial phenol concentrations of 1 and 10 mM, respectively. Approximately 20 mg/L PEG_{3350} were required to minimize the amount of peroxidase needed to treat a 1 mM phenol solution (Nicell *et al.*, 1995; Wu *et al.*, 1993b). This optimum PEG-dose was proportional to the initial phenol concentration (Nicell *et al.*, 1995; Wu *et al.*, 1993b; Kinsley and Nicell, 2000) and depended on the type of phenol treated (Wu *et al.*, 1993a).

Kinsley and Nicell (2000) investigated the usefulness of PEGs for the treatment of phenol using the peroxidase from soybeans (SBP). The effectiveness of PEG increased with increasing molecular weight; with PEG_{35000} providing the greatest enzyme protection, while PEGs of an average molecular weight below 1500 Da were ineffective. However, enzyme requirements could only be reduced 4-fold, which is substantially lower than what was achieved with HRP (Wu *et al.*, 1993b; Nicell *et al.*, 1995). Also, higher PEG doses were required to achieve optimum enzyme protection.

When optimum PEG_{35000} doses were used, 77 % of the initial PEG had precipitated along with the phenolic polymer after treatment (Kinsley and Nicell, 2000). This value was independent of the initial concentrations of PEG or phenol. Nakamoto and Machida (1992) quote a similar number. On the other hand, Wu *et al.* (1997) report that essentially no PEG_{3350} stayed in solution after treatment of a solution containing 1 mM 3-methylphenol. Both studies show that when more PEG was added than was required for full enzyme protection, the excess PEG remained in the solution. The partitioning of PEG into the solid phase is a critical requirement for its potential application, since the release of substantial amounts of high molecular weight PEGs could have an adverse effect on the receiving water bodies due to the low biodegradability of these polymers (Davidson, 1980).

It is generally believed that PEG exerts its protective effect towards the enzyme by interaction with the reaction products (Nakamoto and Machida, 1992; Wu *et al.*, 1997, 1998; Kinsley and Nicell, 2000). The finding that the amount of PEG required to reduce

enzyme inactivation depended on the type of phenol treated and not on the amount of enzyme (Wu *et al.*, 1993a) may support that view. Furthermore, under conditions when PEG was the limiting factor, PEG and the phenolic polymer co-precipitated at a constant mass ratio (Kinsley and Nicell, 2000). Also, under these conditions the quantity of phenol removed was proportional to the amount of PEG added. The first observation implies that there was a stoichiometric relationship between the quantity of phenolic polymers created and the quantity of PEG associated with these polymers. Furthermore, based on the second observation, Kinsley and Nicell (2000) conclude that the protective capacity of PEG is lost once it has interacted with phenolic products.

PEGs form complexes with phenols and various phenolic resins and this ability increases with increasing molecular weight of the PEG polymer (Davidson, 1980). Taken together with the observation that the effectiveness of PEGs increased with their molecular weight (Kinsley and Nicell, 2000) this lends further support to the view that the way high molecular weight PEGs interact with phenolic reaction products is at the root of their ability to protect the enzyme from inactivation. These products are likely to be polymers or oligomers since PEG as well as another protective additive, chitosan, were ineffective when the phenolic substrate treated was pentachlorophenol (PCP) (Zhang and Nicell, 2000), which does not form polymers but only reacts to dimers (Kazunga *et al.*, 1999).

In conclusion, substantially less peroxidase is required when the treatment is conducted in the presence of high molecular weight PEGs. However, indications exist that some PEG will remain in the treated effluent, which is a major drawback for its full-scale application. Moreover, PEG proved to be less effective for the treatment of real wastewaters (Cooper and Nicell, 1996) and enhances the level of soluble reaction products (Nicell *et al.*, 1995; Ghioureliotis and Nicell, 1999). Thus, alternatives to PEG are being explored.

Tatsumi *et al.* (1994) studied HRP-catalyzed phenol removal in the presence of different coagulants. The coagulants tested were two cationic polymers containing amino groups (hexamethylenediamine epichlorhydrin polycondensate [HX] and polyethyleneimine [PEI]), the non-ionic polymer polyacrylamid and the inorganic coagulant aluminium sulfate (alum). Polyacrylamid and alum did not have an impact on

phenol conversion; however, enhanced phenol removal was achieved when the reaction was carried out in the presence of the cationic polymer coagulants HX and PEI. For example, at an initial phenol concentration of 5 mM, approximately 60 % of the initial phenol was removed without additives while with the same enzyme dose complete treatment was accomplished in the presence of 20 mg/L of HX. The dose at which HX became effective increased with increasing phenol concentration. The authors also noticed that the presence of HX improved color removal by enhancing precipitation of reaction products.

In a later study, the same group included chitosan among the coagulants investigated for the treatment of a mixture of chlorophenols with HRP (Ganjidoust *et al.*, 1996). Chitosan proved to be similarly effective in promoting chlorophenol and AOX removal as was HX. The optimum chitosan dose for the treatment of the 0.55 mM chlorophenol solution was 2 mg/L, while higher chitosan doses had an adverse effect on treatment efficiency. Ganjidoust *et al.* (1997) also used chitosan as a coagulant to remove color and TOC from black liquor alkaline wastewaters. The authors found chitosan to be superior to the other coagulants tested, including alum, HX and PEI; however, at least 600 mg/L of chitosan were required to achieve maximum treatment of the high strength wastewater.

Chitosan (see Figure 2.4) is a polyglucoseamin that is usually prepared from chitin through alkaline deacetylation. Chitin is the major component of the exoskeleton of most invertebrates and is present in the cell walls of many fungi and yeasts (Muzzarelli, 1977).

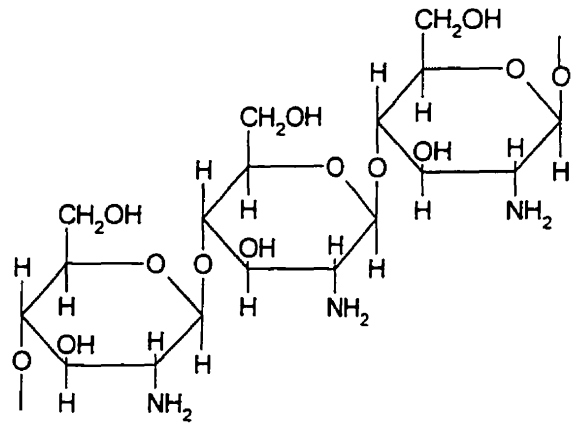


Figure 2.4: Structure of the chitosan polymer.

The major source of commercial chitin and chitosan are the shells of crustaceans, which are generated in high amounts as by-products of the crab and shrimp industry. Chitosan is biodegradable, non-toxic and is already applied as a coagulant in some water treatment applications (Kawamura, 1991). Beside its ability to promote coagulation, chitosan has also been used as a chelating agent to remove metal ions from solution (Muzzarelli, 1977).

Another characteristic of chitosan is its ability to bind quinones (Sun *et al.*, 1992). Binding of quinones to chitosan is rapid and accompanied by a large adsorption enthalpy indicating that covalent bonds are formed (Sun *et al.*, 1992). This ability of chitosan was used by Sun *et al.* (1992) to remove quinones from solution that were formed as products of tyrosinase-catalyzed oxidation of 4-methylphenol. The authors observed that the enzymatic reaction proceeded faster and over a longer period of time when carried out in the presence of chitosan. To explain this observation, Sun and co-workers suggest that quinones were to some degree responsible for tyrosinase inactivation presumably through their ability to cross-link proteins (Letham *et al.*, 1980). Thus, chitosan was able to reduce inactivation of tyrosinase by sequestering the quinones as soon as they were formed. In contrast to Ganjidoust *et al.* (1996 and 1997), who apparently used dissolved chitosan in the form of a stock solution in hydrochloric acid, Sun *et al.* (1992) employed chitosan as a solid adsorbent in suspension. Therefore, chitosan was added to the reaction mixture at a very high concentration of 5 % w/v (approximately equivalent to 50 g/L).

Arseguel and Baboulene (1994) studied the impact of an inorganic additive, talc, at a concentration of approximately 10000 mg/L on HRP-catalyzed phenol oxidation. Talc is a hydrated magnesium phyllosilicate mineral with the overall formula $Mg_3Si_4O_{10}(OH)_2$. The authors observed that the addition of talc after the start of the reaction eliminated the residual absorption peak at 400 nm, which they attributed to being due to quinones. Also, the presence of hydrophobic talc during the reaction resulted in a reduction of the quantity of polyphenols formed (as quantified through HPLC analysis) as well as in an increase in the amount of phenol that was removed by a given dose of enzyme (Arseguel and Baboulene, 1994). These results indicate that this mineral was able to adsorb reaction products, some of which contributed to enzyme inactivation. However, HRP was inactivated when pre-incubated in the presence of the hydrophobic talc. Reduced levels of

polyphenols as well as increased phenol transformation were also observed when the reaction was carried out in the presence of kaolin. On the other hand, hydrophilic calcinated talc did not reduce the concentration of polyphenols, but enhanced phenol removal to an even greater extent than the hydrophobic talc and kaolin (Arseguel and Baboulene, 1994).

2.3 Reaction Products and Toxicity

Free radicals that are formed in the catalytic cycle are highly reactive compounds. They may follow two major reaction paths (Ryu *et al.*, 1993): radical coupling according to reaction (1.16) and radical transfer according to reaction (1.17)

$$R\cdot + R\cdot \longrightarrow R\text{-}R \qquad (1.16)$$

$$R\cdot + \acute{R}\text{-}H \longrightarrow R\text{-}H + \acute{R}\cdot \qquad (1.17)$$

Radical coupling results in the formation of dimers (R-R) through the creation of new C-C or C-O bonds, but not O-O bonds (Neta and Steenken, 1981). In general, this would lead to the formation of five possible dimers, which are shown in Figure 2.5.

Some dimers have been found to be substrates of HRP (Sawahata and Neal, 1982; Yu *et al.*, 1994), which are oxidized to dimer radicals that may combine to form higher molecular weight oligomers.

Radical transfer is essentially a one-electron redox reaction that is driven by the redox potential difference between the two reactants (Neta and Steenken, 1981). The transfer of a hydrogen radical between a monomer radical (R·) and an oligomer (Ŕ-H) leads to the generation of oligomer radicals (Ŕ·) that may combine to form even larger polymers. The overall result is the formation of high molecular weight compounds that tend to precipitate out of the aqueous solution (Dec and Bollag, 1990). In most cases, however, a residual amount of low molecular weight reaction products will remain in the aqueous phase (Ghioureliotis and Nicell, 1999).

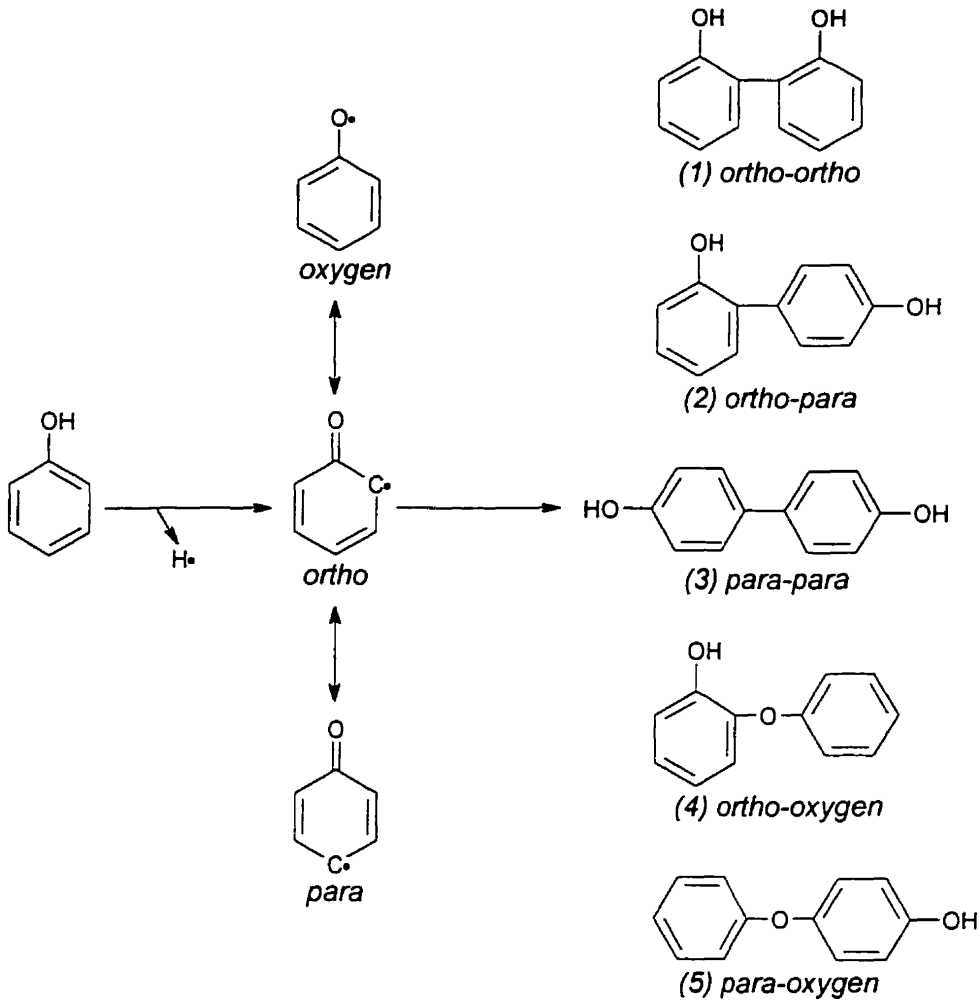


Figure 2.5: Formation of 5 phenolic dimers through the coupling of monomer radicals.

The types and relative quantities of the reaction products formed from enzyme-catalyzed oxidations of phenolic substrates are affected by numerous factors. These include the type of enzyme used, the reaction pH (Aitken *et al.*, 1994), the molar ratio of H_2O_2 to substrate (Griffin, 1991) and the initial substrate concentration (Maloney *et al.*, 1986). Also, other components of the reaction solution such as dissolved oxygen (Griffin, 1991), organic solvents (Pietikäinen and Adlercreutz, 1990) or hydrophilic polymers such as PEG (Ghioureliotis and Nicell, 1999) have an impact on the final composition of the reaction mixture. The presence of more than one substrate increases the complexity of the product distribution due to a variety of possible reactions between the different species that are formed (Simmons *et al.*, 1989; Bollag, 1992).

2.3.1 Reaction products from phenol

In an early study, Danner *et al.* (1973) observed the formation of *o,o'*-biphenol (dimer (1) in Figure 2.5) as the sole product of HRP-catalyzed oxidation of phenol. However, they also show that *o,o'*-biphenol was further oxidized by the enzyme. In contrast, using GC/MS analysis Sawahata and Neal (1982) identified *o,o'*-biphenol as well as *p,p'*-biphenol (dimer (3) in Figure 2.5) as products. They also observed the formation of a yellowish reaction product with the peak absorbance wavelength at 400 nm upon incubation of 0.1 mM phenol with 0.12 mM H_2O_2 and 0.03 nM HRP at pH 7.4. A similar peak appeared when *p,p'*-biphenol was oxidized by HRP, but not when *o,o'*-biphenol was transformed. The observed absorption spectrum was identical with that of *p*-diphenoquinone. The authors suggest that *p,p'*-biphenol is further oxidized by HRP to *p*-diphenoquinone via a semiquinone intermediate. Both of these studies do not mention the formation of a precipitate thereby suggesting that only the initial stages of the reaction were examined.

Results reported by Zou and Taylor (1994) show that 98 % of the removed phenol was converted to water insoluble precipitates when the reaction was carried out in 0.1 M phosphate buffer at pH 7.4 with initial phenol concentrations of 1 or 4 mM. Approximately, 60% to 75% of the precipitate was also insoluble in acetone. Furthermore, using GC/MS, all five dimers shown in Figure 2.5 as well as the trimer 4-(4-phenoxyphenoxy)phenol were identified in the supernatant (Zou and Taylor, 1994). In addition, *p*-quinone was detected at the initial stage of the reaction. A number of unidentified products were also present. The main dimer detected was *o,o'*-biphenol when the initial phenol concentration was 4 mM; however, with 1 mM initial phenol *p,p'*-biphenol was found in higher concentrations.

The same five dimers were also identified as products when Yu *et al.* (1994) conducted the reaction in the presence of PEG. These authors also observed that as the reaction proceeded, more and more *o,o'*-biphenol, *p,p'*-biphenol and *p*-phenoxyphenol (dimer (5) in Figure 2.5) became associated with the precipitate while their concentration in the aqueous phase declined. After a 2-hour reaction time the dimers accounted for 6.7 % of the total dry mass of the precipitate. Among these three dimers *o,o'*-biphenol was the most predominant. However, according to the authors this resulted not so much from

a preferred formation of *o,o'*-biphenol. Rather, this compound was accumulating because it was much slower oxidized by HRP than the other two dimers.

2.3.2 Reactions products from hydroxy-, methyl- and methoxyphenols

Hydroxylated phenols such as hydroquinone and catechol are oxidized by HRP to the corresponding quinones (Park *et al.*, 1999).

When 4-methylphenol was oxidized with HRP the main low molecular weight reaction product was Pummerer's ketone (Hewson and Dunford, 1976). The authors propose a reaction scheme for the formation of this compound, as shown in Figure 2.6.

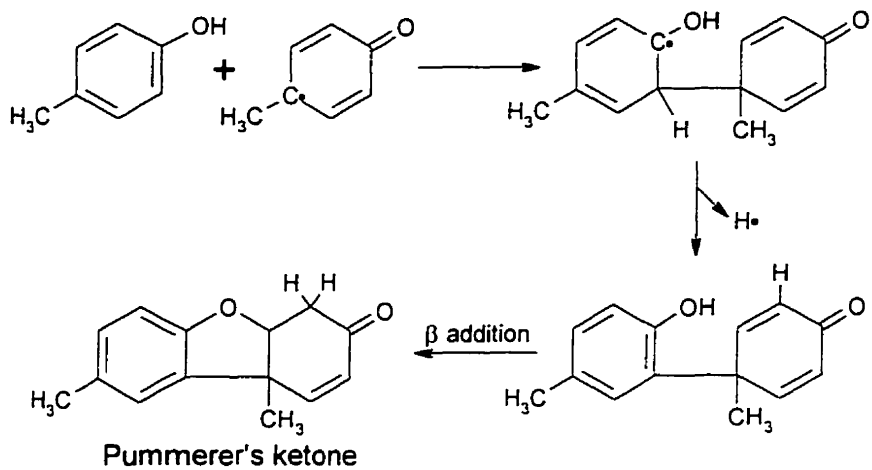


Figure 2.6: Formation of Pummerer's ketone from 4-methylphenol.

The first step is the electrophilic attack of 4-methylphenol by a 4-methylphenoxy radical. The resulting dimer radical is further oxidized by another 4-methylphenoxy radical. After final beta-addition Pummerer's ketone is formed.

In addition to Pummerer's ketone, Pietikäinen and Adlercreutz (1990) also identified the *o,o'*-dimer and a trimer as low molecular products of HRP-catalyzed oxidation of 4-methylphenol. The yield of these three products together was 15 % after 98 % conversion of a 10 mM 4-methylphenol solution in pH 7.0 phosphate buffer. When the reaction was carried out in water-saturated solvents using HRP immobilized on Celite the *o,o'*-dimer was the predominant reaction product.

Simmons *et al.* (1988) studied the reaction products resulting from the incubation of 2-methoxyphenol (guaiacol) with HRP and H_2O_2. Five dimers and two trimers were isolated and identified using thin-layer chromatography, mass spectrometry and NMR. Out of the five possible dimers shown in Figure 2.5, the compound resulting from *ortho*-oxygen coupling was missing presumably due to steric hindrance between the *ortho*-methoxy and the hydroxyl group. The main product was the dimer resulting from *para-para* coupling, followed by the *ortho-ortho* coupled product. Additionally, an intensely orange *ortho*-quinone dimer was present. The authors suggest that this compound resulted from a nucleophilic substitution of a hydroxide ion or water at the *ortho*-quinone position of the quinoide form of the *para-para* coupled dimer. In total, 88% of the product mixture arose from C-C coupling, suggesting that C-C linked oligomers were preferentially formed.

Simmons and co-workers were only interested in the first products that appear during the initial stage of the polymerization process. Thus, low enzyme concentrations and short reaction times were employed resulting in only 16% substrate conversion. When greater enzyme concentrations were used, additional high-molecular-weight compounds were formed as evidenced by a brown precipitate and the appearance of HPLC peaks with long retention times.

2.3.3 Reaction products from chlorinated phenols

Using radiolabeled 2,4-dichlorophenol, Dec and Bollag (1990) report that 88 % of the initial 2,4-dichlorophenol was incorporated in the precipitate that formed when 0.01 M 2,4-dichlorophenol reacted with 2 M H_2O_2 in pH 6.6 phosphate-citrate buffer via the catalytic action of HRP. The average molecular weight of the dioxan-soluble fraction of the precipitate was 800 Da indicating that it mainly consisted of tetra-and pentamers. The dioxane insoluble fraction likely contained compounds of higher molecular weights (Dec and Bollag, 1990). Also, using radiolabeled 2,4-dichlorophenol, Maloney *et al.* (1986) observed that only approximately 50% of the radioactivity was retained by a 0.45 μm filter suggesting that a large fraction of the reaction products was in water-soluble or colloidal form.

Dec and Bollag (1990) also report that chloride ions were released into the solution during the HRP-catalyzed transformation of 2,4-dichlorophenol. In a later study, Dec and Bollag (1994b) examined in detail the oxidative dehalogenation of several chlorophenols. They found that in the case of 2,4-dichlorophenol and 2,4,5-trichlorophenol, on average one chlorine molecule was released per two molecules of phenolic substrate transformed, while less dehalogenation occurred with less chlorinated phenols. Dehalogenation was thought to occur when a free radical having a chlorine atom attached to an aromatic carbon which carries the unpaired electron couples with another radical. Thus, for example, in the case of 2,4-dichlorophenol, coupling of two radicals at the *para*-position would lead to the release of two chloride ions. The authors propose a model to predict how much chloride is liberated per mole chlorophenol oxidized which is based on the number of possible different coupling events between two radicals. Based on this model, Dec and Bollag also predict that in the case of 2,4-dichlorophenol soluble dimers, trimers and tetramers would constitute 24.7 % of all the products formed, while the remaining 75.3 % would be insoluble pentamers. This calculation is based on the assumptions that only the monomers are oxidized by HRP and that higher oligomers are not formed.

Furthermore, in the same study, enhanced levels of dehalogenation were observed when chlorophenols were coupled to a high molecular weight humic acid through the catalytic action of HRP (Dec and Bollag, 1994b). Dehalogenation was also reported to occur with chlorinated anilines (Dec and Bollag, 1995) and other oxidative enzymes such as lignin peroxidase (Hammel and Tardone, 1988), laccase (Dec and Bollag, 1990; 1995) and tyrosinase (Dec and Bollag, 1995).

The loss of chlorine atoms through enzymatic transformation is considered to be an important phenomenon since aromatic compounds that contain less chlorine are usually also less toxic.

When aqueous pentachlorophenol was oxidized with HRP, the predominant reaction product was reported to be tetrachloro-*p*-benzoquinone (chloranilin) (Samokyszyn *et al.*, 1995). However, in a recent study Kazunga *et al.* (1999) found that the main product was an insoluble dimer which was formed by direct coupling of two pentachlorophenoxyl radicals. The authors show that this compound is easily converted to chloranilin when incubated in several organic solvents, which are commonly used in analytical work to

extract reaction products. The formation of a dimer was also postulated by Zhang and Nicell (2000) based on their observation that 0.5 moles of peroxide were required for the oxidation of 1 mole of pentachlorophenol. Furthermore, these authors found that only 2.8 % of chlorines were released into solution when pentachlorophenol was oxidized by HRP. The low degree of dechlorination is also consistent with the dimer identified by Kazunga *et al.* (1999) being the major product, since this molecule has retained all 10 chlorine atoms.

2.3.4 Reaction products from reactions involving more than one substrate

It can be expected based on the high reactivity of HRP-generated radicals that mixtures of substrates will give rise to mixed oligomers through cross-coupling of radicals or through other reactions involving different substrate or product molecules. This was confirmed by Simmons *et al.* (1989) who isolated and identified 5 mixed oligomers generated from a HRP-catalyzed co-oligomerization of guaiacol (2-methoxyphenol) and 4-chloroaniline.

Mixed oligomers were also identified when laccase enzymes were used to catalyze the oxidation of various chlorophenols in the presence of humic phenolic compounds such as syringic or vanillic acids, orcinol and vanillin (Bollag *et al.*, 1980; Bollag and Liu, 1985). Since the primary oxidation products of laccases are identical to those of peroxidases it seems reasonable to assume that similar compounds would also form when peroxidase enzymes were used as catalysts (Simmons *et al.*, 1989).

2.3.5 Polychlorinated dibenzodioxins and dibenzofurans

In an early study Maloney *et al.* (1986) identified polychlorinated dibenzodioxins-and furans (PCDD/Fs) among the by-products formed when 0.6 mM 2,4-dichlorophenol was incubated with 5 mM H_2O_2 and HRP. The PCDD/Fs present contained up to 4 chlorine atoms per molecule.

PCDD/Fs are highly toxic compounds (Environment Canada, 1990). They are released into the environment through human activities such as the burning of chlorinated wastes or pulp bleaching. The burning of wood and natural forest fires may be additional sources of PCDD/Fs in the environment (Thomas and Spiro, 1996). The finding that the

enzymatic transformation of chlorophenols may be another route for the formation of these compounds has motivated further investigations into this process.

Öberg and Swanson (1987) detected no PCDD/Fs after lactoperoxidase-catalyzed oxidation of pentachlorophenol, 2,3,4,6-tetrachlorophenol and 2,4-dichlorophenol. In the case of 2,4-dichlorophenol the discrepancy between these results and the observations of Maloney *et al.* (1986) may be partially due to the fact that only PCDD/Fs containing four or more chlorines were analyzed for.

Svenson *et al.* (1989) studied the formation of polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs) through oxidation of 0.1 mM 2,4,5-trichlorophenol with HRP and 9 mM H_2O_2 in pH 7.0 phosphate buffer. After 72 % of the parent phenol had been transformed, approximately 30 different congeners of PCDD/Fs were identified in the product mixture. Congeners having four, five and six chorines per molecule constituted the bulk of the PCDD/Fs found, while those with 7 or 8 chlorines accounted for less than 0.5 % of the total PCDD/Fs in the product mixture. In total, 10 µmol of tetra through hexa PCDD/Fs per mol of 2,4,5-trichlorophenol had been formed.

In a later study, Öberg *et al.* (1990) confirmed the formation of PCDD/Fs from 2,4,5-trichlorophenol. Similar amounts of PCDD/Fs were formed when lactoperoxidase instead of HRP was used as the catalyst. Importantly, only minor differences in the amount or the pattern of PCDD/Fs isomers were observed when the oxidation of 2,4,5-trichlorophenol was carried out with 0.2 or 9 mM H_2O_2 suggesting that the initial peroxide concentration had a minor impact on PCDD/F formation. Under identical reaction conditions 10 % more PCDD/Fs were formed when 3,4,5-trichlorophenol was oxidized by lactoperoxidase than when 2,4,5-trichlorophenol was transformed. To explain this observation, the authors propose that radicals that possess two unsubstituted *ortho*-groups are more likely to combine to those dimers that will form dibenzodioxins-and furans. They also suggest that the failure to detect PCDD/Fs from PCP and 2,3,4,6-tetrachlorophenol in their previous study (Öberg and Swanson, 1987) was partially due to the fact that these compounds are chlorinated in both *ortho*-positions.

However, other investigators report that a relatively high proportion (approximately 4%) of the initial pentachlorophenol was converted to octachlorodibenzo-*p*-dioxin upon HRP-catalyzed oxidation (Morimoto and Tatsumi, 1997). Differences in reaction

conditions, such as the reaction pH, or in the analytical techniques used may be responsible for these disagreements.

2.3.6 Toxicity studies

The studies described in the previous section have demonstrated that the enzymatic transformation of chlorinated phenols can result in the formation of toxic compounds. These results have motivated research into the assessment of the acute toxicity and mutagenicity of phenolic solutions treated by peroxidases, since the enzymatic treatment should only be implemented if the reaction products are less hazardous than the starting material.

So far, the Microtox™ assay was used in all studies conducted to evaluate the acute toxicity of peroxidase-treated solutions. This assay involves the exposure of a bioluminescent marine bacterium to a toxic sample and the subsequent measurement of the decrease in light output after a specified exposure time. These bacteria produce light through the oxidation of the reduced form of flavinmononucleotide ($FMNH_2$) to a product in an excited electronic state (most likely a hydroxy derivative of FMN) which decays to FMN thereby releasing a molecule H_2O and emitting light (Zubay, 1993). Simultaneously, a long chain aliphatic aldehyde is oxidized to the carboxylic acid. Molecular oxygen functions as the electron acceptor in these redox reactions and the enzymes catalyzing these transformations are referred to as "luciferases" (Zubay, 1993). The bacterial luciferase system is coupled to the respiratory pathway through nicotinamide adenine dinucleotide (NADH) and the flavin nucleotide (Hastings, 1978). Toxicants interfering with this vital metabolic pathway can cause a reduction in the light output in a concentration dependent manner.

It was observed that for high levels of peroxidase treatment (>95% parent phenol transformation) 3-chlorophenol, 2,6-dichlorophenol and 4-methylphenol solutions were almost completely detoxified, while high residual toxicities remained in the cases of phenol, 2-methylphenol, 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol (Ghioureliotis and Nicell, 2000). In particular, several studies report that solutions of phenol, 2-chlorophenol and 2-methylphenol were more toxic after HRP-treatment than before (Heck *et al.*, 1992; Aitken *et al.*, 1994; Ghioureliotis and Nicell, 2000). Although,

solutions of 3-methylphenol (Ghioureliotis and Nicell, 2000) and pentachlorophenol (Zhang and Nicell, 2000) were detoxified to a high extent (approximately 80%), more than 95% phenol conversion was achieved in these cases, thus indicating the presence of toxic reaction products. The observed toxicity levels did not depend upon whether treatment was conducted with the peroxidase from horseradish (HRP) or from soybeans (SBP) (Ghioureliotis and Nicell, 2000).

Ghioureliotis and Nicell (1999) studied the impact of the initial phenol concentration and the degree of phenol conversion on residual toxicity and the concentration of soluble reaction products using HRP and SBP. At high initial phenol concentrations (5 and 10 mM) the toxicity declined steadily at intermediate levels of phenol removal up to a point when 90 to 95 % phenol transformation was reached, but increased sharply when full treatment was approached. Similarly, at lower initial phenol concentrations (1 mM) toxicity levels were highest at highest levels of phenol conversion; however, in this case toxicity did not decline at intermediate levels of treatment. In contrast, the concentration of soluble reaction products (as measured using radiolabeled phenol) increased at intermediate levels of treatment, but declined sharply, once full treatment was achieved. Also, higher concentrations of soluble reaction products were present when phenol transformation was catalyzed by HRP as compared to soybean peroxidase (SBP); however, this did not translate into higher toxicities.

Interestingly, for complete treatment (more than 99% phenol transformation) there was no relationship between the initial phenol concentration and the residual toxicity, suggesting that the concentration of toxic compounds in the aqueous phase was limited by their solubility (Ghioureliotis and Nicell, 1999). However, the concentration of soluble reaction products was proportional to the initial phenol concentration, indicating that the solubility limit for the bulk of soluble compounds had not yet been reached. Furthermore, when treatment was conducted in the presence of PEG, the percentage of the initial phenol that stayed in solution in the form of its reaction products after complete phenol conversion increased from 1.6 to 5.5 %. Yet, this did not seem to have a significant impact on toxicity levels (Ghioureliotis and Nicell, 1999). Overall, the lack of correlation between the concentration of reaction products in the solution and its toxicity suggests

that among the bulk of the soluble compounds only some specific species were those responsible for the elevated toxicity levels.

Massey *et al.* (1994) tested the mutagenicity of reaction products from the oxidation of a number of phenols by different enzymes, including HRP, lignin peroxidase and polyphenol oxidase. The Ames *Salmonella typhimurium* plate incorporation assay with two different strains possessing different mutational target sites was used to identify genotoxic effects. Some of the reaction product mixtures were also pre-incubated with S9 microsomal rat liver preparation in order to test whether they contained compounds that could be transformed into mutagens *in-vivo*. The phenols tested included *ortho* and *para* substituted monochloro and methylphenols as well as pentachlorophenol. Also, the oxidation of 2-and 4-nitrophenol by lignin peroxidase was examined. Except for the nitrophenols oxidized by lignin peroxidase, none of the product mixtures was found to be mutagenic under the conditions tested (Massey *et al.*, 1994). Also, the parent phenols were not mutagenic in this assay.

Previously, Subrahmanyam and O'Brien (1985) observed that products from the peroxidase-catalyzed oxidation of phenol bind to DNA when the DNA was present in the reaction mixture during the reaction, but not when added after the reaction reached completion.

Cross-coupling of reaction products from different substrates has important implications on the types and quantities of reaction products formed and thus on the toxicity of treated solutions. For example, Morimoto and Tatsumi (1997) report that the formation of octachlorodibenzo-*p*-dioxin from the oxidation of PCP was suppressed when the reaction was carried out in the presence of certain naturally occurring phenolic acids.

Similarly, Ghioureliotis and Nicell (2000) observed reduced toxicity levels when 2,4-dichlorophenol was treated in the presence of a number of phenolic co-substrates. These results are in agreement with the observation made by Nicell *et al.* (1993) that the UV absorbance of HRP-treated 2,4-dichlorophenol solutions was more effectively reduced when the treatment was conducted in the presence of another phenolic compound.

2.4 Treatment of Real Wastewaters

Several studies give brief accounts of the treatment of industrial effluents with HRP and H_2O_2. For example, Klibanov *et al.* (1983) report that HRP-treatment of a coal conversion wastewater having a phenol concentration of 400 mg/L (~4 mM) with 14 mM H_2O_2 resulted in 97 % phenol removal after a one-hour reaction time. These authors also relate that components of coal-conversion wastewaters such as ammonia, cyanide, chloride and thiocyanate may inhibit the peroxidase, but claim that this inhibition is insignificant at high, practically important concentrations of phenol. Similarly, a wastewater sample from a triaryl phosphate production plant was adjusted to pH 7 and treated with HRP and 2.5 mM H_2O_2 at 4°C resulting in 96.5 % phenol removal (Alberti and Klibanov, 1982). This wastewater contained 105 mg/L (~1 mM) total phenols as well as triaryl phosphates and inorganic phosphate. Nakamoto and Machida (1992) treated desorption water from activated carbon that had been used to treat waste gas from an integrated circuit manufacturing process and contained 9100 mg/L (~90 mM) phenol at pH 7.6. Phenol removal efficiency was 97 % after treatment with 0.1 M H_2O_2, 10 mg/L HRP and 4 g/L PEG. High concentrations of 2-propanol and butanone present in this sample exhibited no inhibitory effect. Also, hydrogen fluoride did not interfere with phenol removal unless its concentration exceeded 200 mg/L.

A study performed with a landfill leachate and wastewaters from wood preservation (Rejaee and Nicell, 1994) indicated that wastewaters that contain high amounts of oil and grease may require a pretreatment step in order to remove the non-aqueous phase before employing the enzymatic phenol removal process.

Recently, Cooper and Nicell (1996) conducted a study on the treatment of a foundry wastewater containing 3.8 mM total phenols at pH 7.8. Treatment of the foundry wastewater required higher peroxide and HRP doses than treatment of a synthetic solution that contained the same concentration of total phenols. Also, higher doses of the protective additive PEG were needed to maximally reduce enzyme requirements for the treatment of the real wastewater compared to the synthetic phenol solution.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Horseradish peroxidase HRP (EC 1.11.1.7, RZ 1.1) and catalase (EC 1.11.1.6) were purchased from Sigma Chemical Co., St Louis, MO. The nominal activity of HRP was quoted by the supplier as being 87 units/mg dry solid, where one unit of activity corresponds to the formation of 1 mg purpurogallin from pyrogallol in 20 s at 20°C and pH 6. HRP stock solutions (5 mg/mL) were prepared by dissolving the solid enzyme in distilled deionized water and were stored at 4°C.

Nominal 30 % w/w hydrogen peroxide (31 % as assayed by the manufacturer) and potassium ferricyanide were obtained from Fisher Scientific Co., Montreal, QC. 4-aminoantipyrine (4-AAP) (98 % purity) was purchased from Aldrich Chemical Co., Milwaukee, WI. ABTS (2,2`-Azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid] diammonium salt) of 98 % purity and Folin and Ciolcalteau's phenol reagent (2.0 normal) were acquired from Sigma Chemical Co., St. Louis, MO.

Buffers were prepared using American Chemical Society (ACS) grade sodium bicarbonate, sodium carbonate and monobasic and dibasic sodium phosphate, which were obtained from Fisher Scientific Co., Montreal, QC as well as using tris(hydroxymethyl) aminomethane (Trizma®Base, reagent grade) and anhydrous citric acid, which were bought from Sigma Chemical Co., St. Louis, MO.

Phenol (99.5 % + purity) was purchased from Fluka Chemical Corporation (Ronkonkona, NY). All other phenolic compounds (purity 98 % and greater) were purchased from Aldrich Chemical Co., Milwaukee, WI.

Polyethylene glycol (PEG) (average molecular weight 35000) was purchased from Sigma Chemical Co., St Louis, MO. Chitosan samples were kindly donated by Vanson, Redmond, WA. Technical grade alum ($Al_2(SO_4)_3 \cdot 18H_2O$) was obtained from Fisher Chemicals, Fair Lawn, NJ.

Silica gel was purchased from Aldrich Chemical Co., Milwaukee, WI. According to information provided by the supplier, it had the following characteristics: 500 m^2/g BET surface area, 0.75 cm^3/g pore volume, 200-400 mesh, 60 Å. Microgranular cellulose was bought from Sigma Chemical Co., St. Louis, MO. Bentonite, (lab grade powder), kaolin (lab grade powder) and activated carbon powder were obtained from Anachemia Canada Inc., Montreal, QC. Peat moss was bought from a local gardening store.

Alkali lignin (synonymous to Kraft lignin) (weight-average molecular weight ca. 28000, number-average molecular weight ca. 5000) was purchased from Aldrich Chemical Co., Milwaukee, WI.

Sodium iodide, (NaI; molecular biology grade), sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$; Fisher certified reagent) and sodium sulfide ($Na_2S \cdot 9H_2O$; reagent grade) were acquired from Fisher Scientific Co., Fair Lawn, NJ. Sodium nitrite crystals ($NaNO_2$; ACS reagent), anhydrous sodium sulfite (Na_2SO_3; ACS reagent) and sodium cyanide (NaCN; ACS reagent) were bought from Anachemia Canada Inc., Montreal, QC. Sodium thiocyanate crystals (NaSCN; ACS reagent) were purchased from Spectrum Quality Products Inc., Gardena, CA.

Zinc sulfate ($ZnSO_4 \cdot 7H_2O$; Fisher certified reagent), nickel sulfate ($NiSO_4 \cdot 6H_2O$; ACS reagent), manganese sulfate ($MnSO_4 \cdot H_2O$; ACS reagent), cupric(II) sulfate ($CuSO_4$), and chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$; ACS reagent) were obtained from Fisher Scientific Co., Fair Lawn, NJ. Ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$; ACS reagent) and cobalt(II) nitrate ($Co(NO_3)_2 \cdot 6H_2O$; ACS reagent) were bought from Sigma Chemical Co., St. Louis, MO.

Sodium chloride (NaCl; ACS reagent) was purchased from Fisher Scientific Co., Fair Lawn, NJ and calcium chloride ($CaCl_2$) (minimum 96%) from Sigma Chemical Co., St. Louis, MO. Ammonium chloride (NH_4Cl; ACS reagent) and ammonium sulfate crystals ($(NH_4)_2SO_4$; ACS reagent) were bought from Anachemia Canada Inc., Montreal, QC. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$; ACS reagent) was obtained from BDH Inc., Toronto, ON.

Toluene (certified ACS) and hexanes (certified ACS, contains a mixture of isomers) were bought from Fisher Scientific Co., Fair Lawn, NJ. Phenanthrene (98% purity)

originated from the Aldrich Chemical Co., Milwaukee, WI. HPLC-grade methanol was purchased from Sigma Chemical Co., St. Louis, MO.

3.1.2 Equipment

A Hewlett Packard 8453 diode array spectrophotometer (wavelength range 190-1100 nm with 2 nm resolution) was used for all absorbance measurements. Samples were centrifuged in an IEC Centra-8 centrifuge purchased from the International Equipment Company of Needham Heights, MA. Centrifugation was carried out at 3500 rpm corresponding to approximately 3000 g.

The pH was measured with an Orion pH/ISE meter (model 710 A) equipped with a Ross® combination pH electrode (model 81-02), which were manufactured by Orion, Research Inc., Beverly, MA or alternatively with an Ion 83 Meter equipped with a combined electrode (model GK2401C), produced by Radiometer Copenhagen, Denmark.

Dissolved oxygen concentrations (mainly for BOD determinations) were quantified using a dissolved oxygen electrode manufactured by Orion Research Inc., Beverly, MA. Ammonia concentrations were determined with the Radiometer Copenhagen Ion 83 Meter combined with an ammonia selective electrode (ORION model 95-12).

Samples for COD measurement were digested in a COD reactor (model 45600) manufactured by the Hach Chemical Co., Loveland, CO. After the digestion of the samples the absorbance at a wavelength of 600 nm was measured using the Spectronic $20D^+$ spectrophotometer made by the Milton Roy Co., Rochester, NY.

Acute toxicity measurements were made with the Model 500 Toxicity Analyzer manufactured by Azur Environmental, Carlsbad, CA.

For semi-batch experiments, a Harvard 22 syringe pump from Harvard Apparatus, South Natick, MA was used to deliver concentrated solutions of the reagent (HRP or H_2O_2) into the reaction mixtures.

The Shimadzu spectroflourophotometer (model RF-540 V-4.0), manufactured by the Shimadzu Corporation, Kyoto, Japan, was used for fluorescence measurements.

3.2 Analytical Methods

3.2.1 Horseradish peroxidase activity assays

The activity of the HRP stock solution (5 mg of dry solid/mL) was measured routinely using a colorimetric assay containing 2.4 mM 4-AAP, 10 mM phenol and 0.2 mM H_2O_2 in 0.1 M phosphate buffer at pH 7.4. Prior to significant substrate depletion, activity was proportional to the rate of formation of a colored product that absorbs light at a peak wavelength of 510 nm with an extinction coefficient of 7100 M^{-1} cm^{-1} based on the conversion of H_2O_2 (Nicell and Wright, 1997). One unit of activity is defined as the number of micromoles of H_2O_2 consumed per minute at pH 7.4 and 25°C. The concentration of HRP in the activity assay was 0.25 mg/L (based on the weight of the dry solid). All activity measurements were carried out in triplicate. The standard deviation between triplicate analyses was usually below 5 %. The volumes of the stock solution to be supplied to the reactions were calculated based on the determined stock activity. The HRP preparation used contained 119 4-AAP-units per mg dry solid.

The molar concentration of the HRP stock solution was determined by measuring its absorbance (20-fold diluted) at a wavelength of 403 nm within 24 hours after the stock solution was made. The specific activity of the HRP preparation used was 18.9±0.6 U/nmol based on the extinction coefficient of the native HRP of 102 mM^{-1} cm^{-1} (Dunford and Stillman, 1976).

The 4-AAP method cannot be used to measure the activity over the course of a reaction due to interference by reaction products (Buchanan, 1996). Therefore, the activity of HRP during batch reactions was monitored using the ABTS (2,2`-Azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid] diammonium salt) based activity assay (Pütter and Becker, 1983), which was not affected by the presence of reaction products in the assay solution. The assay mixture (total volume 1.2 mL) contains 1.7 mM ABTS and 0.83 mM H_2O_2 in 0.067 M phosphate buffer at pH 6.0. The concentration of HRP in the activity assay was approximately 0.02 mg/L (based on the weight of the dry solid). Activity is proportional to the rate of formation of the resonance-stabilized radical ABTS· that absorbs light at 405 nm with an extinction coefficient of $1.86 \cdot 10^4$ M^{-1} cm^{-1}. One unit of ABTS activity is defined as the number of micromoles of H_2O_2 consumed per minute

at pH 6.0 and 25°C under the assay conditions. One unit based on the 4-AAP assay is equivalent to 1.2 units based on the ABTS assay.

3.2.2 Hydrogen peroxide assays

The HRP activity assays were modified in order to allow the measurement of hydrogen peroxide concentrations. The modified assay mixtures contained excess amounts of HRP and initially no H_2O_2. Following the addition of a sample containing limiting quantities of H_2O_2, reaction products formed that absorbed light in the visible range. The increase in the absorbance was recorded after a specified time period. All H_2O_2 assays were performed in duplicate.

The H_2O_2 assay that was based on the enzymatic oxidation of phenol in the presence of 4-AAP was performed under the following conditions: The concentration of HRP in the assay solution was 100 mg/L, while those of the other reactants (except peroxide) were as cited above for the 4-AAP activity assay. The absorbance at 510 nm (A_{510}; in absorbance units, au) was recorded 6 minutes after the addition of a sample containing H_2O_2 and converted to peroxide concentrations in the assay solution using the following calibration line ($R^2 = 0.997$) based on 6 standard concentrations:

$$[H_2O_2] = 0.163 \text{ mM/au} \times A_{510} \quad (3.1)$$

This calibration is valid up to a concentration of 0.07 mM of peroxide in the assay solution. Beyond this upper limit, the concentrations of other reagents in the assay begin to limit the quantity of color that can be formed in the assay within the incubation time.

The H_2O_2 assay that was based on the enzymatic oxidation of ABTS was conducted as follows. The concentration of HRP in the assay mixture was 4.2 mg/L, while those of the other reactants (except peroxide) were as cited above for the ABTS activity assay. The absorbance at 405 nm (A_{405}; in au) was recorded 6 minutes after the addition of a sample containing H_2O_2. The increase in the absorbance was proportional to the hydrogen peroxide concentration, provided this concentration did not exceed 0.02 mM in the assay solution. The following calibration line ($R^2 = 0.997$, based on 10 standard concentrations) was used to convert absorbance reading to H_2O_2 concentration in the assay solution:

$$[H_2O_2] = 0.0275 \text{ mM/au} \times A_{405} \quad (3.2)$$

3.2.3 Total phenol assay

The concentration of total phenols was measured using a colorimetric assay in which phenolic compounds react with 4-aminoantipyrine (4-AAP) and potassium ferricyanide under alkaline conditions to form a red quinone-type dye that absorbs light with a peak wavelength of 510 nm (Faust and Mikulewicz, 1967). The assay solution was composed of 2.08 mM 4-AAP and 8.34 mM potassium ferricyanide in 0.25 M sodium bicarbonate solution. The extent of color generation at 510 nm (in au) after a 6-minute incubation time was proportional to the concentration of phenols in the assay solution provided that their concentration did not exceed 0.1 mM as phenol. Absorbance readings were converted to total phenols concentration using the following calibration line based on phenol:

$$\text{Concentration of phenol in the assay solution} = F \times (A_{510}) \qquad (3.3)$$

The proportionality factor F ranged from 0.099 to 0.113 mM/au in five calibration procedures performed during this study, while the linear least square regression coefficients ranged from 0.984 to 1.000. The samples were analyzed in duplicate. The standard deviation of duplicate measurements was generally below 2 %. The lower level of detection for this assay was estimated to be 0.001 mM of phenol in the assay mixture. Similar calibration lines were also developed for the chlorinated and methylated phenols used in this study, except for 4-methylphenol (*p*-cresol) which does not react to a colored compound with 4-aminoantipyrine.

This assay is a modified version of the standard analytical procedure for phenols (APHA, 1998). It employs higher concentrations of 4-AAP and potassium ferricyanide, which allows for the measurement of higher phenol concentration than under the standard method while using smaller sample volumes.

The presence of chitosan, PEG, nitrite, iodide, thiosulfate, sulfite, sulfide, cyanide, thiocyanate, hexanes, toluene and phenanthrene at concentrations that were used in the experiments performed in this study did not interfere with the phenol measurement. Solutions containing 1 mM Co^{2+}, Mn^{2+} and Zn^{2+} or more than 0.15 M Ca^{2+} and Mg^{2+} had to be pretreated before the phenol analysis. Details are given in Section 3.3. All other salts used in this study did not interfere with the assay.

3.2.4 Phenol UV calibration

For some experiments, phenol concentrations were also measured based on the UV absorbance maximum of phenol at the wavelength of 269 nm (A_{269}; in au). The following calibration line ($R^2 = 0.999$) was obtained based on 10 standard concentrations:

$$[\text{phenol}] = 0.73\ \text{mM/au} \times A_{269} - 0.02\ \text{mM} \qquad (3.4)$$

This relationship is valid for phenol concentrations up to 1.1 mM.

3.2.5 Toxicity assay

Prior to toxicity analyses, samples were treated with catalase enzyme to remove any residual H_2O_2 and, when required, the sample pH was adjusted to between pH 6.0 and 8.0 using 0.1 N HCl and 0.1 N NaOH. Additionally, suspended solids were removed by centrifugation for 15 minutes at approximately 3000 g. Previous results have shown that neither catalase, nor phosphate buffer solution are toxic to the microorganisms used in the toxicity assay of this study (Ghioureliotis, 1997)

Acute toxicity was determined using the 5-min Microtox™ assay with the Model 500 Toxicity Analyzer according to the procedures for the Basic Test recommended by the instrument manufacturer (Azur Environmental, Carlsbad, CA).

The assay involves the recording of the light output of the luminescent marine bacterium *Photobacterium phosphoreum* after 5 minutes incubation with various dilutions of the sample. The test sample was adjusted to 2% NaCl by adding osmotic adjustment solution (22% NaCl in reagent water). Four serial 1:2 dilutions with diluent (2% NaCl in reagent water) were prepared of this sample and allowed to stabilize to 15±0.5°C. 0.5 mL aliquots of these sample dilutions were added each to 0.51 mL of bacteria suspension (also prepared in diluent and pre-incubated at 15±0.5°C). Thus, the volume fraction (VF) of the sample in the bacterial suspension (in total 1.01 mL) is calculated according to

$$VF = \frac{V_{sample}}{D \times (V_{sample} + V_{bacteria})} \qquad (3.5)$$

where V_{sample} is the volume of the diluted sample added to the bacteria (0.5 mL), $V_{bacteria}$ is the volume of the bacteria suspension (0.51 mL) and D is the pre-dilution factor of the sample.

The light output of the bacteria was recorded immediately before and 5 minutes after the addition of the sample to the bacteria suspension. A control was run concurrently with each test to account for the normal drop of the light output during the 5 minutes incubation time. Gamma (Γ), the ratio of light lost to light remaining, is calculated according to the following expression:

$$\Gamma = \frac{C \times I_0 - I_t}{I_t} \tag{3.6}$$

where, I_0 is the light reading before sample addition, I_t the light level after 5 min, and C is the control ratio, which is the fraction of light remaining in the control after 5 minutes. The control ratio C was between 0.75 and 0.9 during this study, which is within the normal range as specified by the instrument manufacturer. The volume fraction of the sample in the final diluted mixture that causes a 50% decrease in light output is reported as the effective concentration, or EC_{50} value; the corresponding inverse number is the TU_{50} value. A plot of Γ versus the volume fraction of the sample produces a curve from which the EC_{50} can be determined. That is, the EC_{50} corresponds to a sample concentration at which gamma is equal to one.

The recorded light output values have to be corrected for samples exhibiting significant coloration in the EC_{50} concentration range. The color correction procedure was carried out according to a method supplied by Azur Environmental. This method involves using a spectrophotometer to record the transmission at the wavelength of 480 nm for each sample dilution. These recorded transmission values are multiplied with the respective light readings before sample addition (I_0) in order to obtain corrected I_0 values which are inserted into equation (3.5) to yield the corrected Gamma values.

Using the basic test procedure, the highest volume fraction of the sample for which the Γ-value can be determined is 0.45 according to equation (3.4), because the lowest possible value for the pre-dilution factor D is 1.1 due to the required addition of the osmotic adjustment solution. Consequently, EC_{50} values higher than 0.45, corresponding to a $TU_{50} < 2.2$, can only be estimated through extrapolation from the dose-response curve. Toxicity estimations become less accurate for samples of low toxicity, since it becomes difficult to distinguish a trend from background noise, particularly for very flat

dose-response curves. A TU_{50} value of less than 1 indicates that the sample has to be concentrated to become sufficiently toxic to reduce the light output of the bacteria by 50%.

The quality of the measurements was monitored routinely by assaying a 100 mg/L phenol standard solution in diluent. Sixteen measurements yielded an EC_{50} of 21.2 ± 2.1 mg/L (95% confidence interval) for phenol. This value is within the normal range according to the instrument manufacturer.

3.2.6 Biochemical oxygen and chemical oxygen demands

The 5-day biochemical oxygen demand (BOD_5) and the chemical oxygen demand (COD) were determined according to Method 5210 B and Method 5220 D in Standard Methods (APHA, 1998), respectively.

3.2.7 Solids

Total solids (TS) and total suspended solids (TSS) were determined according to Methods 2540 B and 2540 D in Standard Methods (APHA, 1998), respectively.

3.2.8 Lignin assay

The lignin content of the foul condensate wastewater from Kraft pulping was determined according to Method 5550 B (Tannin and Lignin) in Standard Methods (APHA, 1998). Folin and Ciolcalteau reagent (0.02 mL) and carbonate-tartrate reagent, which was prepared according to Method 5550 B, (0.2 mL) were added to a 1 mL solution consisting of 0.1 mL of sample and 0.9 mL of distilled deionized water. The extent of color generation at 700 nm after 30 minutes incubation at 25°C is a measure of the content of hydroxylated aromatics in the sample. The extent of color generation was converted to an equivalent phenol concentration using a calibration curve based on pure phenol.

3.2.9 Sulfide

The concentration of sulfide in the petroleum refinery wastewater was measured according to Method 4500-S^{2-}F in Standard Methods (APHA, 1998).

3.2.10 Ammonia

The concentration of ammonia was measured using an ammonia selective electrode attached to a voltmeter. Four standard solutions in distilled-deionized water were prepared containing 0.1, 1, 10 and 100 mg/L ammonia nitrogen (in form of NH_4Cl). 1 mL 10 N NaOH was added to 100 mL of these solutions in order to convert the ammonia to ammonium. The samples were stirred and the voltage was recorded yielding the following calibration line ($R^2 = 0.999$):

$$\text{Log ([ammonia-N], in mg/L)} = -0.0184 \times \text{Voltage (mV)} + 2.21 \qquad (3.7)$$

The concentrations of ammonia nitrogen in the refinery wastewater were determined using an identical procedure and calculated based on this calibration line.

3.2.11 Phenanthrene

Phenanthrene concentrations were determined with the help of a spectroflourometer by recording the light emitted at the peak wavelength of 346 nm (E_{346}; in emission units, eu) after excitation at a wavelength of 250 nm. The following instrument parameters were used: abscissa scale - ×2; ordinate scale ×2; sensitivity - high; emission slit - 2 nm; excitation slit - 10 nm; scan speed - medium. The wastewater matrix has a significant impact on the intensity of the fluorescence due to the interference of compounds that quench the emitted light. Therefore, separate calibration lines were constructed for wastewater samples that have or have not undergone HRP-treatment by adding 100 to 600 µg/L phenanthrene to either untreated or HRP-treated and coagulated wastewater samples and recording the light output at 346 nm using an excitation wavelength of 250 nm. For the samples that had not been treated with HRP, the following calibration line ($R^2 = 0.998$) was obtained:

$$\text{Phenanthrene (µg/L)} = 23.0\ \text{(µg/L)/eu} \times E_{346} - 269.2\ \text{µg/L} \qquad (3.8)$$

For the samples that had been treated with HRP, the calibration line ($R^2 = 1.000$) was as follows:

$$\text{Phenanthrene (µg/L)} = 12.9\ \text{(µg/L)/eu} \times E_{346} - 77.7\ \text{µg/L} \qquad (3.9)$$

3.3 Experimental Procedures

Synthetic wastewaters were prepared by adding phenolic stock solutions to defined volumes of buffer or reagent grade water. The concentration of the phenolic stock solutions was 400 mM in the case of phenol and 20 mM in the case of the other phenols used in this study. They were prepared by dissolving the solid phenols in reagent grade water. Small amounts of methanol were added to chlorophenol stock solutions in order to enhance their solubility.

Most batch experiments were conducted in tightly closed borosilicate glass vials of 20-mL capacity. For experiments requiring larger reaction volumes, glass beakers of 100 to 150 mL capacity were used. The beakers were sealed with parafilm. Reaction solutions were stirred using magnetic stirring bars. Unless otherwise stated, reactions were carried out in a constant temperature room maintained at 25 ± 1 °C.

All buffers were prepared by the methods of Gomori (1955).

3.3.1 The use of chitosan as an additive

Chitosan stock solutions were made by dissolving 1 g dry powder in 100 mL 1 % acetic acid (Kawamura, 1991) and stored at 4°C. Reaction mixtures containing chitosan were prepared by adding chitosan stock solutions into 0.1 M sodium phosphate buffer (pH 7.0) containing phenol. Reactions were initiated by the addition of concentrated solutions of HRP and H_2O_2. The reactions were allowed to go to completion by providing a 20 hours reaction time.

3.3.2 Toxicity reduction

Reaction mixtures were prepared by dissolving stock solutions of phenols, H_2O_2, and additives (where applicable) in 0.1 M sodium phosphate buffer at pH 7.0.

Batch reactions were initiated by adding an aliquot of peroxidase stock solution (5 mg/mL) to the reaction mixture. The initial reaction volume was 50 to 100 mL. Experiments demonstrated that 3 hours was sufficient reaction time to ensure maximal phenol conversion. Thus, after 3 hours the batch was divided into 20 mL sub-samples that were placed in tightly sealed borosilicate glass vials of 20-mL capacity. When indicated, samples were filtered through 0.45 μm filters (Millipore, Bedford, MA).

Semi-batch reactions were carried out in 312 mL-polypropylene jars (sealed with screw caps) filled with 250 mL phenol solutions containing HRP or H_2O_2. Concentrated peroxide or enzyme solutions were continuously delivered into the reaction mixture using a syringe pump connected to feed inlets placed near the bottom of each reactor.

To examine the effect of ultraviolet illumination on reaction products, a collimated beam apparatus with a low-pressure mercury lamp emitting UV light at 254 nm was used to illuminate HRP-treated phenol solutions from which more than 98% phenol had been removed. Samples were stirred constantly during illumination.

The average radiation intensity (I_{avg}) inside an irradiated volume of a stirred sample was calculated according to the method of Morowitz (1950):

$$I_{avg} = I_0 \left(\frac{1 - e^{\left(-\frac{d}{l} \times \ln \frac{1}{T}\right)}}{\frac{d}{l} \times \ln \frac{1}{T}} \right) \tag{3.10}$$

where

I_0 = incident radiation intensity (mW/cm^2), measured with a radiometer;

d = depth of the sample under UV irradiation (cm);

l = the cell path length (cm);

T = transmittance of the sample at 254 nm.

The following values were used: I_0 = 1.7 mW/cm^2, d = 2 cm, l = 1cm, T = 0.83.

To convert the average radiation intensity (I_{avg}) into the corresponding volumetric radiation intensity (I_{vol}) with the units of milliwatts per liter (mW/L) the following equation was employed:

$$I_{vol} = I_{avg} \frac{A}{V} \tag{3.11}$$

where A stands for the illuminated surface area of the sample and V for the sample volume (A = 2.54 cm^2, V = 0.01 L).

3.3.3 Impact of wastewater parameters

Phenol removal reactions were initiated by the addition of concentrated solutions of HRP (5 mg/mL) and subsequently H_2O_2 (200 or 400 mM) to solutions containing 1 mM phenol and the wastewater constituent that was to be tested. Unless otherwise stated, a 20-hour reaction time was provided to ensure that the enzymatic reactions had gone to completion.

Samples containing suspended solids were prepared by directly weighing the appropriate amount of material into 20 mL-glass vials. Subsequently, the vials were filled with 15 mL of 1.0 mM phenol solutions and stirred vigorously. In the experiments involving aged suspensions, the solid suspensions (10000 mg/L) were stirred overnight in their respective buffers. Solids were removed from suspensions by centrifugation for 15 minutes at 3000 g. Pre-incubations of HRP or H_2O_2 were carried out in 0.05 M sodium phosphate buffer (pH 7.0) in the absence of phenol.

For experiments involving reducing anions, concentrated (10 g/L) solutions of the anions were prepared in distilled-deionized water. The appropriate volumes of these solutions were added to the reaction mixtures. Fresh concentrated solutions of the sodium salts of iodide, nitrite, sulfite, thiosulfate and sulfide were prepared before each experiment. The concentrated solutions of sodium cyanide and sodium thiocyanate were prepared once and stored at room temperature. When measuring phenol concentrations over the course of the reaction, aliquots of catalase enzyme solution (final concentration 0.05 mg/mL) were added to quickly halt the phenol oxidation reaction. Catalase accomplishes this by rapidly catalyzing the transformation of hydrogen peroxide to oxygen and water. UV absorbance measurements were performed after centrifugation.

For the experiments with metal ions, 100 mM-stock solutions of the nitrate or sulfate metal salts were prepared in distilled-deionized water and stored at 4°C. Shortly before the experiment, these solutions were added to 0.05 Tris-HCl buffer (pH 7.2) or distilled-deionized water containing 1.0 mM phenol. The phenol/metal solutions prepared in water were adjusted to pH 5.0 using solutions of 0.1 N NaOH, 1.0 N NaOH and/or 0.1 N H_2SO_4. The amount of sodium introduced in this manner into the reaction mixtures was approximately 3 mM and 2 mM in the case of the solutions containing Fe(III) and Cr(III), respectively, and below 0.1 mM for the other solutions, while the maximum amount of sulfate introduced was 0.1 mM. The presence of Co^{2+}, Mn^{2+} and Zn^{2+} ions caused

precipitation during the phenol assay. Therefore, these ions were removed through sulfide precipitation prior to the analysis of residual phenol. Sulfide was added to the samples at 3.8 mM in the form of a concentrated solution of Na_2S. The coagulation of the metal sulfide particles was enhanced by the addition of chitosan (64 to 127 mg/L) and NaOH (0.64 mM). In the case of the samples containing Co^{2+}, coagulation of the black colloidal CoS additionally required the use of alum ($Al_2(SO_4)_3$) at 97 mg/L.

For experiments involving organic compounds, stock solutions of toluene (10 g/L in 60% methanol), hexanes (10 g/L in 80% methanol) and phenanthrene (1.24 g/L in 100% methanol) were prepared. The appropriate volumes of the toluene and hexanes stock solutions were added to 0.1 M sodium phosphate buffer (pH 7.0) followed by the addition of phenol at 1.0 mM. The phenanthrene stock solution was diluted further to obtain solutions of 25, 50, 75 and 100 mg/L in methanol, which were used to prepare 0.25, 0.5, 0.75, 1.0 and 5.0 mg/L solutions in 0.1 M sodium phosphate buffer (pH 7.0), which was followed by the addition of phenol at 1.0 mM. In order to avoid adsorption of phenanthrene to plastic pipette tips, glass pipettes were used for transferring solutions containing phenanthrene.

Samples containing NaCl, $CaCl_2$, $MgCl_2$, NH_4Cl and $(NH_4)_2SO_4$ were prepared by dissolving the appropriate amount of salts in distilled-deionized water containing 1.0 mM phenol. Subsequently, the pH of these solutions was adjusted to 5.9 using 0.1 M NaOH and 0.2 M HCl. No precipitation of phenolic reaction products occurred in samples containing low amounts of salts. In these cases, the reaction products were coagulated using 90 mg/L alum and low amounts of NaOH. Samples containing high concentrations of Ca^{2+} and Mg^{2+} caused precipitation in the phenol assay. Therefore, the content of these ions was reduced prior to phenol analysis by precipitation using a 0.6 M solution of $NaHCO_3$.

3.3.4 Treatment of a foul condensate wastewater

The reaction volume for the batch reactions was 10 mL. Wastewater samples were allowed to equilibrate to the reaction temperature (25°C) before hydrogen peroxide was added as a concentrated solution (200 mM). Reactions were initiated by adding an aliquot of HRP stock solution to the stirred reaction mixture. The vials were immediately closed

to avoid volatilization and the solutions were stirred for 15 min. A minimum 3-hour reaction time was provided to ensure that the reaction had gone to completion. Prior to phenol analysis, samples were coagulated with alum (74 to 82 mg $Al_2(SO_4)_3$ /L) and centrifuged for 15 minutes at approximately 3000 g.

For experiments in which the foul condensate was spiked with 2 mM pure phenol, aliquots of a 400 mM phenol stock solution were used.

During time-course experiments, aliquots were removed from the reaction solution and diluted 5 to 10 times. 50 µL of the diluted samples were subsequently added into the ABTS activity assay mixture. In order to facilitate the measurement of phenol concentration over the course of the reaction, aliquots of catalase enzyme solution were added to samples at 0.05 mg/mL to quickly halt the phenol oxidation reaction. Catalase accomplishes this by rapidly catalyzing the transformation of hydrogen peroxide to oxygen and water.

2.2 g/L of alkali lignin were added to wastewater samples or to aqueous solutions for the preparation of reaction mixtures containing lignin. After stirring the suspensions overnight, particulate lignin was removed by filtration (0.45 µm pore size, Millipore, Bedford, MA) and the pH was adjusted to the desired level.

In experiments designed to study the effect of wastewater components and/or of lignin, coagulation was carried prior to the start of the reaction. Particulate matter was removed by filtration (0.45 µm pore size, Millipore, Bedford, MA) and the pH was adjusted to the required level with 0.1 N NaOH before phenol was added.

Control samples were treated concurrently with either peroxide or HRP alone. No changes in the phenol concentrations were observed in these samples.

3.3.5 Treatment of a petroleum refinery wastewater

Two samples were obtained from a petroleum refinery plant downstream of a dissolved air flotation unit. Sample A originally contained 0.47 mM (44.6 mg/L) total phenols as phenol and was initially stored at 4°C. After the phenol concentration had dropped to 0.41 mM, the remaining sample was preserved by freezing at –20°C. Sample B, with 0.35 mM total phenols was stored at –20°C upon arrival in the lab.

The reaction volume in the batch experiments was 15 mL. Wastewater samples were allowed to equilibrate to room temperature (20±2°C) before HRP was added as a highly concentrated stock solution. Enzymatic reactions were initiated by adding an aliquot of 200 mM H_2O_2 solution to the stirred reaction mixture. The vials were immediately closed to avoid volatilization and the solutions were stirred for 15 min. Unless otherwise stated, a 12-hour reaction time was provided to ensure that the reaction had gone to completion.

In order to remove the colored colloidal reaction products, the solutions were coagulated with 55 mg $Al_2(SO_4)_3$/L and centrifuged prior to phenol analysis.

In the case of the semi-batch experiments, a syringe pump was used to continuously deliver a concentrated peroxide solution to 75 mL of stirred wastewater to which enzyme had been added.

For experiments involving the co-precipitation of phenanthrene, this compound was added to wastewater samples in the form of a stock solution of 1 g/L in methanol. In order to minimize phenanthrene adsorption, glass pipettes were used instead of plastic pipette tips for transferring solutions containing phenanthrene.

Control samples were treated concurrently with either peroxide or HRP alone in the presence or absence of additives. No changes in the phenol concentrations were observed in these samples.

4. THE USE OF CHITOSAN AS AN ADDITIVE

The study of chitosan for the use as an additive was partially motivated by the observation of Ganjidoust *et al.* (1996) that very low concentrations (2 mg/L) of this material were effective in reducing enzyme requirements. Also, the abundance of this natural polymer, its inherent biodegradability, and the possibility that it may adsorb some toxic reaction products such as quinones were important reasons to conduct this investigation.

4.1 Results

4.1.1 Impact of chitosan type

Chitosans of viscosity grades ranging from 10 to 5700 centipoises (cps) were tested for their ability to enhance enzymatic phenol removal. The results of this experiment are shown in Figures 4.1(a) and (b). All chitosans substantially increased phenol conversion when added to the reaction solutions at 50 mg/L. However, the chitosans were much less effective when used at 10 mg/L. Chitosan of a medium viscosity (420 cps) was found to have the strongest effect and was therefore used in all further experiments. Phenol concentrations did not decrease when only chitosan and H_2O_2 were added to the reaction mixtures. Also, the addition of chitosan after the enzymatic reaction reached completion (usually after 3 hours) did not result in additional phenol conversion. Thus, chitosan enhanced the enzymatic phenol oxidation but was not able to accomplish phenol removal in the absence of active HRP.

4.1.2 Optimum chitosan dose

The minimum chitosan dose that had an optimum effect in promoting phenol removal was determined in batch reactions using 0.025, 0.5 U/mL and 1.0 U/mL HRP at an initial phenol concentration of 1.0 mM (Figure 4.2).

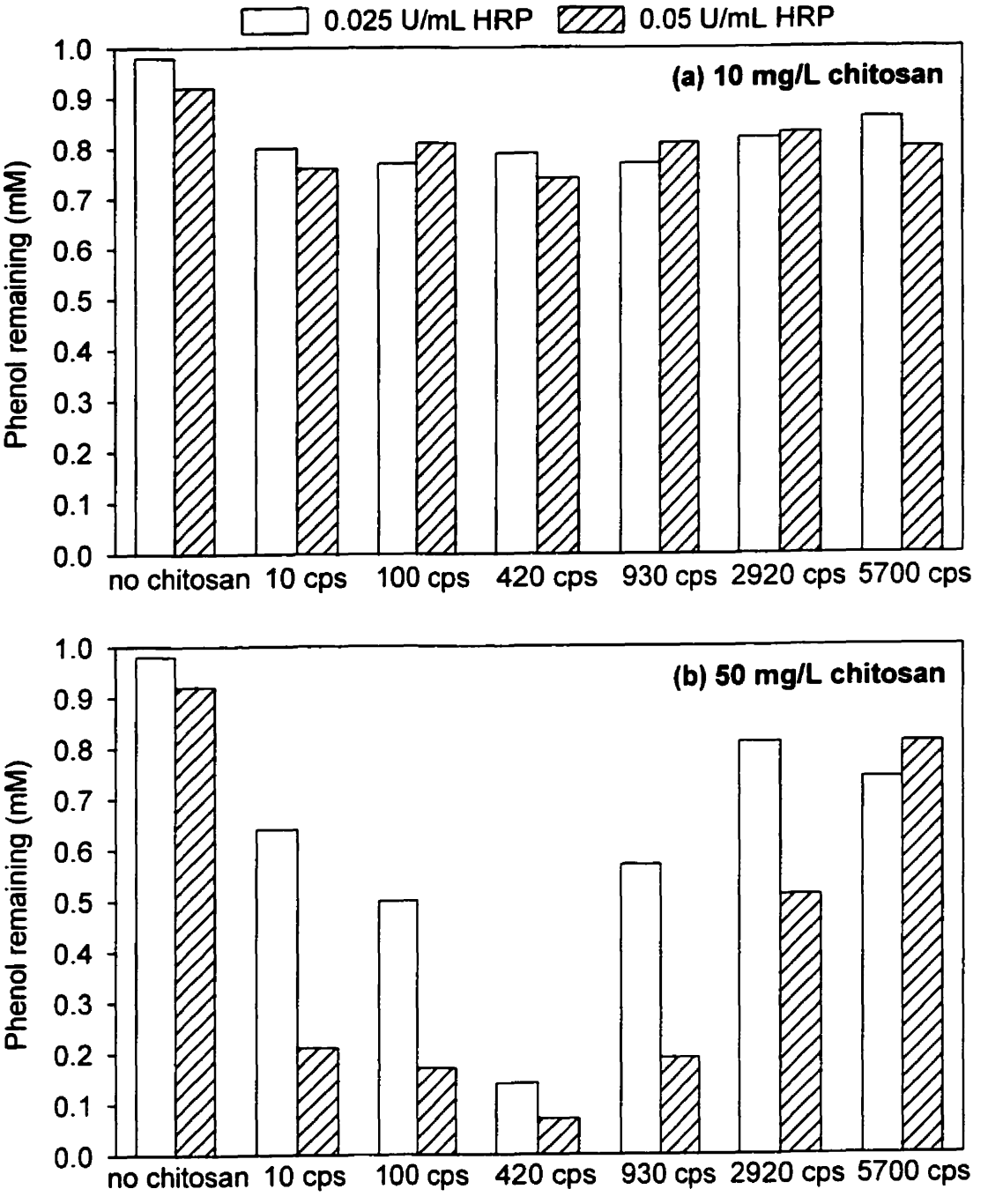


Figure 4.1: Impact of the type of chitosan on phenol removal at (a) 10 and (b) 50 mg/L. $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.2$ mM.

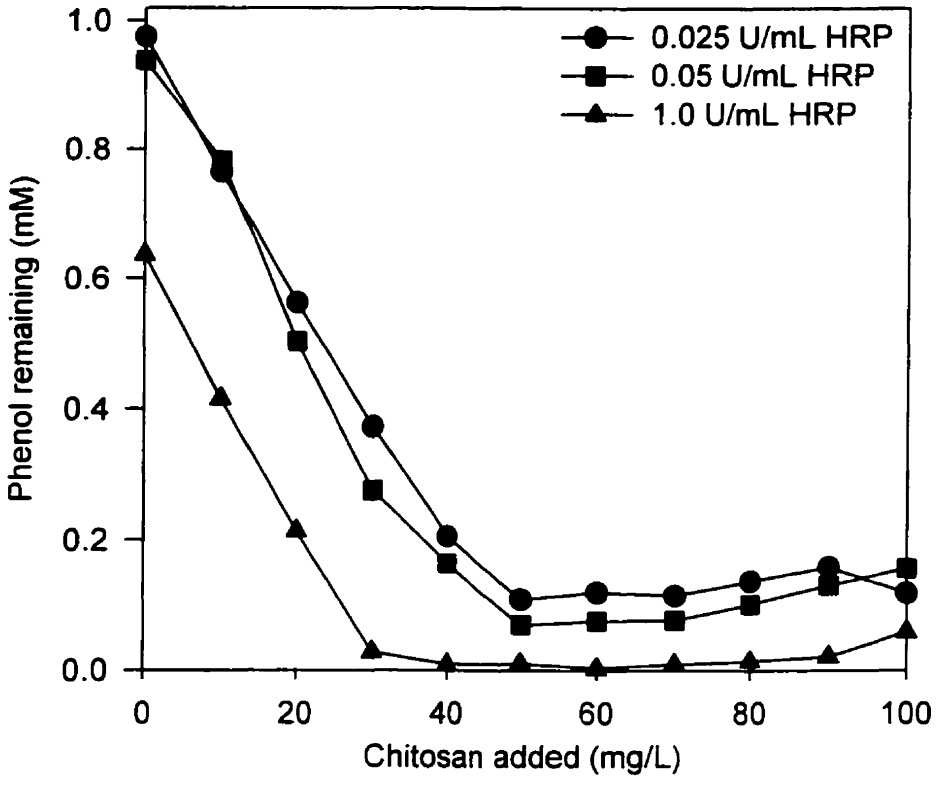


Figure 4.2: Phenol remaining as a function of the amount of chitosan added. $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.2$ mM.

From this figure it was determined that 50 mg/L was the optimum dose. Chitosan doses exceeding 70 mg/L had an adverse effect on phenol removal. For example, when the chitosan concentration was increased to 200 mg/L at 0.5 U/mL the residual phenol concentration increased to 0.2 mM (see Figure B.1 in Appendix B); however, no further deterioration in treatment efficiency was observed when the chitosan dose was raised further to 600 mg/L.

Similar reactions were carried out with initial phenol concentrations up to 10 mM (see Appendix B). From the resulting graphs, the minimum chitosan concentrations that would lead to a maximum enhancement of phenol removal were determined for each initial phenol concentration. These values were plotted as a function of the initial phenol concentration as shown in Figure 4.3. A linear relationship between the optimum chitosan dose and the initial phenol concentration was obtained.

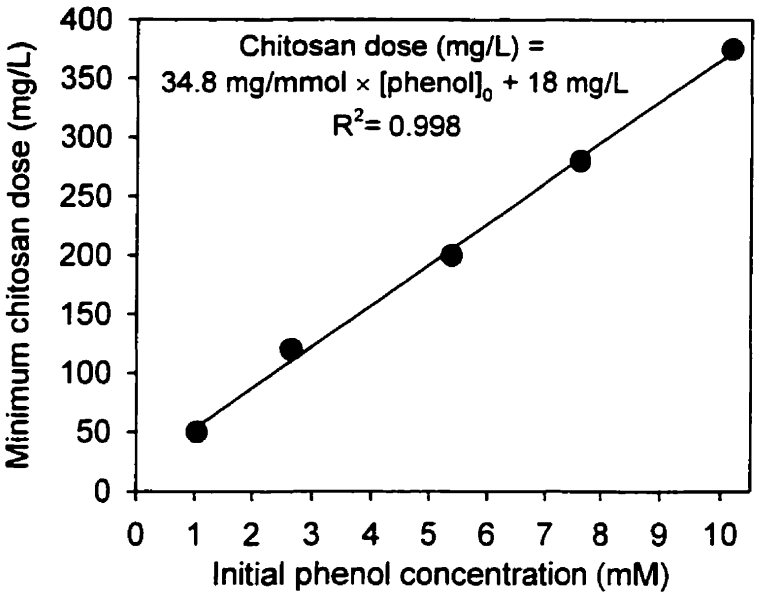


Figure 4.3: Minimum chitosan dose required to achieve maximum phenol conversion as a function of the initial phenol concentration.

When limiting quantities of chitosan were used, the amount of phenol removed increased linearly with increasing chitosan doses. This can clearly be seen from Figure 4.4, which shows a plot of the amount of additional phenol removed due to chitosan as a function of the quantity of chitosan provided to the reaction mixture for different initial phenol concentrations.

The additional phenol that was removed due to the presence of chitosan was calculated by subtracting from each data point the quantity of phenol that was removed under identical conditions in the absence of chitosan, which amounted to 3 to 30% of the initial phenol concentration, depending on the enzyme dose employed. Figure 4.4 shows that the amount of phenol removed increased linearly until reaching a plateau level. The location and height of the plateau depended on the initial phenol concentration and the HRP dose applied. When excess amounts of HRP were used, the amount of phenol removed due to chitosan leveled off at lower chitosan doses, since a large portion of phenol was removed already in the absence of the additive. Least square linear regression analysis of data points lying below their respective plateau levels yielded a slope of 0.027 mmol phenol removed/mg chitosan, which compares well with the inverse slope of

Figure 4.3 (0.029 mmol/mg). This suggests that there was a stoichiometric relationship between the phenol removed and the concentration of chitosan in the reaction mixture.

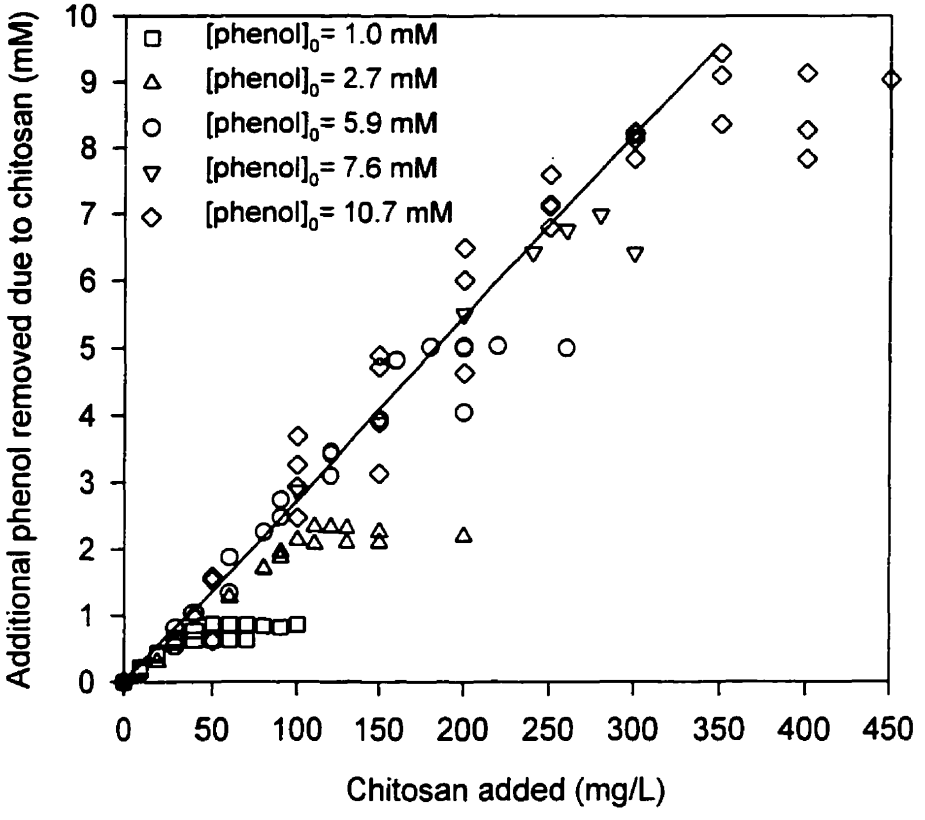


Figure 4.4: Phenol removed due to chitosan as a function of the chitosan dose.

However, a closer examination of Figure 4.4 reveals that the data points obtained with low initial phenol concentrations tended to lie below the straight line calculated by regression. Thus, the value of the stoichiometric relationship between phenol removed and chitosan added may be dependent on the initial phenol concentration or possibly on the enzyme dose used. In order to test these possibilities, the slopes of the linear portions of the data in Figure 4.4 were calculated separately for each data series. Figure 4.5 presents these slopes along with their respective 95% confidence intervals as a function of the initial phenol concentration. The mean value of all 13 slopes was 0.025±0.003 mmol phenol removed/mg chitosan. These slopes were independent of the enzyme dose for data obtained with identical initial phenol concentrations. However, statistical analysis indicated that there was a correlation between the slopes and the initial phenol concentrations. Nevertheless, the wide confidence intervals for each data point make the

validity of this correlation questionable. Therefore, the hypothesis that the amount of phenol removed per quantity of chitosan added is independent on the initial phenol concentration cannot be rejected based on the available data.

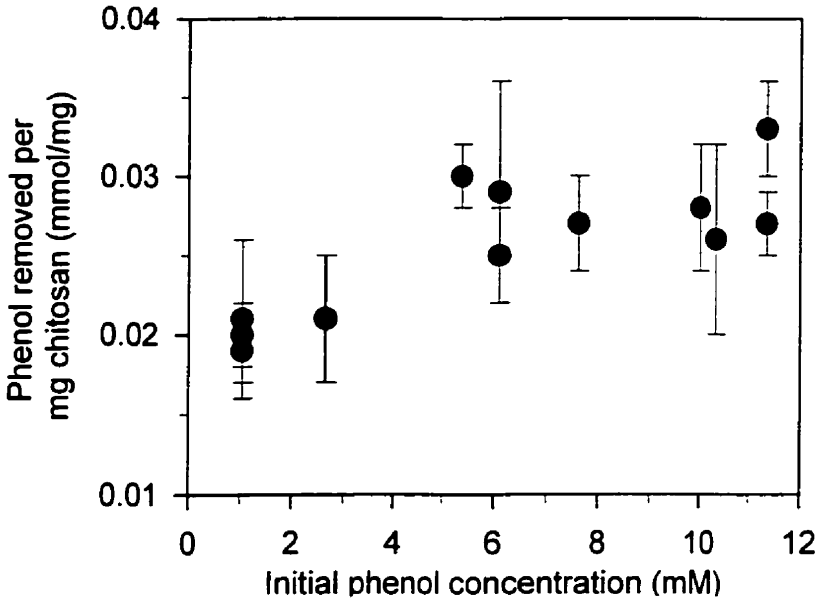


Figure 4.5: Values of the slopes of phenol removed per mg chitosan with their respective 95% confidence intervals.

Chitosan was also effective for the treatment of chloro-and methylphenols as shown in Figure 4.6. During the treatment of 2-methylphenol and 2,4-dichlorophenol, 40 mg/L of chitosan was sufficient.

However, the treatment of 2-chlorophenol and 4-chlorophenol required approximately 70 to 80 mg/L of chitosan. Thus, as in the case of polyethylene glycol with a molecular weight of 3350 Daltons (PEG_{3350}) (Wu *et al.*, 1993a), the amount of chitosan required to achieve a maximum effect was dependent on the type of phenol treated.

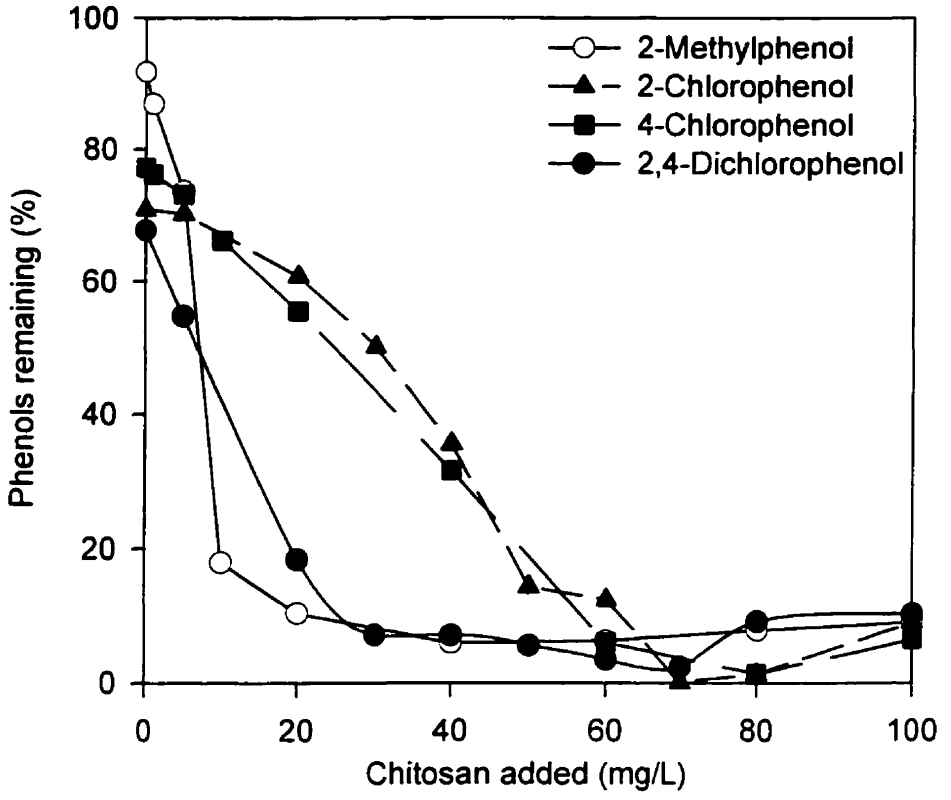


Figure 4.6: Removal of substituted phenols as a function of the chitosan dose. $[phenol]_0 = 1$ mM; $[H_2O_2]_0 = 1.2$ mM; $HRP_0 = 0.1$ U/mL for 2-MP, 2-CP and 2,4-DCP; 0.05 U/mL for 4-CP.

4.1.3 HRP requirements in the absence and presence of additives

Further experiments were conducted to quantify the HRP doses required for treatment conducted in the presence and absence of chitosan. In order to compare the effectiveness of chitosan with that of another polymeric additive, enzyme requirements in the presence of PEG_{35000} were also estimated using experimental results reported by Ghioureliotis (1997).

Figure 4.7 shows the fraction of phenol removed as a function of the relative enzyme dose for initial phenol concentrations ranging from 1 to 10 mM in the absence of additives.

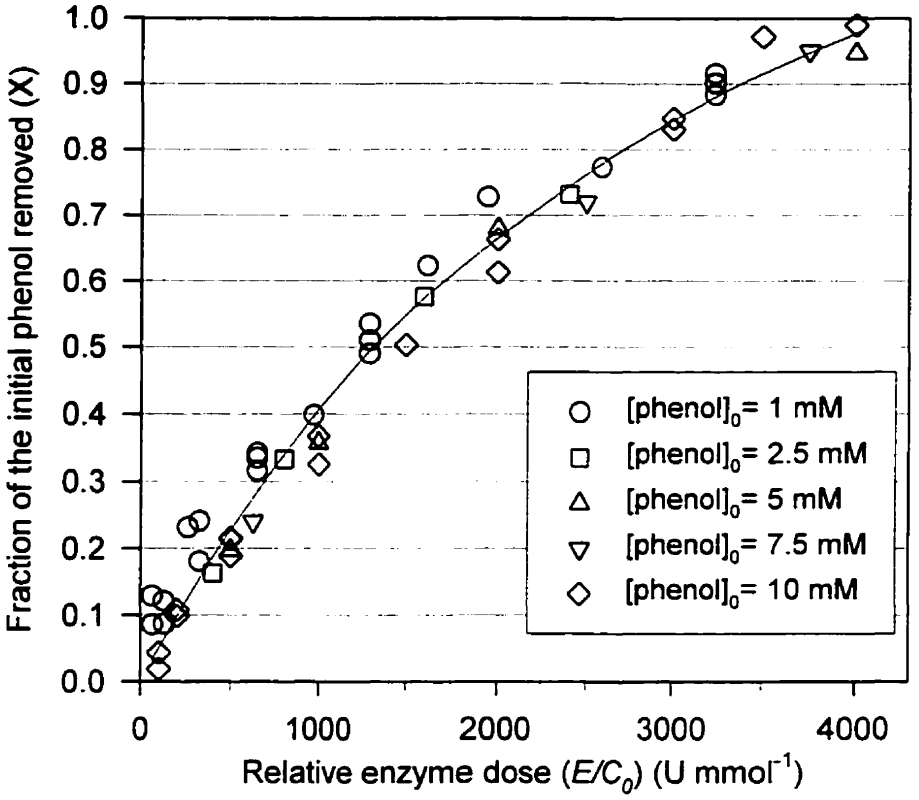


Figure 4.7: Fraction of phenol removed as a function of the relative enzyme dose in the absence of additives. $[H_2O_2]_0$ /$[phenol]_0$ = 1.2

The relative enzyme dose (E/C_0) is defined here as the amount of HRP added per initial phenol concentration and is expressed in units of U·mmol^{-1}. The excellent overlay of the data points obtained with different initial phenol concentrations implies that the amount of HRP required to achieve a specified degree of treatment was proportional to the initial phenol concentration. The resulting curve was described using the following equation

$$X = \frac{C_0 - C}{C_0} = a\frac{E/C_0}{(E/C_0 + b)} \qquad (4.1)$$

where X = fraction of phenol removed, C_0 = initial phenol concentration (mM), C = phenol concentration after the completion of the enzymatic reaction (mM), E = enzyme dose (U·L^{-1}), and a and b are curve-fit parameters (a = 1.840; b = 3527 U·mmol^{-1}).

Substantially less HRP was needed when treatment was conducted in the presence of additives. Figure 4.8 shows a typical result obtained for the treatment of an initial phenol concentration of 5.2 mM in the presence of 180 mg/L of chitosan.

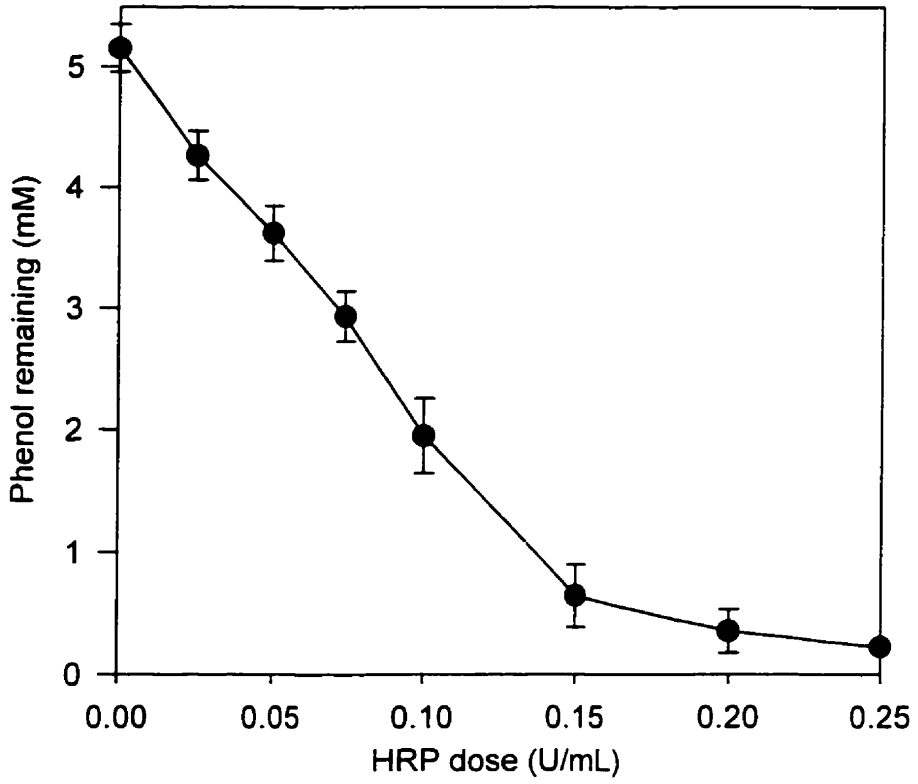


Figure 4.8: Removal of phenol as a function of the HRP dose in the presence of chitosan. $[phenol]_0$ = 5.2 mM; $[H_2O_2]_0$ = 6.0 mM; 200 mg/L chitosan. Error bars stand for the standard deviation of 3 independent experiments.

Similar graphs were prepared for solutions with initial phenol concentrations ranging from 1 to 10 mM phenol and are shown in Appendix B. For intermediate levels of treatment, the relative enzyme dose required to achieve a specified degree of phenol removal was higher at higher initial phenol concentrations. Therefore, as shown in Figure 4.9, an overlay of the data points did not produce a single curve. However, the lines for different initial phenol concentrations converge when the fraction of phenol removed approaches 0.9 (Figure 4.9). This indicates that for higher levels of treatment, enzyme requirements increased linearly with increasing initial phenol concentration.

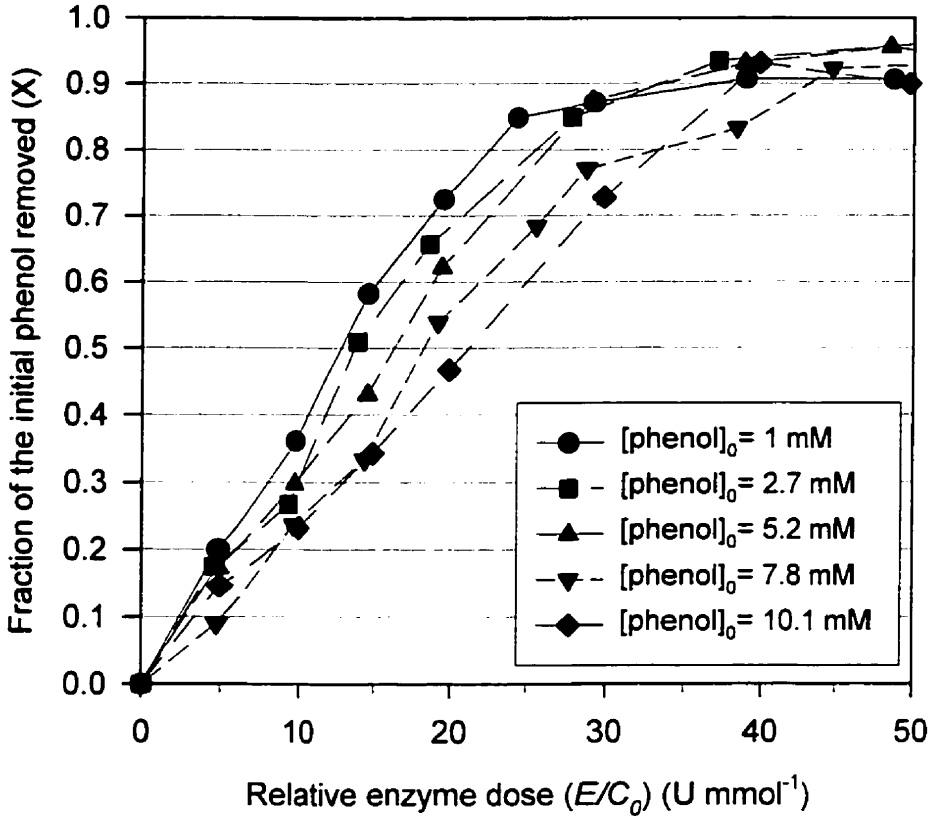


Figure 4.9: Fraction of phenol removed as a function of the relative HRP dose in the presence of the optimum chitosan concentrations (50 to 375 mg/L). $[H_2O_2]_0/[phenol]_0 = 1.2$.

Figure 4.10 shows the fraction of phenol removed as a function of the relative enzyme dose for the treatment of solutions initially containing 0.5 to 10 mM phenol in the presence of PEG_{35000}. Although the data for 0.5 and 1.0 mM phenol are quite scattered, an overlay of all data points produced a single curve as in the case without additives.

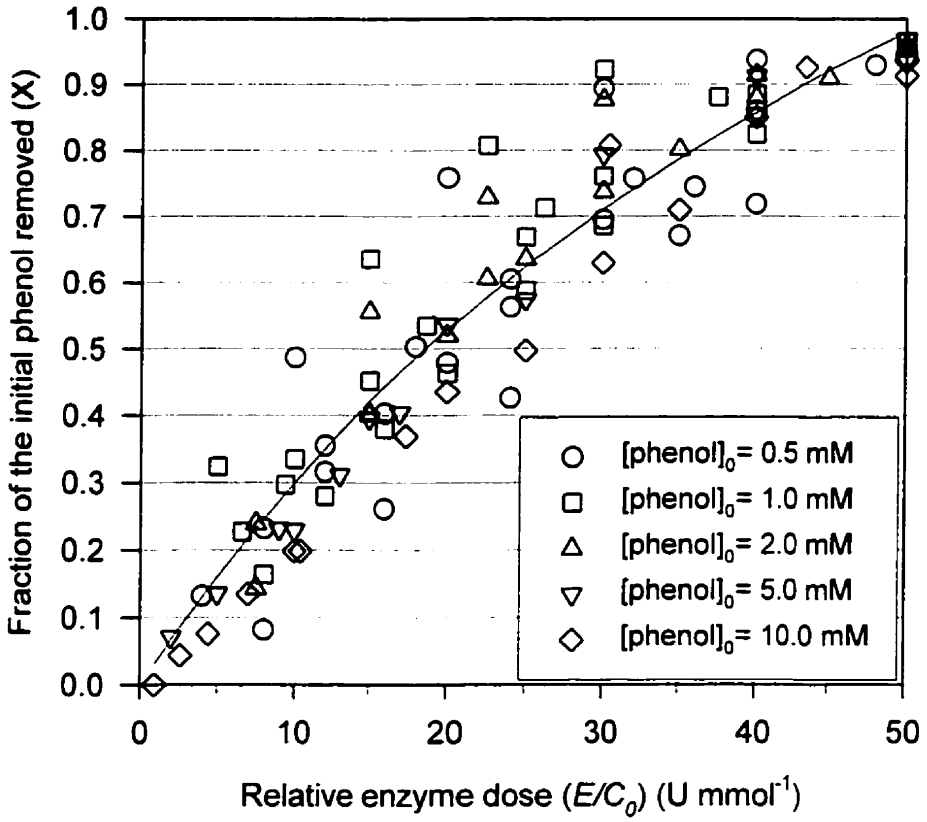


Figure 4.10: Fraction of phenol removed as a function of the relative HRP dose in the presence of 320 mg/L of PEG_{35000}. (Data adapted from Ghioureliotis, 1997). $[H_2O_2]_0/[phenol]_0 = 1.5$

In the absence of protective additives, on average 3400 units of HRP were needed per mmole initial phenol to obtain 90 % phenol removal based on equation 4.1. Enzyme requirements in the presence of chitosan and PEG_{35000} were estimated for each initial phenol concentration and plotted as shown in Figure 4.11 and 4.12, respectively. Using least square regression analysis it was determined that 90 % treatment could be achieved with, on average, 39 ± 3 U·mmol^{-1} in the presence of chitosan. Treatment in the presence of PEG_{35000} required an almost identical quantity of enzyme (42 ± 1 U·mmol^{-1}). Thus, the use of chitosan or PEG_{35000} resulted approximately in an 85–fold reduction in HRP requirements.

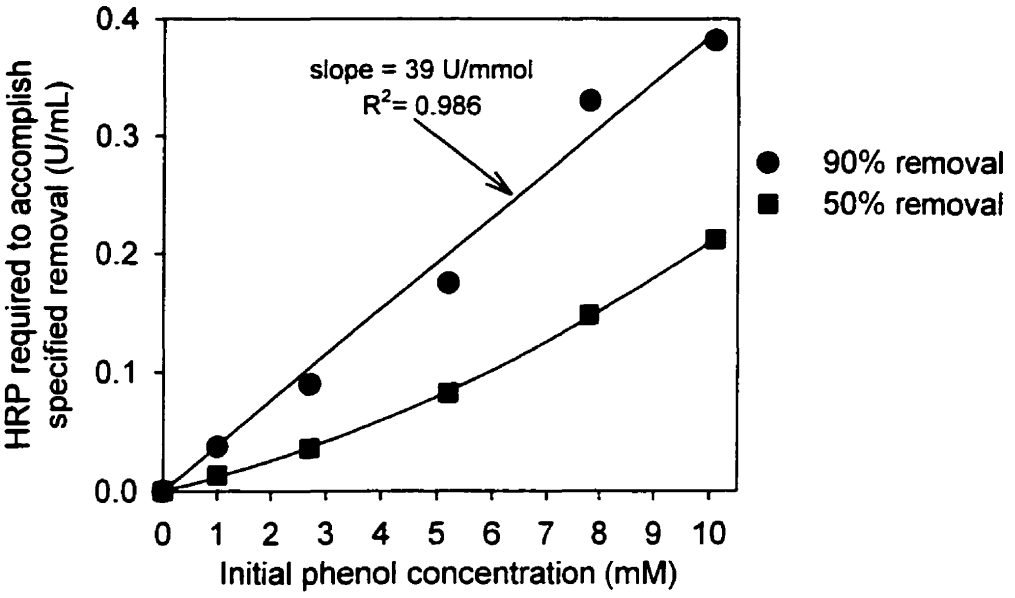


Figure 4.11: HRP requirements for 90 and 50 % phenol removal in the presence of optimum amounts of chitosan.

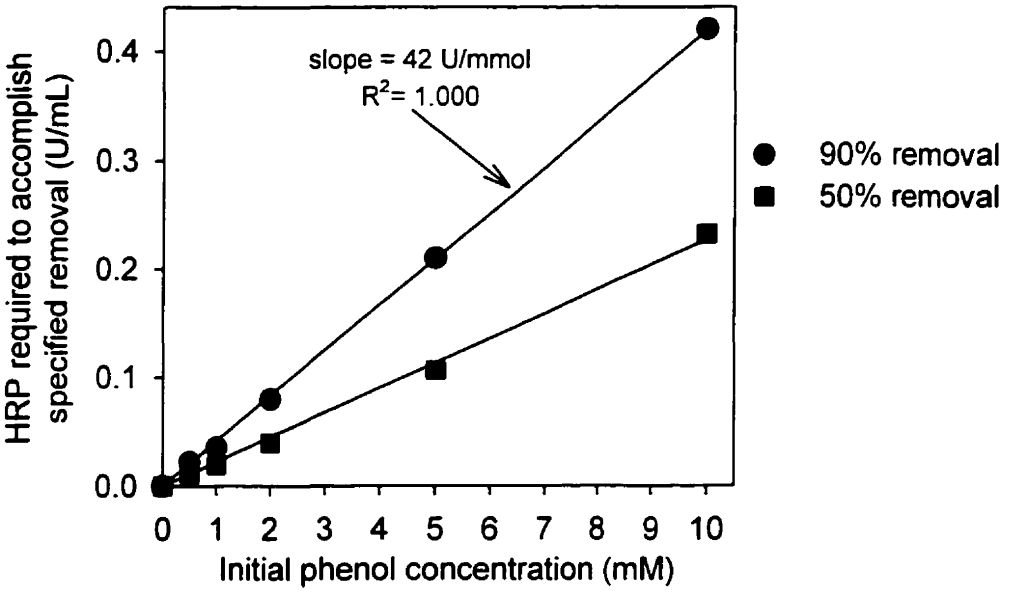


Figure 4.12: HRP requirements for 90 and 50 % phenol removal in the presence of 320 mg/L of PEG_{35000}.

Activity assays (using the 4-AAP assay) carried out in the presence and absence of 50 mg/L of chitosan yielded the same results. Thus, chitosan did not affect the phenol oxidation kinetics. Also, pre-incubation of the enzyme in 400 mg/L of chitosan for 2.5

hours did not result in a significant change in its activity when compared to the controls, indicating that even high concentrations of chitosan did not inactivate the enzyme.

4.2 Discussion

This investigation has shown that the presence of chitosan in the reaction mixture significantly improves phenol conversion. However, the amounts of chitosan required to achieve a maximum effect for the treatment of phenol and other substituted phenols were found to be substantially higher than reported by Ganjidoust *et al.* (1996). When compared to PEG_{3350} (Nicell *et al.*, 1995), optimum chitosan doses for the treatment of phenol were approximately 2 to 3 times higher than the corresponding PEG_{3350} doses. It is recognized that values based on molar concentrations or hydrogen bonding sites may be more appropriate for the comparison of these two additives. However, molar concentrations of the polymer molecules could not be calculated since the required information on the molecular weight distribution of both polymers and on the purity of the chitosan product was not available.

Studies conducted with a variety of polyethylene glycols have shown that the ability of this polymer to promote phenol removal increases with its molecular weight (Nakamoto and Machida, 1992; Kinsley and Nicell, 2000). For chitosans, a high viscosity grade is associated with a high degree of polymerization and a low degree of deacetylation (Muzzarelli, 1977). The degrees of deacetylation of the chitosans used in this study ranged from 76 to 84 % (according to the manufacturer) and did not correlate with the viscosity grade. The degrees of polymerization are unknown; however, since all chitosans are deacetylated to a similar extent it can be assumed that the chitosans of a higher viscosity grade possess also a higher degree of polymerization. Since no clear correlation was found between the viscosity grade and the ability of a chitosan to promote phenol removal it may be concluded that the degree of polymerization may be only one among several factors that have an impact on the effectiveness of chitosan. Interestingly, the chitosan product that performed best (420 cps viscosity grade) was the product that had the lowest degree of deacetylation (76 %) and the highest content of insolubles and ash, thereby indicating that other constituents beside the chitosan itself may have contributed to the observed effect.

As in the case of PEG (Kinsley and Nicell, 2000), the amount of phenol removed increased linearly with increasing concentrations of chitosan in the reaction mixture. Kinsley and Nicell (2000) also observed that PEG and the phenolic reaction products precipitated together at a constant mass ratio. Based on these observations, they hypothesized that the protective capacity of the PEG is lost once it has interacted with the phenolic reaction products. A similar mechanism could explain the stoichiometric relationship between chitosan and the amount of phenol removed. It is postulated that chitosan combines with phenolic reaction products at a constant mass ratio thereby sequestering inactivating species away from the enzyme and preventing its inactivation. Once all the available chitosan has combined with the phenolic products, newly created inactivating species will attack and inactivate the enzyme thus terminating phenol transformation. The interaction of chitosan with the phenolic products must be a very rapid process in order to be fast enough to efficiently minimize the rate of enzyme inactivation. Therefore, a purely physical adsorption may be excluded in favor of a chemisorption process. However, the exact nature of the processes involved needs further investigation.

In the presence of PEG_{35000} or no additives at all, the HRP dose required to achieve a specified degree of treatment was proportional to the initial phenol concentration. In contrast, in the case of chitosan the relative HRP dose was higher at higher initial phenol concentrations for intermediate levels of treatment. A possible explanation could be that the higher chitosan concentrations that were employed at the higher initial phenol concentrations could have increased the viscosity of the reaction mixture. For a 90% phenol transformation, enzyme requirements increased linearly with increasing initial phenol concentration. For this level of treatment, both chitosan and PEG_{35000} reduced enzyme requirements approximately 80-fold. Nicell *et al.* (1995) report that for 90 % phenol removal with PEG_{3350} a 20-fold reduction in HRP requirements was obtained. In comparison with this value, chitosan and PEG_{35000} significantly outperform PEG_{3350} in terms of the degree of enzyme saving that can be expected using these additives.

5. TOXICITY REDUCTION OF PEROXIDASE-TREATED PHENOLIC SOLUTIONS

HRP-catalyzed oxidation and subsequent polymerization of phenolic compounds gives rise to a multitude of polymeric and monomeric products. These products must be characterized in terms of their compatibility with downstream processes or in terms of their potential impact on receiving water bodies before the HRP-based process can be implemented on an industrial scale (Aitken, 1993). Therefore, a number of investigations were carried out to test the impact of HRP-treatment on the toxicity of phenolic solutions. While some phenolic solutions were partially or completely detoxified, the residual toxicities remained high in the cases of treated solutions of 2-methylphenol, 2-chlorophenol, 4-chlorophenol, 2,4-chlorophenol and phenol (Aitken *et al.*, 1994; Ghioureliotis and Nicell, 2000). Also, trace quantities of polychlorinated dibenzodioxins and dibenzofurans were found in the supernatants when polychlorinated phenols such as 2,4-dichlorophenol, 2,4,5-and 3,4,5-trichlorophenols were treated with HRP (Maloney *et al.*, 1986; Svenson *et al.*, 1989; Öberg *et al.*, 1990).

However, recent research also indicates that the particular treatment conditions may significantly influence the quality and quantity of the reaction products that are formed. For example, it has been shown that when PEG was used as a protective additive, there was an increase in the quantity of products that remained in solution following the treatment of phenol (Ghioureliotis and Nicell, 1999). In contrast, Sun *et al.* (1992) report that chitosan is able to covalently bind quinones, which are highly toxic compounds (Walker, 1988) that have been identified as products of HRP-polymerization reactions (Zou and Taylor, 1994; Sawahata and Neal, 1982; Simmons *et al.*, 1988). Therefore, it seems possible that the addition of chitosan to reaction mixtures may result in lower toxicities.

Nevertheless, little is currently known about the impact of reaction conditions on residual toxicity and the stability of soluble reaction products. Therefore, the objective of this study was to investigate the stability of toxic soluble reaction products under ambient

conditions (25°C, pH 7) and to explore ways to prevent and/or eliminate residual toxic compounds following enzymatic treatment.

The ideal way to investigate the impact of reaction conditions on the formation and/or stability of toxic reaction products would be to identify the toxic species and then follow their concentrations over time. For example, the bulk of the soluble reaction products after the enzymatic conversion of phenol are phenolic dimers (Zou and Taylor, 1994). However, these compounds seem not to be mainly responsible for the elevated toxicities since toxicity levels were not correlated with the overall concentration of soluble reaction products (Ghioureliotis and Nicell, 1999). This indicates that the species responsible for the observed toxicity were present in trace quantities or that the toxicity was due to synergistic effects of several species. In both cases the identification of these compounds would require very detailed analytical and toxicological studies. Furthermore, indications exist that reaction products could react further during their isolation (e.g., solvent extraction) (Kazunga *et al.*, 1999) or during analytical procedures (Zou and Taylor, 1994) leading to the formation of artifacts. Thus, the monitoring of toxic species as a function of time could prove to be unfeasible. Therefore, instead of attempting to identify toxic reaction products, in this study the toxicity of phenolic reaction mixtures as a whole was measured as a function of reaction conditions and time.

Toxic effects of phenolic solutions were quantified using the Microtox™ acute toxicity assay. This test has been found to be more sensitive than other microbiological assays, such as the nitrification assay (Reynolds *et al.*, 1987) or the activated sludge respiration assay (Elnabarawy *et al.*, 1988). Toxicity values based on the Microtox™ test correlated well with those based on a number of aquatic species including fathead minnow (*Pimephales promelas*) and the water flea (*Daphnia magna*) (Kaiser and McKinnon, 1993). A good correlation was also found between the Microtox™ EC_{50} and the corresponding rat and mouse LD_{50} values (Kaiser *et al.*, 1994). Thus, the Microtox™ test is sensitive and allows the prediction of toxic effects to other species. In this study this assay was used as a screening tool to compare toxicities remaining after HRP treatment of phenolic solutions under different conditions.

5.1 Results

5.1.1 Toxicities of phenol solutions before and after treatment with HRP

Assays were conducted to determine the toxicities of 1 mM phenol reaction mixtures (22 samples) prior to treatment and solutions from which phenol had been removed after a 3-hour enzymatic reaction (20 samples). The resulting data were subjected to the probability plot correlation coefficient test of normality. TU_{50} values follow a normal distribution (with $r = 0.975$) whereas the corresponding EC_{50} values are log-normally distributed (with $r = 0.988$ for the 1 mM phenol solutions and $r = 0.964$ for the samples from which phenol had been removed). The average TU_{50} value of the untreated phenolic solutions was 8.3 ± 1.0 (95% confidence interval), whereas the average TU_{50} after HRP-treatment was measured to be 15.8 ± 3.1 (95% confidence interval) indicating that HRP-treatment leads to a significant toxicity increase.

5.1.2 Effect of additives and time on the toxicity of HRP treated phenol solutions

Phenol polymerization reactions were carried out with and without the presence of polyethylene glycol (MW 35000) or chitosan (420 cps viscosity grade). In each case, the enzyme and H_2O_2 were added in excess quantities leading to at least 95 % phenol removal after 3 hours of reaction. Toxicities of the reaction mixtures were measured 3, 6 and 24 hours after the start of the reaction. The results of these experiments are presented in Figure 5.1. Three conclusions can be drawn from this graph.

Firstly, toxicities after HRP-treatment tended to be higher when PEG was used as an additive ($p < 0.05$ in paired Student's t-test) while chitosan had no significant impact on the toxicity of the treated solutions after a three-hour reaction period compared to the samples containing no additive.

Secondly, the toxicities of the treated solutions decreased significantly over time, even though no additional phenol removal took place after 3 hours (Figure 1). In the case of the samples treated without additives, the toxicity dropped on average by (45 ± 29) % during the period from 3 to 24 hours after the start of the enzymatic reaction. For the same time interval, a (70 ± 23) % and (75 ± 15) % toxicity drop was observed for the samples treated in the presence of PEG and chitosan, respectively.

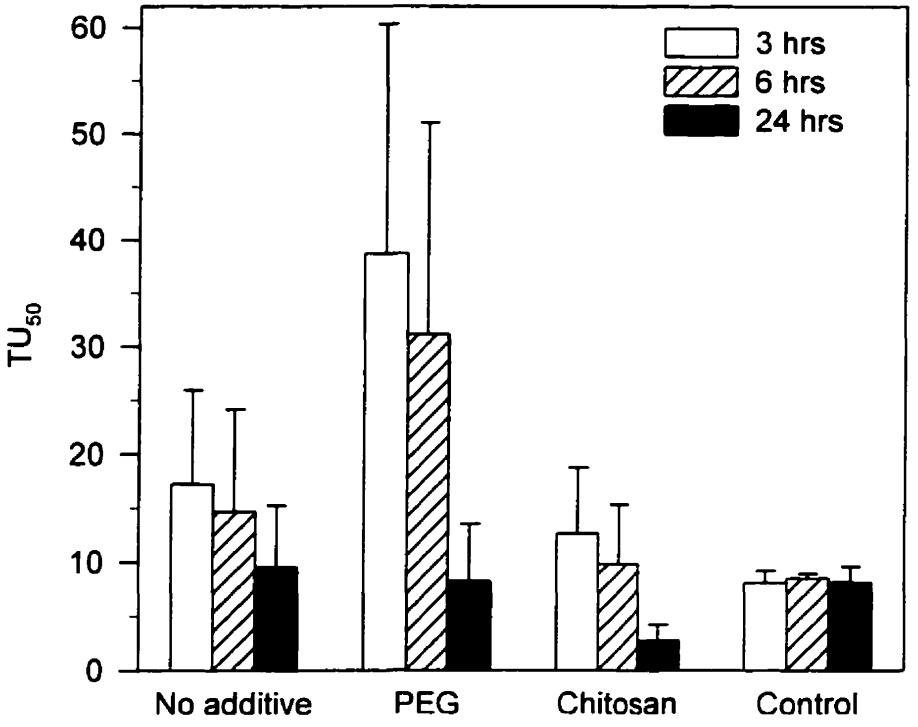


Figure 5.1: Toxicities of phenol reaction mixtures 3, 6 and 24 hours after the start of the reaction. The control represents the reaction solution without HRP and additives. Reaction conditions: $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; PEG = chitosan = 50 mg/L; HRP_0 = 4.5 U/mL (no additive) and 0.25 U/mL (with additives). Error bars stand for the standard deviation of 5 independent experiments.

No toxicity decrease occurred in the case of the untreated reaction solutions, which served as a control (Figure 5.1). Also, the toxicities of the untreated phenol solutions were not affected by the addition of additives (data not shown).

Thirdly, 24 hours after the start of the reaction, the samples that had been treated in the presence of chitosan were significantly less toxic as compared to the samples treated without additives ($p < 0.05$ in paired Student's t-test) (Figure 5.1). Also, at this point of time, samples that had been treated in the presence of PEG were no more toxic than those treated without additives.

Additional experiments were conducted to examine whether the addition of chitosan after the completion of the phenol removal reaction would also lead to a decrease in toxicity, potentially through adsorption of soluble toxic reaction products. Therefore, 50 mg/L of chitosan were added to a reaction mixture 3 hours after the start of the enzymatic

reaction, at which point in time complete phenol conversion had been achieved. The solution was filtered in order to exclude possible interactions between the high molecular weight chitosan and the product precipitates. Toxicities were assayed at this point of time and 6 and 24 hours after the start of the enzymatic reaction (3 and 21 hours after chitosan was added). Due to the high variability of toxicity levels between different experiments, the TU_{50} data are expressed as the percentage of the TU_{50} value of the unfiltered sample assessed immediately after the completion of the enzymatic reaction (3 hours value of the original sample). The results of these experiments are illustrated in Figure 5.2.

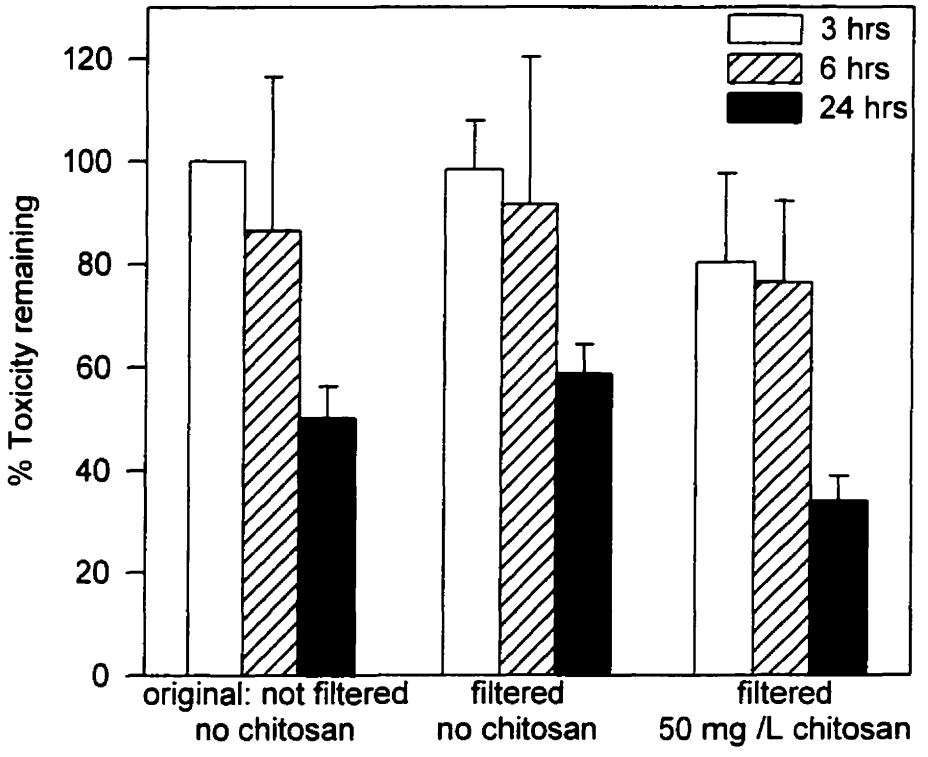


Figure 5.2: Toxicities of phenol reaction mixtures 3, 6 and 24 hours after the start of the reaction as a percentage of the toxicity of the original, unfiltered solution at 3 hours. Chitosan at 50 mg/L was added to the filtered reaction mixture at 3 hours. Reaction conditions: $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 4.5 U/mL. Error bars stand for the standard deviation of 5 independent experiments.

The addition of chitosan to a filtered sample did not change its toxicity immediately. However, the solutions containing 50 mg/L of chitosan were less toxic after 24 hours compared to both controls (original reaction mixture and filtrate). The differences in toxicities were small, but significant ($p < 0.05$ in Tukey's paired comparison test). Note that filtration did not reduce the toxicity of HRP treated samples, thereby indicating that soluble reaction products were likely to be responsible for the elevated toxicity levels.

In order to examine whether mainly hydrophobic reaction products contribute to the high toxicity levels, methanol was added to an HRP-treated sample. Due to its hydrophobic character, this solvent enhances the solubility of hydrophobic compounds that might be adsorbed to the precipitate or to the glass vial. However, no increase of toxicity levels was observed in samples containing 5% or 15% methanol 3 and 21 hours after methanol addition as compared to samples that contained no methanol (data not shown). Note that methanol is only weakly toxic in the Microtox™ assay. Control experiments also revealed that the presence of methanol did not mask the toxicity of reaction products.

5.1.3 Toxicity reduction after addition of hydrogen peroxide

During the course of these experiments it was incidentally observed that, samples from which the H_2O_2 had not been eliminated at three hours after the start of the reaction were less toxic on the following day compared to those samples to which catalase had been added. This observation led to a closer examination of the impact of H_2O_2 on the toxicity of HRP-treated solutions. Therefore, H_2O_2 at concentrations of 1.2 or at 11.6 mM was added to a reaction mixture from which phenol had been removed through a 3-hour enzymatic reaction. Toxicities were assayed at this point of time and 6 and 24 hours after the start of the reaction (3 and 21 hours after H_2O_2 was added). The results of these experiments are presented in Figure 5.3. The addition of H_2O_2 to a HRP-treated sample led to its detoxification ($TU_{50} < 1$) within 6 hours after the start of the reaction when 11.6 mM H_2O_2 were added, or within 24 hours when 1.2 mM H_2O_2 were added.

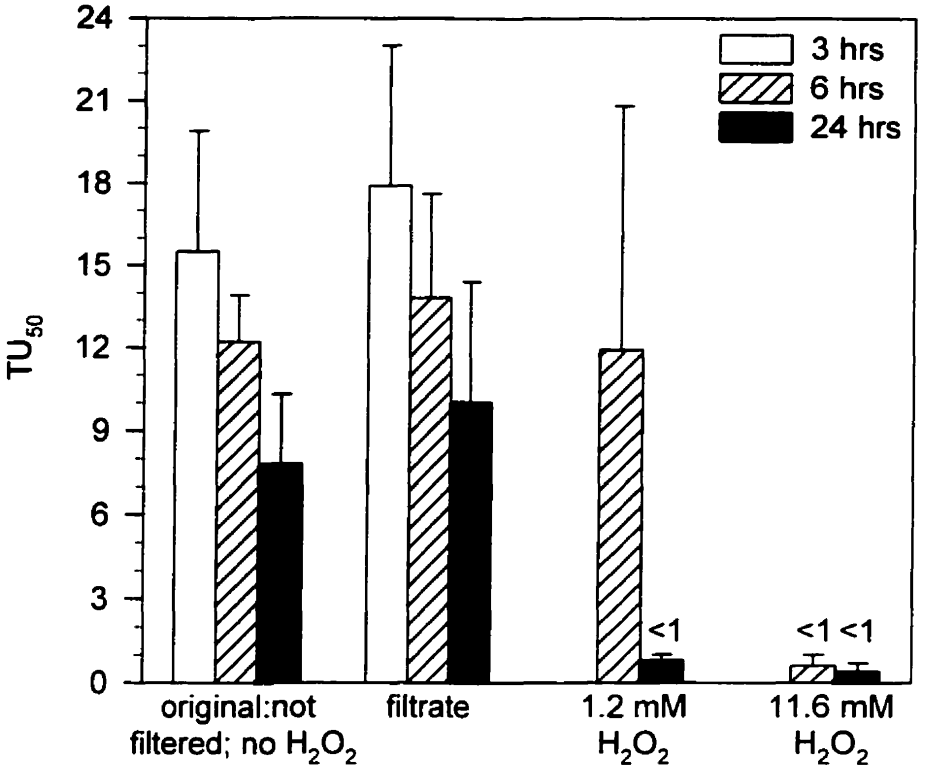


Figure 5.3: Toxicities of phenol reaction mixtures 3, 6 and 24 hours after the start of the reaction as a function of the H_2O_2 added to the filtered reaction mixture at 3 hours. The original represents the unaltered reaction mixture. Reaction conditions: $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 4.5 U/mL. Error bars stand for the standard deviation of 4 independent experiments.

In order to examine how much H_2O_2 is needed for detoxification, an experiment was carried out in which the H_2O_2 concentration was varied from 0 to 11.6 mM. As in the previous experiments, the toxicity of the reaction mixture from which phenol had been removed was measured just before the H_2O_2 was added (3 hrs after the start of the enzymatic reaction). Additional toxicity values were recorded at 6 and 24 hours (3 and 21 hours after the H_2O_2 was added). The results of this experiment are illustrated in Figure 5.4.

At 6 hours, a slight toxicity increase was observed in the sample to which no or low quantities of H_2O_2 (0.6 mM) had been added. However, the samples treated with higher amounts of H_2O_2 were partially or completely detoxified.

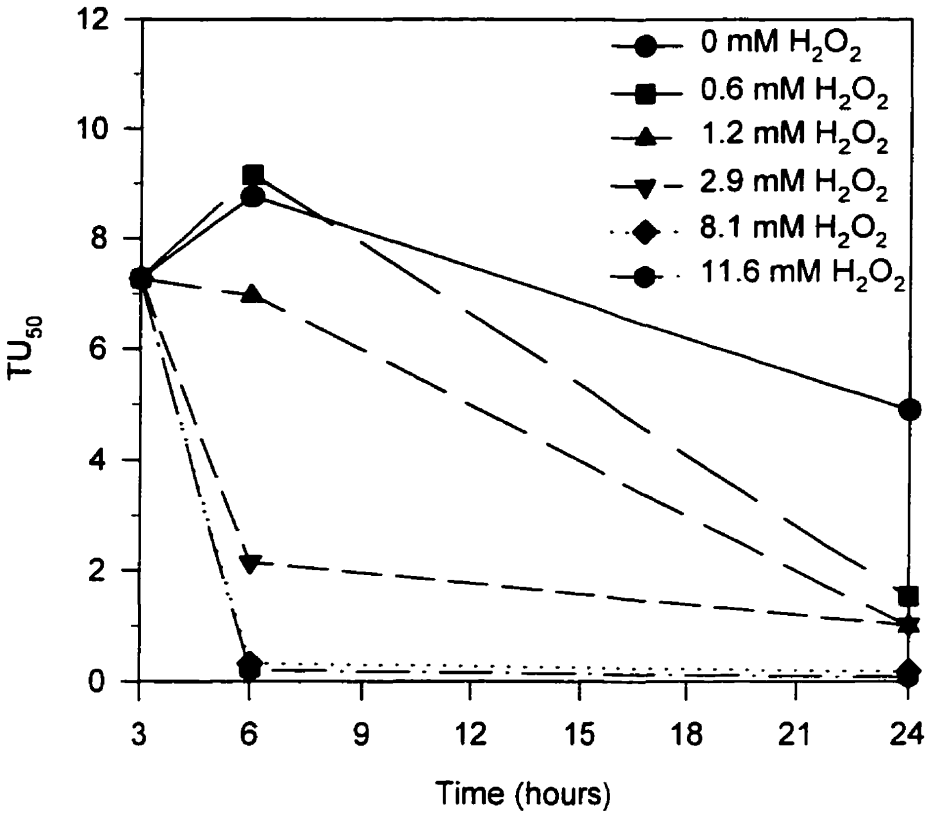


Figure 5.4: Toxicities of phenol reaction mixtures as a function of time and H_2O_2 added. H_2O_2 at different concentrations was added at 3 hours. Reaction conditions: $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.3$ mM; $HRP_0 = 4.5$ U/mL.

The toxicity drop was more pronounced when larger quantities of H_2O_2 were applied. This seemed to result from a faster rate of detoxification, since at 24 hours a TU_{50} of 1-2 was measured in all H_2O_2 treated samples. It should be noted that H_2O_2 addition resulted in detoxification regardless of whether the precipitates were removed from the reaction mixture. No significant drop in the H_2O_2 concentration was measured during the detoxification reaction and no additional phenol was removed (data not shown).

5.1.4 Treatment of other phenols

Similar experiments were carried out with 2,4-dichlorophenol (2,4-DCP), which is highly toxic and could not previously be detoxified upon HRP-treatment (Ghioureliotis and Nicell, 2000). A 0.6 mM solution was treated with HRP to a high degree (>96% 2,4-dichlorophenol removal) for three hours and its toxicity was measured. Subsequently, the

sample was divided into four sub-samples to which H_2O_2 at different concentrations was added. The toxicities of the 4 sub-samples were measured 6 and 24 hours after the start of the enzymatic reaction (3 and 21 hours after H_2O_2 was added). The results of these experiments are illustrated in Figure 5.5.

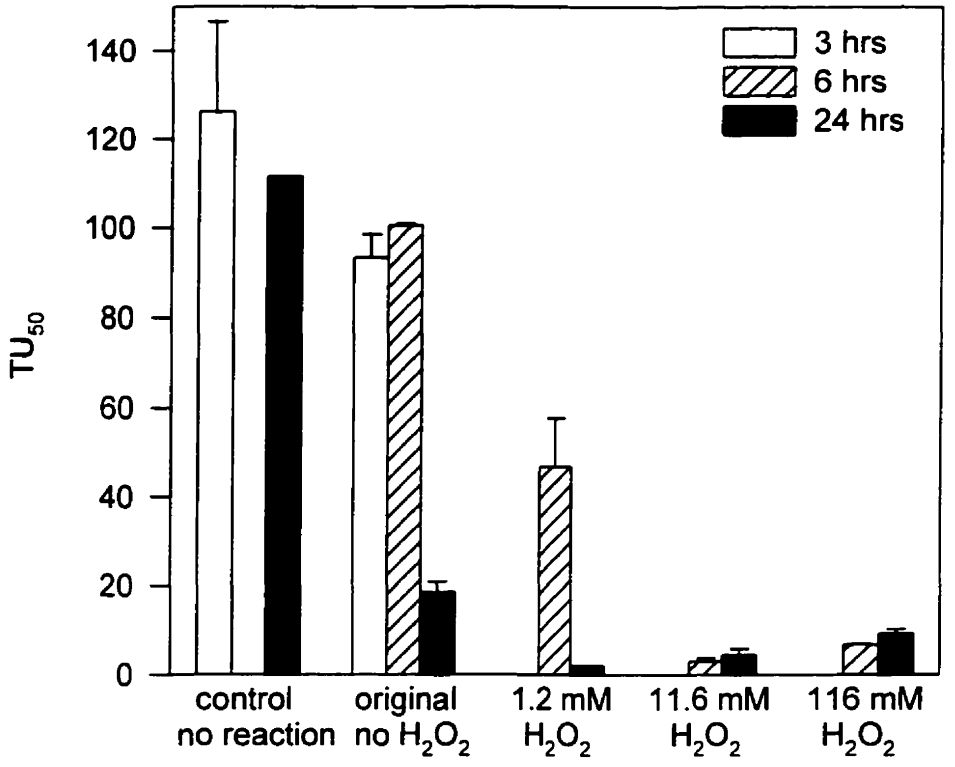


Figure 5.5: Toxicities of 2,4-DCP reaction mixtures 3, 6, and 24 hours after the start of the reaction as a function of the H_2O_2 added to the reaction mixtures at 3 hours. The control represents the initial untreated reaction solution. Reaction conditions: $[2,4\text{-DCP}]_0$ = 0.6 mM; $[H_2O_2]_0$ = 1.2 mM; HRP_0 = 4.5 U/mL. Error bars stand for the range of two independent experiments.

Removal of 2,4-dichlorophenol from the solution did not result in a significant decrease in its toxicity. However, 24 hours after the start of the reaction, the toxicity of the treated solution dropped significantly, whereas the toxicity of the untreated reaction mixture that served as a control remained virtually unchanged. Addition of H_2O_2 to the HRP-treated samples resulted in a large toxicity reduction after 3 hours in the case of 11.6 and 116 mM H_2O_2 solutions and after 21 hours when 1.2 mM H_2O_2 had been added. It seems, however, that toxicities were not as effectively reduced when H_2O_2 was applied in higher concentrations.

Upon the treatment of 2,4-dichlorophenol with HRP and H_2O_2 the reaction solution turned pink due to the formation of reaction products with the peak absorbance wavelength of 496 nm. Parallel to detoxification, a drop in the absorbance at this wavelength as well as at the UV peak absorbance wavelength (285 nm) was observed in the samples containing H_2O_2. The absorbance at both wavelengths increased slightly over time in the original reaction mixture that had not been treated with H_2O_2.

Subsequently, it was investigated whether H_2O_2 treatment would lead to detoxification of HRP-treated solutions of other chloro-and methylphenols, such as 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 2,6-dichlorophenol (2,6-DCP), 2-methylphenol (2-MP), 3-methylphenol (3-MP), and 4-methylphenol (4-MP). The results are summarized in Figures 5.6 and 5.7.

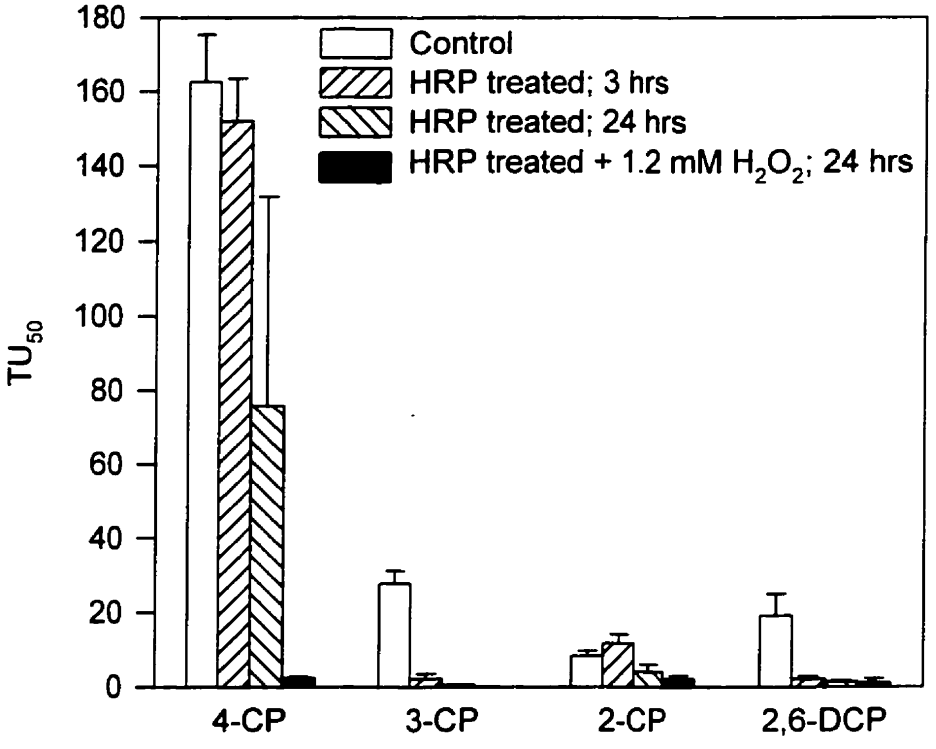


Figure 5.6: Toxicities of chlorophenol reaction mixtures 3 and 24 hours after the start of the reaction. Additional H_2O_2 was added at 3 hours at 1.2 mM. The controls represent the initial untreated reaction solutions. Reaction conditions: $[2\text{-CP}]_0 = [3\text{-CP}]_0 = 0.9$ mM; $[4\text{-CP}]_0 = 0.5$ mM; $[2,6\text{-DCP}]_0 = 1$ mM; $[H_2O_2]_0 = 1.2$ mM; $HRP_0 = 4.5$ U/mL; for 3-CP reactions = 9 U/mL. Error bars stand for the range of two independent experiments.

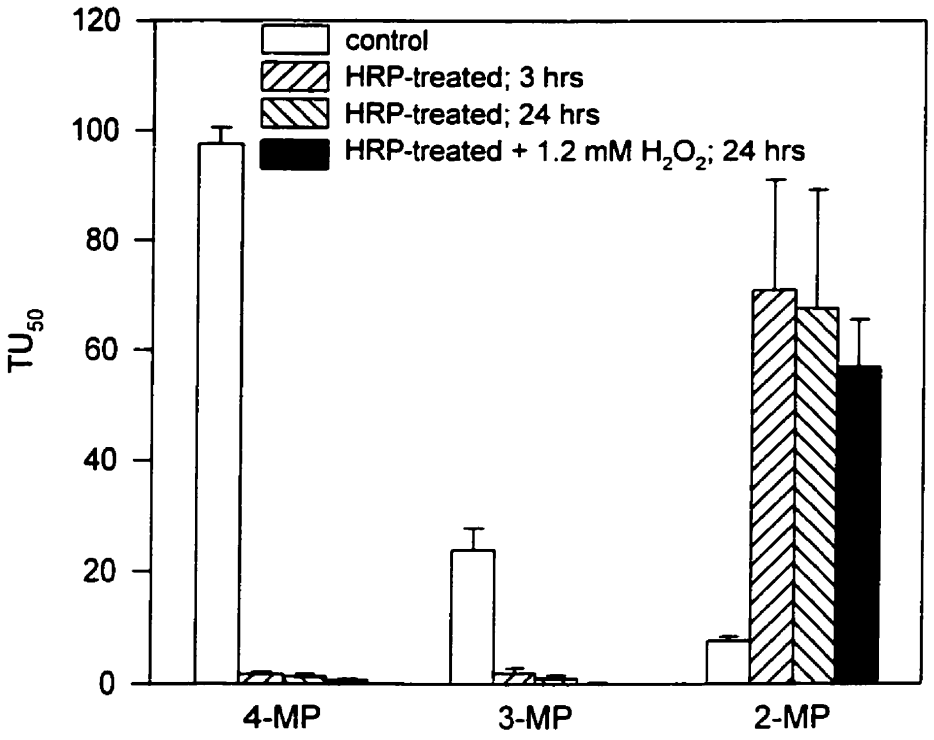


Figure 5.7: Toxicities of methylphenol reaction mixtures 3 and 24 hours after the start of the reaction. Additional H_2O_2 was added at 3 hours at 1.2 mM. The controls represent the initial untreated reaction solutions. Reaction conditions: $[3\text{-MP}]_0$ = 0.9 mM; $[2\text{-MP}]_0 = [4\text{-MP}]_0$ = 1 mM; $[H_2O_2]_0$ = 1.2 mM; HRP_0 = 4.5 U/mL. Error bars stand for the range of two independent experiments.

Except for 2,6-dichlorophenol that was treated to 90% conversion, more than 95% transformation of the parent phenols had been achieved.

A 3-hour enzymatic reaction resulted in substantial detoxification in the case of 3-chlorophenol, 3-methylphenol and 4-methylphenol (Figure 5.6 and 5.7) while the toxicities of the 4-chlorophenol and 2-chlorophenol solutions did not change significantly (Figure 5.6). However, after a total of 24 hours, a toxicity drop was observed in HRP-treated 4-chlorophenol and 2-chlorophenol solutions, which was more pronounced in samples to which 1.2 mM H_2O_2 had been added. The toxicity of the peroxide-treated 2-chlorophenol samples dropped to a TU_{50} of 2.1 after 24 hours (Figure 5.6). However, since the parent 2-chlorophenol is relatively weakly toxic, this rather low value corresponds to approximately 24% of the initial toxicity. In the case of 2-methylphenol, HRP-treatment resulted in an approximately 10-fold increase in toxicity. No toxicity drop

was observed after 24 hours and peroxide treatment did not reduce the toxicity levels significantly as shown in Figure 5.7. An additional experiment in which 11.6 mM H_2O_2 was used confirmed that peroxide treatment was ineffective in reducing the toxicity of HRP-treated 2-methylphenol solutions (data not shown). In all cases, except for 2-chlorophenol and 2-methylphenol, 24 hours after the start of the reaction the toxicities of the peroxide treated samples were at levels corresponding to their residual phenol content indicating that no toxic reaction products were present.

5.1.5 Impact of H_2O_2 and enzyme addition mode on final toxicities

Zou and Taylor (1994) suggested that the mode of H_2O_2 addition during the reaction would have an effect on the nature and distribution of reaction products. To test this hypothesis, a total of 1.3 mM H_2O_2 was added in 10 aliquots over a period of one hour to 1 mM phenol solutions containing 4.5 U/mL of HRP. Similarly, for comparison, reactions were carried out in which all the peroxide was added in a single dose. Three hours after the start of the reaction, the samples were assayed for their toxicity and residual phenol content. Also, the absorbance at 400 nm, which has been previously reported to be the peak absorption wavelength of certain HRP-reaction products from phenol (Sawahata and Neal, 1982; Arseguel and Baboulene, 1994), was recorded. As illustrated in Figure 5.8(a) the stepwise mode of peroxide addition resulted in an approximate 60% decrease in the toxicity and a 40% decrease of the absorbance at 400 nm.

A similar experiment was carried out in order to test whether the mode of enzyme addition might also have an effect on toxicity levels. The experimental procedures were as described above except that this time the enzyme (a total of 4.5 U/mL) was added in 12 aliquots over a period of 1 hour to a 1-mM phenol solution containing 1.3 mM H_2O_2. The results of this experiment are shown in Figure 5.8(b). Contrary to the H_2O_2 step addition, the addition of the enzyme in steps led to an approximate 40% increase in toxicity as compared to the controls. Also, the absorbance at 400 nm was almost four times higher in the samples to which the enzyme was added over a one-hour period. More than 99% phenol removal was achieved in all these experiments.

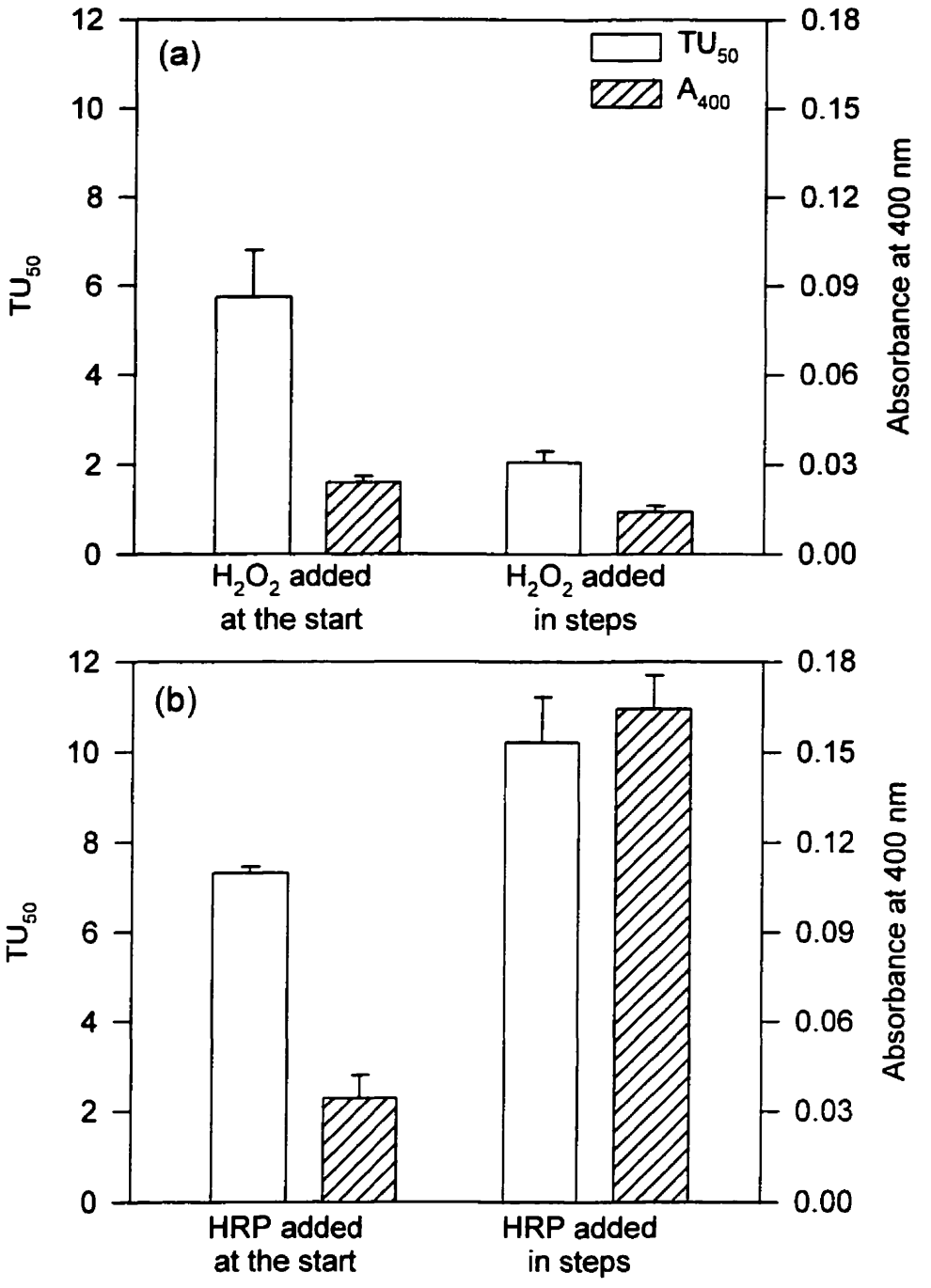


Figure 5.8: Toxicities of phenol reaction mixtures 3 hours after the start of the reaction as function of the H_2O_2 and HRP addition mode. Error bars stand for standard deviations of triplicate reactions. (a) 1.3 mM H_2O_2 added in 10 aliquots over one hour to solutions containing 1.0 mM phenol and 4.5 U/mL HRP. (b) 4.5 U/mL HRP added in 12 aliquots over one hour to solutions containing 1.0 mM phenol and 1.3 mM H_2O_2.

In order to study the effect of continuous reagent addition, a total of 4.5 U/mL of HRP or 1.3 mM of H_2O_2 were fed over a period of one hour to reaction mixtures containing the respective other reagent. Parallel reactions were carried out in which both reagents were added at the start. The toxicities of all three reaction mixtures were monitored at the same time. The results of this experiment are illustrated in Figure 5.9.

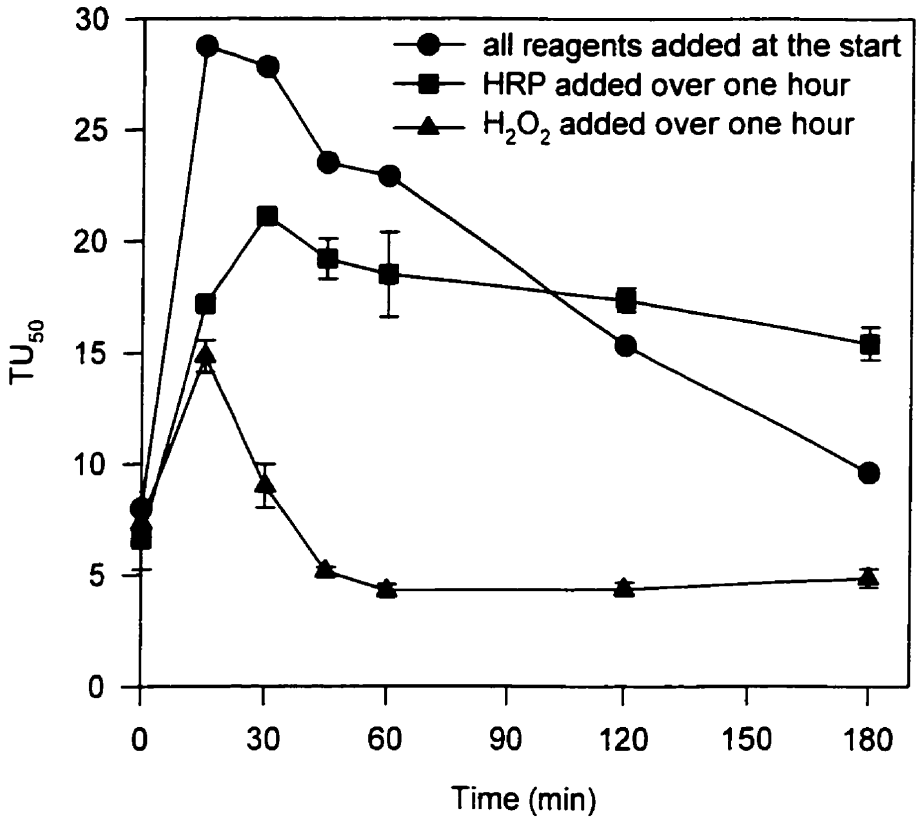


Figure 5.9: Toxicities of phenol reaction mixtures as a function of time and reagent addition modes. Reaction conditions: $[phenol]_0$ = 1.0 mM; total $[H_2O_2]$ = 1.3 mM; total HRP = 4.5 U/mL.

For all three reaction modes, toxicities surged at the start of the reaction. The highest initial toxicity levels were recorded when both reagents were added at once (upper curve), whereas continuous peroxide addition generally resulted in much lower toxicity levels (lower curve). While the toxicity decreased quite rapidly in these two cases, it dropped much more slowly with continuous HRP-addition (middle curve) suggesting that the toxic products formed under these conditions were more stable.

5.1.6 Correlation between toxicity and absorbance at 400 nm for HRP-treated phenol solutions

It was observed that HRP-treated phenol solutions that had a higher absorbance at 400 nm were also more toxic (Figure 5.8). Also, both, the TU_{50} value as well as the absorbance at 400 nm tended to decrease over time. Correlation analysis of 40 data pairs that had been obtained in 8 independent experiments with no additives yielded a Pearson's correlation coefficient of 0.803 having a statistical significance of more than 99.9%. Samples that had been treated with additional H_2O_2 after the completion of enzymatic phenol removal were not included in this analysis. Although all these samples displayed a low absorbance ($A_{400} = 0.011 \pm 0.002$) some samples that had been treated with low amounts of H_2O_2 were highly toxic three hours after H_2O_2 addition (Figure 8.3) indicating that a low absorption at 400 nm did not necessarily translate into a low toxicity.

5.1.7 Impact of UV illumination

Since toxic products appeared to be relatively unstable, experiments were conducted to determine if UV illumination might accelerate the process of detoxification in the presence of H_2O_2 through the formation of highly reactive hydroxyl radicals and to examine whether products of the reaction are susceptible to decomposition through UV light. Therefore, HRP-treated solutions were exposed for different time periods to a low-pressure mercury lamp at a volumetric radiation intensity (I_{vol}) of 0.36 W/L. As illustrated in Figure 5.10, an energy input of about 110 J/L corresponding to five minutes of UV illumination led to detoxification ($TU_{50}<1$) regardless of whether the solution contained H_2O_2 or not.

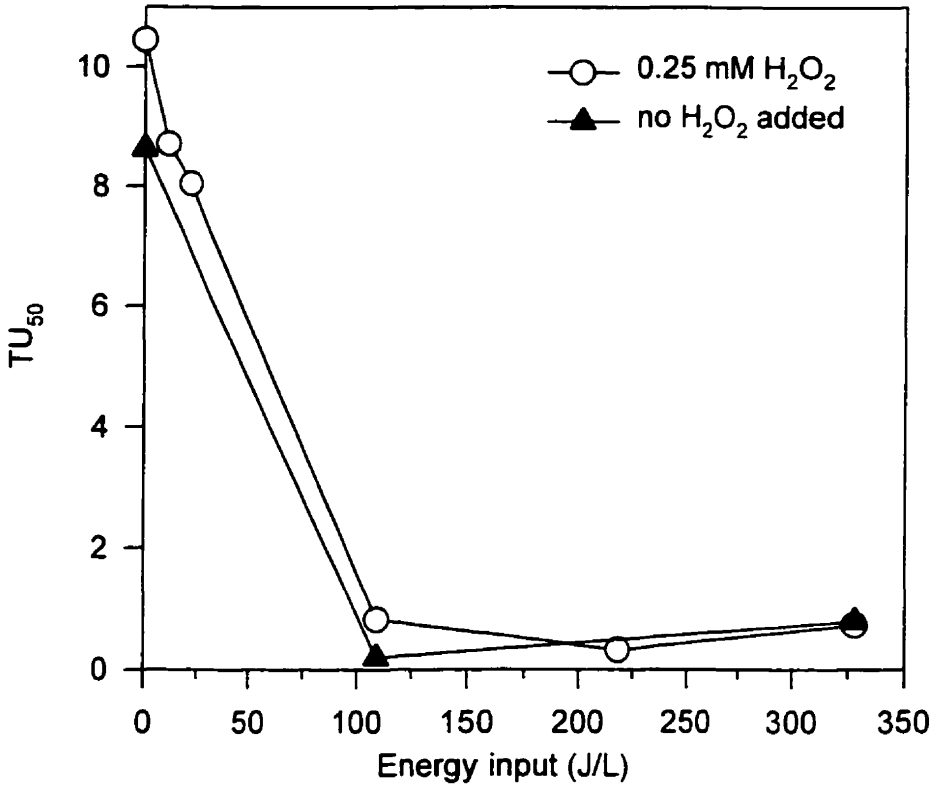


Figure 5.10: Toxicities of HRP-treated phenol solutions as a function of energy input in form of UV illumination at 254 nm. H_2O_2 was added before the start of the UV illumination. Reaction conditions: $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 4.5 U/mL. The reaction time for the enzymatic reaction was 3 hours.

5.2 Discussion

5.2.1 Decrease of toxicity over time

The toxicities of 1 mM phenol solutions increased upon HRP-treatment and then declined within a period of 24 hours. In most cases, the highest toxicity values were measured within three hours after the start of the enzymatic reaction. However, occasionally, such as in the experiment illustrated in Figure 5.4, a higher toxicity was observed at 6 hours. This isolated observation was statistically not significant as can be inferred from Figures 5.1 to 5.3. A significant decline of toxicity over time was also noted in the cases of

solutions of 2,4-dichlorophenol, 2-chlorophenol and 4-chlorophenol that had been treated with HRP.

Two explanations can be considered for this toxicity decline: (1) toxic species could combine to form compounds that have a lower solubility and eventually precipitate out of the solution or (2) they could react over time to non-toxic soluble reaction products. These reactions could be catalyzed by the enzyme or could take place spontaneously. Several studies have demonstrated that products of the enzymatic reaction such as dimers are still substrates of HRP (Sawahata and Neal, 1982; Yu *et al.*, 1994; Danner *et al.*, 1973). Under the reaction conditions applied in this study, 5% of the initial enzyme activity was still present 6 hours after the start of the reaction. Therefore, a further enzymatic reaction seems possible. On the other hand, unstable products of the enzymatic reaction could combine spontaneously or be further oxidized, possibly by the dissolved oxygen, to less toxic compounds. Evidence that HRP-reaction products are able to react independently of the enzyme can be drawn from experiments conducted by Roper *et al.* (1995). It was shown that adding 2,4,5-trichlorophenol to an enzyme-free filtrate that contained reaction products from the HRP-catalyzed oxidation of 2,6-dimethoxyphenol led within 3 hours to a 72% decrease in the 2,4,5-trichlorophenol concentration as well as an additional 30% decrease in the 2,6-dimethoxyphenol concentration. The authors suggest that the removal occurring in the filtrate was primarily due to a chemical reaction between oxidation products of 2,6-dimethoxyphenol and the 2,4,5-trichlorophenol.

Another explanation for the reduction of toxicity over time might be the adsorption of soluble reaction products to the glass material of the reaction vials. This possibility was tested by adding methanol to HRP-treated phenol solutions containing precipitates, which would enhance the solubility of hydrophobic and possibly toxic compounds. However, this did not result in an increase in toxicity and did not prevent the decrease of toxicity over time. Therefore, this explanation is discounted.

5.2.2 The impact of additives

Treatment in the presence of PEG resulted in higher toxicity values at 3 hours compared to the samples treated without additives, however, there was no significant difference between these two treatments 21 hours later (Figure 5.1). A possible explanation for this

observation is that PEG may enhance the solubility of toxic reaction products, which leads to an overall higher concentration of soluble reaction products as observed by Ghioureliotis and Nicell (1999), but does not influence their gradual transformation.

The presence of chitosan resulted in lower toxicity values compared to the controls after an extended period of time (Figures 5.1 and 5.2) regardless of whether chitosan was added before or after the completion of the phenol removal reaction. This toxicity reduction could possibly be due the chemisorption of toxic quinones to the amino groups of chitosan. However, the results of Sun *et al.* (1992) indicate that this reaction takes place within a few minutes suggesting that other effects, such as coagulation and precipitation might also play a role.

5.2.3 The impact of hydrogen peroxide addition after the completion of the enzymatic reaction

This study has demonstrated that solutions of various phenols can be detoxified upon HRP-treatment alone or with subsequent addition of H_2O_2. The sole exceptions were the *ortho*-substituted compounds 2-chlorophenol and 2-methylphenol. Hydrogen peroxide was particularly effective for the detoxification of HRP-treated solutions of 4-chlorophenol, 2,4-dichlorophenol and phenol. The rate of toxicity decrease was greater with higher concentrations of peroxide (Figures 5.3 to 5.5), whereas the final toxicity levels did not depend on the peroxide concentration. In the case of 2,4-dichlorophenol, toxicity was not as effectively reduced when 11.6 and 116 mM H_2O_2 were used (Figure 5.5). This suggests the formation of toxic compounds at high peroxide concentrations. The higher toxicities could also possibly be due to the presence of some residual H_2O_2 in the sample during the toxicity analysis resulting from an incomplete catalytic H_2O_2 decomposition at higher H_2O_2 concentrations.

H_2O_2 is widely used as an oxidant for advanced oxidation of organic pollutants, including phenols, chlorophenols and other aromatics (Chen *et al.*, 1997; Mokrini *et al.*, 1997; Bigda, 1995). Upon activation with an iron catalyst (Fenton's Reagent), UV light or ozone hydroxyl radicals (OH•) are generated from H_2O_2 that have a very high oxidation potential and that can oxidize toxic reaction products to less toxic species such as carboxylic acids (Plant and Jeff, 1994). For certain organics, such as aldehydes and some

nitrogen and sulfur containing compounds, direct treatment with H_2O_2 without a catalyst is also effective (Plant and Jeff, 1994). In the present study, the active oxidizing species was likely the H_2O_2 molecule itself, since no iron catalysts were employed. However, the formation of low quantities of hydroxyl radicals due to the presence of trace amounts of ferrous iron cannot be excluded. Interestingly, after the addition of peroxide to a reaction mixture from which phenol had been removed, the peroxide concentration did not decrease over time, suggesting that only very low amounts of oxidizable reaction products were present. This is supported by results obtained through radiotracer experiments (Ghioureliotis and Nicell, 1999).

Among the phenols tested, only 2-methylphenol showed elevated toxicities after HRP-treatment that did not decrease with time and could not be reduced upon addition of H_2O_2. Likewise, 2-chlorophenol could not be detoxified to a high extent during this study. It seems that *ortho*-substituted phenols have a higher tendency to form stable toxic compounds than their *para* or *meta* substituted counterparts. Maloney *et al.* (1986) reported that a furan-type compound accounted for 13.4% of the yield for the HRP-catalyzed oxidation of 2-methylphenol. However, the formation of dioxin and furan-type compounds was also reported for 4-methylphenol (Brown, 1967) and 2,4-dichlorophenol (Maloney *et al.*, 1986), which were detoxified upon HRP/H_2O_2 treatment. Thus, the reason why reaction products from *ortho*-substituted phenols are more stable remains unclear.

5.2.4 The impact of the mode of H_2O_2 and enzyme addition

The mode of reagent addition had a significant impact on the toxicity of HRP-treated phenol solutions (Figures 5.8 and 5.9). It has been suggested that the rate of phenolic radical formation has an impact on the distribution of reaction products along with other factors such as dissolved oxygen concentration, the ratio of H_2O_2 to phenolic substrate, and the pH (Griffin, 1991). Decreasing the instantaneous amount of H_2O_2 and/or HRP during the reaction leads to a slower rate of radical formation. This, as proposed by Ryu *et al.* (1993), could shift the reaction product distribution to higher molecular weight compounds that would exhibit a less toxic effect due to their lower solubility. This explanation is consistent with the lower level of toxicity observed at the initial stages of

the reaction with continuous HRP and H_2O_2 addition. However, this theory cannot explain why the toxicity declined at a slower rate with continuous enzyme addition as compared to the continuous H_2O_2 addition. The continuous introduction of HRP to the reaction mixture leads to two major differences in comparison to the continuous H_2O_2 addition. Firstly, the instantaneous concentration of H_2O_2 at early stages of the reaction is much higher in this case. It is conceivable that this difference could have an impact on the nature of the reaction products formed. Secondly, using the ABTS activity assay (data not shown) it was observed that when HRP was added continuously, the enzyme activity in the reaction mixture remained high for a longer period of time (approximately 33 % of the total activity remained at 180 min) due to reduced rates of inactivation by reaction products, whereas the activity dropped to 1.4 % when H_2O_2 was introduced slowly to the reaction mixture. It may be hypothesized that some toxic reaction products form from the peroxidase-catalyzed oxidation of phenolic reaction products (e.g., oxidation of dimers to quinones as demonstrated by Sawahata and Neal, 1982). In this case, a high concentration of active enzyme under conditions when the residual concentration of the parent phenol is very low could result in a continuous replenishment of toxic species.

5.2.5 The impact of UV illumination

Detoxification of the HRP-treated solutions was also achieved upon UV illumination at an energy input of 110 J/L corresponding to 5 minutes illumination at a volumetric radiation intensity of 0.36 W/L (Figure 5.10). The required energy input was about an order of magnitude lower compared to values needed for phenol elimination using UV irradiation at 254 nm combined with ozone and hydrogen peroxide as calculated from data provided by Mokrini *et al.* (1997). The presence of H_2O_2 did not reduce the required energy input thereby suggesting that toxic molecules were decomposed directly through UV light and not through the formation of hydroxyl radicals.

5.2.6 Absorbance at 400 nm

In the case of HRP-treated phenol solutions, detoxification was correlated with a drop of the absorbance at 400 nm. Several studies indicate that compounds with a maximum absorbance at 400 nm are formed immediately upon initiation of the reaction (Sawahata

and Neal, 1982; Arseguel and Baboulene, 1994; Ma and Rokita, 1988). The absorbance at this wavelength increases as the reaction proceeds and then diminishes as a precipitate is formed. 400 nm is the characteristic absorbance peak of phenoxyl radicals (Neta and Steenken, 1981) and also of quinones, particularly of *p*-diphenoquinone (Sawahata and Neal, 1982), which has been identified as a product of HRP-catalyzed oxidation of phenol. However, since some samples exhibited a high toxicity despite a low absorption at 400 nm, it cannot be concluded that quinones are the predominant toxic species. Rather, the changes in the absorbance at 400 nm could also be seen as an indicator of the dynamic changes in the distribution of reaction products that take place during and after the enzymatic phenol conversion.

6. IMPACT OF WASTEWATER PARAMETERS

6.1 Suspended Solids

The total solids content of a wastewater is defined as all matter that remains as residue after evaporation at 103 to 105°C (Metcalf and Eddy, 1979). Suspended solids are the fraction of the total solids, which is retained by a filter. The filter is commonly chosen so that the minimum diameter of the retained solids is about 10^{-6} m (1 μm) (Metcalf and Eddy, 1979). Domestic wastewaters contain typically 100 to 350 mg/L of suspended solids (Metcalf and Eddy, 1979), whereas depending on the type of the operation the content in industrial wastewaters may exceed 10000 mg/L (Dyer and Mignone, 1983). Solid particles may vary over a wide range of chemical and physical characteristics. For example they can be of organic or mineral origin.

It is hypothesized that suspended solids could influence the HRP-catalyzed phenol oxidation through at least three mechanisms:

(1) The enzyme could become immobilized onto the particles, mainly through adsorption. The immobilization on the solid surface causes a change in the microenvironment of the enzyme. Depending on the type of support, this may result in either a reduced or an increased stability of the biocatalyst (Kennedy and Roig, 1995). Immobilization may also change the kinetic properties of the enzymatic reaction and the affinity of the enzyme towards its substrate (Kennedy and Roig, 1995).

(2) Suspended solids could interact with reaction products by physical or chemical adsorption. Since the primary reaction products of the HRP-catalyzed phenol oxidation are very reactive phenol radicals, which were shown to contribute to enzyme inactivation (Ator and Ortiz de Montellano, 1987), an interaction of these or other reactive species with the solid surface could lead to an enhanced stability of the peroxidase during the reaction.

(3) Finally, the solids could interact with the substrate molecules, removing them from the aqueous phase through adsorption.

6.1.1 Characteristics of the investigated solids

In order to investigate the impact of selected suspended solids on peroxidase-catalyzed phenol removal, batch reactions were carried out in the presence of bentonite, kaolin, silica gel, cellulose, peat moss and powdered activated carbon (PAC).

Bentonite, kaolin and silica gel are representative of clays. Clays are the mineral soil particles that are smaller than 2 μm (Tan, 1998). Due to their large surface area they are very important for the chemical reactivity of soils. A large fraction of clays in typical soils consists of colloidal species, defined as particles smaller than 0.2 μm (Tan, 1998).

Kaolinite minerals are hydrated aluminosilicates with the general chemical composition Al_2O_3:SiO_2:H_2O. The crystal is composed of aluminum octahedron sheets stacked above silica tetrahedron sheets, which are held firmly together (Tan, 1998). For this reason, kaolinites do not swell in water and have relatively low surface areas (i.e. approximately 7-30 m^2/g). Owing to the presence of exposed hydroxyl groups, kaolinites have a pH-dependent negative charge resulting in some cation exchange capacity (l-10 mEq/100 g) (Tan, 1998).

Bentonite is the name for commercial grade clay that mainly consists of smectite types of minerals (also referred to as montmorillonites). Like kaolinites, smectites are also hydrated aluminosilicates. However, smectites often contain Mg^{2+} and Fe^{3+} rather than Al^{3+} or Si^{4+} in the crystal sheet. This imparts a permanent negative surface charge and is responsible for the relatively high cation exchange capacity of 70 mEq/100 g for a typical smectite. Ions most often found in exchange position of natural clays are Mg^{2+}, Na^{+}, Al^{3+} and H^{+} (Snell and Ettre, 1971). In comparison to kaolinites, smectites also have a higher specific surface area (700 to 800 m^2/g) and are known to strongly swell in water (Tan, 1998).

Silica gel is a highly porous, non-crystalline form of silica (SiO_2) used to remove moisture from gases and liquids and as support for chromatography. Silica minerals are frequently a significant constituent of the clay fraction of soils. However, in contrast to other clay minerals they are generally considered inert or chemically inactive (Tan, 1998). The silica gel used in this study had a BET surface area of 500 m^2/g.

In this investigation, bentonite, kaolin and silica gel were chosen to represent inorganic suspended solids because these materials possess a range of different

reactivities, starting from silica gel, which is relatively unreactive to bentonite, which exhibits high reactivity. Also, these types of materials are relatively abundant in natural waters and may also be found in industrial wastewaters.

Peat is the brownish, only partially decomposed, fibrous remains of plant tissue. It is naturally formed in areas where plant material accumulates due to an inhibition of residue decomposition by lack of oxygen (Brady and Weil, 1999). Moss peat (or peat moss) is derived from mosses such as sphagnum. It has high water holding capacities and is quite acidic (Brady and Weil, 1999). In order to be classified as peat, soil material must contain at least 35% organic matter. Soil organic matter consists of three components: (1) living biomass, (2) partially decomposed plant and animal material (detritus), (3) a largely amorphous and colloidal mixture of complex organic substances, referred to as soil humus (Brady and Weil, 1999). The soil humus is sub-divided into humic and nonhumic substances. Humic substances comprise the bulk of the soil organic matter (60 to 80%). They are formed during decomposition of plant and animal tissue in a complex process called humification. Humic substances are classified into three groups: humin (insoluble), humic acids (dark brown, only soluble in alkali, MW = up to 300000) and fulvic acids (yellow to red, soluble in acids, MW = 2000-50000) (Brady and Weil, 1999). The major nonhumic substances in soil humus are lignin, carbohydrates (mainly polysaccharides), and lipids. Also, low amounts of low molecular weight organic acids, amino acids, nucleic acids and proteins are present (Tan, 1998).

Cellulose is a carbohydrate polymer that is the main structural element in the cell walls of trees. It is the most abundant natural organic material. Cellulose consists of D-glucose monomer units, which are joined by β-1,4-glycosidic bonds (Snell and Ettre, 1971). The degree of polymerization varies between 5000 and greater for native celluloses to 200 for some cellulose derivatives (Snell and Ettre, 1971). Celluloses have a very complex and heterogeneous tertiary structure and are essentially insoluble in water (Attala, 1999).

Peat moss and cellulose represent organic suspended solids in this study. Peat moss was chosen because it consists of a very complex mixture of soluble and insoluble organic constituents, including living biomass, partially decomposed organic material and colloidal organic compounds. Industrial wastewaters containing these types of materials

may, for example, originate from the pulp and paper industry. Pulp and paper wastewaters may also contain cellulose and cellulose-derived compounds.

Activated carbons are excellent adsorbents and thus are used to separate a variety of species from the aqueous or gaseous phase. This material is obtained through combustion or thermal decomposition of carbonaceous substances, such as wood, coal or coconut-shells (Bansal *et al.*, 1988). The adsorbent properties of activated carbon are mainly attributable to their large surface area (800-1500 m^2/g of most commercial products) combined with a favorable pore size, which makes the internal surface accessible for adsorption (Bansal *et al.*, 1988). Activated carbon is applied for phenol removal from industrial wastewaters and is reported to have a phenol capacity of 0.5 – 25 kg/100 kg carbon (Patterson, 1985). Powdered activated carbon was included in this study primarily due to its high adsorptive capacity with the objective to test whether adsorption of the enzyme may interfere with enzymatic phenol removal. Also, it is possible that carbon adsorption may be used to treat certain wastewaters prior to HRP-treatment, in which case some activated carbon may be present in the wastewater stream.

6.1.2 Results

(a) Impact on phenol removal

Batch experiments were carried out to investigate the impact of suspended solids on the extent of phenol removal from solutions initially containing 1.0 mM phenol. Since the hydronium ion concentration was expected to affect the way the solids interact with other species present in the reaction mixture, reactions were carried out at three different pH values: 5.0, 7.0 and 9.0 in citrate-phosphate, sodium phosphate and carbonate buffer, respectively. HRP activity drops significantly at pH's lower than 5 and higher than 9 (Nicell *et al.*, 1993). Therefore, most real wastewater treatment applications are likely to be carried out within this pH-range. Sodium hydrogen phosphate was added to the carbonate buffer to obtain a uniform phosphate concentration of 0.05 M under all conditions. An HRP-dose of 2.0 U/mL was supplied to the reactions while the H_2O_2 dose was 1.3 mM. Under these conditions, only partial phenol removal was achieved at pH 5.0 and 7.0, while reactions at pH 9.0 led to almost complete phenol conversion.

Additionally, reactions were also carried out at pH 9.0 using 0.1 U/mL HRP which led to intermediate levels of treatment and made it easier to identify possible negative or positive effects of the tested solids. A reaction time of 20 hours was provided to ensure that the reactions have gone to completion. In order to test for removal of phenol from the aqueous phase through adsorption to the solid surfaces, control batches were included which contained neither HRP nor peroxide. The results of these experiments are shown in Figures 6.1 to 6.6.

Silica gel had generally no effect on the enzymatic reaction at all concentrations tested (Figure 6.1). However, the presence of the other solids had an impact on the extent of phenol conversion.

In the case of bentonite (Figure 6.2), phenol removal was enhanced at the concentrations of 10 and 1000 mg/L at pH 7.0 and 5.0. At pH 9.0, bentonite had no impact on phenol conversion up to a concentration of 1000 mg/L. However, when the bentonite concentration was increased to 10000 mg/L an adverse effect on phenol transformation was observed at all pH levels.

Kaolin (Figure 6.3) and peat moss (Figure 6.4) led to an enhanced phenol conversion at pH 7.0 when present at 1000 and 10000 mg/L. The same effect was observed at pH 5.0 only at a solids concentration of 10000 mg/L. At pH 9.0, kaolin and peat moss decreased phenol conversion when present starting at 10000 and 1000 mg/L, respectively. Note that approximately 0.1 mM phenol was removed in 10000 mg/L peat moss suspensions at pH 7.0 and 9.0 in the absence of HRP and H_2O_2 (Figure 6.4).

Cellulose (Figure 6.5) increased phenol conversion when present at 10000 mg/L at pH 5.0 or pH 7.0. At pH 9.0, cellulose had no impact on residual phenol concentrations.

Powdered activated carbon (PAC) did not have a substantial effect on phenol transformation in the concentration range studied (Figure 6.6). Although some enhanced phenol removal was obtained at pH 7.0 with 100 mg/L PAC, this effect can be attributed to the fact that approximately 14 % (0.15 mM) of the initial phenol had been adsorbed under this condition. Similar to results with other solids, phenol removal was adversely affected at pH 9.0 when the PAC concentration was increased to 50-100 mg/L.

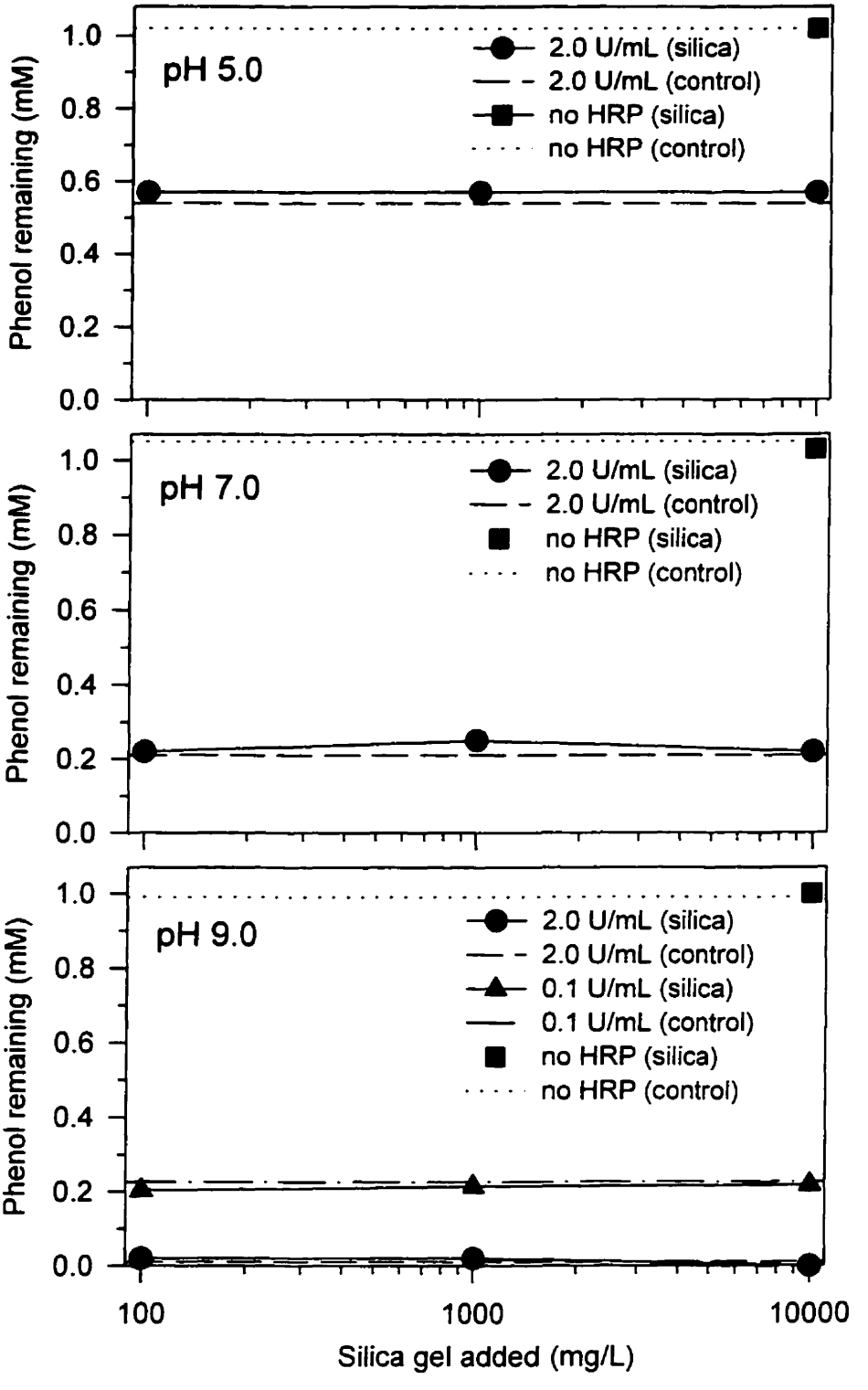


Figure 6.1: Phenol remaining as a function of the amount of silica gel added.

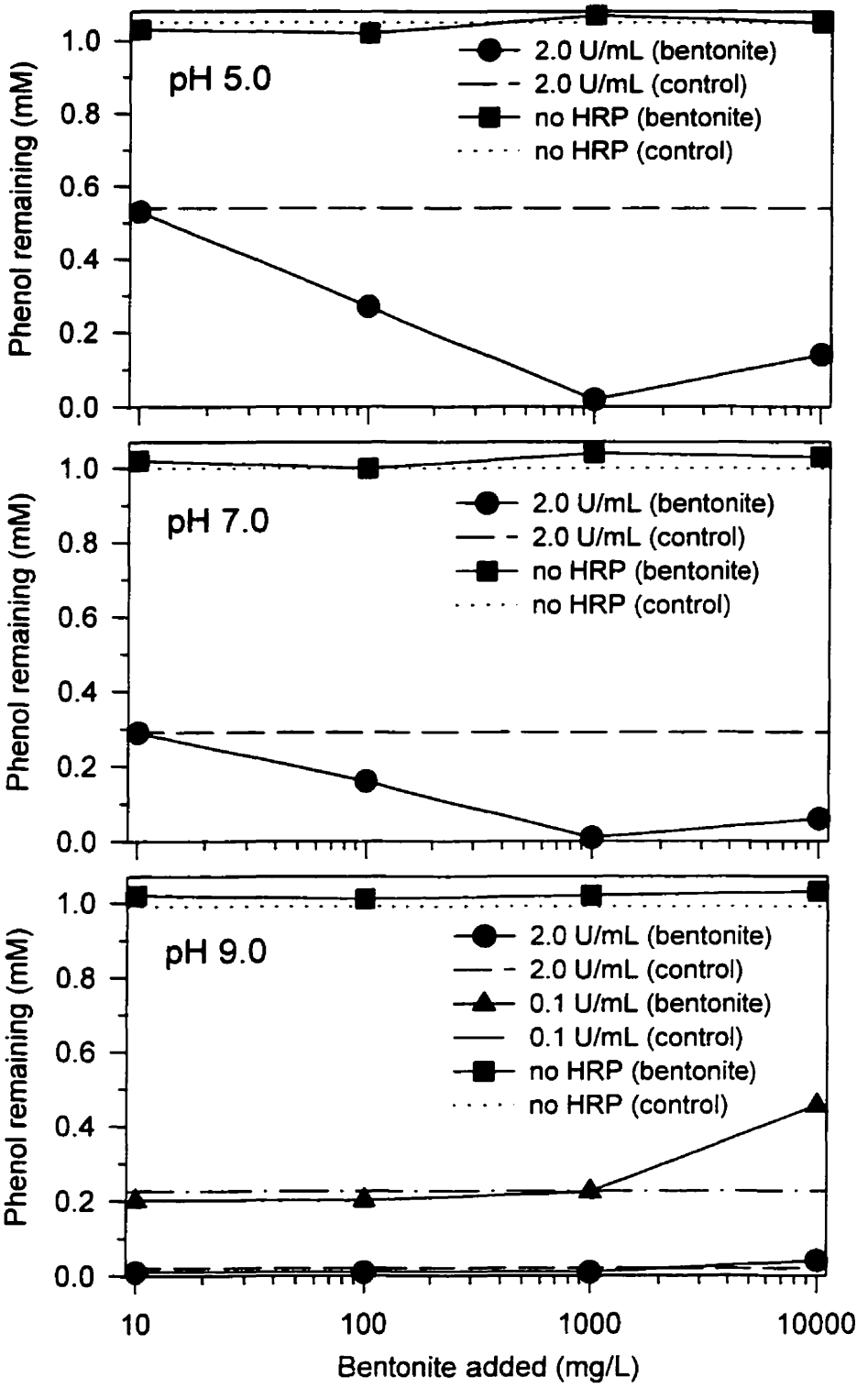


Figure 6.2: Phenol remaining as a function of the amount of bentonite added

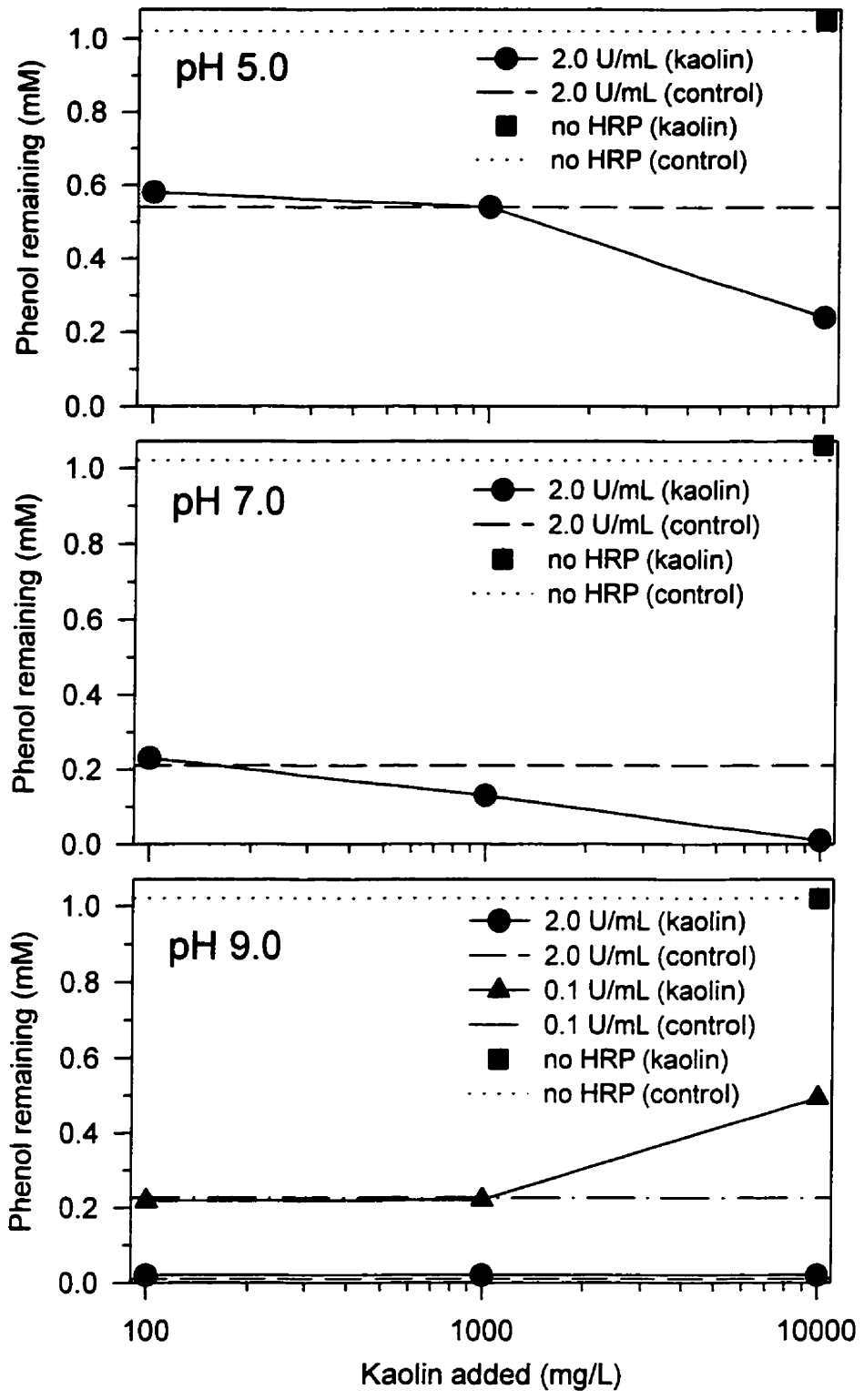


Figure 6.3: Phenol remaining as a function of the amount of kaolin added.

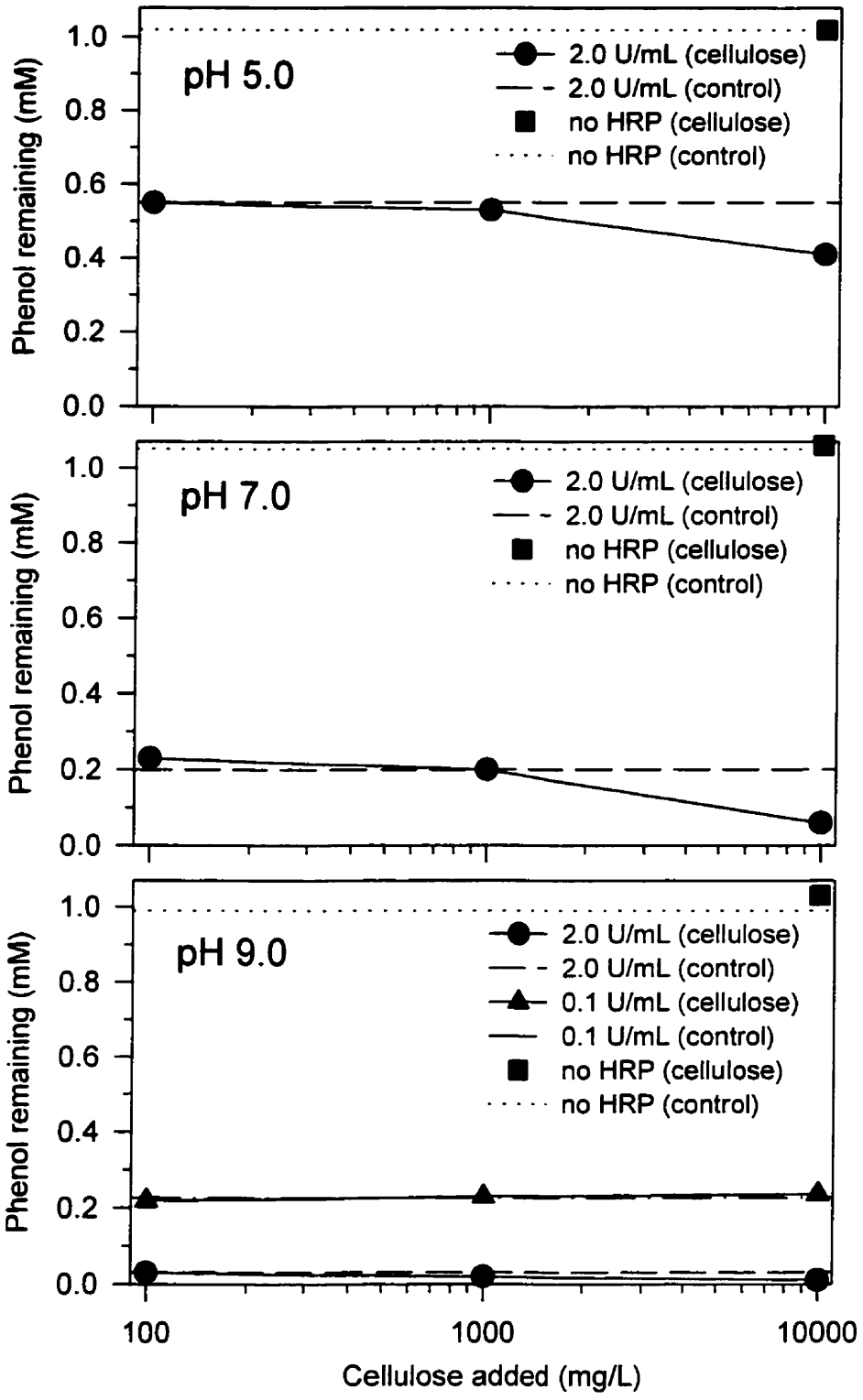


Figure 6.4: Phenol remaining as a function of the amount of cellulose added.

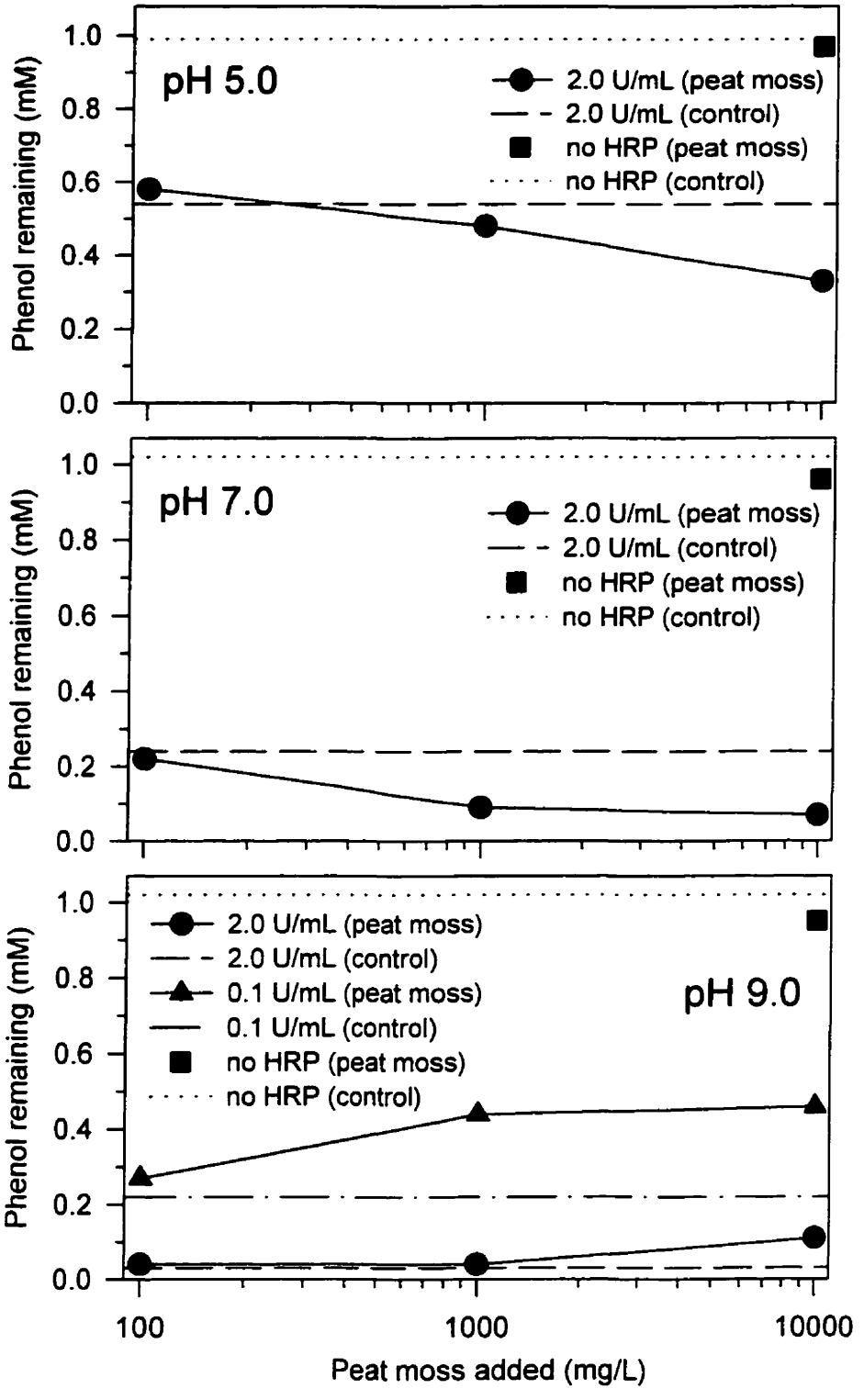


Figure 6.5: Phenol remaining as a function of the amount of peat moss added.

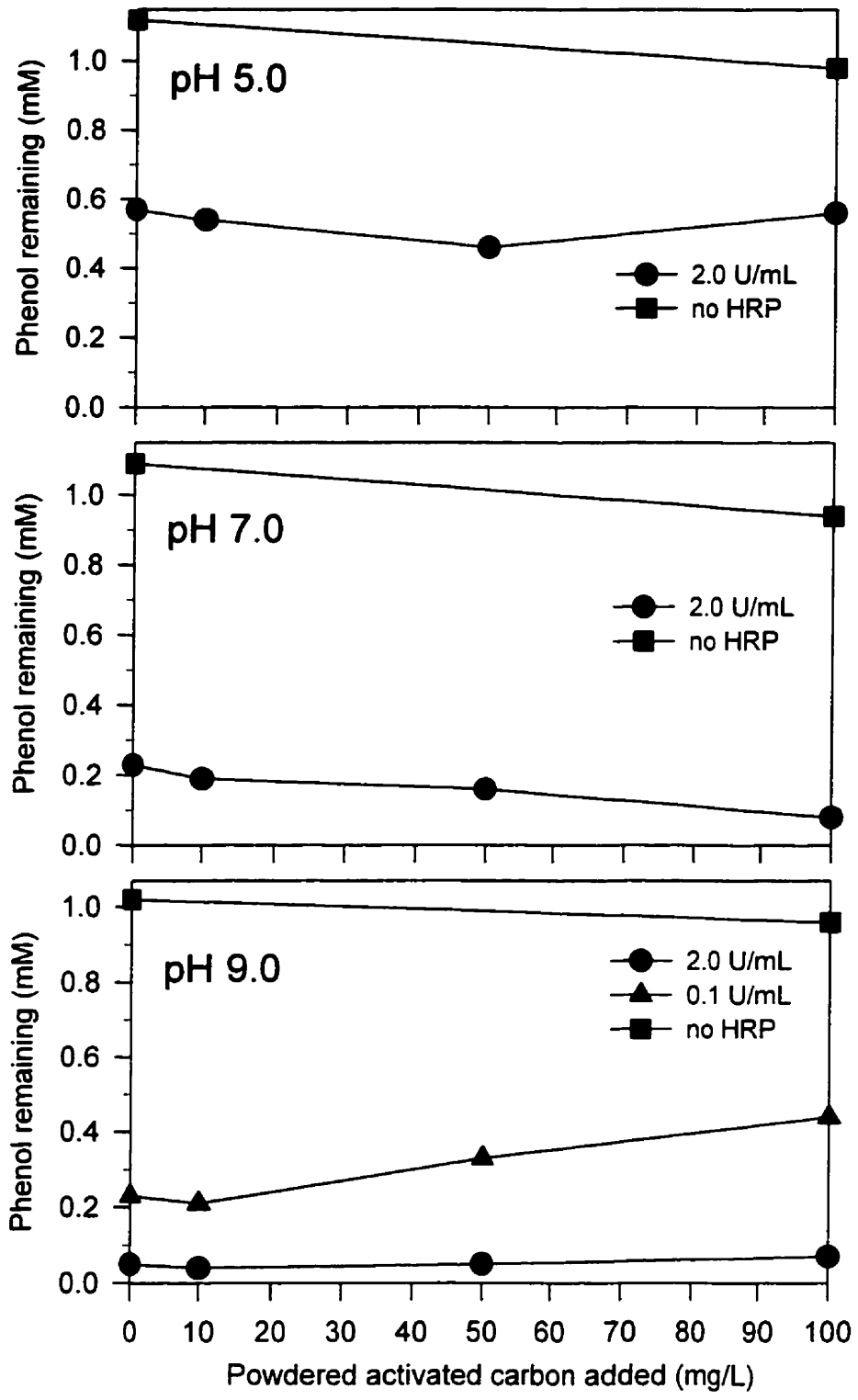


Figure 6.6: Phenol remaining as a function of the amount of powdered activated carbon added.

Tests with 10000 mg/L (except PAC at 100 mg/L) of solids were conducted to check whether these materials could catalyze phenol removal in the presence of 1.3 mM of H_2O_2. No significant phenol transformation was apparent in these experiments.

(b) Impact on enzyme and peroxide stability

Experiments described in the previous section have shown that phenol removal is enhanced in the presence of many solid materials at pH 7.0 and 5.0. However, an enhanced phenol removal does not necessarily mean that the enzyme is stable in the presence of these solids. For example, it was found that HRP was inactivated when incubated with hydrophobic talc (Arseguel and Baboulene, 1994). Nevertheless, this mineral enhanced phenol transformation presumably through adsorption of reaction products, which rapidly inactivate the enzyme once the phenol removal reaction is initiated. Thus, the enzyme may be inactivated through the contact with certain solids, but at the same time these materials may protect the enzyme from product inactivation by adsorbing reactive reaction intermediates. If the rate of inactivation by the solids is relatively slow compared to the inactivation by reaction products, the net effect of the presence of the solids will be to prolong the catalytic lifetime of the enzyme leading to enhanced phenol conversion.

Under the controlled conditions of industrial wastewater treatment, it is unlikely that the enzyme would be exposed to the wastewater matrix over a prolonged period of time before the phenol removal reaction would be initiated. Thus, inactivating species would have little time to exert their detrimental effect. However, for such applications as groundwater treatment, where immediate mixing of the two reagents, HRP and peroxide, might not always be guaranteed, longer term enzyme inactivation by components of the soil system may affect the outcome of the treatment process.

The ideal approach to investigate the stability of the enzyme in the presence of suspended solids would be to monitor its activity in the suspension over time, since the enzyme could become immobilized on the solid surface, while retaining its activity. However, measurement of enzyme activity in suspensions containing high concentrations of fine solids is not feasible because the solids interfere with the spectrophotometric readings. Therefore, inactivation of the enzyme was investigated by pre-incubating the

enzyme in the presence of solids before the reaction was initiated through the addition of phenol and H_2O_2. Thus, a decrease of phenol removal after pre-incubation with the solid would indicate that some inactivation of the catalyst took place.

HRP or H_2O_2 were pre-incubated for a period of one hour at pH 7.0 in the presence of solids (10000 mg/L of bentonite, kaolin, silica, cellulose, and peat moss or 100 mg/L PAC) before the reactions were initiated by the addition of phenol and the respective other reagent. For comparison, reactions were carried out to which both reagents were added at the same time. Control reactions did not include suspended solids. The results of this experiment are shown in Figure 6.7.

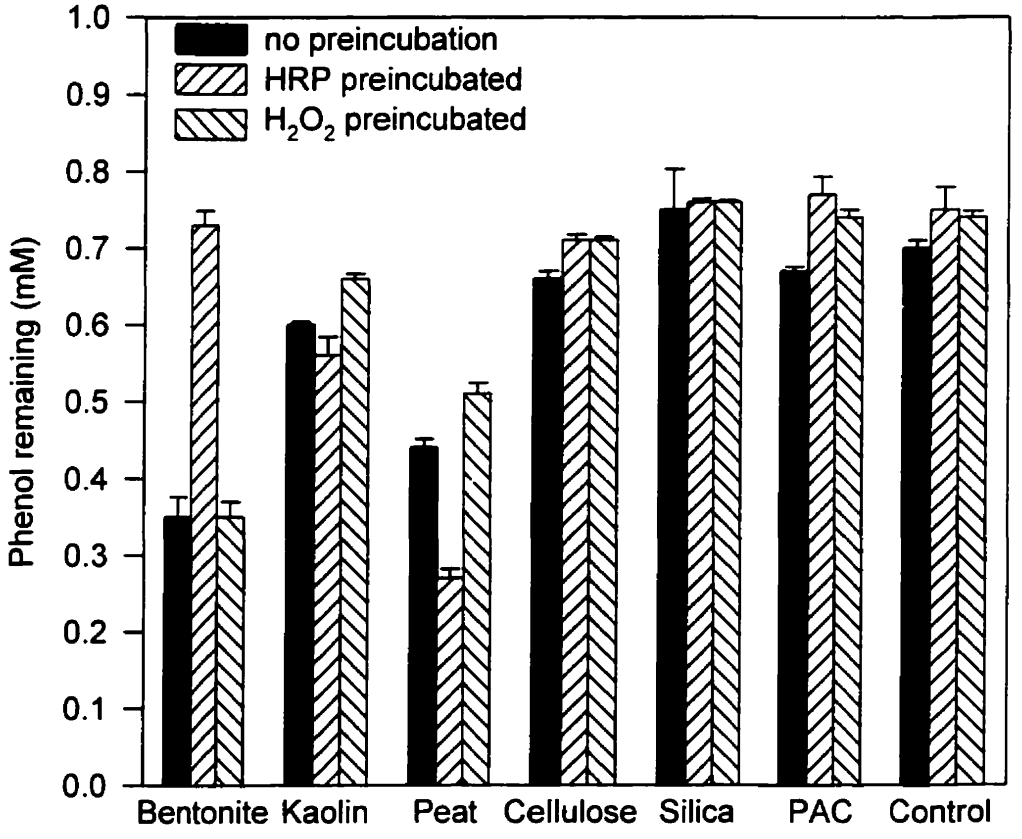


Figure 6.7: Effect of pre-incubation of HRP or H_2O_2 in the presence of suspended solids. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ =1.3 mM; HRP_0 = 0.5 U/mL; pH 7. Error bars stand for the range of duplicate reactions.

Incubation of the enzyme or the peroxide in the presence of 10000 mg/L of cellulose or silica had no significant effect on amount of phenol transformation indicating that the enzyme and the peroxide were stable under these conditions. However, when the enzyme was incubated in the presence of 10000 mg/L of bentonite, phenol was transformed to a substantially lower degree. In contrast, significantly higher phenol removal was achieved when HRP was pre-incubated with 10000 mg/L of peat moss. Pre-incubation of peroxide in the presence of peat moss resulted in a reduced level of phenol removal suggesting that peat moss effects peroxide decomposition.

The changed level of substrate conversion after incubation of the enzyme with the solid might suggest in the case of bentonite that the enzyme was gradually inactivated or in the case of peat moss that it was stabilized through the contact with the solid particles. However, the alternative explanation is that the sample matrix itself could have been changing over time while the solids were exposed to the phosphate buffer. For example soluble or colloidal matter could gradually partition into the aqueous phase. Also, the solid surface could change its characteristic when brought in contact with the liquid, which may affect the way, the enzyme or possibly certain reaction products interact with the solid.

In order to test these possibilities further experiments were carried out with bentonite, kaolin and peat moss. The solids were added at 10000 mg/L to 0.05 M pH 7.0 phosphate buffer and stirred vigorously over night. Subsequently, each suspension was divided into two sub-samples, of which one was centrifuged for 15 minutes at 3000 g to remove the particulate matter. In the following discussion, these sample matrices will be referred to as aged suspensions and its supernatants. Also, fresh solid suspensions were prepared by adding the solid into the buffer shortly before the start of the experiment. Two types of experiments were carried out with these three sample matrices. In one experiment, HRP was pre-incubated for up to three hours in the matrices containing or corresponding to 10000 mg/L solids before reactions were initiated by the addition of 1.0 mM phenol and 1.3 mM H_2O_2. For the other experiment, the aged suspension and its supernatant were diluted with buffer to obtain suspensions and supernatants that would correspond to solids concentrations of 100 and 1000 mg/L (in the case of bentonite also 10 mg/L). Subsequently, phenol removal reactions were carried out with these samples as

well as with freshly prepared suspensions. Treatment was conducted with 0.5 U/mL HRP in the case of bentonite and peat moss and 1.0 U/mL in the case of kaolin. The results of these experiments are presented in Figures 6.8 to 6.10.

The results displayed in Figure 6.8(a) indicate that aged bentonite suspensions and their supernatants were more effective in enhancing phenol removal than freshly prepared suspensions. Moreover, while the phenol removal efficiency was identical in aged suspensions and their supernatants up to a concentration of 1000 mg/L, it decreased in the aged suspension at 10000 mg/L, but remained high in the corresponding particle-free supernatant. This observation was confirmed by the results presented in Figure 6.8(b), which show that at 10000 mg/L consistently higher phenol removal was achieved when the solid particles were separated from the aged suspension. However, prolonged incubation of the enzyme in the aged bentonite suspension or its supernatant did not affect the degree of treatment that was achieved. Thus, the enzyme was stable in both of these matrices. In contrast, residual phenol concentrations were higher the longer the peroxidase was exposed to the freshly made bentonite suspension strongly indicating that this material inactivated the enzyme during the pre-incubation period.

Similarly, in the case of kaolin (Figure 6.9) phenol transformation was more enhanced in the aged than in the fresh suspensions. However, in contrast to what was observed with bentonite, dissolved or colloidal matter that was eventually present in the aged supernatant was not effective. Thus, in the case of kaolin the enhancing characteristics were only associated with the particulate matter. Moreover, the results presented in Figure 6.9(b) indicate that HRP was stable in all three sample matrices.

In the case of peat moss, at 10000 mg/L higher levels of phenol conversion were obtained with the aged supernatant as compared to the fresh and aged suspensions (Figure 6.10(a)) indicating that particulate matter exhibited an adverse effect on enzymatic phenol transformation. Moreover, confirming the previous observation, phenol removal slightly increased when the enzyme had been pre-incubated in the fresh suspension (Figure 6.10(b)). However, pre-incubation of HRP with the aged suspension and its supernatant had no effect.

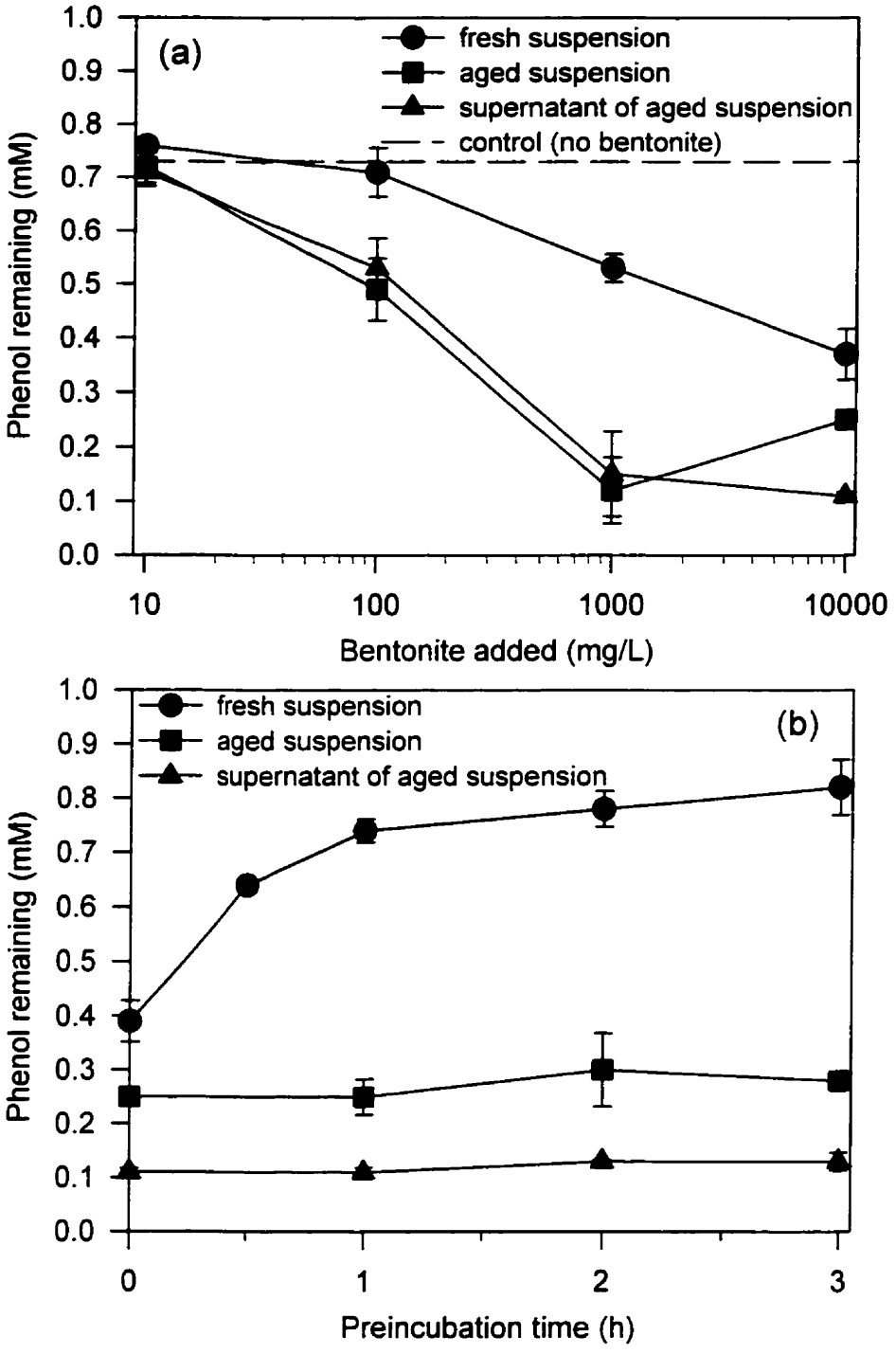


Figure 6.8: (a) Phenol remaining in the presence of fresh and aged bentonite. (b) Effect of HRP pre-incubation in the presence of fresh and aged bentonite at 10000 mg/L. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 0.5 U/mL; pH 7. Error bars stand for the range of two independent experiments.

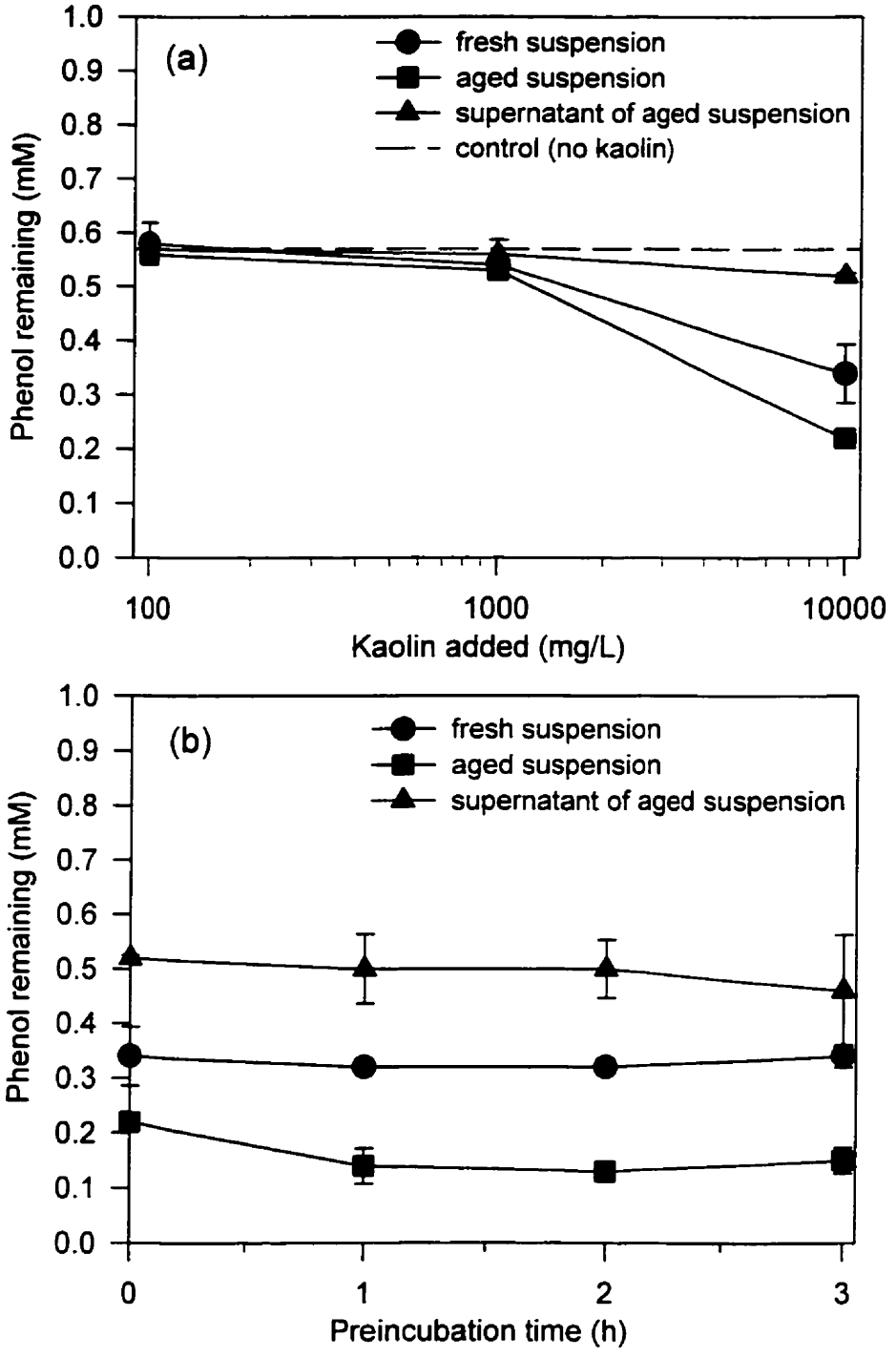


Figure 6.9: (a) Phenol remaining in the presence of fresh and aged kaolin. (b) Effect of HRP pre-incubation in the presence of fresh and aged kaolin at 10000 mg/L. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 1.0 U/mL; pH 7. Error bars stand for the range of two independent experiments.

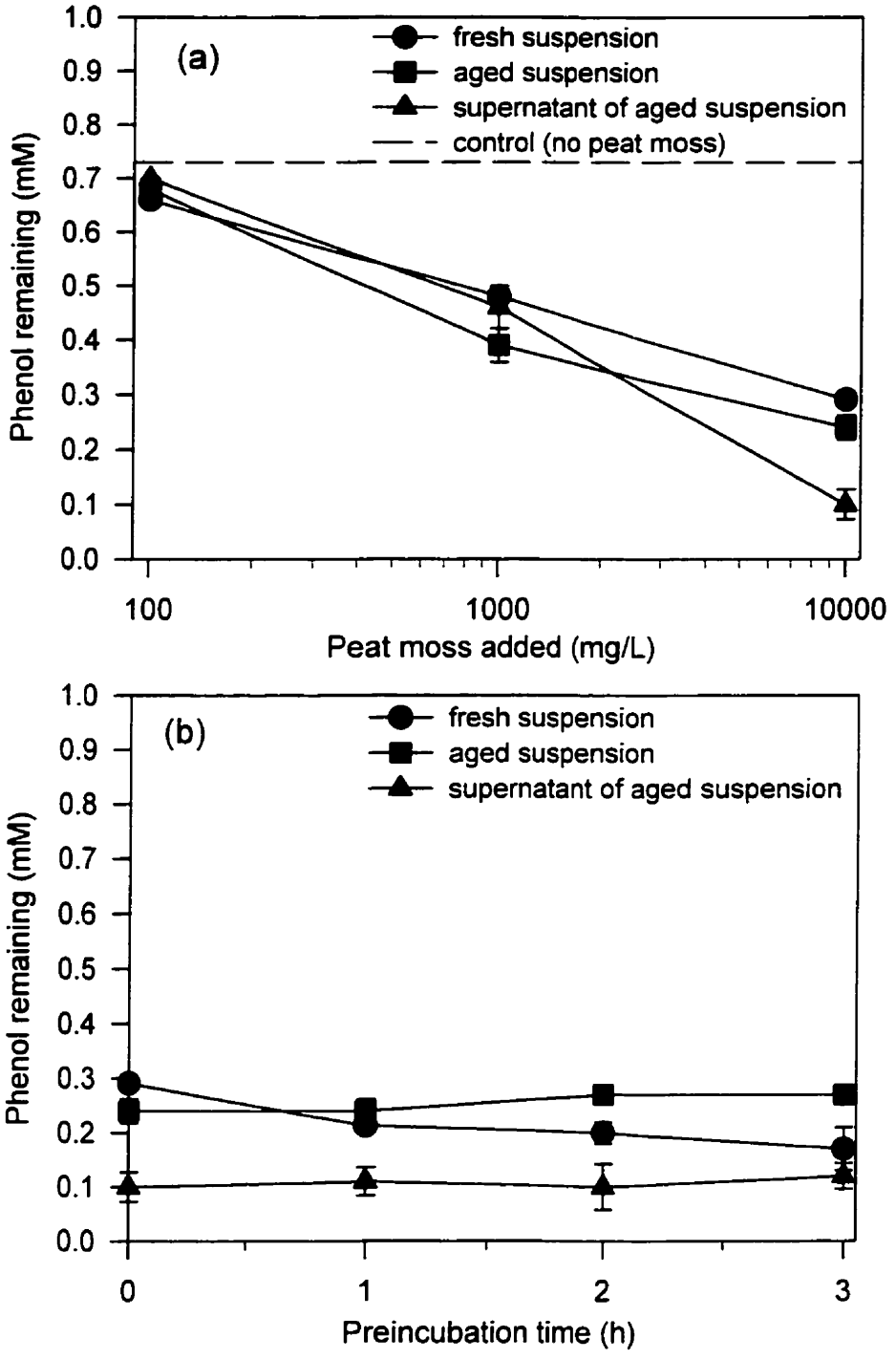


Figure 6.10: (a) Phenol remaining in the presence of fresh and aged peat moss. (b) Effect of HRP pre-incubation in the presence of fresh and aged peat moss at 10000 mg/L. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 0.5 U/mL; pH 7. Error bars stand for the range of two independent experiments.

A H_2O_2 assay was performed on the samples containing or corresponding to 10000 mg/L peat moss. It revealed that no residual hydrogen peroxide had remained even though only limited phenol removal had been achieved. In order to test the stability of H_2O_2 in the presence of peat moss, this reagent was added at 1.2 mM to filtered and unfiltered aged suspensions of 10000 mg/L peat moss and the peroxide concentration was monitored over time. The results of this experiment are shown in Figure 6.11.

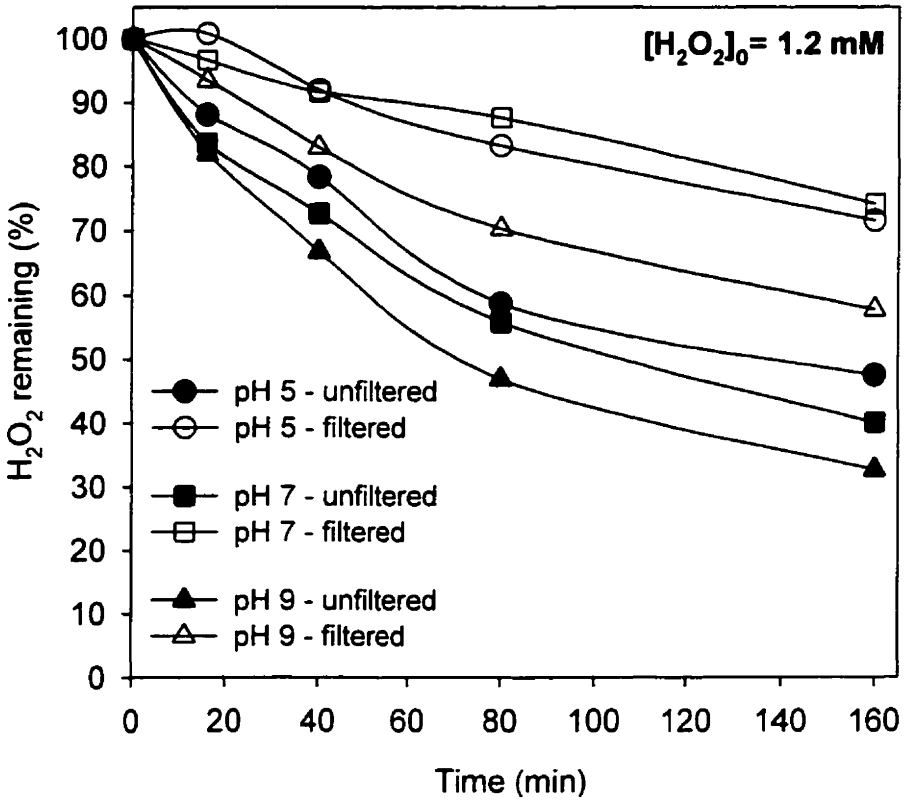


Figure 6.11: Hydrogen peroxide decay in the presence of filtered and unfiltered peat moss material at 10000 mg/L.

Two conclusions can be drawn from the data presented. Firstly, H_2O_2 disappeared faster in the presence of particulate matter at all three pH's. This observation suggests that phenol removal in peat moss suspensions was peroxide limited and thus may explain why higher phenol conversion was achieved when the particles were removed from the aged suspension (Figure 6.10).

Secondly, the rate of H_2O_2 decay was faster at pH 9.0 than at pH 7.0 or 5.0. Extracts obtained at pH 9.0 were also darker in color than those at higher pH levels since they contained higher concentrations of humic constituents, which are more soluble under alkaline conditions.

Experiments were carried out to determine whether phenol removal in solutions containing peat moss could be improved by supplying higher doses of H_2O_2. Peat moss at 10000 mg/L was added to pH 5.0, 7.0 and 9.0 buffers and stirred vigorously overnight in order to extract the soluble material. Subsequently, a part of these suspensions was centrifuged to remove the particulate matter. After phenol was added to these 6 sample matrices, they were subjected to the enzymatic phenol removal reaction using 0.5, 0.75 and 0.1 U/mL HRP for reactions at pH 7.0, 5.0 and 9.0, respectively. The H_2O_2 concentration was varied from 1.3 to 2.3 mM. The results of these experiments are displayed in Figure 12. Generally, phenol removal improved with increasing peroxide doses indicating that the phenol conversion was indeed at least in part limited by the availability of H_2O_2. An exception was the aged supernatant at pH 5.0, in which phenol removal remained constant, while it increased in the whole suspension. The reason for this phenomenon is not clear. However, a possible explanation could be that HRP may be more sensitive to inactivation by H_2O_2 at lower pHs. In this case, increasing H_2O_2 concentrations may not necessarily lead to higher phenol removal efficiencies.

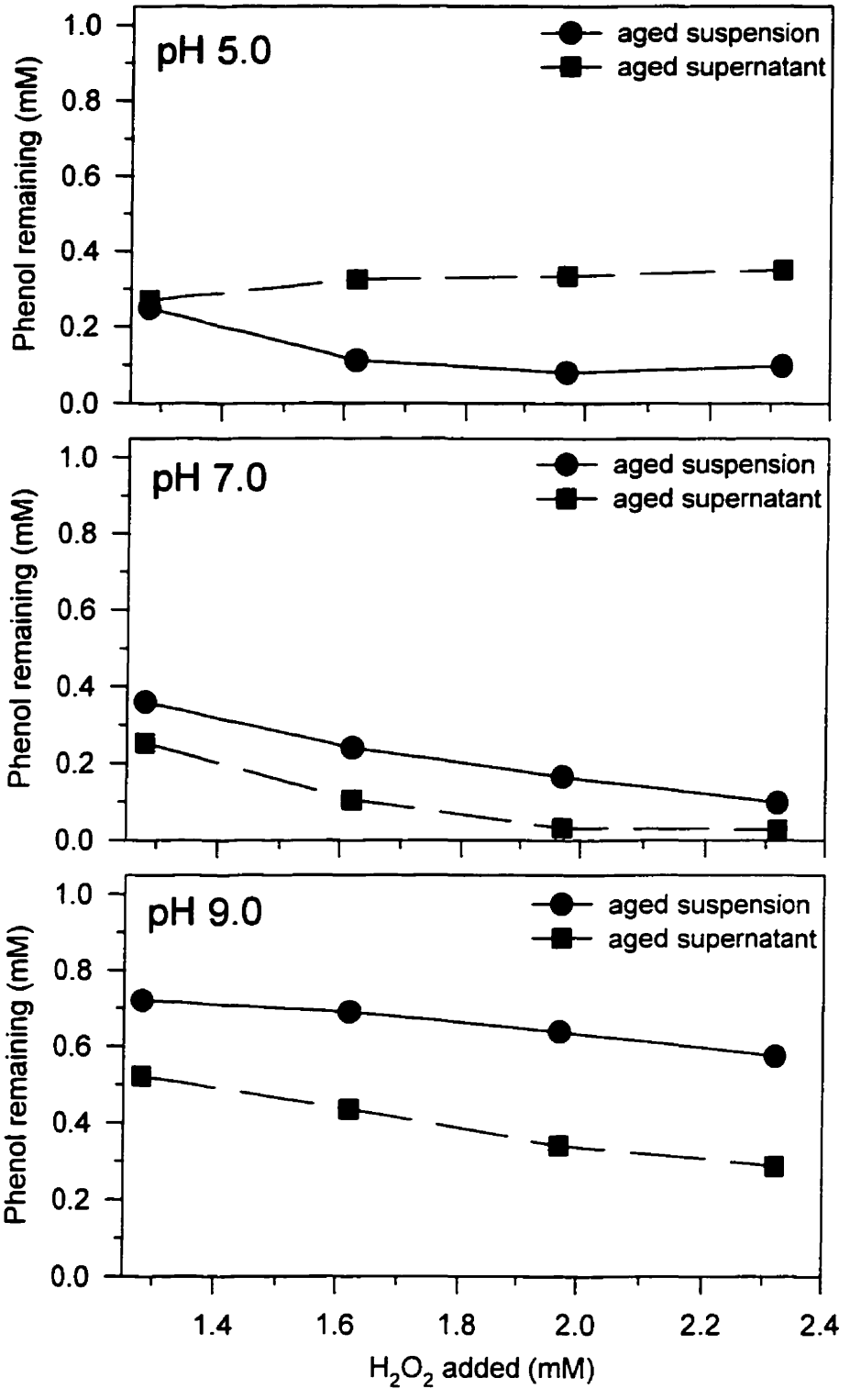


Figure 6.12: Effect of increasing H_2O_2 concentrations on the treatment in the presence of aged peat moss suspensions and supernatants at 10000 mg/L. HRP_0 = 0.75 U/mL for pH 5; 0.5 U/mL for pH 7 and 0.1 U/mL for pH 9.

(c) Impact on toxicity of HRP-treated phenol solutions

In order to test whether the nature and distribution of HRP reaction products would depend on the presence of other components in the waste matrix, 1.0 mM phenol solutions in 0.05 M phosphate buffer (pH 7.0) were treated with HRP and 1.3 mM H_2O_2 in the presence of bentonite, kaolin or filtered peat moss extract (corresponding to 10000 mg/L peat moss). Control samples, which did not contain suspended solids, were treated with 4.5 U/mL HRP. Lower HRP doses were used with the samples that were treated in the presence of suspended solids, since these solids have shown to enhance phenol removal. In each case a high level of phenol transformation was obtained after a 3-hour reaction time. At this point the toxicity of the solutions was measured and compared to toxicities of the control reaction mixtures that were treated concurrently. The TU_{50} of the control reaction mixtures was determined to be 22.5 ± 2.3 (95% confidence interval) using nine samples obtained in four independent experiments. The results of these experiments are summarized in Table 6.1.

Table 6.1: Toxicities of 1.0 mM phenol solutions treated in the presence of suspended solids or filtered peat moss extract.

Solid added	HRP dose (U/mL)	% Phenol removed[a]	TU_{50} ± std[b]	% Difference to controls
Bentonite (10 mg/L)	4.5	99.7	20.1 ± 1.0	-10.9[c]
Bentonite (1000 mg/L)	2.0	99.7	5.3 ± 0.4	-76.5
	2.0	96.5	5.6 ± 0.3	-75.2
	3.0	99.8	3.9 ± 0.3	-82.7
Kaolin (10000 mg/L)	2.0	99.6	13.7 ± 1.1	-39.1
	3.0	99.8	14.6 ± 1.0	-35.1
Peat moss (10000 mg/L)	2.0	98.0	7.1 ± 0.6	-68.5
	3.0	97.3	5.8 ± 0.3	-74.3

[a]Phenol removal in controls was > 99%.
[b]All experiments were carried out in duplicate or triplicate.
[c]Not significantly different from controls (p = 0.207 in Student's t-test)

Bentonite did not exert a significant impact on toxicity levels compared to the controls when present at 10 mg/L. At this concentration bentonite also did not affect the phenol transformation efficiency (see Figures 6.2 and 6.8(a)). However, samples treated in the presence of 1000 mg/L of bentonite were on average 78% less toxic than the reaction mixtures treated without solids. A lower toxicity reduction (approximately 37%) was achieved when treatment was conducted in the presence of 10000 mg/L of kaolin. The presence of dissolved or colloidal material originating from peat moss led to a 71% toxicity drop as compared to the controls.

The toxicity (expressed as TU_{50}) of the solids alone in phosphate buffer was approximately 1 for 10000 mg/L kaolin and less than 0.5 for 1000 mg/L of bentonite and 10000 mg/L of peat moss.

Note that differences in enzyme doses as well as small variations in the residual phenol concentration did not significantly affect the resulting toxicity levels.

In further experiments, bentonite or kaolin were investigated to determine if they exert their effect by adsorbing toxic reaction products after they are formed or by modifying the product distribution during the phenol transformation reaction. 1000 mg/L of bentonite or 10000 mg/L of kaolin were added to a reaction mixture that had been treated with 4.5 U/mL of HRP and 1.3 mM H_2O_2 for 3 hours resulting in more than 99 % phenol removal. The suspensions were stirred vigorously for another three hours before their toxicities were assayed. Controls were treated identically except that no solids were supplied. The results of these experiments are summarized in Table 6.2.

Table 6.2: Toxicities of HRP-treated phenol solutions after a 3-hour incubation with kaolin or bentonite.

Solid added	**$TU_{50} \pm$ std**	**$TU_{50} \pm$ std of controls**	**% Difference to controls**
Bentonite (1000 mg/L)	16.4 ± 2.0	19.2 ± 0.6	-14.4[a]
Kaolin (10000 mg/L)	$18.9. \pm 1.9$	19.2 ± 0.6	-1.1

Results of 4 replicates (n = 4).
[a] Significantly different from controls (p = 0.043 in Student's t-test)

No toxicity reduction was observed when the HRP-treated samples were incubated with kaolin. Thus, kaolin was not able to adsorb toxic reaction products after they were formed. On the other hand, in the presence of bentonite, a small but significant toxicity drop was recorded. However, toxicities resulting from contacting an HRP-treated reaction mixture with bentonite (TU_{50} = 16.4) were much higher than if bentonite was present in the reaction mixture at the start of the reaction ($TU_{50} \approx 5.0$). These results indicate that bentonite was able to interact with precursors of toxic reaction products, thus preventing the formation of more stable toxic compounds.

6.1.3 Discussion

This investigation has demonstrated that peroxidase-catalyzed phenol removal was enhanced in the presence of peat moss at pH 5 and pH 7. Moreover, the efficiency of the fresh peat moss suspension in promoting phenol removal increased when it was pre-incubated with HRP (Figure 6.7). It seems that this effect was not due to a gradual stabilization of the enzyme in the presence of peat moss, since if this were true, pre-incubation in the aged suspension would likely have the same effect. These observations may rather indicate that the fresh peat moss suspension was gradually changing as it was exposed to the buffer. This change likely involved the dissolution and partition of soluble and colloidal peat moss components into the aqueous phase. In fact, the higher degrees of phenol removal obtained with the particle free aged supernatant clearly demonstrate that these peat moss components were the ones responsible for enhanced phenol removal (Figures 6.10 and 6.12). Therefore, an increase in their concentration over time would explain the enhancement in phenol removal in the fresh peat moss suspension after incubation in phosphate buffer. On the other hand, enhanced H_2O_2 decomposition in the presence of particulate matter derived from peat moss (Figure 6.11) could have at least in part been responsible for the adverse effect of peat moss solids on phenol removal.

Low molecular weight humic precursors, such as guaiacol or ferulic acid are known to be excellent substrates for peroxidase enzymes (Park *et al.*, 1999). Also, when incubated in the presence of other phenolic substrates, they frequently enhance their enzymatic transformation (Roper *et al.*, 1997; Morimoto and Tatsumi, 1997; Park *et al.*, 1999). Although peat moss extract likely contained low-molecular weight humic

precursors, their concentration seemed to be relatively low. This is based on two observations. First, the phenol assay of the pH 9.0 peat moss extract revealed that it contained 0.023 mM of total phenols. Lower values were measured with extracts obtained at pH 5.0 and 7.0. Second, in previous studies it was observed that HRP-catalyzed oxidation of humic precursors frequently resulted in the formation of colored products (Simmons *et al.*, 1988; Morimoto and Tatsumi, 1997). Therefore, in order to test whether a reaction would occur leading to the formation of colored products, HRP and peroxide were added to pH 7.0 peat moss extract and the absorbance spectrum of the solution was recorded over the whole UV/Visible range (190 to 800 nm). No significant change of the absorption spectrum occurred, indicating that either no reaction had taken place or that the concentration of phenolic humic precursors was relatively low. In the studies involving co-precipitation of humic substrates with harder-to-remove phenols, at least equimolar concentrations of humic compounds were used (Roper *et al.*, 1997; Morimoto and Tatsumi, 1997; Park *et al.*, 1999). Thus, at low concentrations low-molecular weight humic substrates may only partially be responsible for the enhanced phenol conversion observed in peat moss at pH 5.0 and 7.0.

Sarkar *et al.* (1988) report that precipitation of 2,4-dichlorophenol was enhanced when the enzymatic reaction was carried out in the presence of fulvic acid having an average molecular weight of 800 Da. They also found that 2,4-dichlorophenol reaction products were covalently bound to the high molecular weight fulvic acid. Similarly, Dec and Bollag (1994b) observed that the transformation of several chlorinated phenols was increased when the reaction was carried out in the presence of humic acids. Furthermore, the enhanced release of chloride from the chlorinated phenols indicated that covalent bonds had been formed between the phenolic substrates and the humic material. It is, therefore, likely that phenol radicals or other reaction products derived from phenol could also couple to higher-molecular weight humic material contained in peat moss. Assuming that these reaction products were mainly responsible for enzyme inactivation, their binding to humic material could result in reduced rates of enzyme inactivation and thus increased level of phenol conversion.

However, the findings of Gianfreda and Bollag (1994) seem to contradict the results of this and previous studies. These investigators found that 2,4-dichlorophenol conversion

by free and immobilized HRP and laccase was inhibited in the presence of soil, which was added to the buffered 2,4-dichlorophenol solution (pH 6.0) at 50000 mg/L. In particular, inhibition was correlated to the content of organic matter in the soil matrix. Based on the observation that H_2O_2 is not stable in the presence of soil organic matter, it is possible that in the case of HRP the reduced extent of 2,4-dichlorophenol transformation was at least partially due to non-enzymatic peroxide decomposition. Also, the decrease in phenol transformation at pH 9.0 observed in this study (Figure 6.4) could in part be attributed to higher rates of peroxide decomposition at elevated pH levels (see Figure 6.11).

The lower toxicity levels after phenol transformation in the presence of peat moss extract strongly indicate that phenolic reaction products were able to react with components present in the reaction mixture, presumably humic or fulvic acids, thus altering the product distribution and suppressing the formation of toxic compounds. Similarly, Morimoto and Tatsumi (1997) also observed that the formation of toxic products from pentachlorophenol was suppressed in the presence of naturally occurring humic acids.

The results of this investigation also have shown that under certain conditions bentonite or kaolin enhance enzymatic phenol removal. Also, both minerals affect the nature of the reaction products generated over the course of phenol oxidation as reflected in the toxicity results and as shown for kaolin by Arseguel and Baboulene (1995). It is therefore likely that these minerals enhance phenol removal by adsorbing reaction products that contribute to enzyme inactivation.

In the case of bentonite, dissolved or colloidal matter seems to be solely responsible for enhanced phenol removal since the aged supernatant was at least as effective in enhancing phenol transformation as the aged suspension (Figure 6.8). Moreover, at the highest bentonite concentration tested (10000 mg/L), consistently lower residual phenol concentrations were observed after the particulate matter had been removed from the aged suspension (Figure 6.8(b)). This indicates that this material had an adverse effect on the enzymatic phenol transformation. This effect was not due to direct enzyme inactivation since prolonged incubation of HRP with the particulate matter present in the aged suspension had no impact on the level of residual phenol (Figure 6.8(b)). In contrast,

incubation of the enzyme in fresh bentonite suspensions clearly resulted in enzyme inactivation (Figures 6.7 and 6.8(b)). Thus, prolonged exposure of bentonite to the aqueous phase seemed to have altered the characteristics of the particulate matter resulting in a reduced capacity to inactivate the enzyme. However, the enzyme had to be incubated in the fresh bentonite suspension for more than one hour in order to obtain lower levels of phenol removal than those achieved in the absence of bentonite.

Note that bentonite that had been stirred in distilled-deionized water overnight did not significantly inactivate the enzyme and also enhanced phenol removal in a way comparable with the bentonite suspension aged in buffer (data not shown). Thus, the effects were similar in the absence or presence of phosphate.

In contrast to bentonite, in the case of kaolin only the particulate matter was effective in enhancing phenol transformation as can be inferred from Figure 6.9. Also, kaolin became more effective after prolonged exposure to the phosphate buffer.

As mentioned earlier, bentonite belongs to the smectite group of clay minerals. When in contact with water these minerals expand, meaning that the distance between the crystal layers increases as water penetrates into the intermicellar regions (Tan, 1998). Dissolved organic molecules can also access the interlayer spaces and become adsorbed. In contrast, in the case of kaolinites adsorption can only occur on the outer surfaces. Therefore, smectites have a much higher capacity to adsorb organic molecules than kaolinites. These differences may explain why bentonite was more effective than kaolin in enhancing phenol removal and reducing toxicity levels. Also, it may be speculated that bentonite became more effective after prolonged exposure to the aqueous phase due to a gradual increase in accessible adsorption sites caused by interlayer swelling.

In soils, bentonite or kaolin clays are normally exposed to the aqueous phase over a long period of time. Thus, the suspensions that have been stirred in buffer overnight resemble more closely the real case scenario than the fresh suspensions. Based on the results obtained in this study, it can be concluded that under these circumstances neither bentonite nor kaolin would inactivate the enzyme when present in wastewaters up to a concentration of 10000 mg/L at neutral or acidic pHs.

6.2 Inorganic Anions

The impacts of the following inorganic anions on HRP-catalyzed phenol removal were examined: thiosulfate ($S_2O_3^{2-}$), sulfite (SO_3^{2-}), iodide (I^-), nitrite (NO_2^-), sulfide (S^{2-}), cyanide (CN^-) and thiocyanate (SCN^-). Iodide, sulfite and nitrite were chosen because these species are substrates of HRP and thus could potentially competitively inhibit phenol oxidation (Dunford and Stillman, 1976). Nitrite reduces HRP-I to HRP-II, while iodide and hydrogen sulfite (HSO_3^-) reduce HRP-I directly to HRP, but also react with HRP-II (Dunford and Stillman, 1976).

Thiosulfate, sulfide, cyanide and thiocyanate are frequently found in industrial wastewaters. In particular, they are associated with phenols in coal conversion and petroleum refining effluents. Furthermore, cyanide (Dunford and Stillman, 1976) and sulfide (Theorell, 1951) are known inhibitors of HRP.

Note that all anions were used as the sodium salts.

6.2.1 Results

(a) Thiosulfate, sulfite, iodide and nitrite

(i) Treatment under different conditions

In order to investigate the impact of thiosulfate, sulfite and iodide and nitrite on HRP-catalyzed phenol removal, batch experiments were carried out at low (10 mg/L) and high (200 mg/L) levels of these anions. These concentration levels were chosen since a literature overview has indicated that these are reasonable assumptions for industrial wastewaters. At pH 7, 61% of the sulfite exists as of HSO_3^- and the rest as SO_3^{2-} (calculated based on data from CRC Handbook of Chemistry and Physics, 2000-2001). However, in order to facilitate the discussion all sulfite forms will be referred to as sulfite in this study. Experiments were designed to cover four different conditions leading to:

(A) a high level of phenol removal (~98%) which was limited only by the availability of phenol (4.0 U/mL HRP and 1.3 mM H_2O_2). This condition was used in order to test whether those compounds may interfere with phenol conversion at low residual levels of phenol and thus make a high degree of treatment difficult.

(B) a medium level of phenol removal which was limited by peroxidase enzyme (1.5 U/mL HRP; 1.3 mM H_2O_2). This condition was used in order to test whether the presence of these compounds may enhance or interfere with the activity of the enzyme itself and thus increase the amount of enzyme required to achieve the desired level of treatment.

(C) a medium level of phenol removal which was limited by hydrogen peroxide (4.0 U/mL HRP; 0.58 mM H_2O_2). This condition was used in order to test whether the presence of reducing compounds may increase peroxide requirements.

(D) a medium level of phenol removal which was limited by peroxidase as well as by hydrogen peroxide (1.5 U/mL; 0.58 mM H_2O_2). A comparison of this treatment with (C) may provide information about whether the enzymatic phenol oxidation is competing with reducing compounds for the H_2O_2 that is available.

Table 6.3 summarizes the characteristic appearance of phenolic solutions after treatment at condition (A) in the presence of the salts.

Table 6.3: Appearance of reaction mixtures after HRP-treatment at condition (A) in the presence of the tested salts.

$Na_2S_2O_3$	Na_2SO_3	NaI	$NaNO_3$
No precipitate; dark brown colored liquid; darker color at 10 mg/L	Not different from controls	Violet shimmer and strong smell of iodine at 200 mg/L	Greenish-yellowish supernatant at 200 mg/L

The residual phenol concentrations obtained under the four different conditions are shown in Figure 6.13.

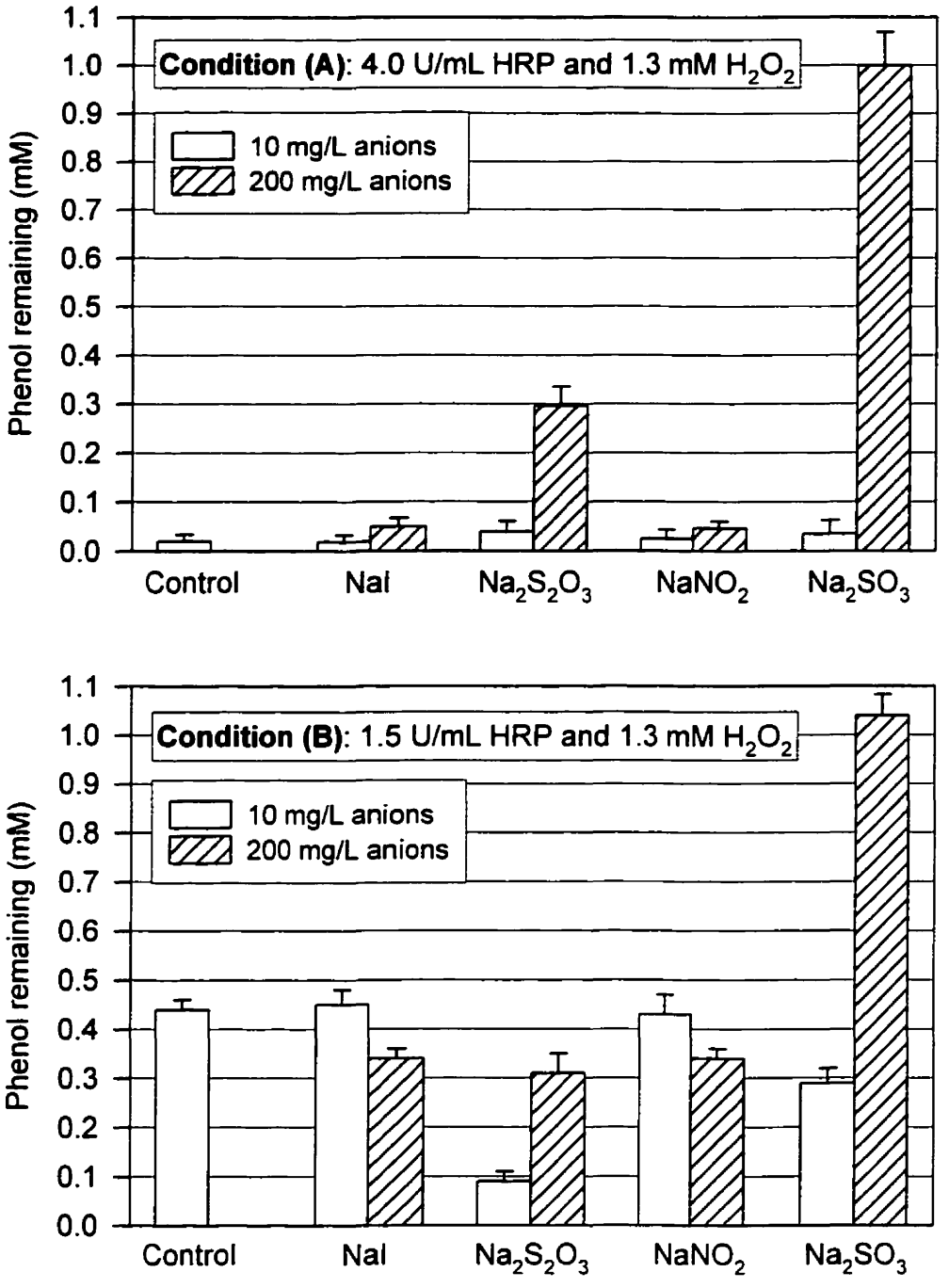


Figure 6.13: Treatment of phenol in the presence of iodide, thiosulfate, nitrite and sulfite under conditions (A) to (D). $[phenol]_0$ = 1.0 mM; pH = 7.0. Error bars stand for the standard deviation of 3 independent experiments.

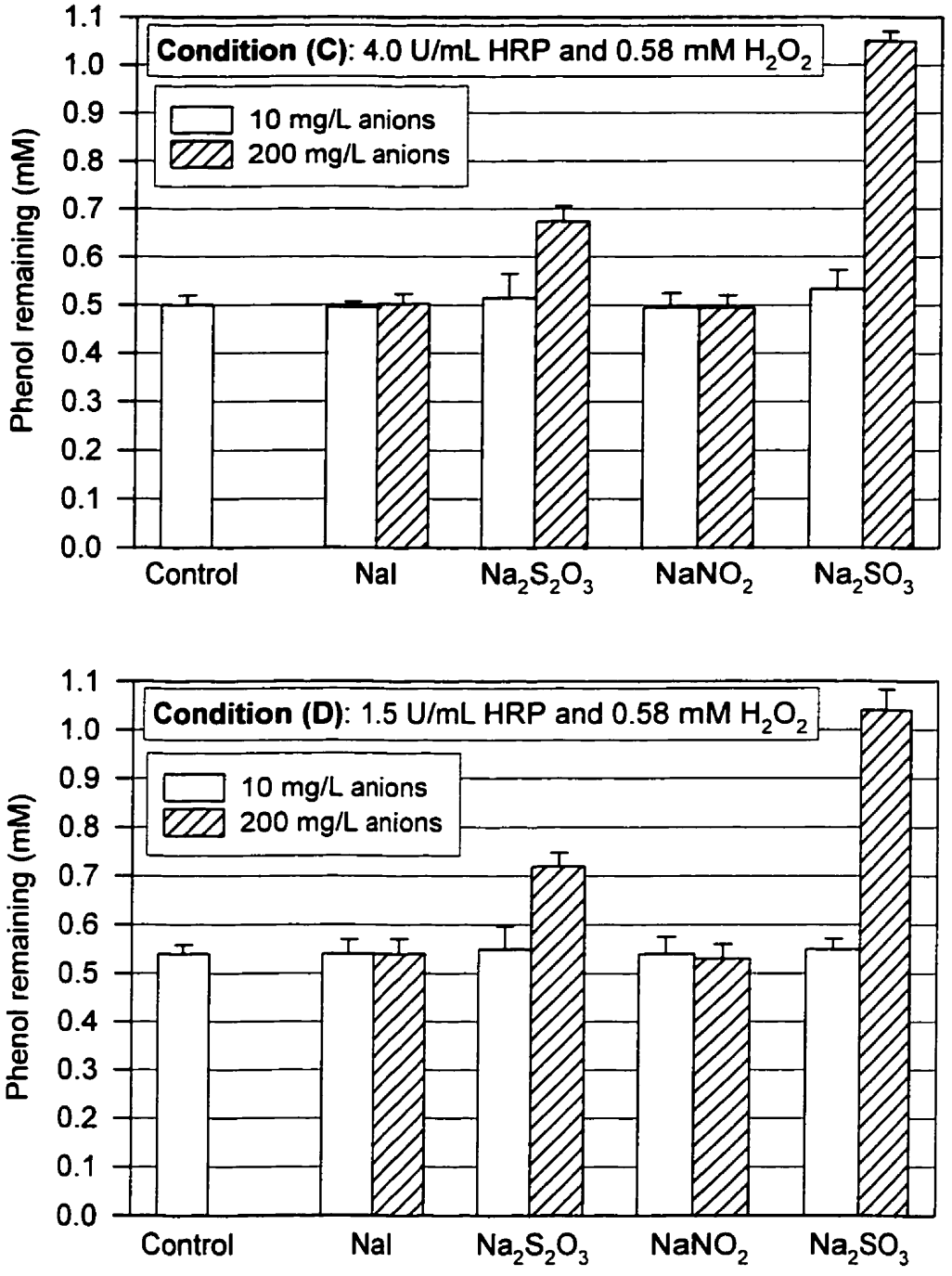


Figure 6.13: (continued)

For all conditions tested (A to D) no phenol transformation took place when reaction mixtures contained 200 mg/L of sulfite.

When reactions were carried out under condition (A) none of the anions tested interfered with the degree of phenol removal when they were present at low concentrations (10 mg/L). However, at 200 mg/L, samples treated in the presence of nitrite and iodide contained slightly more residual phenol. When treatment was conducted in the presence of 200 mg/L of thiosulfate, the residual phenol concentration was significantly higher (0.3 mM) compared to the controls (0.02 mM).

When the extent of phenol conversion was limited by HRP (condition (B)) more phenol was removed in the presence of 200 mg/L of iodide or nitrite and 10 mg/L of sulfite. At 10 mg/L, thiosulfate was particularly capable of enhancing phenol conversion, while removal was lower when the thiosulfate concentration was increased to 200 mg/L.

At condition (C), when peroxide was the limiting reagent, high concentrations of thiosulfate (200 mg/L) had an adverse impact on phenol transformation. Importantly, with 200 mg/L thiosulfate, there were only small differences in the residual phenol concentrations when comparing conditions (C) and (D), and conditions (A) and (B), respectively. These conditions differ in the amount of enzyme employed but are identical in the quantity of H_2O_2 that was provided to the reaction mixture. This suggests that in the presence of 200 mg/L of thiosulfate, phenol transformation was limited by hydrogen peroxide and that an increase in the enzyme dose could not reduce the thiosulfate-induced limitation.

All the other substances tested had no significant effect at conditions (C) and (D).

An additional experiment was carried out with batch reactions containing iodide, nitrite and thiosulfate up to a concentration of 400 mg/L in order to more closely examine the impact of these anions on phenol removal under enzyme-limited conditions. H_2O_2 was employed at 1.3 mM and the enzyme concentration was 1.0 U/mL in this experiment. The results, as displayed in Figure 6.14, show that the more iodide and nitrite was present in the reaction solution, the lower was the residual phenol concentration. At the highest concentration (400 mg/L), both anions enhanced phenol transformation by almost 50 %. However, in the case of thiosulfate the lowest residual phenol concentration was recorded at 10 mg/L and it increased steadily with increasing levels of thiosulfate.

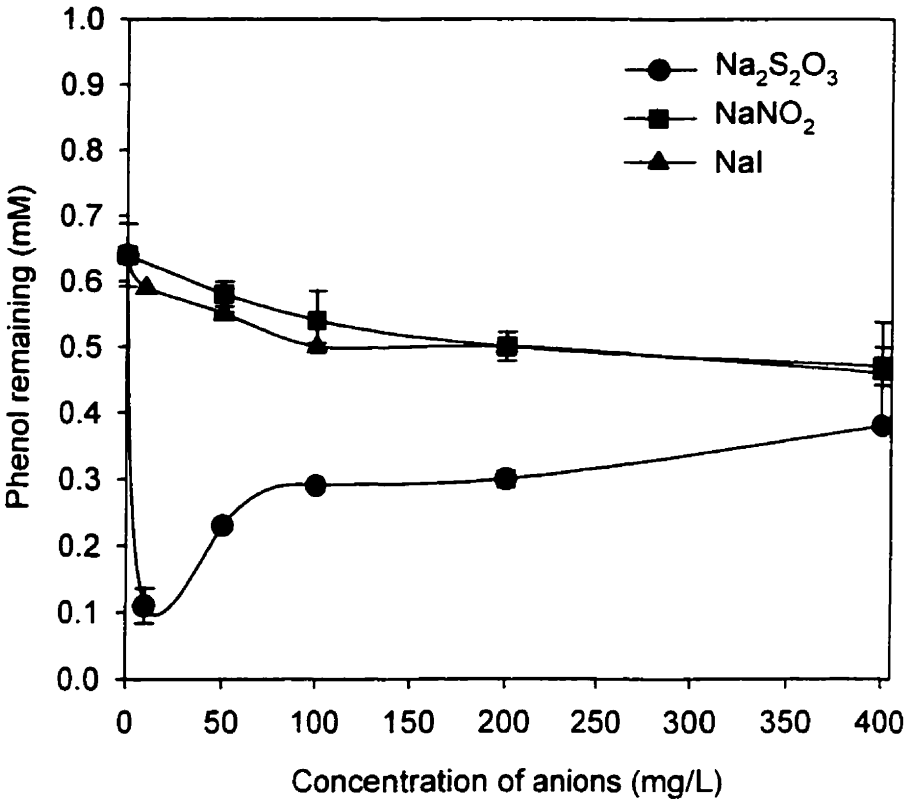


Figure 6.14: Phenol remaining as a function of the concentrations of thiosulfate, nitrite and iodide in the reaction mixture. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 1.0 U/mL; pH 7.0. Error bars stand for the range of 2 independent experiments.

(iii) Impact on peroxide demand

Thiosulfate and sulfite are oxidized by hydrogen peroxide to sulfate and the oligomeric products dithionate, trithionate and tetrathionate; while nitrite is oxidized to nitrate (Schumb *et al.*, 1955). Also, iodide can be oxidized with hydrogen peroxide to iodine under acidic conditions (Schumb *et al.*, 1955). The extent and the rate of these reactions depend on the reaction pH and the presence of catalysts, such as trace concentrations of iron compounds (Schumb *et al.*, 1955).

In order to test whether H_2O_2 would react with these species under the reaction conditions employed in the batch experiments, 1.0 mM hydrogen peroxide was added to pH 7.0 buffer containing 200 mg/L of the inorganic compound. The residual peroxide concentration was recorded after 6 and 33 minutes using the ABTS-H_2O_2 assay. The results showed that no peroxide was consumed in the presence of nitrite. However, after

33 minutes, the peroxide concentration decreased by approximately 5 % in solutions containing 200 mg/L of iodide. Thiosulfate interfered to a low extent with the assay, presumably by reduction of the oxidized ABTS radical. However, after taking this interference into account, it was also observed that thiosulfate slowly reacted with H_2O_2 resulting in approximately 10% peroxide consumption after 33 minutes. In the presence of 200 mg/L of sulfite, no peroxide remained in solution after 6 minutes.

Further batch experiments were carried out in order to examine the impact of thiosulfate, iodide, nitrite and sulfite on the stoichiometry between hydrogen peroxide and phenol for the HRP-catalyzed phenol oxidation reaction. In these experiments the H_2O_2 concentration was varied between 0 and 3.2 mM. The results are displayed in Figure 6.15.

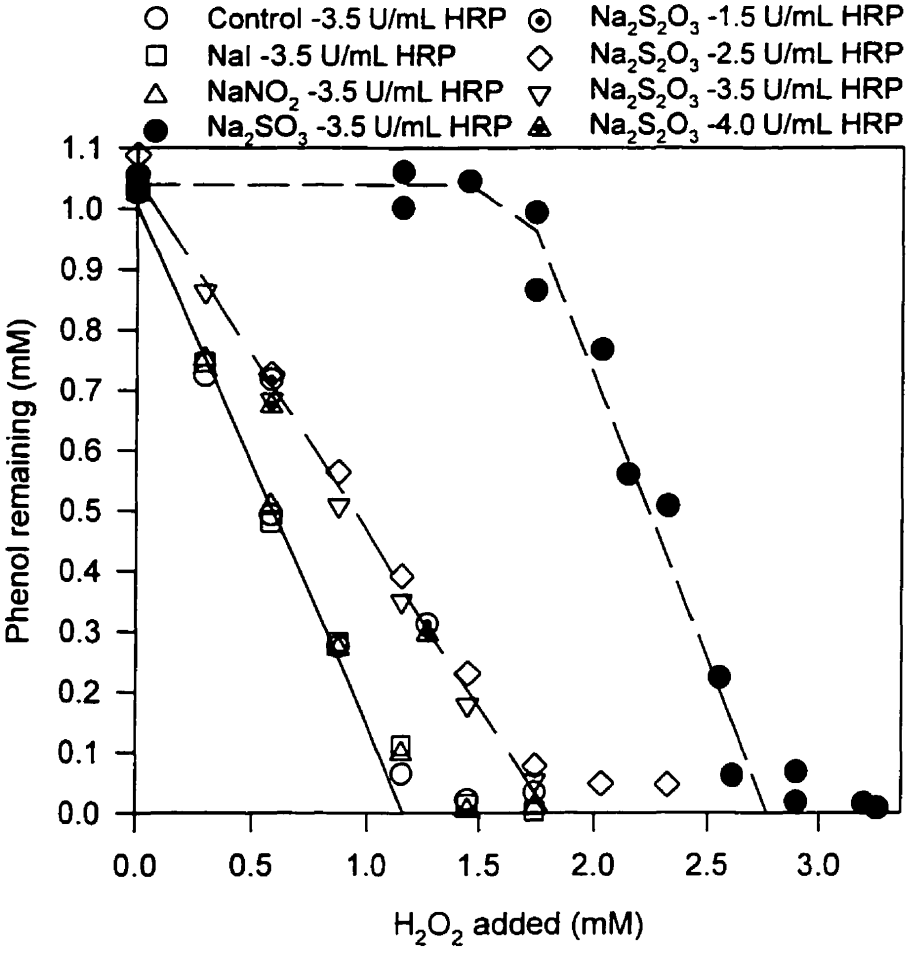


Figure 6.15: Phenol remaining as a function of the quantity of H_2O_2 added in the presence of 200 mg/L of iodide, nitrite, sulfite or thiosulfate. pH = 7.0.

From the linear slopes of phenol remaining versus H_2O_2 added, it was determined that when no reducing anions were present, 1.15 moles of H_2O_2 were consumed per mole of phenol removed. This value did not change when treatment was conducted in the presence of 200 mg/L of iodide or nitrite. However, in the presence of 200 mg/L of thiosulfate, 1.71 mole of peroxide were required to remove 1 mole of phenol. Importantly, as can be seen from Figure 6.15, the amount of phenol removed using a specified H_2O_2 dose was independent on the quantity of enzyme that was employed.

In the case of 200 mg/L of sulfite, no phenol oxidation took place when the initial H_2O_2 concentration was below 1.7 mM. However, the reaction solution turned brown immediately when the concentration of peroxide added was greater than 1.7 mM, indicating that phenol oxidation commenced without delay. When the peroxide dose was above 1.7 mM, the stoichiometry between phenol and peroxide was the same as without additives (1.07 mol H_2O_2/mol phenol). Approximately 3.0 mM H_2O_2 were required to achieve complete (> 98 %) phenol removal.

The 4-AAP H_2O_2 assay was used to further investigate the reaction of sulfite with H_2O_2 at pH 5.0, 7.0 and 9.0. H_2O_2 at different concentrations was added to buffered solutions containing 200 mg/L (2.5 mM) of sulfite. Residual peroxide was recorded after 6 minutes. The results showed that 200 mg/L (2.5 mM) sulfite consumed 2.5 mM H_2O_2 at pH 5.0, 1.9 mM at pH 7.0 and 0.6 mM at pH 9.0. The amount of peroxide consumed was independent of the initial peroxide concentration. Note that the peroxide consumption measured at pH 7.0 (1.9 mM) was in a relatively good agreement with the minimum amount of peroxide required to initiate phenol oxidation as estimated from Figure 6.15 (1.7 mM).

Nitrite and iodide are substrates of HRP. Thus, their enzymatic oxidation also leads to hydrogen peroxide consumption. At intermediate levels of treatment no additional peroxide consumption was recorded (Figure 6.15) suggesting that mainly phenol was oxidized. In fact, the rate constant for the reaction of HRP-I with phenol is approximately 2 to 3 orders of magnitude higher than with iodide or nitrite, while the differences are even greater for reactions with HRP-II (Dunford and Stillman, 1976). However, at lower phenol concentrations when the ratio of phenol to iodide or nitrite becomes very low, these compounds may successfully compete for the enzyme's active site and be oxidized

leading to peroxide consumption. This could explain the higher residual phenol concentrations when treatment was conducted at condition (A) in the presence of 200 mg/L of nitrite and iodide (Figure 6.13).

In order to test whether phenol removal at high levels of treatment in the presence of 200 mg/L iodide or nitrite was in fact peroxide limited, reactions were carried out with higher peroxide concentrations using an amount of enzyme that would lead to nearly complete phenol transformation (~97 %). Reaction rates of nitrite and iodide with HRP-I and HRP-II increase exponentially with decreasing pH (Dunford and Stillman, 1976). Also, the spontaneous oxidation of iodide to iodine is favored at lower pH (Schumb *et al.*, 1955). Thus, reactions were also conducted at pH 5.0 in order to test whether nitrite and iodide would exert more competition with phenol for H_2O_2 at lower pH. The results of these experiments are shown in Figure 6.16. At pH 7.0, more phenol was removed in all cases when the peroxide concentration was increased from 1.2 to 1.4 mM (Figure 6.16(a)). This effect was particularly pronounced with nitrite and iodide. In the presence of these substances further improvements in phenol transformation occurred when 1.7 mM peroxide was provided resulting in 99.3 and 98.0 % removal for nitrite and iodide, respectively. In contrast, in the absence of these compounds the highest level of phenol transformation was 97.1 % for an initial H_2O_2 concentration of 1.4 mM. Thus, it was possible to achieve almost complete phenol transformation in the presence of nitrite or iodide by providing more peroxide.

At pH 5.0 with $[H_2O_2]_0$ = 1.2 mM only 59 % and 87 % of the initial phenol were removed in the presence of iodide and nitrite, respectively, while 92 % transformation was accomplished in the absence of these compounds (Figure 6.16(b)). Thus, iodide and nitrite posed a stronger limitation on phenol transformation at lower pH. In the case of iodide, this limitation could be overcome and complete (99.9 %) phenol removal could be achieved when the H_2O_2 dose was increased to 2.0 mM. In the case of nitrite, higher peroxide doses also led to higher removal. However, phenol removal increased only marginally as the peroxide dose was raised beyond 1.7 mM. Samples treated in the presence of nitrite exhibited a yellow color indicating the presence of a soluble reaction product. It was also noticed when conducting the 4-AAP phenol assay that the absorbance peak of the colored reaction product broadened and shifted from 510 nm, which is characteristic

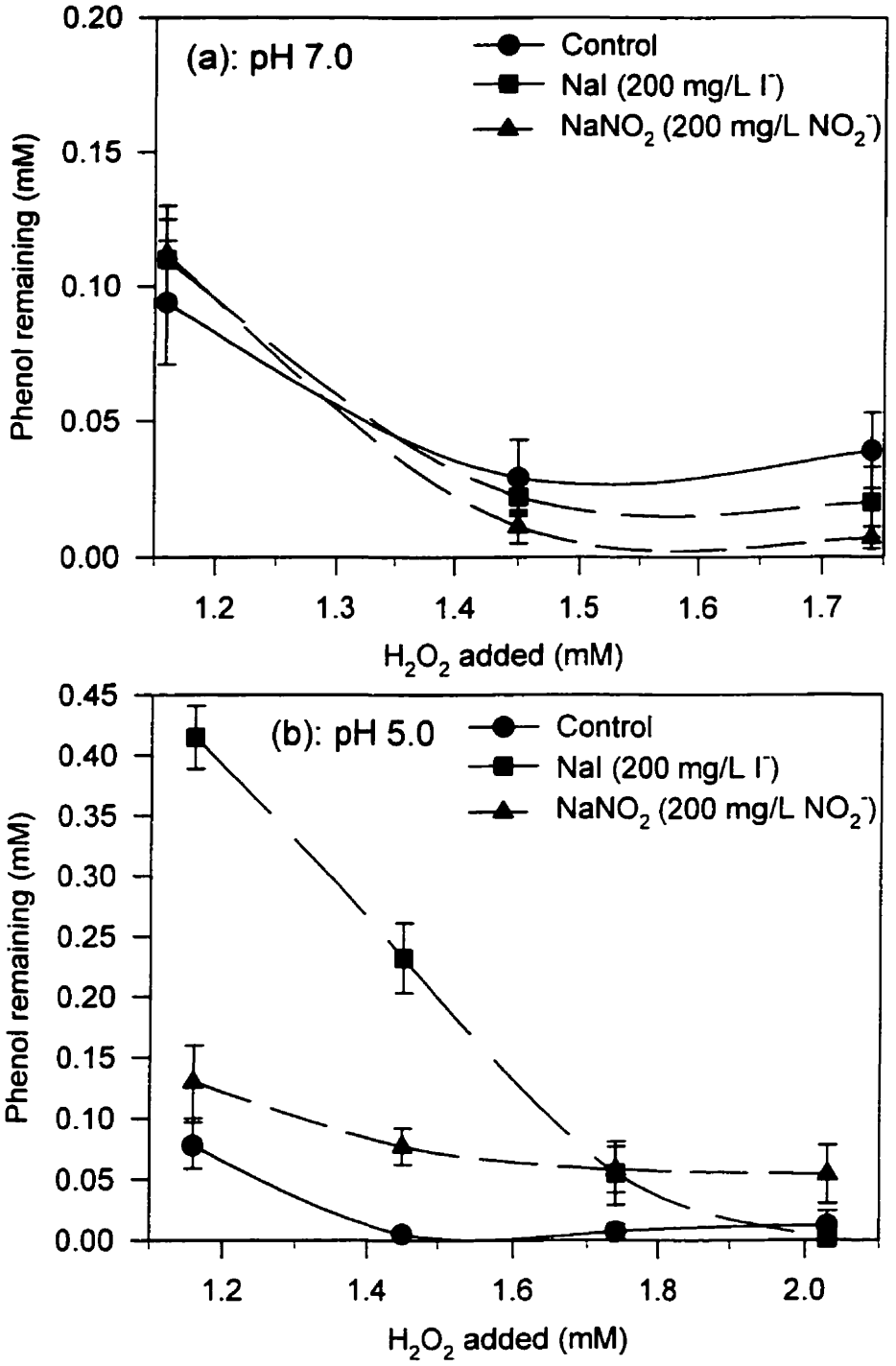


Figure 6.16: Phenol remaining as a function of the quantity of H_2O_2 added in the presence of 200 mg/L of iodide or nitrite. $[phenol]_0$= 1.0 mM. (a) pH 7.0; HRP_0 = 3.5 U/mL. (b) pH 5.0; HRP_0 = 8.0 U/mL. Error bars stand for the standard deviation of 4 (a) or 3 (b) independent experiments.

for phenol, to approximately 550 nm suggesting that the phenolic compound measured in the assay may not have been the original phenol, but possibly a phenolic reaction product that was unreactive with HRP.

(iv) Impact on the rate of phenol conversion

Experiments were performed with 200 mg/L of nitrite, iodide, sulfite and thiosulfate at pH 7.0 in order to test whether these compounds would have an impact on the rate of enzymatic phenol conversion. Hydrogen peroxide was provided at concentrations that ensured that phenol conversion was not limited by the peroxide available, while the HRP dose was equal for all conditions. As can be seen from Figure 6.17, iodide and nitrite had only marginal effects on the reaction rate.

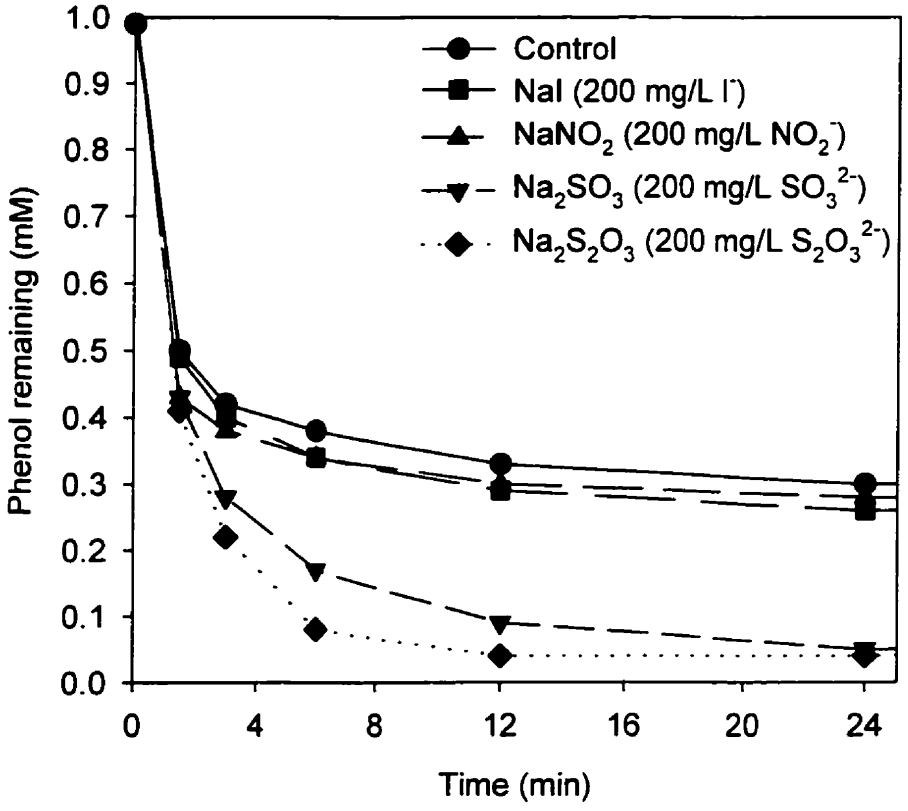


Figure 6.17: Phenol decrease over time during treatment in the presence of 200 mg/L of iodide, nitrite, sulfite or thiosulfate. $[phenol]_0$ = 1.0 mM; HRP_0= 2.5 U/mL; $[H_2O_2]_0$ = 1.3 mM (control, iodide and nitrite), 3.0 mM (sulfite) and 1.9 mM (thiosulfate); pH = 7.0.

However, thiosulfate and sulfite substantially increased the rate and extent of phenol conversion. In the case of thiosulfate, the apparent increase of the reaction rate may have been partially due to the higher peroxide concentrations that were employed. Therefore, a control experiment was conducted using 10 mg/L of thiosulfate in which both the enzyme and the peroxide dose were the same as in the control reaction (data not shown). As in the case of 200 mg/L thiosulfate, in the presence of 10 mg/L thiosulfate the phenol transformation rate as well as the extent of phenol removal were significantly higher than in the control reaction. Thus, the higher phenol transformation rate in the presence of 200 mg/L of thiosulfate was not mainly due to the higher peroxide concentrations that were applied.

(v) Impact on soluble reaction products

A slightly colored solution was obtained when the reaction was carried out in the presence of nitrite. No precipitate had formed in the presence of thiosulfate. These observations suggested that these anions had an impact on the nature of reaction products that were formed. To test this hypothesis, the absorbance at 269 nm, which is the maximum absorbance wavelength of phenol, was recorded for the samples treated with HRP for different periods of time, which were obtained in the experiment described in Section *(iv)*. The absorbance values were converted to corresponding phenol concentrations based on a calibration line. The obtained values were plotted versus those phenol concentrations that were measured using the 4-AAP assay as shown in Figure 6.18. If no soluble reaction products were present, phenol concentrations based on the assay and on the absorbance should be equal and thus all points in this graph should lie on a straight line with a 45° slope. Points lying above this line indicate the presence of reaction products in the aqueous phase.

From Figure 6.18 it can be seen that the initial absorbance was approximately the same for all solutions, thereby indicating that the anions did not absorb light at 269 nm. When no reducing anions were present, phenol concentrations based on absorbance exceeded those based on the 4-AAP assay for intermediate stages of the reaction; however, as the reaction approached completion the points lay closer to the straight line with a 45° slope. A very similar pattern was observed in the presence of iodide. In the presence of nitrite generally slightly higher absorbance values were recorded. Also, as the

reaction reached completion the absorbance did not decrease to levels that corresponded to the residual phenol concentration.

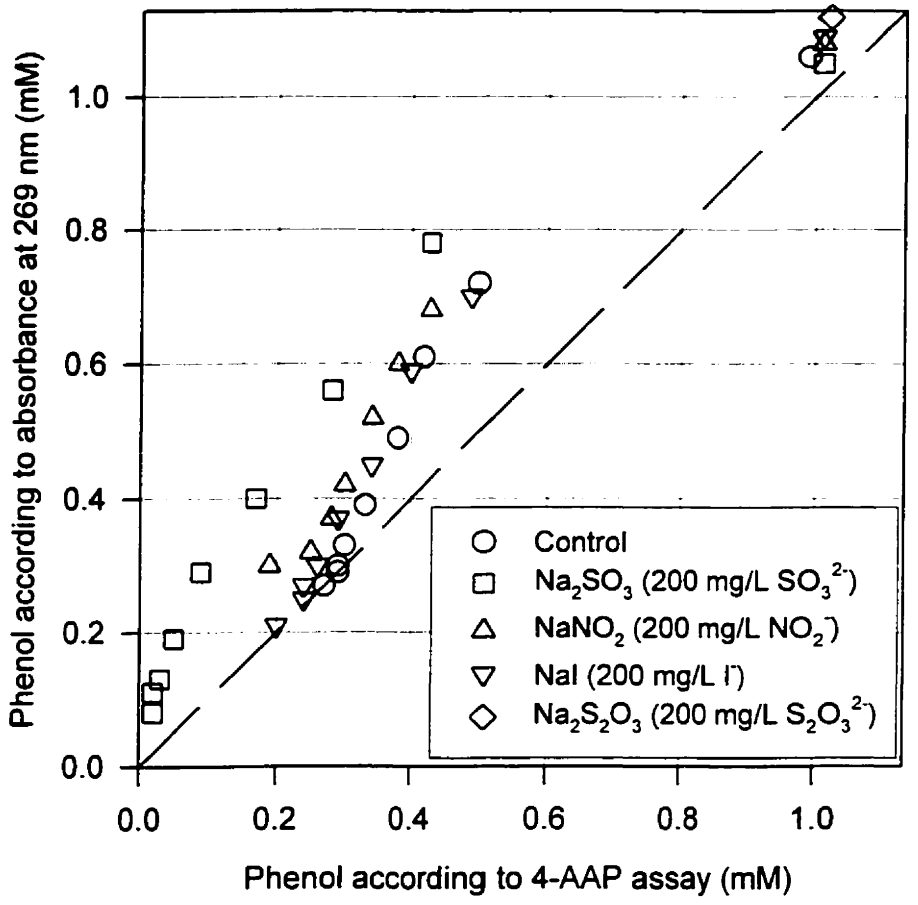


Figure 6.18: Residual phenol concentrations based on the UV absorbance versus concentrations based on the 4-AAP assay after treatment of a 1.0 mM phenol solution in the presence of 200 mg/L of sulfite, nitrite, iodide or thiosulfate.

In the presence of sulfite, the absorbance at 269 nm substantially exceeded the corresponding phenol content. However, at later stages of the reaction the absorbance declined and approached its expected value. Samples treated in the presence of 200 mg/L of thiosulfate exhibited an absorbance exceeding 3 absorbance units (which would correspond to more than 2 mM phenol) and were therefore not included in Figure 6.18. An attempt to coagulate the reaction products with 90 mg/L of chitosan resulted in a precipitate that was easily separated by centrifugation. (Alum coagulation could not be

used due to interfering reactions between it and the phosphate buffer.) However, the resulting supernatant still had an absorbance of 1.2 absorbance units at 269 nm.

(b) Sulfide

Sulfide was supplied to the reaction mixtures in the form of Na_2S. At pH 7.0, 76 % of the total sulfide is present in form of H_2S, and 24 % exists as HS^- (calculated based on data from CRC Handbook of Chemistry and Physics, 2000-2001); thus almost no S^{2-} ions are present. However, in order to facilitate the discussion, the sum of these three species will be referred to as sulfide in this study.

In initial batch experiments containing 55 mg/L of sulfide, 1.0 mM phenol and 1.3 mM H_2O_2 no phenol conversion was observed even when the HRP dose was increased up to 6 U/mL (data not shown). However, in further experiments phenol removal was accomplished when higher peroxide concentrations were employed. The results of these experiments in which the sulfide concentration ranged from 0 to 82.5 mg/L are shown in Figure 6.19.

As in the case of sulfite, no reaction took place below a certain H_2O_2 concentration (Figure 6.19). However, unlike the case of sulfite, even when the peroxide dose that was added exceeded the threshold concentration, phenol oxidation did not start immediately. Rather it took several minutes to up to one hour before phenol oxidation commenced as was evidenced through a change of the color of the reaction mixture to the typical brownish shade (absorbance at 400 nm). In the time period between peroxide addition and the onset of phenol oxidation the originally clear reaction mixture became turbid due to the formation of a white precipitate. Also, the sulfide smell disappeared and a sulfur smell became noticeable. The reactions taking place seemed not to be catalyzed by the enzyme since identical observations were made with reaction mixtures to which no HRP had been added.

As shown in Figure 6.19 the extent of phenol removal in the samples containing sulfide exceeded 90 % when the optimum H_2O_2 dose was supplied, while a maximum of 80 % phenol conversion was achieved in the control samples. This observation indicates that the enzyme was not inactivated by the sulfide. A control experiment was conducted to verify this conclusion. HRP was pre-incubated for one hour in phenol solutions

containing 55 mg/L of sulfide before 5.8 mM of peroxide were added. The same phenol conversion was accomplished in this sample as in the samples to which peroxide was supplied immediately after HRP was added (Figure 6.19).

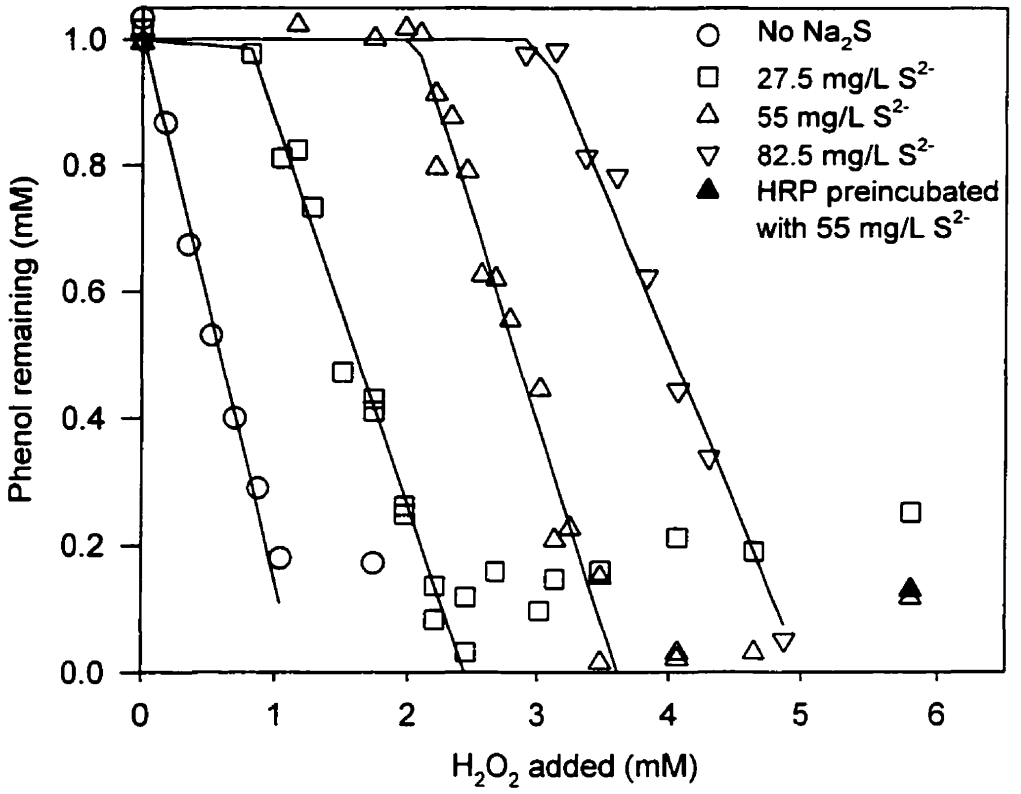


Figure 6.19: Phenol remaining as a function of the amount of peroxide added in the presence or absence of 27.5 to 82.5 mg/L of sulfide. $[phenol]_0$ = 1.0 mM; HRP_0 = 2.5 U/mL; pH = 7.0; reaction time = 20 hours.

A regression analysis was performed on the linear portion of the data of phenol remaining versus H_2O_2 added in order to calculate how much peroxide was consumed per mole of phenol removed once sufficient peroxide was provided. Also, the threshold peroxide concentrations below which no phenol oxidation took place were determined for each initial sulfide concentration from the intersections between the line at [phenol] = 1 mM and the straight lines for the residual phenol as a function of the H_2O_2 added. The results of these calculations are summarized in Table 6.4.

Table 6.4: Results of regression analyses of the data presented in Figure 6.19.

	Sulfide added (mg/L)			
	0	**27.5**	**55.0**	**82.5**
Molar concentration of sulfide (mM)	0	0.86	1.72	2.57
[a]H_2O_2 consumed per phenol removed (mol/mol) ± 95 % confidence interval	1.14 ± 0.16	1.61 ± 0.14	1.52 ± 0.17	1.95 ± 0.29
H_2O_2 threshold concentration ($[H_2O_2]_S$) (mM) ± 95 % confidence interval	0	0.79 ± 0.22	2.06 ± 0.30	3.03 ± 0.49
Ratio of $(H_2O_2)_S$ to sulfide (mol/mol) ± 95 % confidence interval	-	0.92 ± 0.25	1.20 ± 0.16	1.18 ± 0.19

[a]Determined from data points obtained with $[H_2O_2] > [H_2O_2]_S$.

Two observations can be made from the results presented in Table 6.4. First, the H_2O_2 threshold concentration was proportional to the sulfide content in the reaction mixture. On average at least 1.10 mole H_2O_2 per mole sulfide were required to start the phenol oxidation.

The second observation is that even when the peroxide dose that was provided exceeded the H_2O_2 threshold concentration, the peroxide-phenol stoichiometry was approximately 40 % higher for the samples containing sulfide.

In order to elucidate the mechanisms by which sulfide inhibits the enzymatic phenol transformation, an attempt was made to monitor the enzyme activity and the H_2O_2 concentration in the presence of sulfide using the ABTS and the 4-AAP assays. However, these attempts were not successful since sulfide readily reacts with colored compounds produced in these assays presumably reducing them back to the uncolored form. Similarly, when sulfide was added to reaction mixtures during the enzymatic phenol oxidation the colored reaction products were rapidly transformed into uncolored compounds and the phenol transformation was halted.

An additional batch experiment was conducted to record the time course of the phenol oxidation reaction in the presence and absence of sulfide. HRP was added to 1.0 mM phenol solutions containing 0 or 55.0 mg/L sulfide. The reactions were initiated by the addition of sufficient H_2O_2 to accomplish complete phenol conversion under both conditions. The results of this experiment are shown in Figure 6.20.

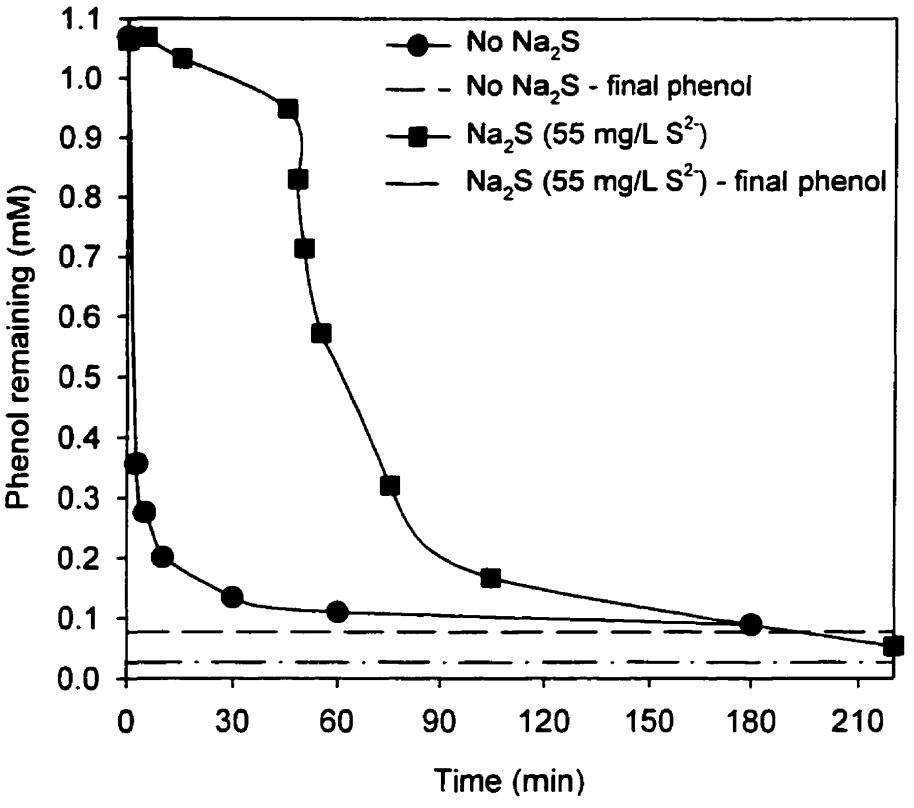


Figure 6.20: Phenol conversion as a function of time in the presence or absence of 55 mg/L sulfide. $[phenol]_0$= 1.0 mM; HRP_0= 3.0 U/mL; $[H_2O_2]_0$= 1.3 mM (for no sulfide) and 4.9 mM (for 55 mg/L sulfide); pH = 7.0.

For the batch mixture containing sulfide it took almost 45 minutes before significant (i.e., more than 10 %) phenol removal was recorded. Also, even after the onset of phenol oxidation the initial rate of the reaction was slower than the rate of the control reaction without sulfide. On the other hand, after 3 hours the residual phenol concentrations reached the same levels in the control reaction and in the batch to which sulfide had been added. Furthermore, as noticed earlier (Figure 6.19), the final level of phenol conversion in the sulfide batch exceeded the conversion that was achieved in the control sample.

In order to test for possible enhanced formation of soluble reaction products in the presence of sulfide, the absorbance at 269 nm was recorded for the samples obtained in the batch experiment. The absorbance values were converted to the respective phenol concentrations and plotted versus the phenol concentrations measured using the 4-AAP assay as shown in Figure 6.21.

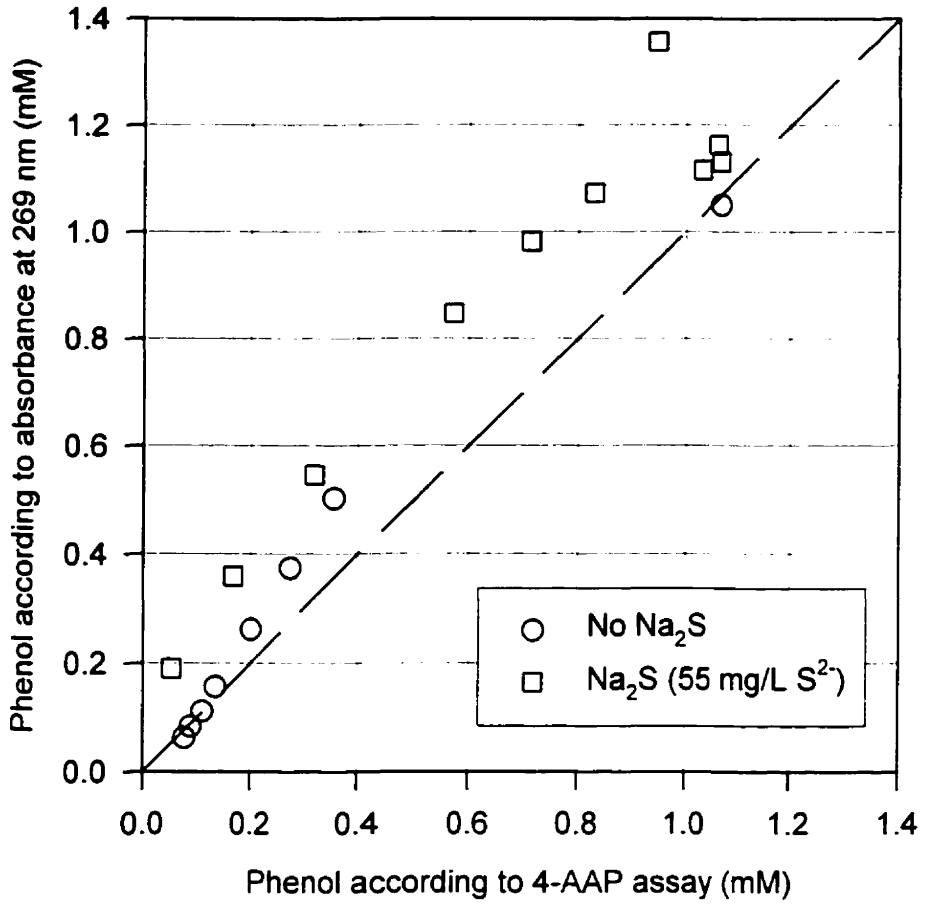


Figure 6.21: Residual phenol concentrations based on the UV absorbance versus concentrations based on the 4-AAP assay after treatment in the absence or in the presence of 55 mg/L of sulfide.

The addition of sulfide itself resulted in slightly increased absorbance values even before the enzymatic reaction was started. Nevertheless, even when taking this into account, samples treated in the presence of sulfide exhibited elevated UV absorbances.

(c) Thiocyanate and cyanide

The impact of thiocyanate (SCN^-) on the enzymatic phenol conversion was tested by the addition of NaSCN to buffered reaction mixtures up to a concentration of 400 mg/L in terms of SCN^-. Phenol removal reactions were conducted under HRP-limited conditions with high (4.0 U/mL) and medium (1.5 U/mL) HRP doses using 1.3 mM H_2O_2. The results of this experiment are presented in Figure 6.22.

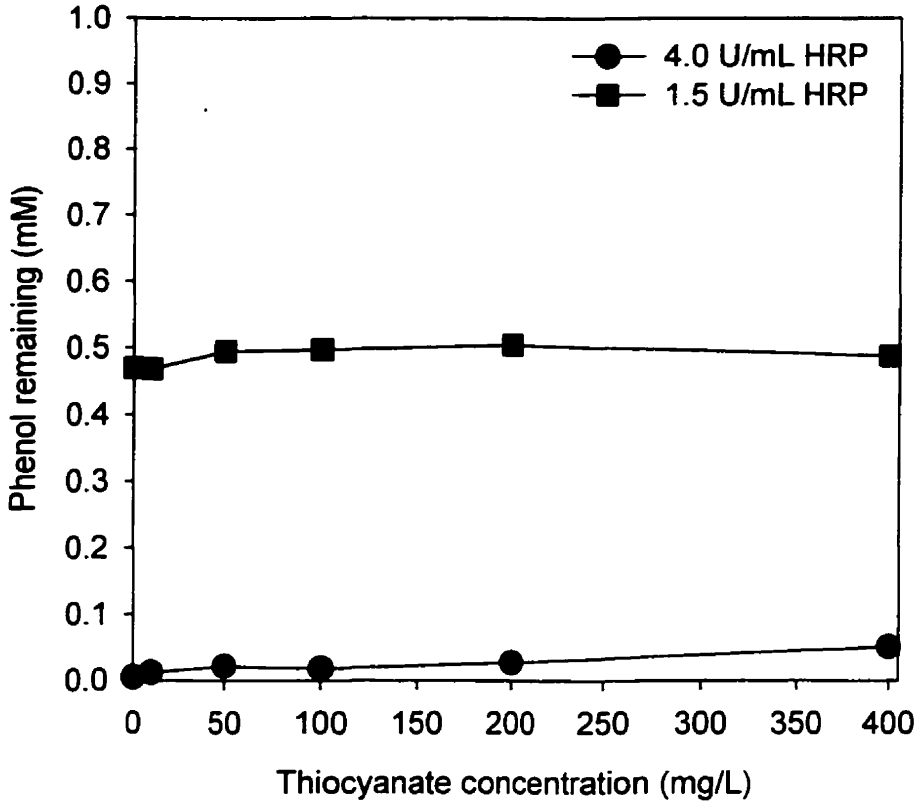


Figure 6.22: Phenol remaining as a function of the amount of thiocyanate added. $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.3$ mM; pH = 7.0.

As can be seen from Figure 6.22, thiocyanate had no impact on phenol transformation at medium levels of treatment (treatment with 1.5 U/mL). However, increasing thiocyanate levels had an inhibiting effect at conditions leading to high levels of phenol transformation when the residual phenol concentrations became relatively low (treatment with 4.0 U/mL). This led to a reduction in the extent of phenol transformation from 99.5 % with no thiocyanate to 95.0 % in the presence of 400 mg/L of thiocyanate.

In order to test whether the presence of SCN^- would increase H_2O_2 requirements for the treatment of phenol, reactions were carried out in which the H_2O_2 dose was varied between 0 and 1.74 mM. The results are shown in Figure 6.23.

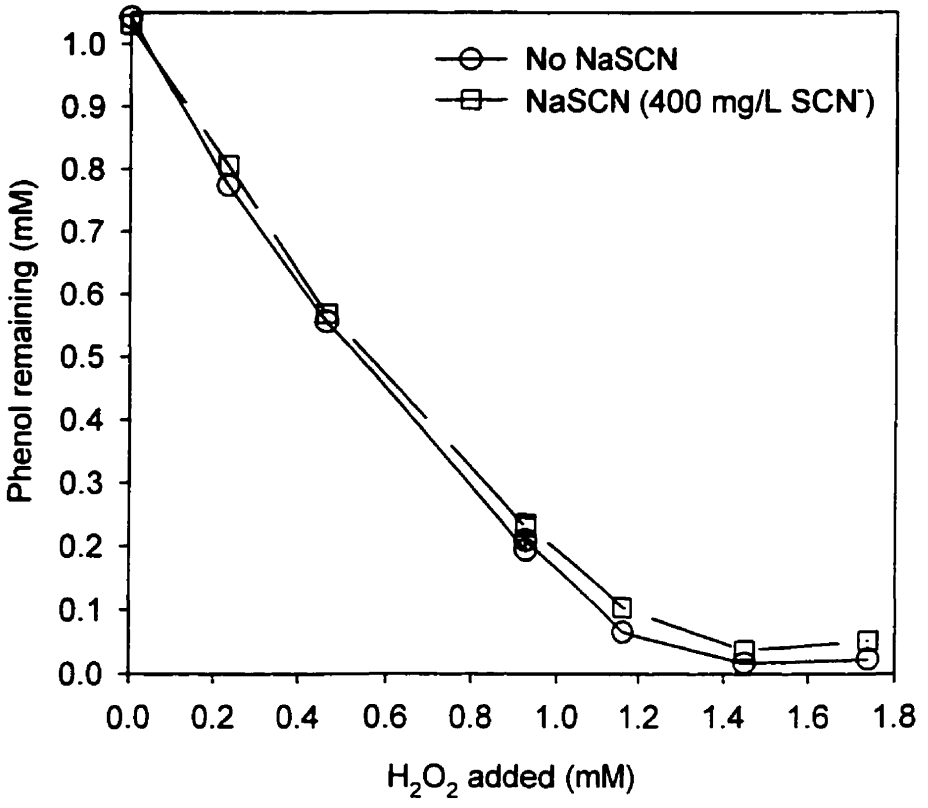


Figure 6.23: Phenol remaining as a function of the amount of peroxide added in the presence and absence of 400 mg/L of thiocyanate. $[phenol]_0$ = 1.0 mM; HRP_0 = 4.0 U/mL; pH = 7.0.

Virtually identical residual phenol concentrations were recorded for H_2O_2 doses in the range from 0 to 0.93 mM. At higher peroxide doses, slightly more phenol was removed in the control batches than in the batches containing 400 mg/L of thiocyanate. However, the impact of thiocyanate on the level of phenol conversion was relatively low, as the maximum removal (achieved at an H_2O_2 dose of 1.5 mM) was only reduced from 98.4 % in the absence of thiocyanate to 96.5 % in the presence of 400 mg/L of thiocyanate.

Cyanide is an effective inhibitor of HRP. It binds tightly to the native enzyme (HRP_N) preventing its reaction with H_2O_2 (Dunford and Stillman, 1976). In order to examine the effect of cyanide on enzymatic phenol removal, batch reactions were carried out containing 1.0 mM phenol and 0.5 to 8.0 mg/L cyanide (expressed as CN^-) which was added in form of NaCN. 1.3 mM hydrogen peroxide and 4.0 or 5.6 U/mL HRP were

supplied to the reaction mixtures. The results of this experiment, which are presented in Figure 6.24, show that the residual phenol concentrations increased linearly with increasing cyanide levels.

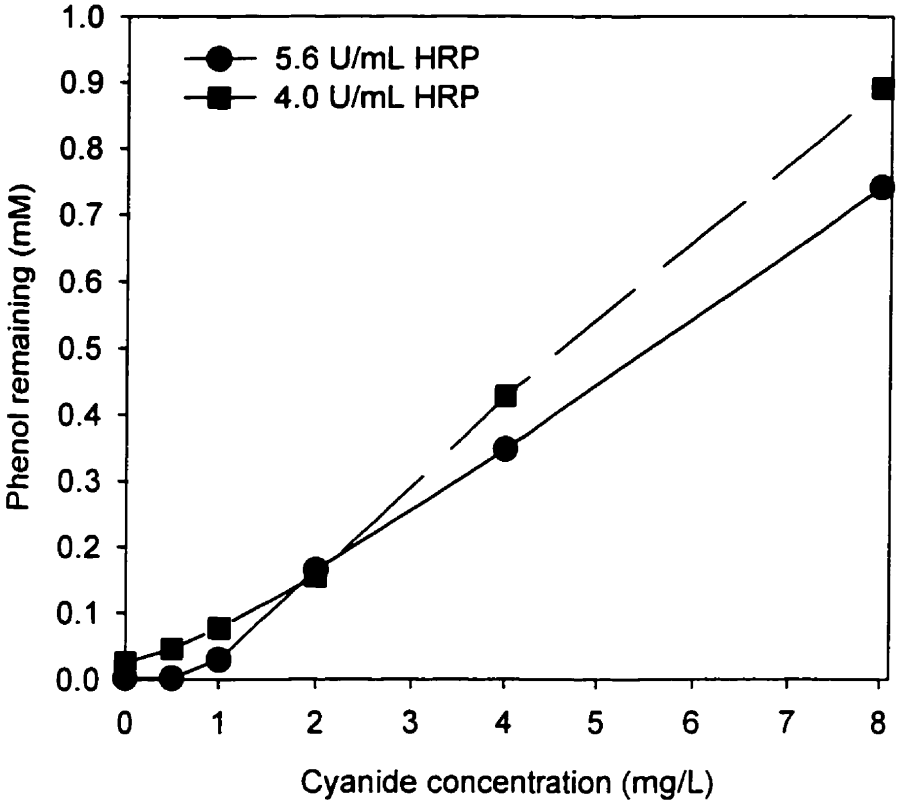


Figure 6.24: Phenol remaining as a function of the amount of cyanide added. $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.3$ mM; pH = 7.0.

Even low cyanide concentrations strongly reduced phenol transformation. For example, 4.0 U/mL HRP were sufficient to remove approximately 97 % of the initial phenol when no cyanide was present. The same amount of enzyme resulted in 55 % phenol transformation with 4.0 mg/L cyanide and in only 10 % removal with 8.0 mg/L cyanide.

Since both species, cyanide and H_2O_2, compete for binding to the native enzyme, it can be assumed that high H_2O_2 concentrations may replace cyanide at the enzyme's active site and thus improve phenol transformation. Therefore, tests were conducted to determine whether an increase in the H_2O_2 dose would diminish the inhibiting effect of

cyanide. Batch experiments were performed with cyanide concentrations ranging from 0.5 to 8.0 mg/L in which the H_2O_2 dose was varied between 1.3 and 7.0 mM. The HRP dose supplied was 4.0 U/mL. A reaction time of 3 hours was provided in this experiment. In addition, selected samples were monitored to determine if an increase in the reaction time to 22 hours would significantly improve phenol transformation. The results of this experiment, presented in Figure 6.25, show that there was no improvement in treatment efficiency upon increasing H_2O_2 concentrations.

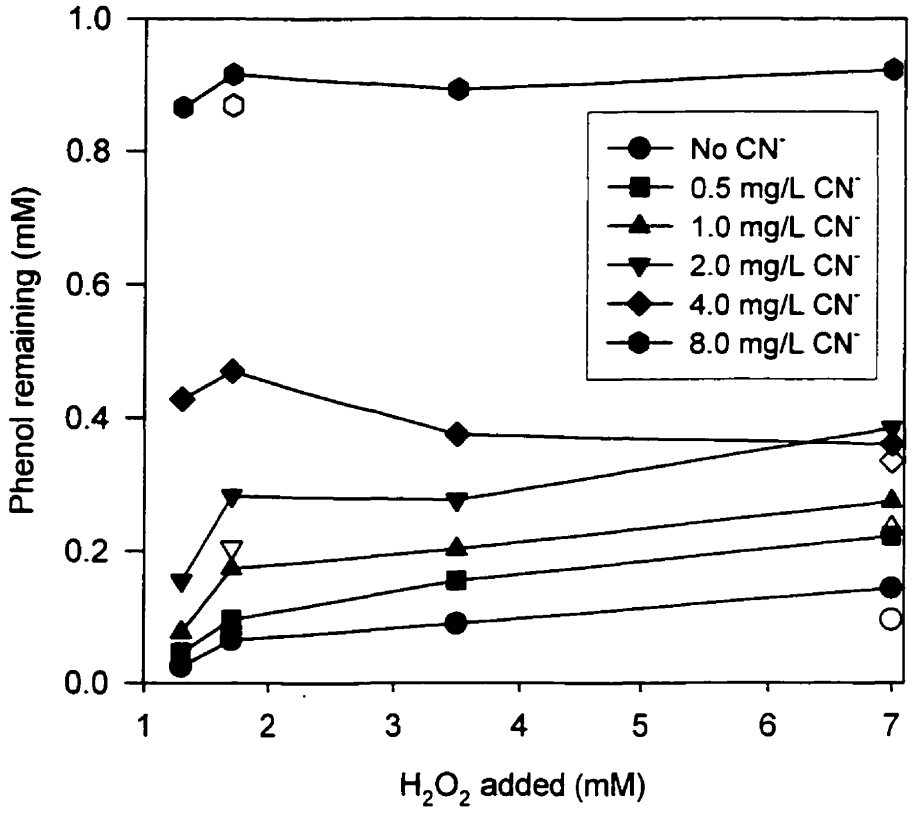


Figure 6.25: Phenol remaining as a function of the quantity of H_2O_2 added for treatment on the presence of cyanide. $[phenol]_0$ = 1.0 mM; HRP_0 = 4.0 U/mL; pH = 7.0; reaction time = 3 hours (filled symbols) or 22 hours (open symbols).

At low cyanide concentrations (0 to 2.0 mg/L) increasing peroxide doses led to rather higher residual phenol levels, which may be attributed to HRP inactivation by H_2O_2. At higher cyanide levels (4.0 to 8.0 mg/L) increasing H_2O_2 doses had no clear impact on

phenol removal. Also an increase in the length of the reaction time (open symbols) only slightly improved phenol transformation.

Further experiments were carried out in order to assess the effect of cyanide on the amount of enzyme required to achieve a high level of treatment of a 1.0 mM phenol solution. The HRP dose was varied between 4.0 and 16.0 U/mL and 1.4 mM H_2O_2 were supplied to the reaction mixtures. The results are shown in Figure 6.26. From this graph, the quantities of HRP required to achieve 95 % phenol transformation were evaluated in the presence of 0.5 to 4.0 mg/L of cyanide. These values are presented in Figure 6.27.

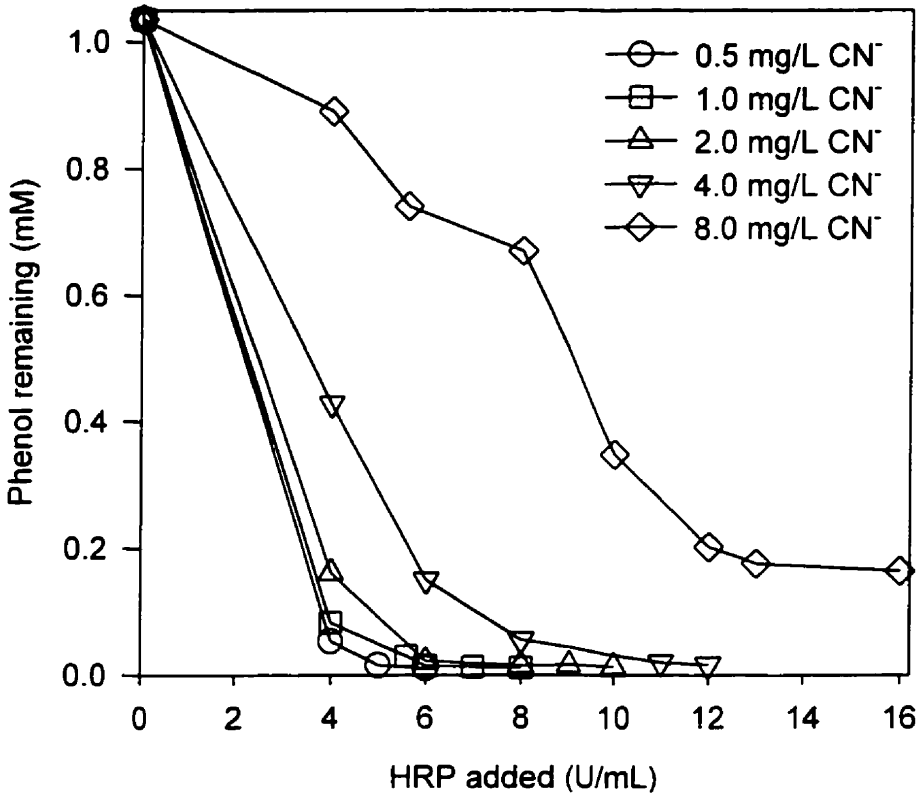


Figure 6.26: Phenol remaining as a function of the enzyme dose in the presence of various concentrations of cyanide. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ =1.4 mM; pH = 7.0; reaction time = 22 hours.

For the highest cyanide concentration tested (8.0 mg/L), no value could be estimated since the maximum phenol transformation achieved was only 84 % with the highest enzyme dose of 16 U/mL. Figure 6.27 shows that within a cyanide concentration range of

0.5 to 4 mg/L an additional 1.1 U/mL of HRP were required per mg/L of cyanide to achieve 95 % phenol transformation.

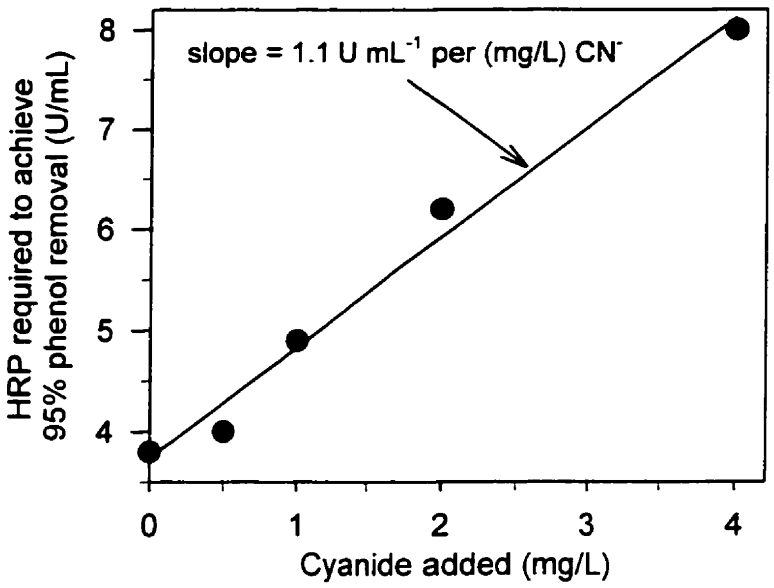


Figure 6.27: HRP required to achieve 95% phenol removal from a 1.0 mM phenol solution containing 0 to 4.0 mg/L cyanide at pH 7.0.

6.2.2 Discussion

The results of this investigation have shown that high concentrations of iodide and nitrite enhance phenol removal under enzyme-limited conditions (see Figures 6.13 and 6.14). The mechanisms underlying this phenomenon are unknown. However, it seems possible that the presence of these substances may have an impact on the product distribution and thus indirectly affect the stability of the enzyme over the course of the phenol transformation reaction.

Even though iodide and nitrite are substrates of HRP, their oxidation is relatively slow compared to the oxidation of phenol (Dunford and Stillman, 1976). Also, at pH 7.0 and under the reaction conditions applied in this study iodide and nitrite were not spontaneously oxidized by H_2O_2 at any significant rate. This explains why hydrogen peroxide requirements did not increase for intermediate levels of treatment (Figures 6.13 and 6.15). Nevertheless, the batch experiments conducted under condition (A) suggested that at low residual phenol concentrations the enzymatic oxidation of iodide and nitrite

might compete with the oxidation of phenol and thus reduce its transformation (see Figure 6.13(A)). At pH 7.0 the limitation posed by iodide and nitrite could be completely overcome by increasing the H_2O_2 dose (Figure 6.16(a)). Besides its practical implications, this observation also indicates that the enzyme is not inactivated when catalyzing the transformation of its inorganic substrates. If this would be the case, then not only an increase in the H_2O_2 concentration but also an increase in the HRP dose would be required to achieve the same level of phenol removal as in the absence of the inorganic substrates.

Iodide and nitrite had a stronger inhibiting effect at pH 5.0, which can be explained by faster rates of their reaction with H_2O_2 in acidic environments (Dunford and Stillman, 1976; Schumb *et al.*, 1955). In the case of nitrite, the phenol transformation could not be increased beyond 95 % by raising the H_2O_2 dose (Figure 6.16(b)). A possible explanation for this may be the formation of a soluble phenolic reaction product that, similar to phenol, would react to form a colored compound in the 4-AAP phenol assay but would only possess a low reactivity with HRP. This explanation is supported by the observation that phenolic solutions that were treated in the presence of nitrite exhibited a yellowish color as well as elevated UV absorbance values, particularly when the reactions had been conducted at pH 5.0. It is recommended that detailed analytical studies be performed to identify these reaction products since it seems possible that toxic nitrophenols could form when phenolic radicals are generated in the presence of high concentrations of nitrite or its oxidation product nitrate.

The presence of the reduced sulfurous anions thiosulfate, sulfite and sulfide or possibly their oxidation products increased phenol transformation provided sufficient H_2O_2 was supplied (Figures 6.13, 6.17 and 6.19). Thiosulfate was particularly effective in enhancing phenol removal even at low concentrations (Figure 6.14). However, phenolic solutions treated in the presence of these compounds tended to exhibit elevated UV absorbances (Figures 6.18 and 6.21). Generally, an increase in the absorbance may either be due to the presence of a larger quantity of soluble or colloidal reaction products or to the formation of products having a higher extinction coefficient. The particularly high UV absorbance of solutions treated in the presence of thiosulfate was mainly due to colloidal particles since no precipitate had formed. These particles could be coagulated with high

doses of chitosan (90 mg/L). However, the UV absorbance was not reduced to low levels, which partially may have been due to residual dissolved chitosan. The formation of soluble or colloidal phenolic products in the presence of reduced sulfuric compounds could have a negative effect on attempts to reduce the organic load and/or the toxicity of industrial effluents using the HRP-based treatment process.

Although all three sulfurous anions that were tested increased the H_2O_2 demand, characteristic differences were observed. HSO_3^-/SO_3^{2-} almost instantaneously reacted with hydrogen peroxide. Therefore, no phenol oxidation occurred before the peroxide demand of sulfite had been satisfied (Figure 6.15). At pH 7.0 this demand was approximately 0.75 mmole H_2O_2 per mmole HSO_3^-/SO_3^{2-} (or 0.009 mmole H_2O_2 per mg SO_3^-). This value was obtained through direct measurement of the peroxide remaining after exposure to sulfite and was in good agreement with results obtained in the batch reactions (Figure 6.15). At lower pH values, sulfite was probably completely oxidized to sulfate resulting in a one-to-one stoichiometry between peroxide and sulfite. At higher pHs, the less oxidized species dithionate ($S_2O_6^{2-}$) may have formed to some extent, thereby reducing the stoichiometry between peroxide and sulfite.

Thiosulfate, on the other hand, reacted only slowly with H_2O_2. Therefore, this reaction may not have been responsible for the higher peroxide requirements associated with treatment in the presence of high concentrations of this compound. Also, if a fraction of the available peroxide were in fact consumed in the side reaction with thiosulfate, increasing the rate of enzymatic peroxide utilization should lead to more phenol removal for a given H_2O_2 dose. The rate of enzymatic peroxide utilization increases with increasing enzyme concentrations. However, the amount of phenol transformed for a given peroxide dose did not increase significantly as more HRP was provided to the reaction mixtures (Figure 6.15). These observations suggest that an alternative explanation may be required to account for the higher peroxide demand.

No precipitate had formed upon HRP-treatment of phenolic solutions containing 200 mg/L of thiosulfate. This indicates that the reaction products formed in the presence of this compound differed from those formed in its absence. The nature of reaction products may affect the stoichiometry between phenolic substrates and H_2O_2, as was demonstrated in the case of 4-methylphenol (Hewson and Dunford, 1976). It therefore seems possible

that thiosulfate may also modify the product distribution and thus indirectly affect the stoichiometry between H_2O_2 and phenol.

Interestingly, the presence of thiosulfate and hydrogen sulfite (or rather its oxidation products) increased not only the extent but also the rate of phenol transformation (Figure 6.17). This does not necessarily mean that the presence of these compounds increased the intrinsic rate constants of the enzymatic reactions with H_2O_2 or phenol. It is rather more likely that the apparent increase in the reaction rate resulted from a reduced rate of enzyme inactivation. Thus, the concentration of active enzyme remained higher for a longer period of time resulting in a slower decline in the phenol transformation rate.

Sulfide exhibited the most interesting behavior of the three sulfur-containing anions tested. Firstly, the presence of sulfide clearly inhibited enzymatic phenol conversion. This is evidenced by the delay that occurred between the addition of the reagents and the onset of phenol oxidation (Figure 6.20). According to Schumb *et al.* (1955), sulfide is oxidized to sulfur by hydrogen peroxide in acid aqueous solutions. At alkaline conditions the reaction can proceed further to sulfate, although elemental sulfur once formed is quite unreactive with hydrogen peroxide. Debellefontaine (1996) reported that at pHs below 8.5 sulfide is oxidized by hydrogen peroxide to sulfur with a one-to-one stoichiometry. This reaction goes to completion within several tens of minutes. The approximate 1 to 1 stoichiometry between sulfide and H_2O_2, the appearance of a white solid and the smell of the reaction mixture are consistent with the oxidation of sulfide to elemental sulfur. If, in fact, the oxidation of sulfide stopped at the level of sulfur, then it can be concluded from the observed approximate one-to-one stoichiometry between sulfide and H_2O_2 (Table 6.4) that the sulfide concentration was reduced to a very low level before phenol oxidation commenced. Thus, sulfide is a strong inhibitor of peroxidase-catalyzed phenol oxidation.

Secondly, as determined from the slopes in Figure 6.19, more peroxide was consumed per mole phenol removed even after the peroxide demand of the sulfide had apparently been satisfied (Table 6.4). This may indicate that certain reaction products originating from sulfide may still consume H_2O_2. Alternatively, a similar explanation may apply as in the case of thiosulfate; thus, the nature of the phenolic reaction products formed in the presence of compounds originating from sulfide may enhance the enzymatic peroxide consumption.

The nature of the inhibition of phenol conversion by sulfide is not fully understood. Keilin and Hartree (1951) state that one molecule of peroxidase reacts reversibly with one molecule of H_2S to form a spectroscopically well-defined compound. Unfortunately, no data are shown and no references are given to support this statement. In this study, no change in the absorption spectrum of HRP was detected after the addition of an equimolar amount of sulfide. However, it was observed that sulfide rapidly decolorized oxidized compounds, presumably transforming them back to their reduced forms. Based on this observation it can be hypothesized that sulfide, which has strong reducing characteristics (E^0 = 0.142 V for H_2S(aq) and –0.478 V for SH^-; CRC Handbook of Chemistry and Physics, 2000-2001) may inhibit phenol transformation by reducing the forming radicals back to phenol. Subrahmanyam *et al.* (1990) proposed the same explanation for the observed inhibition of enzymatic phenol oxidation by glutathione, which is also a strongly reducing compound.

Since sulfide itself readily reacts with H_2O_2, the inhibition can be overcome by increasing the peroxide dose by the amount required to accomplish sulfide oxidation. In fact, oxidation with hydrogen peroxide is a common treatment process for the elimination of sulfide from industrial wastewaters (Debellefontaine, 1996). Thus, the sulfide could be treated first by the addition of the appropriate amount of H_2O_2 for its oxidation. Subsequently, additional peroxide and HRP could be introduced into the same reactor in order to treat the phenolic compounds. Sulfide concentrations should be closely monitored in wastewaters where they tend to fluctuate to a high degree in order to avoid phenol breakthrough due to inadequate H_2O_2 doses and/or retention times. Moreover, for wastewaters containing high sulfide concentrations, it may prove to be more advantageous to reduce the sulfide content by a different process such as air stripping before applying hydrogen peroxide and HRP to treat the phenolic compounds.

Thiocyanate had no impact of phenol removal at intermediate levels of treatment but at high concentrations (400 mg/L) it reduced phenol transformation slightly at higher levels of treatment (Figures 6.22 and 6.23) when higher concentrations of H_2O_2 or HRP were applied.

As expected, cyanide proved to be the most problematic anion among those tested in this investigation. Due to its high affinity to the native enzyme ($K_D = 2 \cdot 10^{-6}$ M; Dunford

and Stillman, 1976) only 5 % of the HRP molecules remain unoccupied in the presence of 1 mg/L of cyanide at pH 7.0. The observed cyanide-inhibition cannot be overcome by applying longer reaction times or by increasing the H_2O_2 concentration to more than 5 times of the value required for full treatment in the absence of cyanide (Figure 6.25). The only option remaining to accomplish treatment in the presence of cyanide is to increase the enzyme dose. However, this results in substantially higher enzyme requirements even at low cyanide concentrations (Figure 6.27). Therefore, it is recommended that cyanide concentrations should be reduced below 1 mg/L before applying the HRP-based treatment process.

6.3 Organic Compounds

In order to determine whether the presence of organic compounds, which are not substrates of HRP, would have an impact on the extent of phenol removal, the enzymatic reaction was conducted in the presence of methanol, toluene, phenanthrene and a mixture of hexanes. These compounds were selected to represent groups of organic pollutants that are often encountered in industrial wastewaters. Methanol is the major alcohol contained in pulp and paper wastewaters (LaFleur, 1996); while toluene, hexane and phenanthrene represent aliphatic, aromatic and polycyclic aromatic hydrocarbons present in wastewaters produced by the petrochemical industries (Dyer and Mignone, 1983).

The concentrations supplied to reaction mixtures ranged from 10 to 200 mg/L for toluene and hexane, from 0.25 to 1 mg/L for phenanthrene and from 1 to 3 % (vol/vol), corresponding to 7.9 g/L to 23.7 g/L, for methanol. According to Dyer and Mignone (1983) these concentrations are very high and in the case of phenanthrene exceed the liquid solubility of this compound. Treatment was conducted with 2.0 U/mL HRP and 1.3 mM H_2O_2 in pH 7.0 phosphate buffer. The results of these batch experiments as presented in Figure 6.24 show that the residual phenol content of the samples treated in the presence of the organic compounds did not significantly differ from the residual phenol content of the 7 control samples (p-value in ANOVA single factor test = 0.332). Thus, the organic compounds did not interfere with phenol transformation. Dunnett's method of multiple comparisons with a control also indicated that the differences between the measurements were not significant ($p > 0.05$).

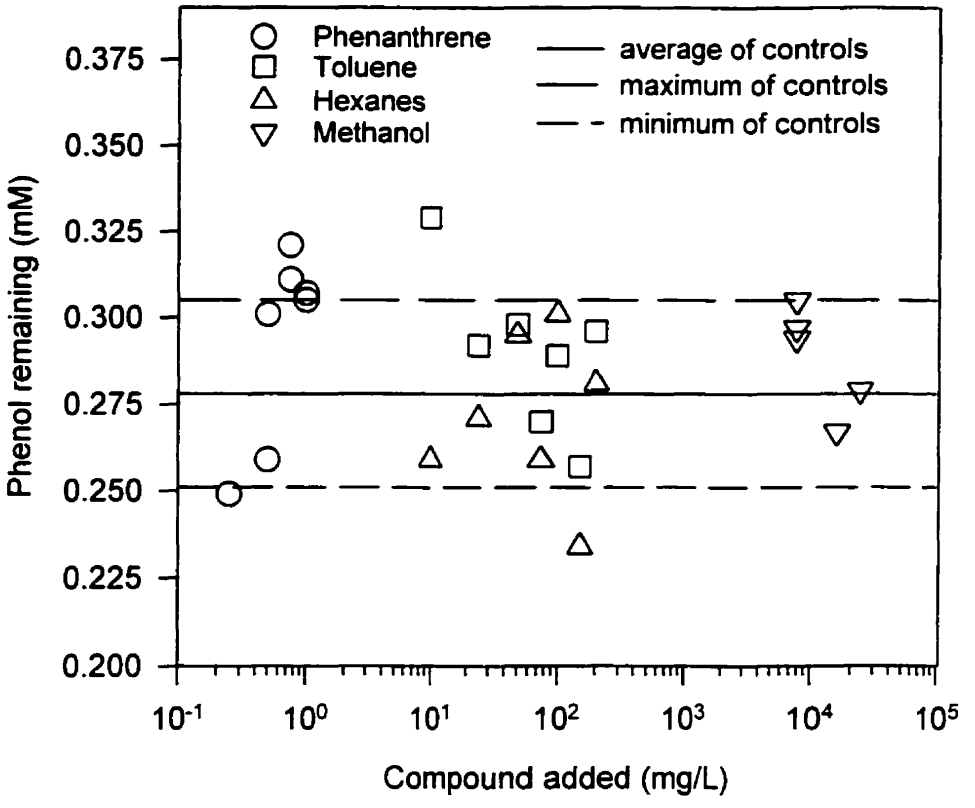


Figure 6.28: Phenol remaining as a function of the amount of toluene, hexanes, phenanthrene or methanol present in the reaction mixture. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 2.0 U/mL.

An additional experiment was conducted with 4.0 U/mL HRP in order to test whether the organics may interfere with phenol transformation at high levels of treatment, when the residual phenol concentration becomes very low. All reactions were conducted in triplicate. Phenol oxidation was stopped after 3 hours with the aid of catalase and the residual phenol concentrations were recorded. The results of this experiment are summarized in Table 6.5.

Table 6.5: Phenol remaining after treatment of a 1.0 mM phenol solution with 4.0 U/mL HRP and 1.3 mM H_2O_2 in the presence of organic compounds.

Sample	Control	Hexane	Toluene	Phenanthrene	Methanol
Concentration	-	200 mg/L	200 mg/L	5 mg/L	5 % (vol/vol)
Phenol remaining (μM ± std)	15.6 ± 4.1	12.9 ± 10.2	9.2 ± 0.6	13.8 ± 3.5	8.9 ± 0.8

The data presented in Table 6.5 show clearly that there was no interference with phenol removal due to organic compounds even at high levels of treatment. These results exemplify the ability of the HRP-based treatment to remove phenol in the presence of high concentrations of aliphatic, aromatic and polycyclic aromatic hydrocarbons and alcohols.

6.4 Metals

Dissolved heavy metal ions are common pollutants in industrial wastewaters. According to the National Pollutant Release Inventory (NPRI) (Environment Canada, 1999) in the year 1999 the Canadian industry released, disposed or recycled zinc, chromium, copper and manganese and their compounds each in quantities exceeding 20000 tonnes. Nickel was used in lower quantities; however, it is a common pollutant in pulp and paper, petroleum refining and explosives manufacturing wastewaters (Dyer and Mignone, 1983), which are potential targets of the HRP-based phenol removal process. Although, iron and its compounds are not listed as pollutants by the NPRI, iron compounds are present in high concentrations in a variety of industrial settings (Dyer and Mignone, 1983).

Metal cations are known to be able to modulate (increase or inhibit) the activity of many enzymes (Fullbrook, 1996). For example, in the case of HRP, manganese and cobalt are reported to have an inhibiting effect at concentrations as low as 10^{-5} M (Guilbault *et al.*, 1966). The abundance of heavy metals in industrial wastewaters and their potential effects on enzymatic catalysis makes it crucial to test the possible impacts of these substances on peroxidase-based phenol removal. Therefore, the following metal ions were investigated in this study: chromium (III), manganese (II), iron (III), cobalt (II), nickel (II), copper (II) and zinc (II).

6.4.1 Results

All the metals were used in the form of their sulfate or nitrate salts. Phenol removal in the presence of the metal ions was tested under five different conditions ((A) to (E)). Conditions (A) to (D) correspond to those that were used to investigate the impact of reducing inorganic anions (see Section 6.2.1(a) on page 128). However, certain modifications were made in order to meet the specific treatment goals at each condition. Additionally, tests were also conducted to determine whether phenol removal could be accomplished without the addition of peroxidase (condition (E)). The reaction conditions applied are summarized in Table 6.6.

Table 6.6: Reaction conditions applied to test the impact of metal ions on phenol removal.

Condition	**(A)**	**(B)**	**(C)**	**(D)**	**(E)**
Distilled-deionized water; pH 5.0					
HRP dose	4.5 U/mL	2.0 U/mL	8.0 U/mL	2.0 U/mL	0
H_2O_2 dose	1.3 mM	1.3 mM	0.58 mM	0.58 mM	1.3 mM
0.05 M Tris-HCl buffer; pH 7.2					
HRP dose	4.5 U/mL	1.5 U/mL	4.5 U/mL	1.5 U/mL	0
H_2O_2 dose	1.3 mM	1.3 mM	0.58 mM	0.58 mM	1.3 mM

Preliminary experiments with 0.01 mM solutions revealed no differences with control reactions. Therefore, the metal ions were tested at a concentration of 1 mM in subsequent experiments. This value corresponds to 52 to 65 mg/L depending on the metal tested, which is very high for most industrial wastewaters (Dyer and Mignone, 1983). All reactions were carried out in distilled-deionized water at pH 5.0 and in 0.05 M tris(hydroxymethyl) aminomethane - hydrochloric acid (Tris-HCl) buffer at pH 7.2. These sample matrices were chosen for the reasons summarized below.

All the metal salts tested decreased the pH when added to distilled-deionized water at 1 mM. When alkali was added to these solutions to increase the pH to neutral conditions precipitation occurred in most cases. Therefore, in order to be able to conduct treatment at neutral conditions, a buffer was used that could stabilize the solution, thus preventing the formation of precipitates. The Tris-HCl buffer fulfilled this requirement for all solutions

except for those containing Cr(III) and Fe(III) salts, which showed some precipitation. The prevention of precipitation in the Tris-HCl buffer is likely to be due to a complexation of the metal ions. In order to test the effect of the metal ions at conditions where no complex formation would occur, reactions were also carried out in distilled-deionized water. In this case, the metallic solutions were adjusted to pH 5.0 using concentrated solutions of NaOH and H_2SO_4. Sodium chloride (NaCl) was added at 1 mM to the control solution at pH 5.0 in order to increase the salt concentration.

The results of these experiments are presented in Figures 6.29 and 6.30. The Dunnet's method for multiple comparisons with a control (Berthouex and Brown, 1994) was used to evaluate the statistical significance of the observed differences between the samples. The significance level was set to 0.05.

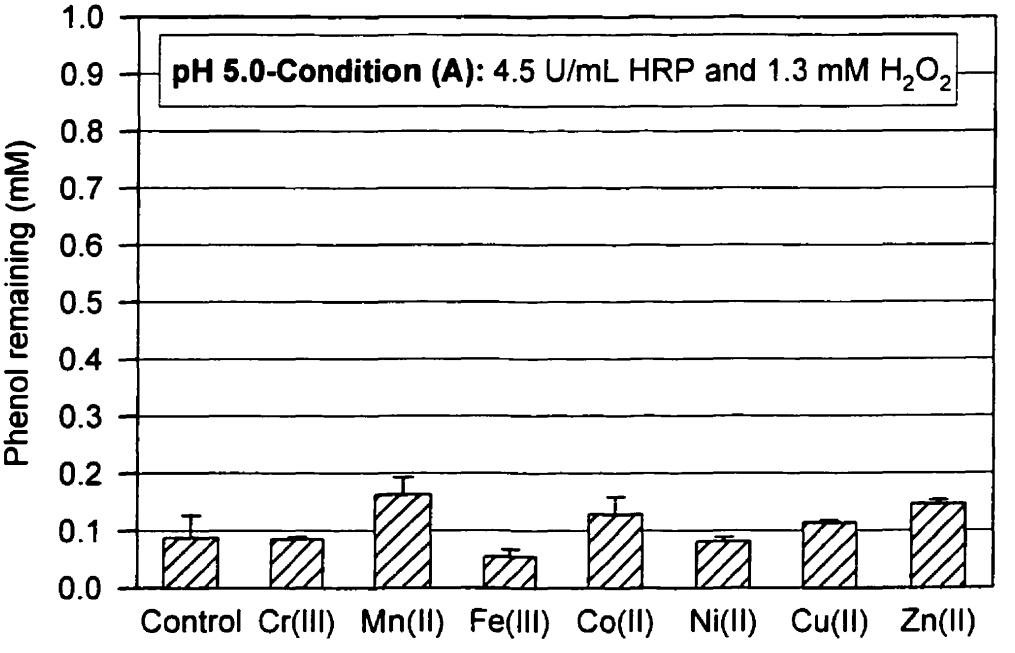
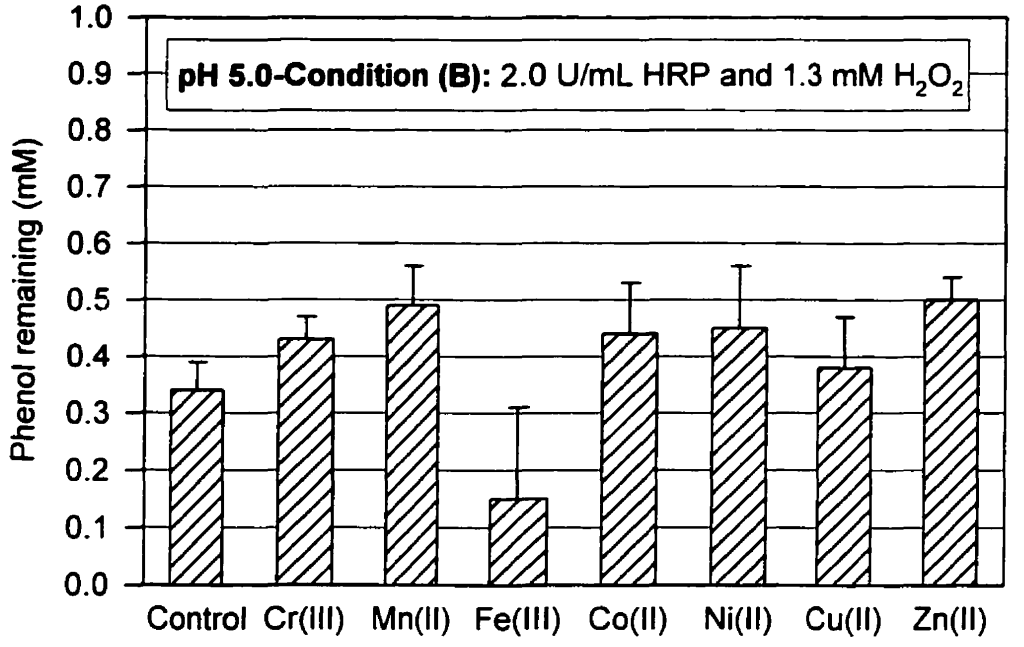


Figure 6.29: Phenol removal at conditions (A) to (E) in the presence of 1mM of metal cations in water at pH 5.0. $[phenol]_0 = 1.0$ mM. Error bars stand for the standard deviation of 3 independent experiments.



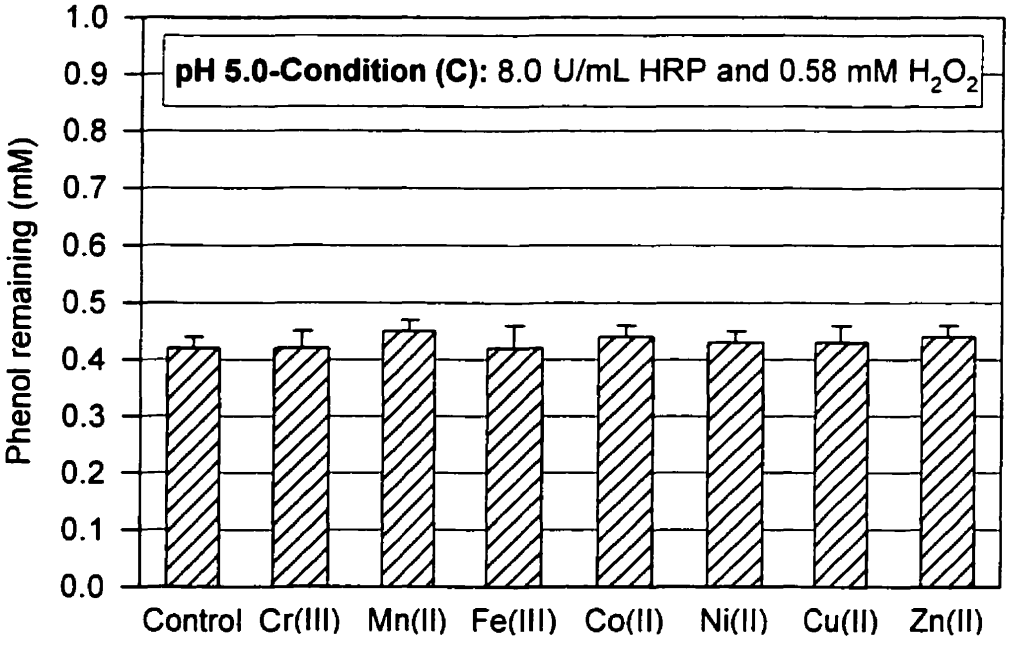


Figure 6.29: (continued)

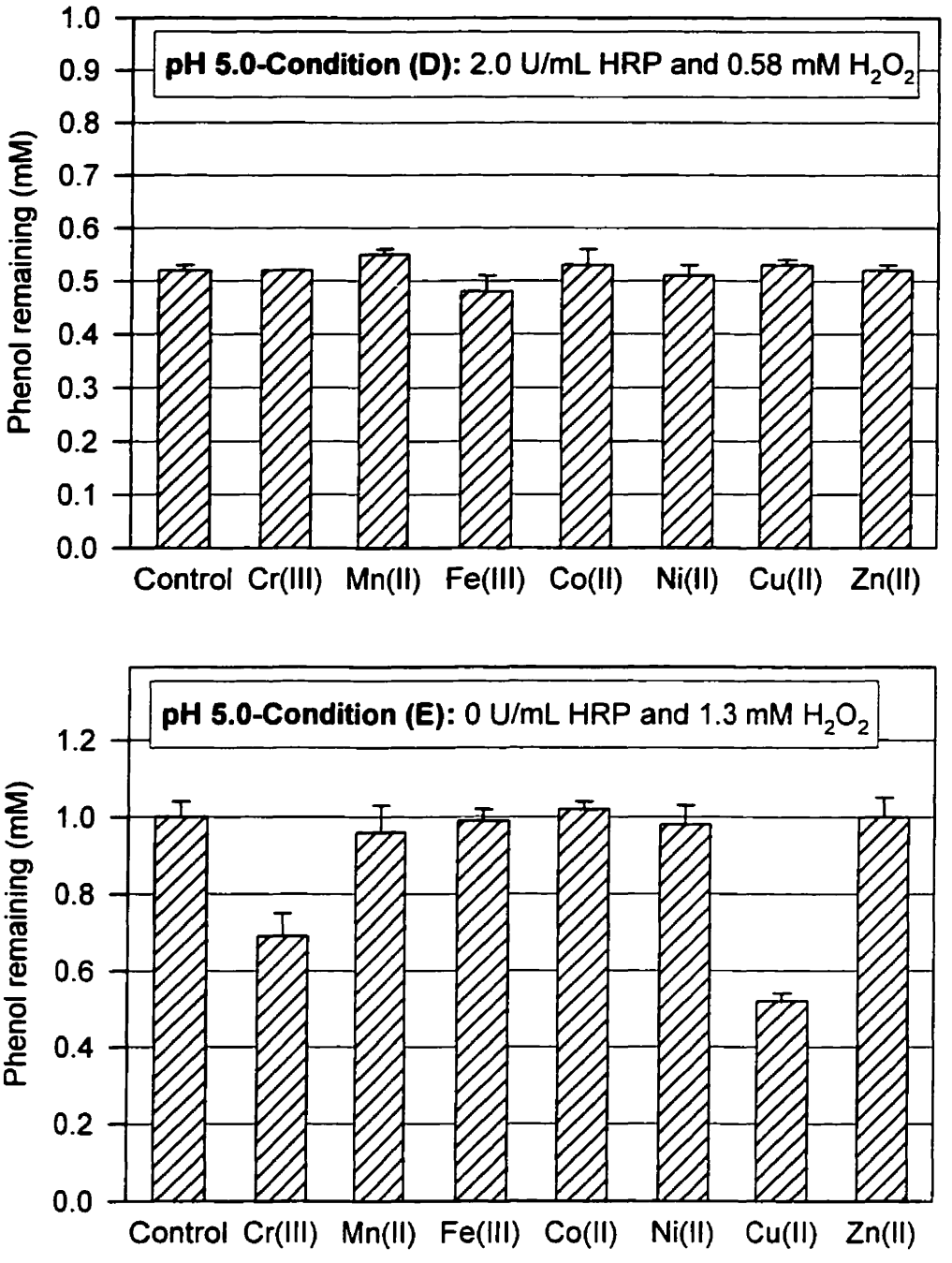


Figure 6.29: (continued)

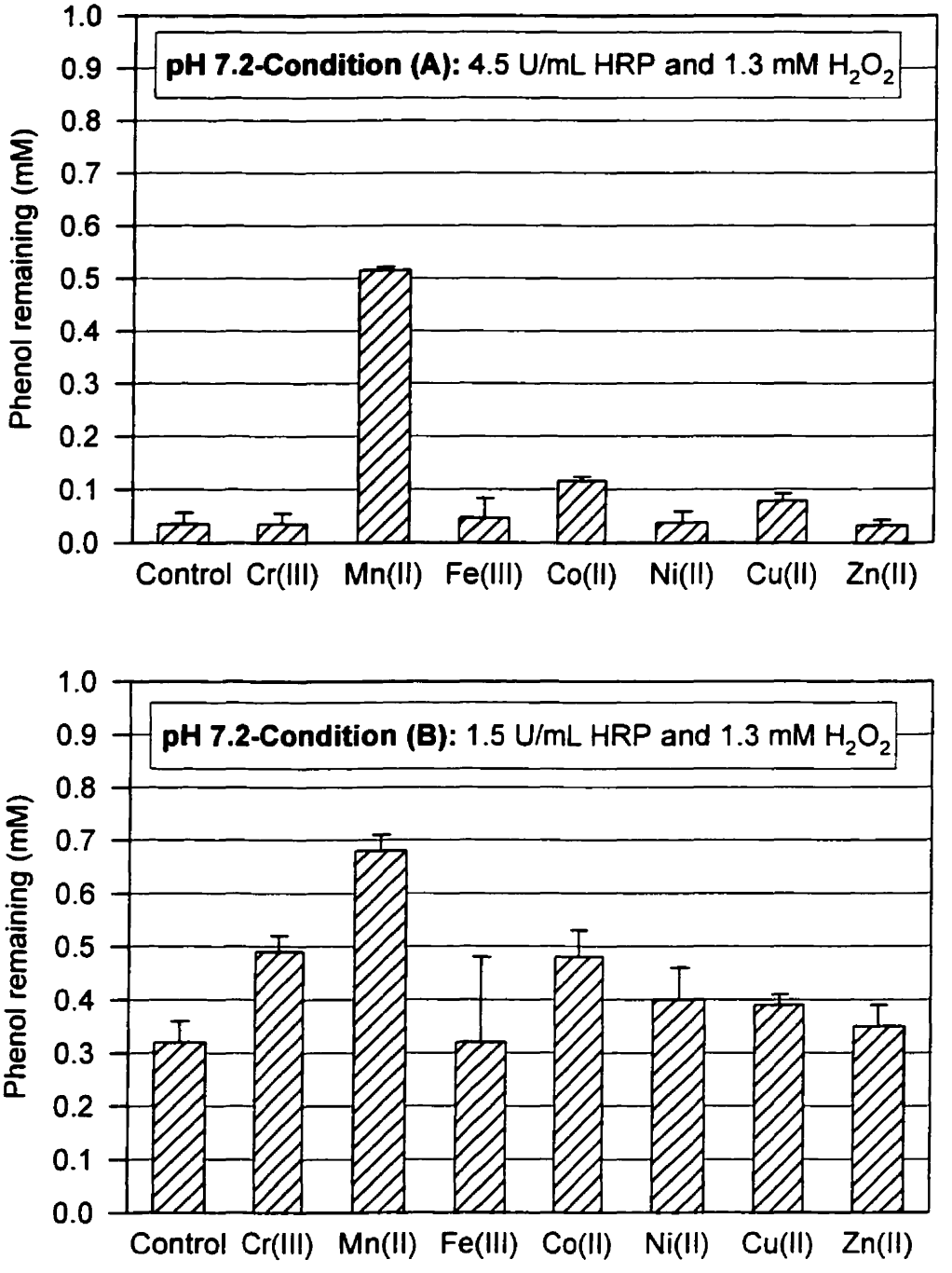
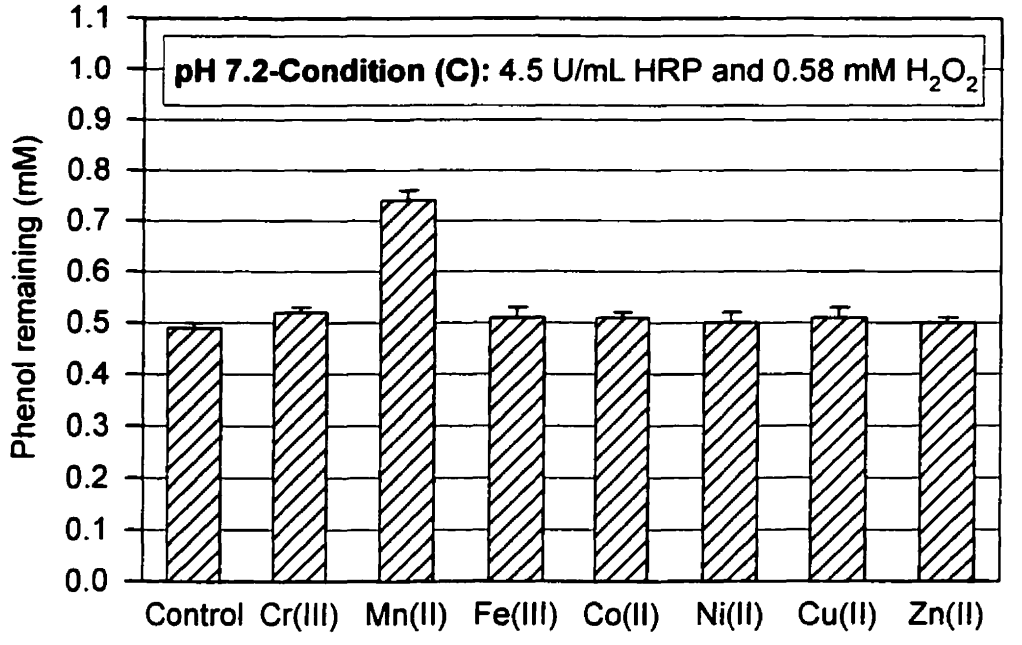


Figure 6.30: Phenol removal at conditions (A) to (E) in the presence of 1 mM of metal cations in Tris-HCl buffer at pH 7.2. $[phenol]_0$ = 1.0 mM. Error bars stand for the standard deviation of 3 independent experiments.



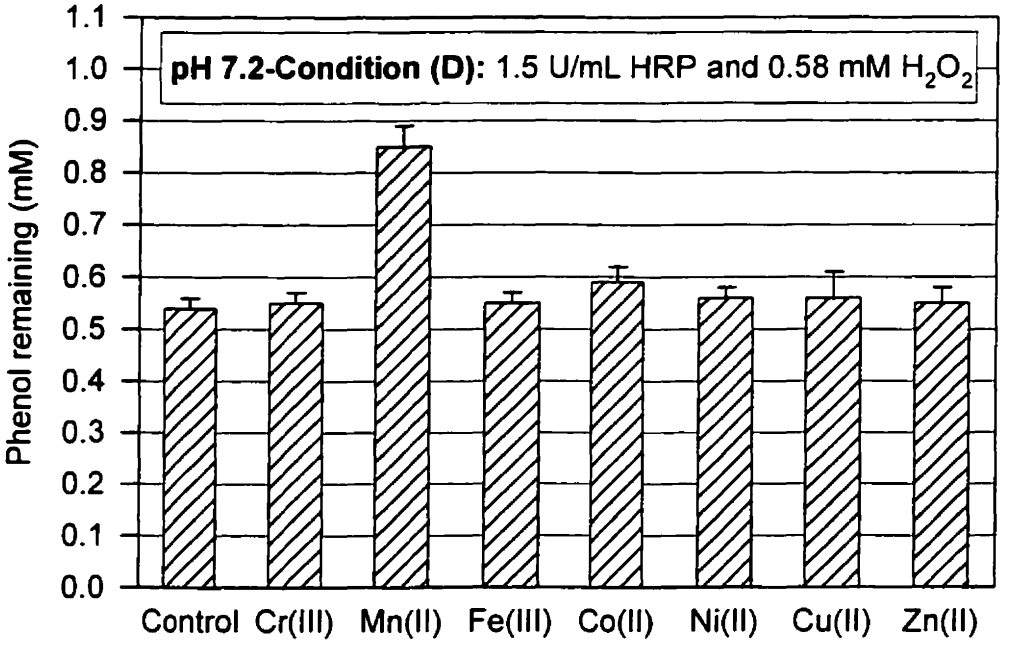


Figure 6.30: (continued)

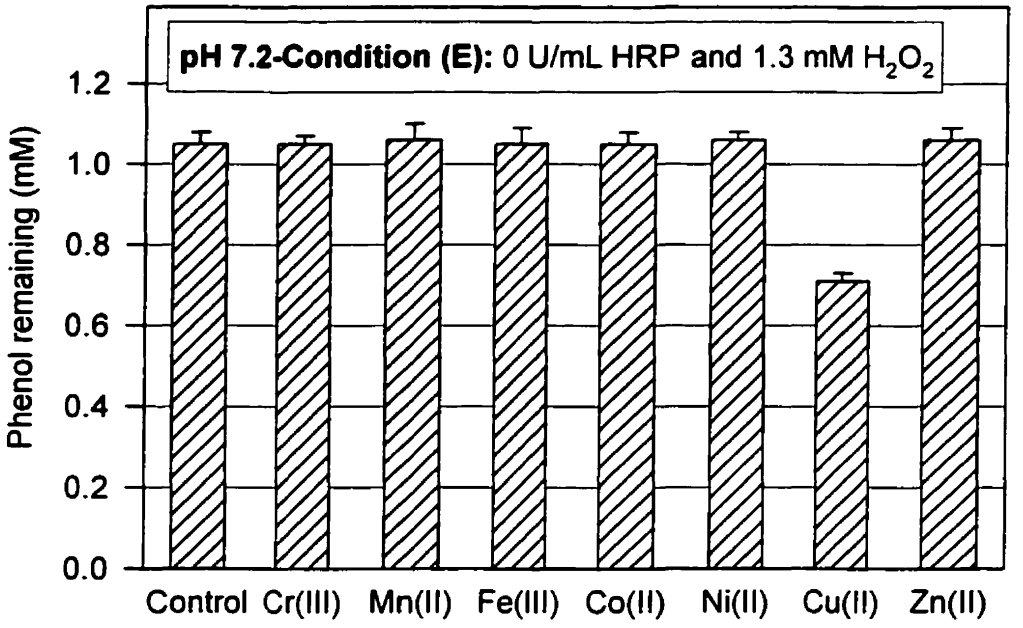


Figure 6.30: (continued)

At condition (A), corresponding to a high level of phenol removal, and pH 5.0, only solutions treated in the presence of Mn(II) and Zn(II) exhibited residual phenol levels that were significantly higher than those of the control samples (Figure 6.29). Treatment in Tris-HCl buffer at condition (A) (Figure 6.30) resulted in lower levels of phenol conversion with Mn(II) and Co(II).

At condition (B), in which phenol removal is limited by HRP, and pH 5.0 (Figure 6.29), none of the tested metals yielded results that were significantly different from the controls when using Dunnet's test. However, when the data for each metal were compared one by one with the control data using Student's t-test, then significant differences were obtained in the case of Mn(II), Cr(III) and Zn(II). When treatment was conducted in the Tris-HCl buffer at condition (B) (Figure 6.30) Mn(II), Cr(III) and Co(II) were found to significantly interfere with phenol conversion.

The results obtained with Fe(III) at condition (B) showed a great deal of variation. In several instances residual phenol values below 0.15 mM were measured after treatment in the presence of this metal, which was much lower than the corresponding control values. However, these observations were difficult to replicate. When NaOH was added to the ferric solutions in distilled-deionized water to adjust the pH from originally 3.0 to 5.0,

flocs of ferric hydroxide slowly started to form. The floc formation started earlier when higher amounts of salts (NaOH and H_2SO_4) were added for pH adjustment. Similarly, when the Fe(III) salt was added to the pH 7.2 Tris-HCl buffer, iron hydroxide flocs formed when the solution was allowed to stand. Interestingly, it was observed that no enhancement of phenol removal took place if the floc formation occurred before the enzymatic phenol removal was initiated by the addition of HRP and H_2O_2. However, if the reaction was started while the ferric hydroxide was still in the form of colloidal particles then significant improvement of phenol removal was obtained. Therefore, it is hypothesized that the variation of the results obtained under condition (B) is due to a variation in the state of the ferric solution.

At conditions (C) and (D), when phenol removal was mainly limited by the available H_2O_2, none of the metals tested had an effect on residual phenol when treatment was conducted in distilled-deionized water at pH 5.0 (Figure 6.29). However, in the Tris-HCl buffer, Mn(II) interfered with phenol removal at both conditions (C) and (D) (Figure 6.30).

Some metals were able to catalyze phenol removal when the treatment was conducted without the addition of HRP, i.e., condition (E). At pH 5.0, 30 % and 50 % phenol removal was observed in the presence of Cr(III) and Cu(II), respectively (Figure 6.29). In the Tris-HCl buffer, only Cu(II) was able to effect a reduction in the phenol concentration by 30 % (Figure 6.30). No phenol removal took place in the absence of H_2O_2. Thus, these metals catalyzed phenol oxidation with H_2O_2 in a manner similar to HRP. Also, the solutions in which phenol transformation had taken place exhibited a brownish color, which is characteristic of the presence of phenolic oxidation products. Note that Cr(III) and Cu(II) did not promote phenol removal when HRP was present in the reaction mixture. This is probably due to the slow rates of the reactions catalyzed by the metal ions compared to rate of the peroxidase-catalyzed phenol oxidation.

Guilbault *et al.* (1966) reported that Mn(II) and Co(II) reduced the activity of HRP by 50 % when present at approximately 0.05 and 0.08 mM, respectively. The activity assay was conducted using homovanillic acid as a reducing substrate at approximately 0.3 mM and 2 mM H_2O_2 in pH 8.5 Tris buffer. In order to test whether the reduction of phenol removal was due an inhibition of the enzyme activity, the 4-AAP activity assay

was carried out in the presence of up to 1 mM of these ions. The assay was modified in so far as 0.05 M Tris-HCl buffer (pH 7.4) was used instead of 0.1 M sodium phosphate buffer, since the metal salts possess only a very low solubility in the presence of phosphate. It was found that under the conditions tested, the presence of Mn(II) had no effect on enzyme activity even when the phenol concentration was decreased and the H_2O_2 concentration increased to more closely match the conditions employed by Guilbault *et al.* (1966). No reduction in enzyme activity was also observed with cobalt (II). However, this metal interfered with the assay at 1 mM as some color developed in the absence of peroxidase.

6.4.2 Discussion

In summary, Mn(II) inhibits phenol removal. Particularly strong inhibition was observed at near neutral pH in the Tris-HCl buffer under every condition tested. Cr(III) affected phenol removal in both sample matrices (pH 5.0 and 7.2) only under the purely enzyme-limited condition (B). Co(II) dissolved in Tris-HCl buffer at pH 7.2 and Zn(II) dissolved in distilled-deionized water at pH 5.0 both had an effect under the treatment conditions (A) and (B), under which conversion was mainly limited by the availability of the enzyme.

In contrast to the results reported by Guilbault *et al.* (1966), Mn(II) and Co(II) were not able to reduce enzyme activity under the conditions of the 4-AAP activity assay. This discrepancy is probably due to differences between the assay conditions since Guilbault and co-workers used a higher pH and a different reducing substrate than is employed in the 4-AAP assay. According to Guilbault *et al.* (1966), Fe(III), Cu(II) and Ni(II) also inhibited HRP at relatively low concentrations. However, these ions had no significant impact on phenol removal as demonstrated in this study. These differences indicate that observations made using enzymatic assays at steady-state conditions may not provide a good indication of whether a substance is able to affect treatment efficiency under conditions which prevail in industrial applications.

Compared to the effect of Mn(II), solutions treated in the presence of Co(II), Zn(II) and Cr(III) exhibited only slightly elevated residual phenol levels. Therefore, these ions are not expected to pose major problems for the treatment of real wastewaters.

The mechanisms of how metallic ions interfere with phenol conversion or inhibit enzyme activity are not fully understood. Bryska *et al.* (1997) state that certain metal ions including Mn(II) interact with oxygen-containing ligands, such as carboxyl groups present on the enzyme globule. These interactions could modify the affinity of the enzyme towards its substrates or could render the enzyme more vulnerable towards inactivation by substrates or reaction products.

6.5 Salinity

The content of inorganic salts or, in other words, the ionic strength of the reaction matrix could affect enzymatic phenol removal in a number of ways. For example, such an effect could results from changes in intrinsic enzyme rate constants (e.g., by changes in ionization of critical amino acid residues on the enzyme molecule). The ionic strength could also influence the solubility and/or the stability of intermediate reaction products, which could lead to changes in the rate of enzyme inactivation. Finally, the structural stability of the enzyme molecule could also be affected by high salt concentrations.

In this study, sodium chloride (NaCl), calcium chloride ($CaCl_2$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), ammonium chloride (NH_4Cl) and ammonium sulfate ($(NH_4)_2SO_4$) were tested for their possible impacts on HRP-catalyzed phenol conversion. The effects of these salts were investigated in the concentration range of 0.001 to 0.6 M in terms of the concentration of the cation. These concentrations correspond to 0.06-35.1 g/L of NaCl, 0.11-66.6 g/L of $CaCl_2$, 0.10-57.1 g/L of $MgCl_2$, 0.05-32.1 g/L of NH_4Cl and to 0.07-39.6 g/L of $(NH_4)_2SO_4$. The value of 0.6 M was chosen as an upper limit because in the case of sodium chloride this concentration is equal to the salinity of seawater (35.1 g/L).

6.5.1 Results

For the experiments, the salts were dissolved in distilled-deionized water containing 1.0 mM phenol. The pH of the 1.0 mM phenol solution prior to the addition of salts was 5.9. Since this solution served as the salt-free control, the pHs of the solutions containing salts were adjusted, as necessary, to the same value with 0.1 M NaOH and 0.2 M HCl. The amount of NaCl introduced through pH adjustment was below 0.5 mM for the solutions

containing the highest salt concentration. Phenol removal experiments were carried out in these sample matrices using 1.5 and 3.5 U/mL HRP and 1.3 mM H_2O_2. The reaction time exceeded 20 hours. The results of these experiments are presented in Figure 6.31.

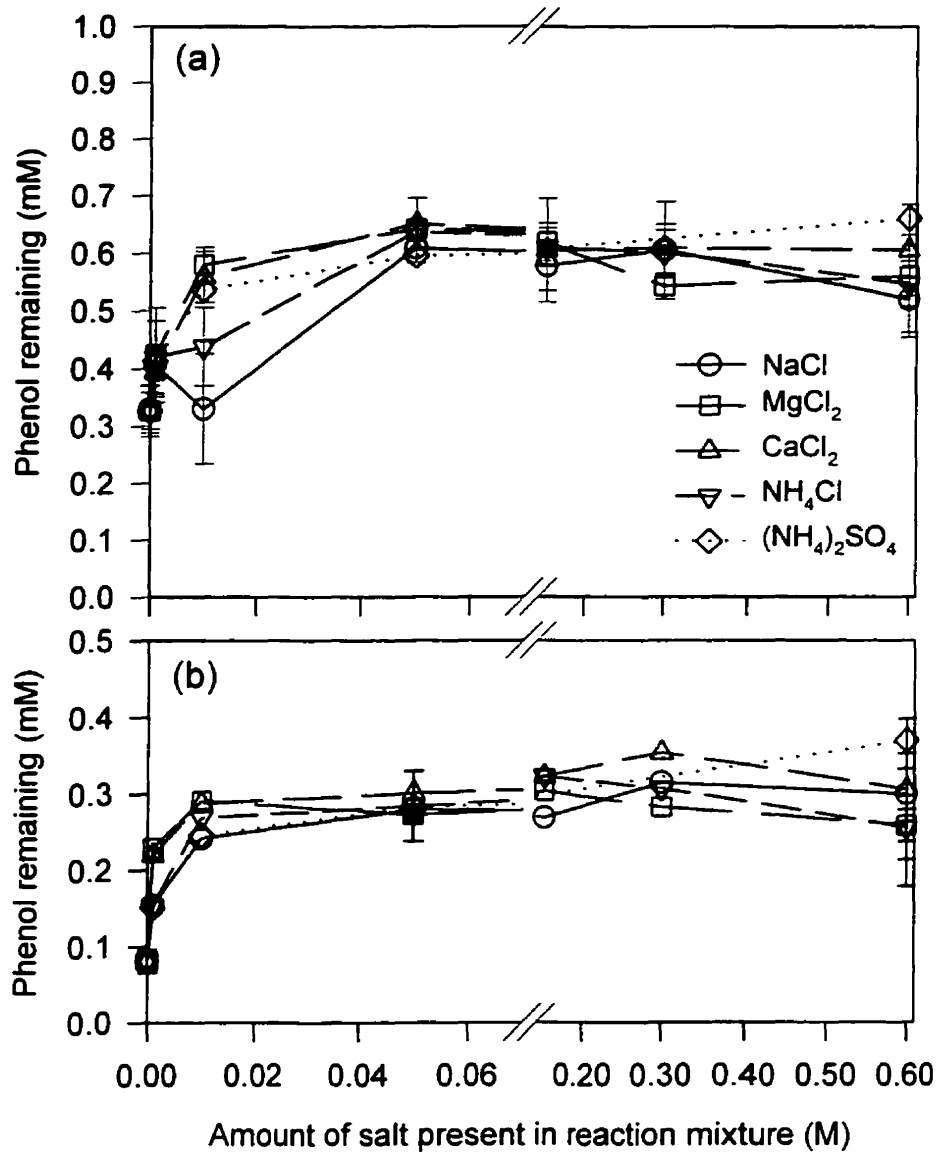


Figure 6.31: Phenol removal as a function of the amount of salts present in the reaction mixture with (a) 1.5 U/mL HRP or (b) 3.5 U/mL HRP at pH 5.9. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM. Error bars stand for the standard deviation of 3 (a) or 2 (b) independent experiments.

The part of the graph close to the abcissa axis has been expanded in order to see more clearly the values obtained at low salt concentrations.

The results displayed in Figure 6.31 clearly show that the residual phenol levels increased with increasing salt concentrations. When 1.5 U/mL HRP were used, residual phenol levels increased from 0.33 mM, measured in the salt-free controls, up to a maximum of approximately 0.60 mM that was reached in the presence of 0.01 to 0.05 M of salts (Figure 6.31(a)). Similarly, when 3.5 U/mL of HRP were used, the phenol concentration increased from 0.08 mM to 0.30 mM (Figure 6.31(b)). Increasing the salt content beyond 0.05 M had no additional impact on phenol removal. These maximum residual phenol levels did not depend on the type of salt tested. However, the salt concentration at which the maximum impact on phenol removal would occur was a function of the type of salt. With 1.5 U/mL HRP (Figure 6.31(a)), the maximum effect was reached at a salt concentration of 0.05 M in the case of NaCl and NH_4Cl. In the case of $CaCl_2$, $MgCl_2$ and $(NH_4)_2SO_4$, a salt concentration of 0.01 M was sufficient to exert the same effect. When a higher enzyme concentration was applied (Figure 6.31(b)), the threshold concentration seemed to shift to lower values for all salts tested.

The phenolic reaction products did not precipitate in the salt-free control reaction solutions. Precipitation occurred in the NaCl, NH_4Cl and $(NH_4)_2SO_4$ solutions at a salt concentration of 0.01 M and higher. Solutions treated in the presence of $CaCl_2$ and $MgCl_2$ showed precipitation already at 0.001 M, which was the lowest salt concentration tested.

The relatively high levels of phenol removal in the distilled-deionized water at pH 5.9 were unexpected since the same enzyme doses led to less phenol conversion when the reactions were carried out at a more favorable pH of 7.0 in phosphate buffer (compare with Figure 6.13(B)). Also, similar observations made with the different salts led to the hypothesis that dissolved ions had a rather unspecific effect on the enzymatic phenol conversion. In order to examine this effect more closely, an experiment was carried out using 0.05 M solutions of NaCl and $(NH_4)_2SO_4$. For the purpose of comparison, a reaction was also conducted in 0.05 M sodium phosphate buffer at pH 5.9. Distilled-deionized water served as a control. After 1.5 U/mL HRP and 1.3 mM H_2O_2 were supplied to the reaction solutions, the change in the phenol concentrations was monitored as a function of time. The result of this experiment is presented in Figure 6.32.

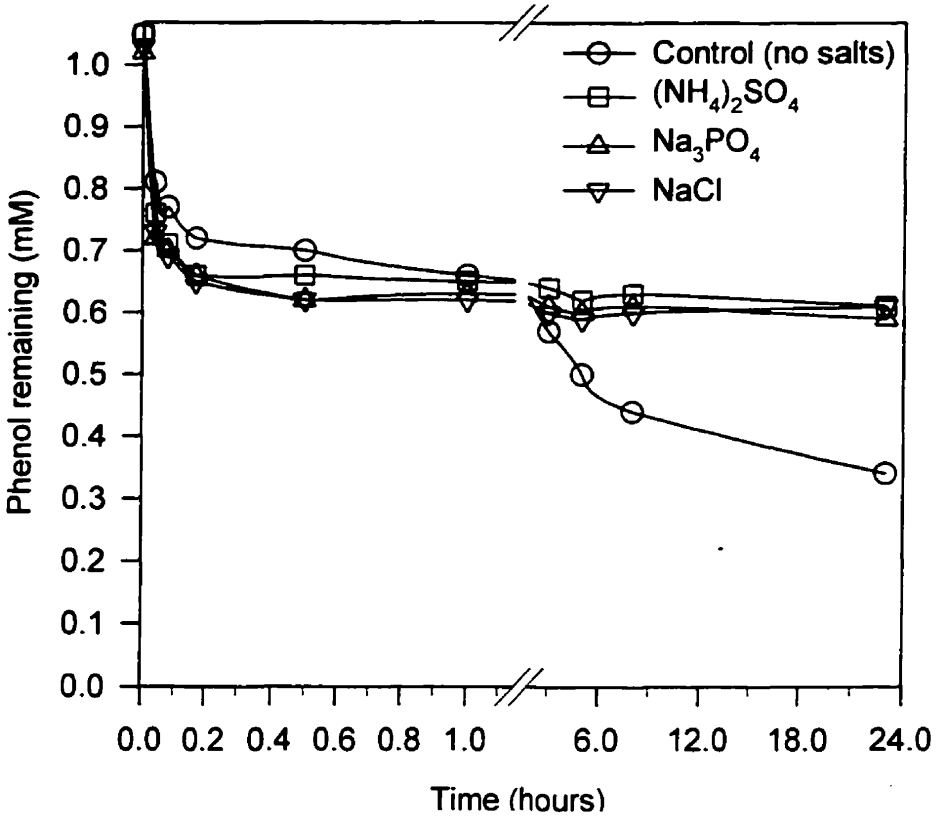


Figure 6.32: Phenol removal as a function of the reaction time in presence or absence of salts at 0.05 M. $[phenol]_0$ = 1.0 mM; $[H_2O_2]_0$ = 1.3 mM; HRP_0 = 1.5 U/mL; pH 5.9.

During the initial hour of the reaction phenol conversion was somewhat slower in the salt-free control solution than in the other three reaction matrices. At three hours phenol removal reached approximately 40 % in the NaCl, $(NH_4)_2SO_4$ and phosphate buffer solutions. No further phenol conversion took place after this point of time, which was likely due to complete inactivation of the enzyme. However, in the case of the salt-free control, phenol conversion proceeded beyond this point to reach almost 70 % at 23 hours, suggesting that a fraction of the enzyme was still active. As in the previous experiments, no precipitation occurred in the salt-free control as opposed to the other three reaction matrices. This may indicate that precipitation of reaction products was responsible for the higher degree of enzyme inactivation in the presence of salts.

6.5.2 Discussion

The above results show that all the salts tested had similar effects when present above a certain threshold concentration. Thus, neither the metal cations Na^+, Ca^{2+} or Mg^{2+} nor the anions chloride or sulfate exerted a specific effect on enzymatic phenol conversion. Also, ammonium, which is a common pollutant in industrial and municipal wastewaters, did not affect phenol removal up to a concentration of almost 11 g/L (0.6 M) under the reaction conditions tested when compared to other cations.

However, significantly higher levels of phenol conversion were obtained when the reactions were carried out in salt-free water. This effect was not due to an increase in the rate of the phenol oxidation as can be seen from Figure 6.32. Rather, it seems that under this condition, the enzyme was able to retain its catalytic activity for a longer period of time. Moreover, treatment in salt-free water also prevented the formation of precipitates. Precipitation occurred in solutions containing divalent cations (Ca^{2+} and Mg^{2+}) at a concentration as low as 0.001 M (1 mM or 24-40 mg/L). In the presence of salts containing the monovalent cations (Na^+ and NH_4^+), precipitation started at higher salt concentrations. Also, the threshold concentration at which the salts exhibited their maximum effect in decreasing phenol conversion tended to be higher for the sodium and ammonium chlorides as compared to their calcium and magnesium counterparts (Figure 6.31). Taken together, these observations suggest that the unspecific effect of salts may be attributable to their ability to promote precipitation.

It was previously suggested that the protective action of borate towards the enzyme might be due to its ability to prevent the precipitation of polymeric reaction products (Nicell, 1991). This would preclude the enzyme from being trapped and precipitated along with the polymers, which could lead to its inactivation. In fact, experiments conducted by Nakamoto and Machida (1992) show that the majority of the enzyme becomes attached to the phenolic polymers as they form. It seems, therefore, possible that the process of precipitation, which involves the formation of large and relatively dense particles, could inactivate the enzyme that is attached to the polymers.

Studies on the HRP-catalyzed production of high molecular weight phenolic polymers indicate that the limited solubility of the phenolic reaction products restricts the formation of higher molecular weight products (Ayyagari *et al.*, 1996). Therefore,

polymers with higher molecular weights can be obtained when the reaction is carried out in water/solvent mixtures. Thus, it is possible that in the reactions carried out in this study larger molecular weight products were formed under the salt-free conditions that prevented precipitation. This may have had an affect on the stability of the enzyme over the course of the phenol removal reaction.

7. TREATMENT OF A FOUL CONDENSATE FROM KRAFT PULPING

Phenolic compounds in the Kraft pulping process mainly originate from the decomposition of lignin during the cooking of wood chips in the digester (LaFleur, 1996). Phenol derivatives formed during the delignification reaction are mainly phenol, catechol and syringyl structures as well as compounds with formyl, acetyl and carboxyl groups that are *para* to the hydroxy group (LaFleur, 1996). High levels of phenols can be found in foul condensates from the evaporators and the recovery furnace in which the black liquor is concentrated and finally burned. In addition to phenolic compounds, low molecular weight alcohols, aldehydes, ketones, terpenes and sulfur-bearing compounds have been identified in foul condensates (LaFleur, 1996).

Condensates are one of the main sources of wastewater in a Kraft mill (LaFleur, 1996). Although steam stripping substantially reduces the organic load of condensates, mainly by removing methanol (LaFleur, 1996), it might be advantageous to remove the phenolics as well. This would be particularly important if this water is to be reused for other applications, such as in bleaching where chlorinated recalcitrant organics can be formed from the phenolic compounds (LaFleur, 1996).

A suitable in-plant treatment technology for the removal of specific pollutants from process waters must be simple to operate and selective for the targeted compound. Technologies such as adsorption and chemical oxidation have the advantage of short retention times and often require relatively simple equipment. However, they are not highly selective in terms of the pollutants removed during treatment (Aitken, 1993). In addition, due to the lack of selectivity, the cost of these treatment methods can become prohibitive as the total concentration of organic compounds in the waste mixture increases. Biological degradation processes can be used to treat the bulk of organic contaminants in a waste stream. However, they often fail to reduce the concentration of recalcitrant compounds below target levels, particularly if these are toxic (Aitken, 1993).

Up to this point, this study has shown that horseradish peroxidase is able to selectively remove phenols from complex mixtures under a variety of reaction conditions. In addition, previous research has demonstrated that HRP-catalyzed phenol removal can be carried out in simple reactors under mild conditions and with short retention times

(Buchanan *et al.*, 1998). Therefore, a HRP-based treatment process might be a viable approach for the removal of phenolics from foul condensates.

There were three primary objectives to this investigation: (1) to study phenol removal from a foul condensate with HRP and H_2O_2; (2) to assess the effect of treatment on chemical oxygen demand and toxicity which are two important bulk wastewater quality parameters; and (3) to evaluate the impact of the wastewater matrix on the enzymatic process by comparing the treatment of real and synthetic wastewaters.

Most of the results presented in this chapter were published in an article by Wagner and Nicell (2001a).

7.1 Results

Two grab samples of a foul condensate from the scrubber of a recovery furnace were obtained from a hardwood Kraft pulp and paper mill and stored at 4°C in the dark. This stream was selected because it was the source with the highest concentration of phenols in the mill. The wastewater samples were characterized in terms of pH, total phenol concentration total and suspended solids, COD and lignin content. The values obtained for these parameters are summarized in Table 7.1.

Table 7.1: Characteristics of foul condensate samples.

Sample	**Total phenols**	**Suspended Solids**	**Total Solids**	**pH**	**Lignin**	**COD**
	(mM as phenol)	(mg/L)	(mg/L)		(mM as phenol)	(mg/L)
A	0.152	18	186	9.4	n.d.	1580
B	0.114	n.d.	n.d.	9.1	0.26	1130

n.d. : not determined

For the purpose of discussion, a target treatment level of 1.0 mg/L or 0.011 mM (as phenol) of residual total phenols was used in the treatment experiments described below. Although this target is within the range of common regulatory discharge limits for phenols (Fuller and Tomlin, 1988), it was chosen with the assumption that the bulk of the total phenols would be removed by the enzymatic process, either as a pretreatment step or

for in-plant recirculation of waters, and any residual phenols would be treated in a secondary system before being discharged from a given facility.

7.1.1 Impact of reagent doses on removal of total phenols

Experiments were conducted to determine the relationship between the degree of phenol treatment and HRP and hydrogen peroxide concentrations. In experiments designed to investigate the impact of peroxide, the H_2O_2 concentration was varied between 0 and 1.0 mM. Excess quantities of peroxidase (2.0-6.0 U/mL) were added to ensure that the conversion of phenols was only limited by the availability of H_2O_2.

The results of these experiments for samples A and B are illustrated in Figure 7.1.

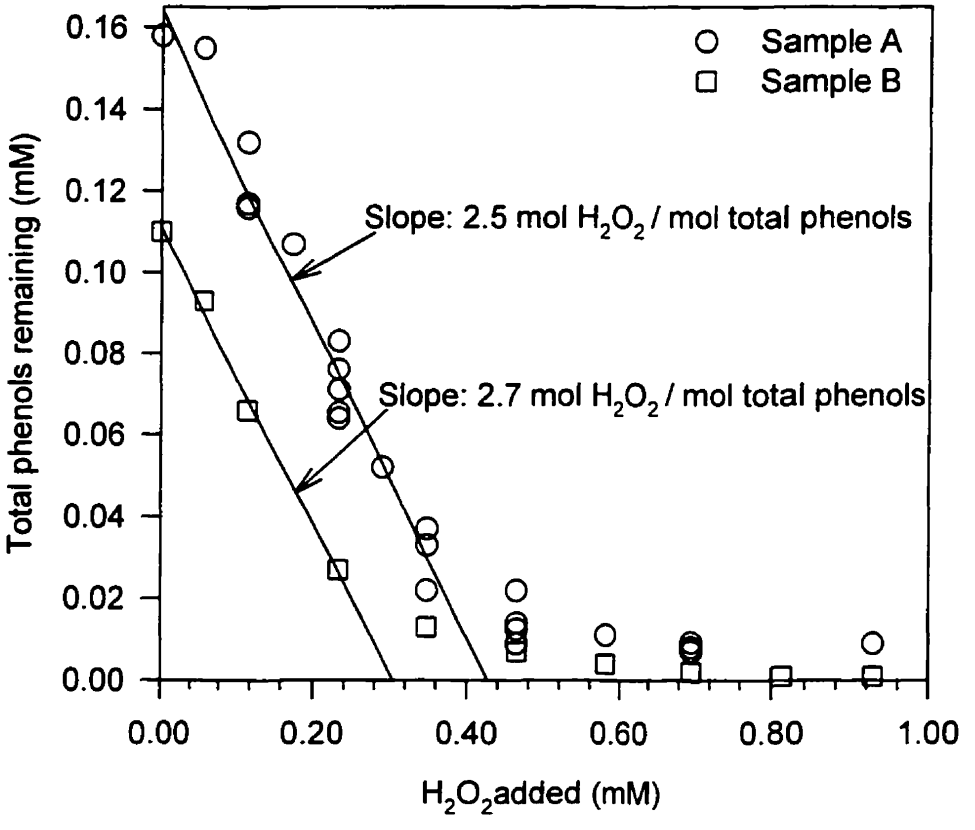


Figure 7.1: Total phenols remaining in the foul condensate as a function of the amount of H_2O_2 added. HRP_0 = 2.0–6.0 U/mL; no pH adjustment.

In the regions where peroxide limited the conversion of phenol, the relationship between total phenols remaining and peroxide added was linear. The slopes of the two curves (± 95% confidence intervals) were measured to be 2.5 ± 0.3 (r^2 = 0.967) and 2.7 ± 0.5 (r^2 = 0.996) mM H_2O_2 per mM of total phenols for wastewater sample A and B, respectively. Approximately 0.70 mM and 0.46 mM H_2O_2 were needed to achieve a reduction of the total phenols concentration to levels less than 1.0 mg/L (equivalent to 0.011 mM) as phenol for samples A and B, respectively.

The amount of enzyme required to achieve a desired level of phenol removal was determined by performing batch reactions in which the enzyme dose ranged from 0 to 3.2 U/mL. Sufficient H_2O_2 (0.70 mM) was supplied in order to ensure that the conversion of phenols was only limited by the availability of the enzyme catalyst. The residual concentration of total phenols as a function of enzyme added is presented in Figure 7.2.

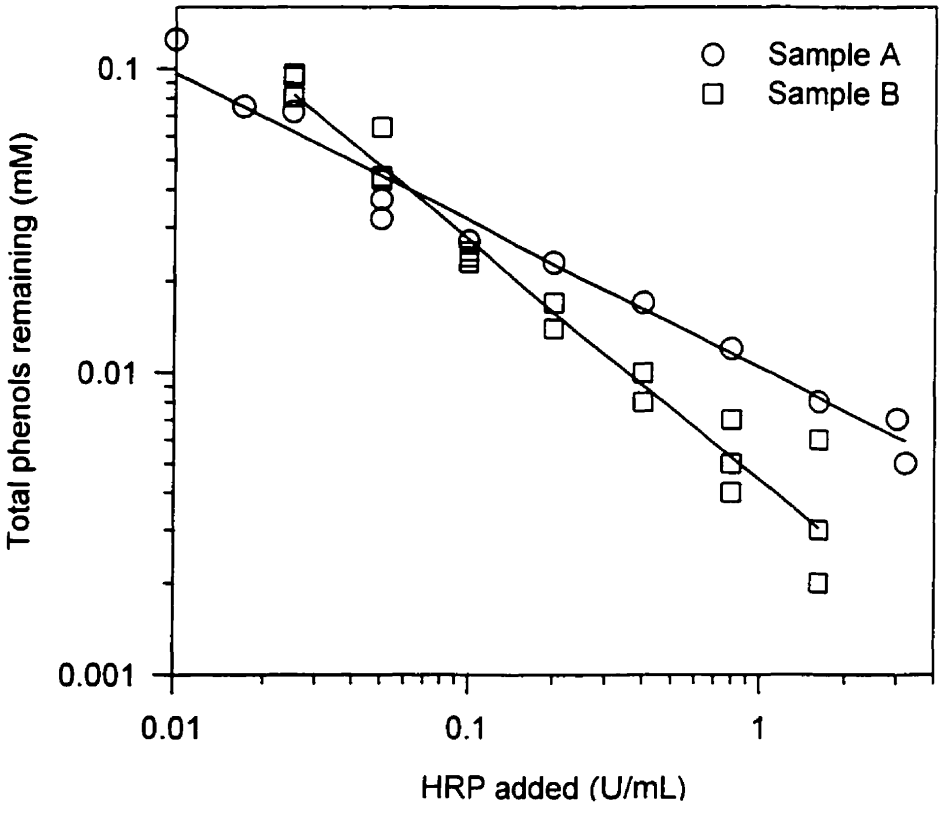


Figure 7.2: Total phenols remaining in the foul condensate as a function of the amount of HRP added. $[H_2O_2]_0$ = 0.70 mM; no pH adjustment.

Linear relationships were found to exist between the logarithms of the residual total phenols concentration of the enzyme dose. A least-squares regression yielded regression coefficients (r^2) of 0.973 and 0.963 for the two wastewater samples.

Minimum HRP doses of 1.0 U/mL and 0.3 U/mL were required to reduce total phenol concentrations below 1 mg/L for wastewater samples A and B, respectively. A further reduction of the total phenols concentration to 0.5 mg/L required 4.0 and 0.8 U/mL for samples A and B, respectively. Thus, a slight improvement in the removal of total phenols comes at the cost of disproportionately higher enzyme requirements.

7.1.2 Impact of pH on phenol removal

In order to determine the impact of reaction pH on enzymatic phenol removal in the condensate, three sets of reactions with 0.017, 0.05 and 0.10 U/mL were conducted in which the pH was varied from 5.0 to 9.2. Sample pHs were adjusted using 0.10 M HCl. The results of these experiments are shown in Figure 7.3.

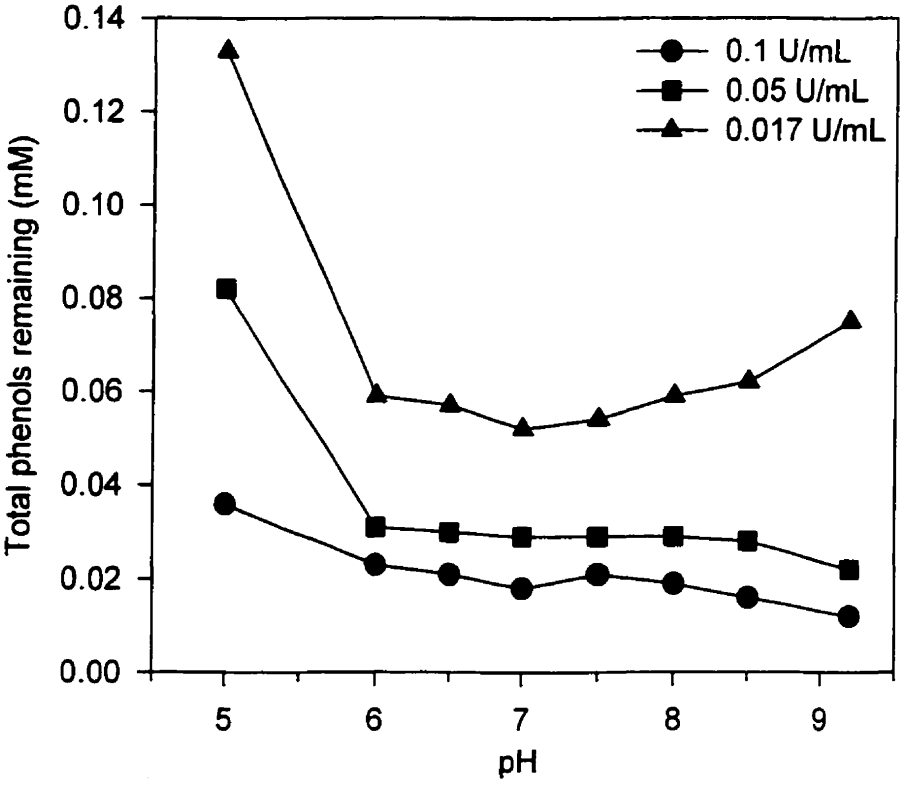


Figure 7.3: Total phenols remaining in the foul condensate as a function of the reaction pH and the amount of HRP added. $[H_2O_2]_0$ = 0.70 mM; waste sample A.

Significant phenol removal was obtained at all pH levels tested when sufficient HRP was provided. However, for limiting enzyme doses, the removal efficiency decreased significantly at pHs below 6.0. The optimum pH was 7.0 for the lowest HRP dose of 0.017 U/mL. For pHs above 6.0 with high doses of enzyme, treatment efficiency was only moderately influenced by reaction pH. Here, the lowest residual phenol concentration was obtained at the original pH of the wastewater.

7.1.3 COD reduction

Alum coagulation resulted in 23 % and 12 % reduction of the initial CODs of samples A and B, respectively. HRP-treatment had only a marginal effect on the COD concentration of both wastewater samples. Negligible COD removal was observed following HRP-treatment of sample A even when sufficient enzyme (3.2 U/mL) and peroxide (0.70 mM) were added to reduce the total phenols concentration below 1.0 mg/L. The treatment of sample B with 1.6 U/mL HRP and 0.70 mM peroxide resulted in a maximum of 5% (60 mg/L) additional COD removal beyond that removed by coagulation.

7.1.4 Toxicity reduction

The impact of peroxidase treatment on the acute toxicity of the condensate was assessed using the Microtox™ assay. Sample B was treated with 0.70 mM peroxide and 1.6 U/mL HRP for three hours and then assessed for toxicity before and after the colloidal reaction products were removed by coagulation. Also, the toxicities of the untreated wastewater and a sample that had been treated by coagulation alone were measured. The results of these experiments are presented in Table 7.2.

Table 7.2: Acute toxicity remaining of foul condensate before and after HRP-treatment and coagulation. HRP_0 = 1.6 U/mL; $[H_2O_2]_0$ = 0.70 mM.

Treatment options	Original wastewater (sample B)	Alum coagulation	HRP-treatment	HRP-treatment followed by alum coagulation
$TU_{50} \pm$ std[a]	5.5 ± 0.4	5.9 ± 0.4	3.0 ± 0.2	3.5 ± 0.3

[a] Average of three independent experiments

HRP-treatment led to a 46 % toxicity reduction with respect to the untreated wastewater. Alum coagulation of the untreated wastewater and the HRP-treated wastewater resulted in slight toxicity increases. A control experiment, in which a sample of distilled water was subjected to the coagulation process, indicated that the elevated toxicity of the coagulated samples was likely due to residual coagulant.

7.1.5 Effect of wastewater components on enzymatic treatment

Experiments were conducted in order to evaluate the effect of the wastewater matrix upon the phenol removal reaction. Comparisons were made between the degree of substrate conversion during batch treatments conducted using synthetic and real wastewaters. Since the identities and quantities of individual phenols in the condensate were unknown, it was not possible to create a synthetic wastewater of an identical composition. Therefore, in order to minimize the influence of the endogenous phenolic compounds, the total phenol concentration in the wastewater sample was spiked to approximately 2 mM by the addition of pure phenol. This corresponds to approximately a 20-fold increase in the total phenols concentration. Thus, attention was focused on the treatment of pure phenol in a real wastewater matrix.

(a) Phenol removal from spiked condensate and synthetic wastewater

A synthetic wastewater sample was prepared by dissolving 2.0 mM phenol in 0.1 M sodium phosphate buffer. The buffer was adjusted to pH 8.0 since this corresponds to the optimal pH for the treatment of pure phenol (Nicell *et al.*, 1992). Thus, for the purpose of comparison in these experiments, the pH of the spiked wastewater was also adjusted to pH 8.0.

Batch reactions were carried out in parallel to treat the synthetic wastewater prepared in 0.1 M phosphate buffer and the spiked condensate. The enzyme activity provided ranged from 0 to 4.0 U/mL. In initial experiments, a hydrogen peroxide dose of 2.3 mM was supplied to more than satisfy the expected one-to-one stoichiometric requirements for the oxidation of 2.0 mM phenol (Nicell *et al.* 1992). In subsequent experiments, the peroxide concentration was increased to 3.5 mM. Also, 0.1 M phosphate (as Na_2HPO_4)

was added to a series of spiked condensate samples to examine the impact of phosphate buffer salts on the treatment. The results are illustrated in Figure 7.4.

Immediately following the addition of enzyme, the spiked wastewater turned dark black but no precipitate formed. However, during coagulation with alum, the dark material precipitated as a black solid leaving a colorless solution. A precipitate spontaneously formed in the phosphate buffer, thereby eliminating the need for coagulation of these samples.

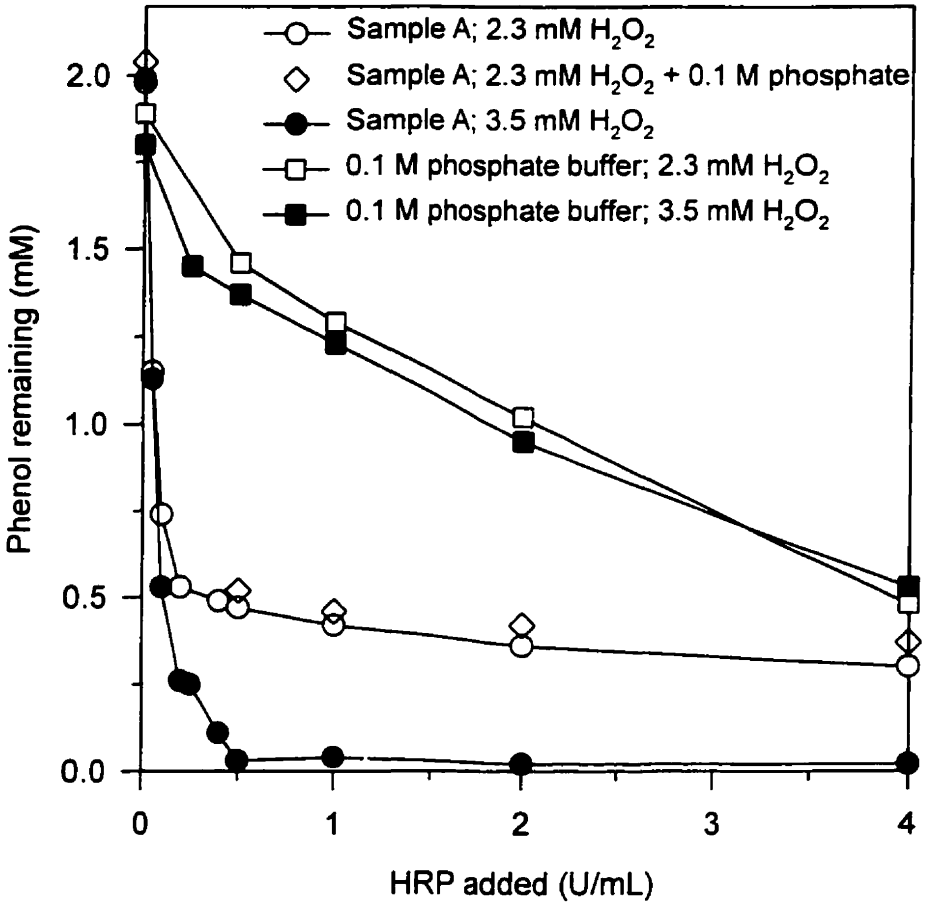


Figure 7.4: Phenol remaining in foul condensate and 0.1 M phosphate buffer as a function of the amount of HRP added and two H_2O_2 concentrations (condensate was spiked with 2.0 mM phenol; pH 8.0).

The degree of treatment obtained with 2.3 mM peroxide was considerably different for synthetic wastewater and spiked condensate. Substantial phenol removal from the condensate was achieved with a low dose of enzyme but treatment leveled off at a

residual of 0.3 mM total phenols. In contrast, the treatment of synthetic wastewaters required substantially more peroxidase to achieve the same level of phenol removal; however, full conversion of phenol in this phosphate buffer solution could be achieved when sufficient enzyme was provided.

The addition of phosphate buffer salts to the spiked condensate resulted in the formation of precipitate during the reaction; however, as shown in Figure 7.4, it did not affect the conversion of phenol as a function of enzyme dose. Therefore, these salts were not responsible for the differing enzyme requirements of the synthetic waste and condensate.

2.3 mM peroxide was sufficient to meet the stoichiometric demands for the treatment of the synthetic wastewater. Since hydrogen peroxide was quite stable in the condensate, the significant quantity of residual phenols in the condensate treated with 2.3 mM peroxide reflects the higher peroxide demands for the oxidation of endogenous phenols (see slopes in Figure 7.1). Thus, a significant amount of the endogenous phenols was converted at the same time as phenol. In order to slightly exceed the peroxide requirements of the endogenous phenols and the pure phenol, a peroxide dose of 3.5 mM was selected for further experiments.

Almost 99% phenol removal was observed in the spiked wastewater at an HRP dose of 0.5 U/mL and 3.5 mM H_2O_2. In contrast, more than 12 times this amount of enzyme (> 6 U/mL, data not shown) was required to remove the same amount of phenol from the phosphate buffer solution. Since phosphate buffer salts did not exert a detrimental effect on the treatment of the spiked condensate, these results suggest that components of the condensate matrix enhanced the oxidation of phenol by peroxide and HRP.

(b) Effect of coagulation

Preliminary experiments revealed that the quantity of enzyme required to transform the phenols in the spiked condensate was increased if the colloidal wastewater components were first removed by coagulation. Filtration alone had no significant effect on treatment efficiency. Therefore, it was suspected that the colloidal particles might contribute to the enhanced treatment of phenols in the spiked condensate.

In order to characterize the effect of colloidal particles, an experiment was conducted in which un-coagulated and coagulated spiked condensates were treated. Wastewater sample B was coagulated by the addition of alum and the resulting precipitate was removed by filtration through a 0.45 μm membrane filter. The pH of the filtered solution was adjusted back to the pH of the original sample with 0.1 M sodium hydroxide and the phenol concentration was increased to 2 mM. Similarly, the concentration of phenol in a sample of wastewater B was also raised to 2 mM. All treatments were conducted with a slight excess of peroxide (3.5 mM). However, a limiting quantity of HRP (0.3 U/mL based on the 4-AAP assay) was used in order to study any difference in phenol conversion that could be achieved by a given dose of enzyme due to the presence of colloidal materials. The reaction was initiated by the addition of an aliquot of concentrated H_2O_2 solution. The phenol concentration and enzyme activity were monitored for a period of 64 minutes. The results of this experiment are presented in Figure 7.5.

Figures 7.5(a) and 7.5(b) demonstrate that a higher degree of phenol conversion and lower rates of enzyme inactivation occurred in the un-coagulated sample. After 64 minutes, the same dose of enzyme achieved 66% and 90% removal of phenol from coagulated and un-coagulated samples, respectively. Also, 4 minutes after the start of the reaction, 50% of the enzyme was inactivated in the coagulated sample, whereas it took 12 minutes to reach 50% inactivation when the un-coagulated sample was treated.

The log-log plot of Figure 7.5(b) reveals that the phenol removal reaction proceeded in two distinct phases. In the first few minutes of the reaction, enzyme inactivation was slow and the phenol removal rate was constant and identical in both wastewater samples (with a slope of 0.23 mM/min from the curves of Figure 7.5(a)). Rapid enzyme inactivation and a substantial decrease in the phenol removal rate characterized the second phase of the reaction. Notably, the two samples differed in the length of the first phase of the reaction. Whereas the first phase lasted only 2 minutes in the coagulated sample, it took 4 minutes before the onset of significant (>10%) enzyme inactivation when the reaction was conducted in the un-coagulated wastewater matrix.

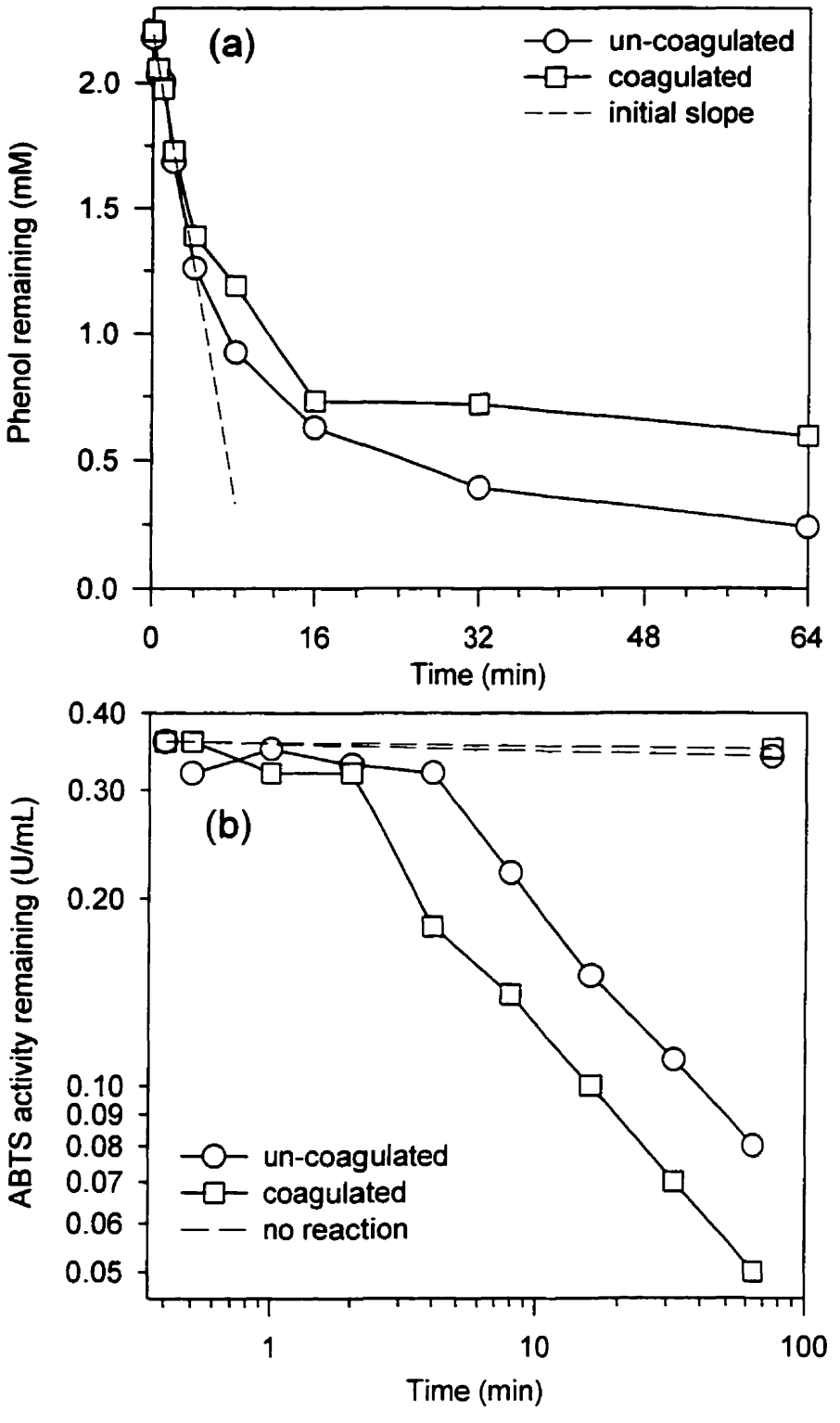


Figure 7.5: (a) Phenol and (b) activity remaining as function of reaction time during the treatment of un-coagulated and coagulated foul condensates (condensate was spiked with 2.0 mM phenol; HRP_0 = 0.3 U/mL; $[H_2O_2]_0$ = 3.5 mM; pH = 9.0; waste sample B).

Since the length of the first phase was doubled in the original wastewater sample, about twice as much phenol was removed (0.92 mM, 42%) as in the coagulated wastewater (0.48 mM, 22%) before the reaction entered into the second phase. During this phase, the amount of enzyme inactivated per phenol removed was approximately the same (~ 0.2 U/mL per mM phenol) for both wastewater samples.

When no peroxide was used to initiate the enzymatic reaction (dotted line in Figure 7.5 (b)), virtually no enzyme inactivation took place in either the un-coagulated or coagulated samples within the same time period as the treatment experiments. This indicates that the enzyme inactivation during phenol treatment (solid lines in Figure 7.5(b)) was a result of the catalytic action of HRP.

Experiments were also conducted in order to test whether residual dissolved aluminum species could enhance enzyme inactivation. Two 2.0 mM solutions of phenol were prepared in distilled water and one was pretreated with alum. Subsequently, the samples were treated with identical doses of enzyme and peroxide. Results demonstrated that there was no adverse effect of aluminum species on treatment efficiency. Thus, the use of alum did not cause the reduced treatment efficiency and increased rate of enzyme inactivation observed in the coagulated samples discussed above.

(c) Effect of lignin on treatment efficiency

Black liquor contains 30 to 40% lignin as a percentage of dry solids (Venkatesh and Nguyen, 1985). Since the foul condensate was an effluent stream arising from scrubbing of gases produced during the evaporation and burning of black liquor, it was suspected that the compounds that were removed by coagulation could be colloidal lignin particles. Therefore, the lignin content was measured before and after wastewater constituents were removed by coagulation using the folin assay. On average, 14 ± 3 % (standard error of the mean with seven samples) of the original lignic compounds could be precipitated by alum coagulation from the foul condensate. It should be noted that phenolic compounds also report as lignin in the lignin assay. However no phenols, as determined by the 4-AAP assay, were removed during this procedure. This suggests that it was lignic compounds that were removed by coagulation. Moreover, scans in the UV absorbance region of lignic

compounds revealed an average 33% decrease in the peak absorbance (273 nm) after coagulation.

To test the hypothesis that lignin could be responsible for the observed protection of the enzyme from inactivation, batch treatments of spiked condensate were conducted with and without the addition of lignin. Commercially available alkali (Kraft) lignin was selected for this purpose because it was produced through the same process as the lignin present in black liquor. 2.2 g/L of alkali lignin were added to un-coagulated and coagulated wastewater samples and stirred overnight. Subsequently, particulate lignin was removed by filtration and the phenol content of these samples was increased to 2 mM. Since the pH of the condensate had dropped to 7.4 by this time, the pHs of all samples were also adjusted to this value. The results are presented in Figure 7.6.

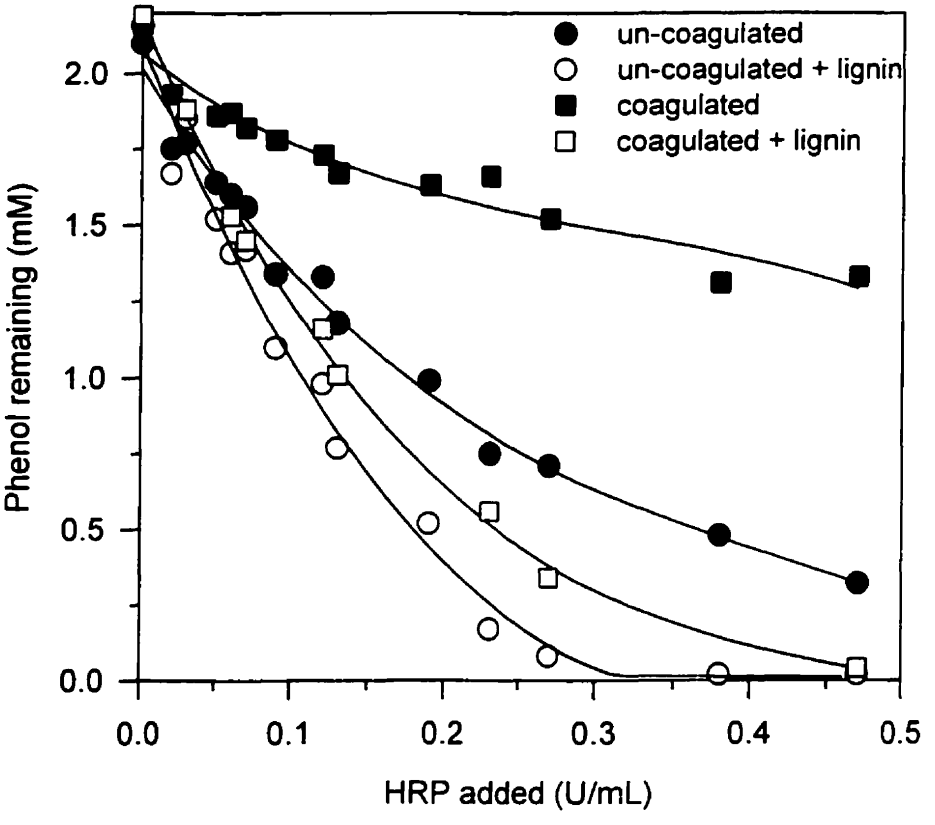


Figure 7.6: Phenol remaining in un-coagulated and coagulated foul condensates as a function of HRP added (condensate was spiked with 2.0 mM phenol; $[H_2O_2]_0 = 3.5$ mM; pH 7.4; waste sample B)

A comparison of un-coagulated and coagulated samples without lignin addition reveals that the removal of colloidal compounds resulted in a dramatic drop of the treatment efficiency (top curve in Figure 7.6). The addition of lignin to the coagulated wastewater sample improved treatment efficiencies beyond the levels found for the treatment of the un-coagulated wastewater (Figure 7.6). Also, further improvements in treatment were achieved when lignin was added to the un-coagulated wastewater.

Aqueous samples of 2.0 mM phenol were prepared in distilled deionized water with and without the addition of lignin. A batch containing 2.2 g/L alkali lignin was subdivided into three samples: one was filtered, another was coagulated and then filtered, and a third remained unfiltered and un-coagulated. It should be noted that phosphate buffer was not used in these experiments because phosphate ions interfere with alum coagulation; however, reductions in enzyme requirements were also obtained when lignin was added to phenol solutions prepared in phosphate buffer (data not shown).

As shown in Figure 7.7, the addition of lignin to the aqueous phenol samples yielded similar results as obtained for the treatment of the foul condensate. Samples treated in the absence of lignin (top curve) were much more resistant to enzymatic treatment than samples treated in the presence of lignin (bottom three curves).

In the presence of un-coagulated lignin (bottom two curves of Figure 7.7), only 0.4 U/mL were needed to achieve more than 97% phenol removal. This is very close to the values obtained for the un-coagulated and the coagulated wastewater samples to which lignin had been added (0.38 and 0.47 U/mL, respectively, from bottom two curves of Figure 7.6).

These results also show that the presence of the filterable fraction of lignin did not contribute to enhanced treatment. Notably, phenol removal efficiencies were lower in samples from which lignin had been coagulated prior to the reaction. In these experiments, coagulation resulted in a 38% lignin removal based on the folin assay. However, the removal efficiencies observed in these solutions were significantly higher than in solutions without lignin. Thus, a portion, but not all, of the lignin that enhances treatment can be removed by coagulation.

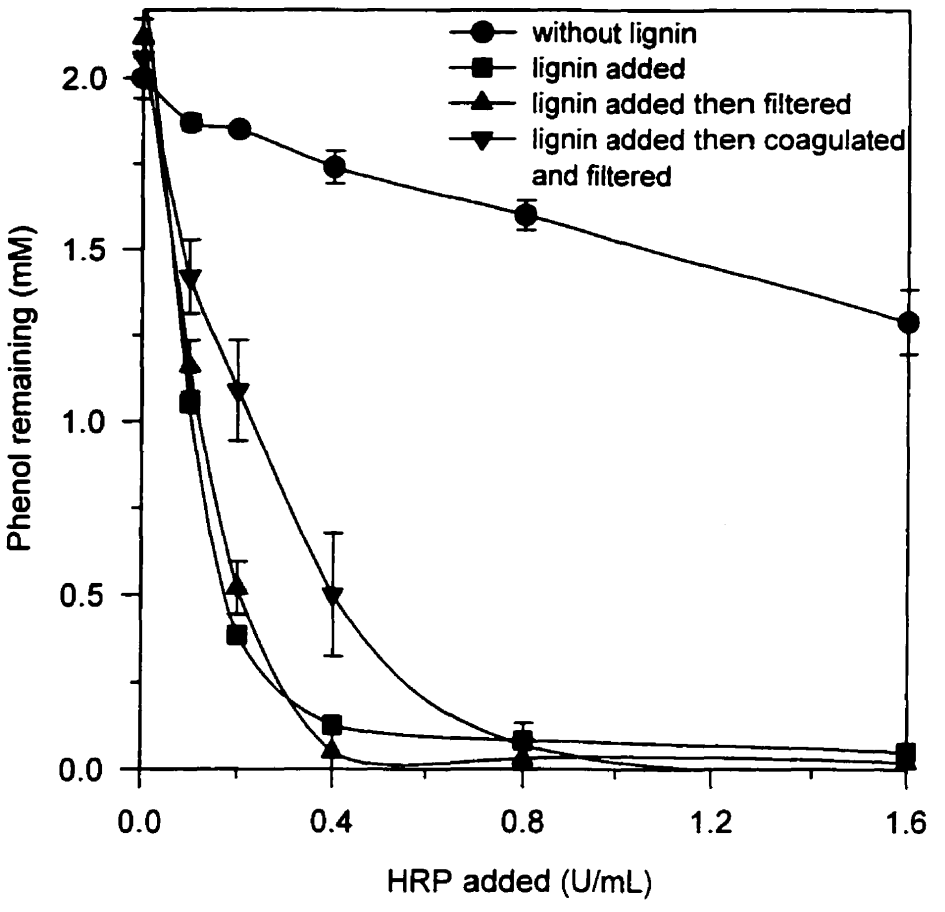


Figure 7.7: Phenol remaining in aqueous phenol solutions as a function of HRP added. ($[H_2O_2]_0$ = 2.3 mM; pH 7.4). Error bars represent the standard deviation of 3 independent experiments.

Note that no reduction in phenol concentration was observed in samples containing peroxide and lignin alone without the addition of enzyme. Therefore, enhanced phenol removal noted in the presence of lignin was not due to an association between lignin and phenol.

The UV absorbance peak of the un-coagulated condensate at 273 nm closely matched the peak at 277 nm of aqueous lignin. In addition, the coagulation of these solutions caused similar decreases in the peak absorbance. Thus, in general, for a given dose of enzyme, consistently better treatment was obtained for synthetic and real wastes when the absorbance in the range of 273 to 277 nm was higher.

Experiments were performed with real and synthetic wastewaters in order to test whether lignin acts as a co-substrate in the reaction. The presence of a co-substrate should

increase the amount of H_2O_2 consumed per quantity of phenol removed. However, as shown in Figure 7.8, when peroxide was added in limited quantities to reaction mixtures, the degree of phenol conversion was unchanged for the coagulated and un-coagulated condensate as well as for the treatment of aqueous phenol with and without the presence of lignin. Therefore, the presence of lignin in the samples had no effect on reaction stoichiometry and, thus, lignin did not act as a co-substrate.

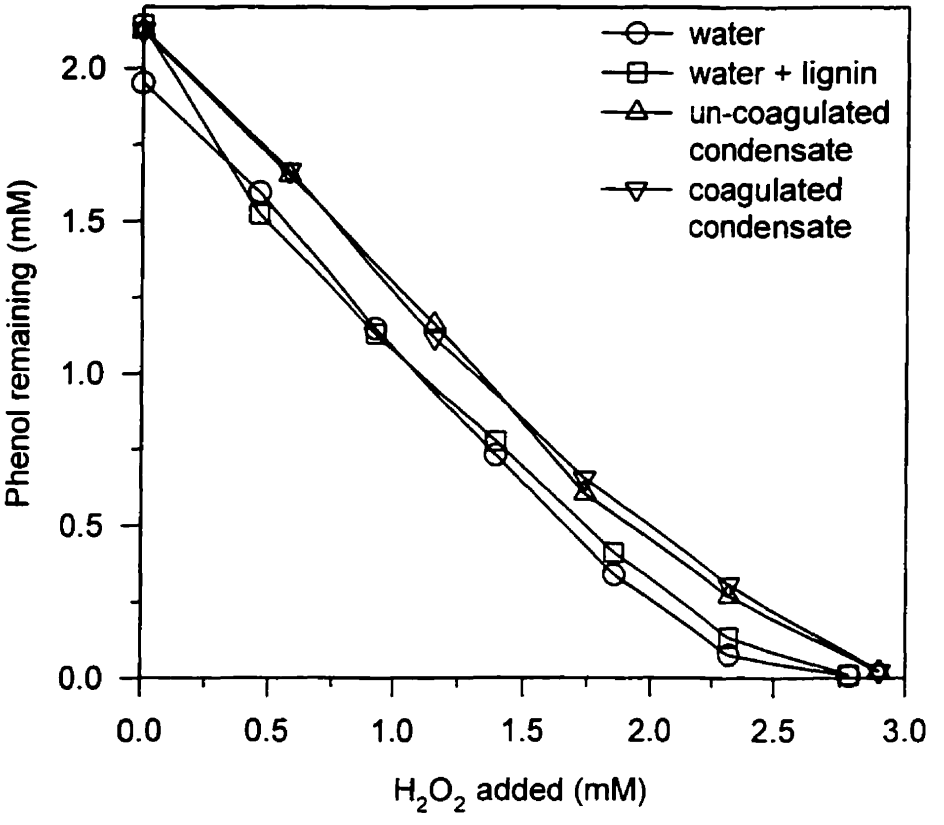


Figure 7.8: Phenol remaining in aqueous phenol solutions treated with and without the presence of lignin and in spiked un-coagulated and coagulated condensates as a function of hydrogen peroxide added. HRP_0 = 9 U/mL; pH 7.4, aqueous phenol solutions; pH 9.0, spiked condensates.

(d) Effect of lignin on the toxicity of HRP-treated phenolic solutions

In order to test whether the presence of lignin would have an impact on the residual toxicity of phenolic solutions after HRP-treatment, 2.2 g/L of lignin were added to distilled-deionized and stirred over night. After the particulate lignin was removed by

filtration phenol was added to these solutions at 1.0 mM. A 1.0-mM phenol solution in distilled-deionized water served as a control. The pH of the lignin containing samples and the controls was adjusted to approximately pH 7.2 using a NaOH solution. Subsequently, these samples were treated with HRP and H_2O_2 for three hours and their toxicities were recorded. The results of three independent experiments are summarized in Table 7.3. Note that the lignin solution by itself, without the addition of phenol, exhibited a toxicity of 2.0 TU_{50} units.

Table 7.3: Residual toxicities after HRP-treatment of a 1.0 mM phenol solution in the presence and absence of lignin.

Treatment in the presence of lignin			**Treatment in the absence of lignin**		
HRP dose (U/mL)	Phenol removed (%)	$TU_{50} \pm$ std[a]	HRP dose (U/mL)	Phenol removed (%)	$TU_{50} \pm$ std[a]
0.4	98.4	5.9 ± 0.1	4.5	99.5	34.0 ± 1.3
0.4	93.6	9.5 ± 0.2	4.5	99.5	14.5 ± 0.0
1.0	99.1	7.5 ± 0.1	4.5	99.5	15.3 ± 2.6

[a]All experiments were carried out in duplicate.

The results presented in Table 7.3 show clearly that the presence of lignin significantly reduced the toxicity resulting from the HRP-treatment of phenol.

7.2 Discussion

7.2.1 Treatment of the foul condensate with HRP and hydrogen peroxide

The stoichiometry between the phenol removed and the H_2O_2 consumed deviates substantially from the one-to-one stoichiometry found for the treatment of many phenolic compounds (Nicell *et al.*, 1992). This difference might be to due to an underestimation of the molar concentration of phenolic compounds in the total phenol assay since some phenolic compounds (e.g., 4-methylphenol) do not generate color in the total phenol assay. Thus, such compounds would consume peroxide during treatment but would not be included in the total phenol assay results. Therefore, the actual total phenols

concentration could be up to 2.4 times higher than the value obtained using the 4-AAP assay.

The stoichiometry between peroxide and total phenols was consistent between condensate samples A and B (see Figure 7.1), suggesting that the relative amounts of phenolic substrates in these samples were the same. However, the quantities of enzyme required to treat the two wastes were substantially different (see Figure 7.2), even when their different initial total phenols concentrations were taken into account. This may be due to differences in the composition of other wastewater components, which could enhance or impede the enzymatic phenol removal reaction. UV scans also suggested that the organic content of these waste samples was quite different.

The enzymatic treatment of scrubber condensate can be carried out over the entire pH range tested (5.0 to 9.2). Slightly better treatment with higher enzyme doses was obtained at alkaline pHs that are typical for Kraft process effluents (Figure 7.3). However, the optimum pH for the low enzyme concentration was pH 7.0. This apparent discrepancy might reflect a decreased stability of the enzyme at elevated pH values coupled with a slightly enhanced reactivity towards certain phenolics in this pH range. That is, applying low enzyme concentrations decreases the reaction rate; hence environmental factors such as pH and temperature have more time to destabilize the enzyme and to significantly affect the total substrate conversion that can be achieved. At higher enzyme concentrations, and thus higher reaction rates, the enzyme will be inactivated primarily by reaction products. Consequently, other destabilizing factors will have a lower impact on the overall substrate conversion.

The low COD reduction (60 mg/L or 5%) reflects the fact that phenols constitute only a very small fraction of the oxidizable compounds in foul condensates. During the COD experiment, the quantity of total phenols removed was 0.108 mM, based on phenol. The theoretical oxygen demand of this quantity of phenol is 24 mg/L. If it were assumed that the colorimetric assay for phenols under-reports the quantity of total phenols by a factor of 2.5, the theoretical oxygen demand would be increased to 60 mg/L. Even if the calculations are based on phenolic compounds that have higher oxygen demands, such as guaiacol (methoxyphenol) or methylphenol, this value only increases by approximately

20%. Thus, the COD reduction achieved (60 mg/L) was within the range that could be expected from theoretical considerations.

In contrast, HRP-treatment resulted in a substantial toxicity reduction (46 %), regardless of whether colloidal reaction products were removed by coagulation or remained in the sample (Table 7.2). The COD and toxicity results indicate that while the phenols represent only a small fraction of the total organic content of the waste, they constitute a large fraction of the total toxicity. Thus, peroxidase-based treatment selectively targets a significant portion of the toxic components of the wastewater transforming them into non-toxic products. These products are presumably higher molecular weight polymers with low solubilities. In previous studies, it has been shown that the treatment of pure aqueous solutions of certain phenols can result in solutions that are more toxic than the untreated sample (Ghioureliotis and Nicell, 2000). However, when these phenols were treated in the presence of other phenols, the toxicities of treated solutions were reduced (Ghioureliotis and Nicell, 2000) and the formation of specific toxic species was suppressed (Morimoto and Tatsumi, 1997). These results suggest that the distribution of reaction products from enzymatic treatment depends on the types and relative quantities of phenolic substrates in the waste mixture. In addition, in experiments involving the treatment of aqueous phenol in the presence and absence of lignin, solutions treated with lignin were substantially less toxic (Table 7.3). Thus, the presence of non-substrates may also have an effect on the distribution of reaction products. However, the impact of other wastewater components on residual toxicity remains unknown. Therefore, it is necessary to conduct studies with actual wastewaters in order to assess the efficacy of the peroxidase treatment for toxicity reduction. In addition, toxicity testing with other species such as *Daphnia magna* or fathead minnow is recommended.

7.2.2 Effect of the wastewater matrix on treatment efficiency and enzyme inactivation

In experiments involving spiked wastewaters, higher treatment efficiencies were obtained when phenol treatment was conducted in the wastewater matrix as compared to phosphate buffer or pure water (Figures 7.4 to 7.6). This suggests that wastewater components are responsible for enhanced treatment efficiencies. Reduced treatment efficiencies observed

for samples from which certain components were first partially removed by coagulation indicate that those components were the ones responsible for the observed effect.

There are a number of ways in which these wastewater components can improve treatment efficiency. They could provide a reactive co-substrate thereby facilitating the removal of the less reactive phenol (Roper *et al.*, 1995). They could also affect the enzyme by reducing inactivation due to environmental factors, by enhancing its catalytic activity (e.g., surface active agents [Flock *et al.*, 1999]), or by protecting the enzyme from inactivation by reaction products (e.g., hydrophilic polymers, [Wu *et al.*, 1998]). From the results obtained in this study, three of these explanations can readily be excluded. Firstly, since a co-substrate would be directly oxidized through the catalytic action of the enzyme, its involvement in the reaction would result in a higher H_2O_2 consumption for the same degree of substrate conversion. The identical H_2O_2-phenol stoichiometry of the coagulated and the un-coagulated samples (see Figure 7.8) makes co-polymerization an unlikely explanation. Secondly, in the absence of peroxide, the stability of the enzyme was constant in coagulated and un-coagulated wastewater samples (see Figure 7.5(b)); thus, the wastewater components did not have the opportunity to protect the enzyme from environmental factors. Thirdly, an enhancement of enzymatic activity would lead to higher rates of substrate conversion for the same initial enzyme dose. However, phenol removal rates in both the un-coagulated and the coagulated wastewater were found to be identical during the first phase of the reaction (see Figure 7.5(a)) before significant enzyme inactivation took place (see Figure 7.5(b)). The latter experiment supports the fourth explanation for enhanced treatment in the waste matrix. That is, the duration of the initial phase of high activity was twice as long during the treatment of the un-coagulated waste (see Figure 7.5(b)). The delayed onset of significant enzyme inactivation in the un-coagulated sample clearly indicates that wastewater components protect the enzyme from inactivation by reaction products. Wu *et al.* (1998) and Kinsley and Nicell (2000) suggest that protective hydrophilic polymers are consumed over the course of the phenol removal reaction due to interactions with reaction products. Based on this theory, enzyme inactivation would be suppressed by wastewater components during the initial phase of the reaction. As the reaction proceeds, more inactivating products are formed which interact with protective species and deplete their protective effect. Once these species are

consumed, the enzyme would be exposed to reaction products and then be rapidly inactivated. The longer duration of the initial phase in the sample containing all wastewater components would then reflect a higher concentration of protective species.

The protective species are likely to be dissolved and colloidal lignin derivatives. This hypothesis is supported by the data of Figures 7.6 and 7.7. First, coagulation resulted in a lower content of lignic compounds (as indicated by the folin assay and by the reduction in UV absorbance at 273 nm) and at the same time resulted in an increase in enzyme requirements. Second, higher treatment efficiencies in real and synthetic wastewaters were obtained in the presence of colloidal or dissolved lignin derivatives.

Lignin is a branched polymer that is formed by enzyme-initiated dehydrogenative polymerization of a mixture of phenolic monomers; e.g., coniferyl and sinapyl alcohol in hardwood (Kringstad and Lindström, 1984). Alkali or Kraft lignins are heterogeneous mixtures of compounds that differ in their hydrophobicities as well as in their molecular weights (Forss *et al.*, 1989). The water-soluble and colloidal fractions of alkali lignin will consist mainly of low molecular mass compounds as well as hydrophilic high molecular mass species that might be bound to carbohydrates (Forss *et al.*, 1989). It is conceivable that, due to their aromatic character, some of these compounds might be able to interact with phenol radicals and other products that are formed during the enzymatic reaction. Further studies are required to elucidate the nature of the interactions between phenolic reaction products, peroxidase and lignin derivatives.

8. TREATMENT OF A PETROLEUM REFINERY WASTEWATER

Phenols in petroleum refinery wastewaters originate mainly from crude oil fractionation and thermal or catalytic cracking (Dyer and Mignone, 1983). In addition to phenolic compounds, this type of effluent usually contains a variety of other organic and inorganic pollutants such as polyaromatic hydrocarbons (PAHs), benzene and its derivatives, cyanides and sulfides (Dyer and Mignone, 1983). Generally, biological treatment is used to treat the majority of these contaminants. However, biological degradation usually requires a long retention time and is easily upset by temperature changes and the periodic occurrence of toxic contaminants (Lanouette, 1977). The treatment of phenols through enzyme-catalyzed oxidation may be a feasible alternative to traditional biological treatment processes.

The objectives of this study were (1) to investigate the treatment of a petroleum refinery wastewater with HRP and H_2O_2; (2) to assess the effectiveness of additives in a real waste matrix; (3) to study the effect of the enzymatic treatment on wastewater quality parameters including toxicity, chemical (COD) and biochemical (BOD) oxygen demands; and (4) to assess the impact of selected organics on the treatment efficiency.

Most of the results presented in this chapter have been published in an article by Wagner and Nicell (2001b).

8.1 Results

Table 8.1 summarizes the wastewater characteristics as determined in the lab or according the information provided by the refinery.

Table 8.1: Characteristics of the petroleum refinery wastewater.

Sample	pH	Total phenols	COD	BOD_5	Oil and Grease[a]	SS	NH_4^+	S^{2-}
		(mM)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
A	8.6	0.41-0.47	282-314	n.d.	<15	17	8.7	4.4
B	8.6	0.35	306	94	<15	n.d.	6.0	2.2

SS: suspended solids; n.d.: not determined; [a] Values provided by the refinery.

8.1.1 Hydrogen peroxide and enzyme requirements

Regulative authorities have imposed a monthly average discharge limit of 14 kg total phenols per day on this particular refinery. With an average daily flow of 11×10^6 L, this translates to an allowable concentration of 1.27 mg/L of total phenols (corresponding to 0.013 mM as phenol) in the effluent stream. Batch experiments were conducted in order to minimize the amount of HRP and peroxide required to achieve this target level of treatment. It should be noted that in all these experiments, phenol transformation resulted in the formation of colloidal products that required subsequent coagulation with alum to accomplish their precipitation.

In the experiments carried out to determine the minimum amount of H_2O_2, HRP was added in excess (i.e., 7 U/mL) in order to ensure that peroxide would be the only reagent that would limit the quantity of total phenols removed. The results of these experiments are presented in Figure 8.1.

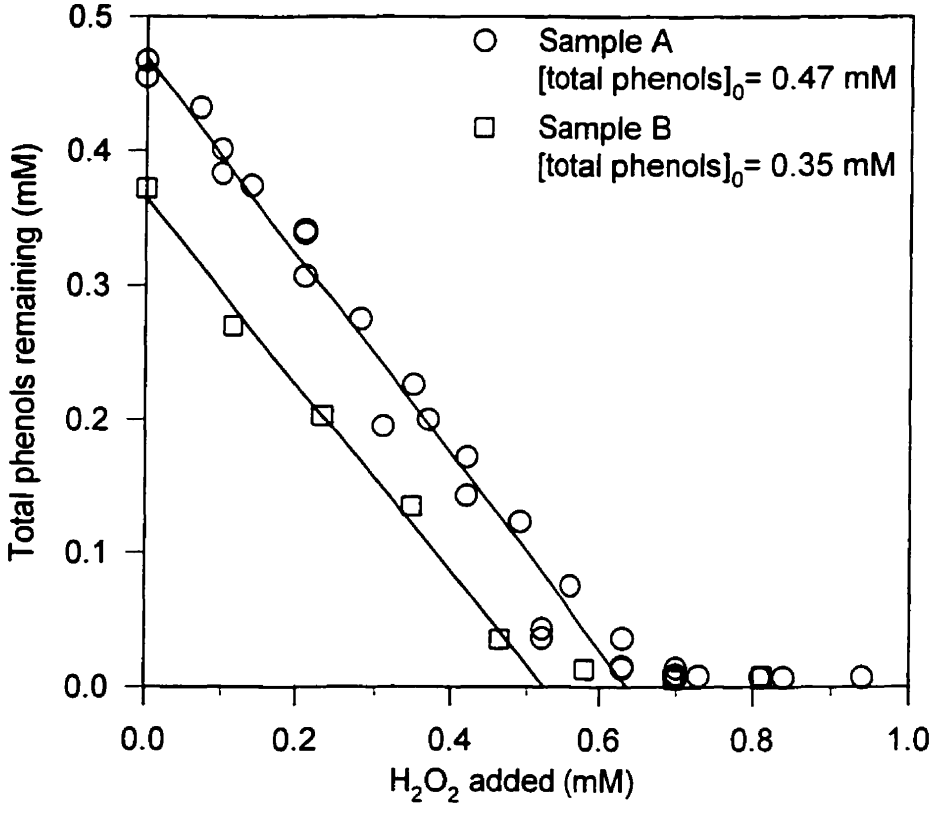


Figure 8.1: Total phenols remaining as a function of the H_2O_2 dose. HRP_0 = 7 U/mL.

Regression analyses from the linear portions of the plots yielded an apparent stoichiometry of 1.3 ± 0.1 and 1.4 ± 0.2 mole H_2O_2 per mole of total phenols for samples A and B, respectively. The minimum amount of H_2O_2 required to achieve the discharge limit was 0.7 mM for both samples.

Batch reactions were conducted to evaluate phenol removal as a function of the quantity of enzyme added. The peroxide supplied was the minimum amount required (i.e., 0.7 mM) as determined from Figure 8.1. As shown in Figure 8.2, a linear relationship was found between the percentage of total phenols remaining after treatment and the logarithm of the ratio of HRP to initial phenol.

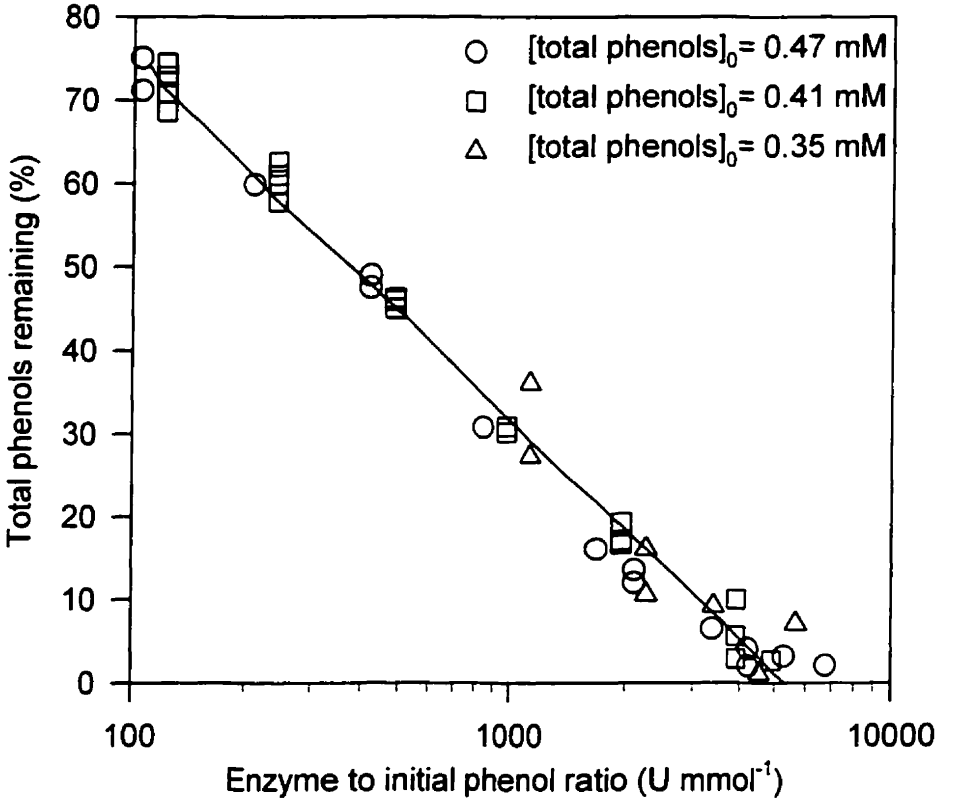


Figure 8.2: Total phenols remaining as a function of the enzyme to initial phenol ratio. $[H_2O_2]_0 = 0.7$ mM.

The minimum enzyme dose required to reduce the total phenols concentration below 0.013 mM (as phenol) was 1.6 to 2.2 U/mL for samples with initial total phenols concentrations ranging from 0.35 to 0.47 mM, respectively. This corresponds to an

approximately 97 % phenol conversion using 4500 HRP units per mmole of initial phenols. In comparison to that, 15 % less HRP (i.e, 3900 $U \cdot mmol^{-1}$ as calculated using equation 4.1) was required to accomplish the same degree of treatment of pure phenol when the reaction was carried out in phosphate buffer at pH 7.0.

It was observed that H_2O_2 spontaneously disappeared (about 70 to 80% within the first 30 minutes) when added to the wastewater even when no HRP was present (data not shown). The H_2O_2 disappearance was at least partially due to decomposition into O_2 and H_2O since a rapid O_2 evolution was observed immediately after H_2O_2 was added, as measured using a dissolved oxygen electrode. For samples that had been filtered to remove suspended solids, the peroxide concentration dropped by about 0.24 mM within the first 30 seconds and then stabilized. The initial drop was likely due to the oxidation of dissolved reduced contaminants such as sulfides. The stabilization of the peroxide concentration in the filtered samples suggests that the suspended solids catalyze H_2O_2 decomposition. An elemental analysis revealed that the sample contained small quantities of suspended iron and zinc minerals, which might be responsible for the observed H_2O_2 decomposition. Based on these observations, H_2O_2 was always the last reagent added during treatment experiments in order to minimize non-enzymatic peroxide consumption.

Since the enzymatic reaction must compete with the spontaneous decomposition reaction for available H_2O_2, it was suspected that phenol removal could possibly be limited by the non-enzymatic disappearance of H_2O_2. For high concentrations of HRP (e.g., the 7 U/mL used in Figure 8.1), the rate of spontaneous peroxide decomposition is slow compared to the enzymatic phenol oxidation. However, spontaneous H_2O_2 decomposition would be expected to have a significant impact on the extent of phenol removal at lower enzyme concentrations when the enzymatic reaction is much slower.

The top curve of Figure 8.3 shows the phenol removal that can be achieved using a range of doses of HRP under conditions of 0.7 mM peroxide, which is just sufficient to satisfy the stoichiometric requirements of the enzymatic reaction (from Figure 8.1). However, the enzyme can be used more effectively if the quantity of peroxide is increased up to an amount that meets the demands of both the enzymatic reaction and the losses through the spontaneous decomposition of peroxide. This is shown in the middle curve of Figure 8.3 where the peroxide dose has been optimized at each point such that

sufficient peroxide has been provided to allow the enzyme (the most expensive component of the treatment) to be used to its fullest extent. These H_2O_2 concentrations ranged from 1.4 mM for an enzyme dose of 1.6 U/mL to 2.4 mM for 0.05 U/mL. In order to achieve a residual total phenols concentration of 0.013 mM (as phenol), 2.0 U/mL of HRP was needed when using 0.7 mM H_2O_2 (top curve). However, as shown by the middle curve, the same level of treatment can be obtained using 20% less enzyme but at the cost of a 100% increase in the peroxide dose (i.e., 1.6 U/mL with 1.4 mM H_2O_2).

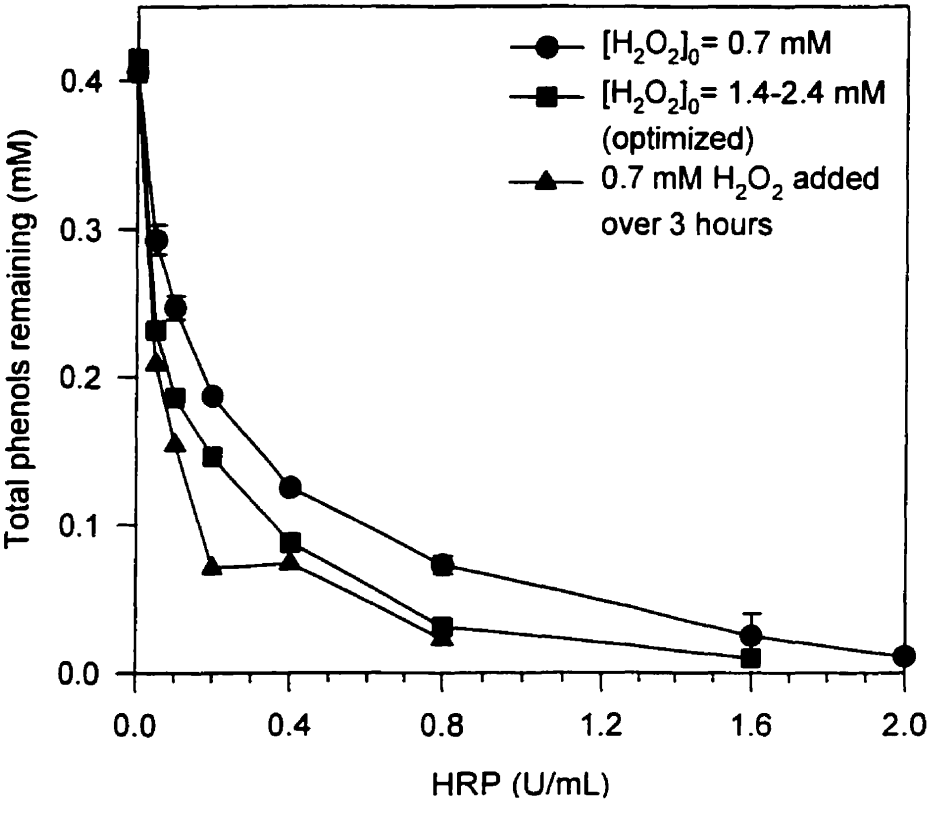


Figure 8.3: Impact of H_2O_2 dose and addition mode on phenol removal. $[phenol]_0$= 0.41 mM (sample A).

According to the kinetic model developed by Buchanan and Nicell (1997), the rate of the enzymatic H_2O_2 utilization ($r_{H_2O_2}$) can be expressed as (assuming a 1:1 stoichiometry between phenol and H_2O_2):

$$r_{H_2O_2} = \frac{-(E_0 - E_{iii} - E_{inact})}{\left[\frac{1}{k_1[H_2O_2]} + \frac{1}{[P]}\left(\frac{k_2 + k_3}{k_2 k_3}\right)\right]} \quad (8.1)$$

with $[H_2O_2]$ = concentration of H_2O_2 (M) at time, t; [P] = phenol concentration (M) at time t; E_0 = total HRP concentration (M); E_{iii} = Compound III concentration (M) at time t; E_{inact} = total concentration of inactivated enzyme species (M) at time t; and k_1, k_2 and k_3 = kinetic parameters (see values in Table 2.1).

An analysis of this expression reveals that this rate is relatively insensitive to changes in the instantaneous H_2O_2 concentration, since $1/k_1$ is approximately 60 times smaller than $(k_2+k_3)/(k_2 \cdot k_3)$. For example, reducing the instantaneous peroxide concentration by 50 % from an amount that is equal to the concentration of phenol will only reduce the rate of enzymatic H_2O_2 consumption by 2 %. On the other hand, kinetic analysis of the rate of the spontaneous H_2O_2 decay in the refinery wastewater showed, that a 50 % reduction in the initial H_2O_2 concentration resulted in an estimated 50 % reduction in the rate of the peroxide decomposition. Thus, the rate of spontaneous decomposition is significantly more dependent on the instantaneous H_2O_2 concentration than the rate of the enzymatic peroxide utilization.

Therefore, a way to ensure that the peroxide would be predominantly used by the enzyme and less of it lost through decomposition would be to keep the instantaneous H_2O_2 concentration low by adding it to the reactor over an extended period of time. The result of this approach is shown in the bottom curve of Figure 8.3. A comparison of the two bottom curves of Figure 8.3 shows that the slow addition of peroxide allowed the enzyme to achieve an even slightly higher phenol removal with just the stoichiometrically required peroxide dose of 0.7 mM. However, the reduced reagent requirements come at the expense of a longer retention time due to the need for the slow addition of peroxide to the batch mixture.

8.1.2 The impact of reaction time and pH on phenol removal

Wastewater sample B was supplied with 2.0 U/mL and 0.9 mM H_2O_2 and the subsequent decrease in the total phenol concentration was recorded over a period of three hours. The results are displayed in Figure 8.4. Approximately 90 % of the phenols were removed

within the first 15 minutes of the reaction, while nearly 60 minutes were required to reduce the total phenols concentration below the discharge limit of 0.013 mM as phenol.

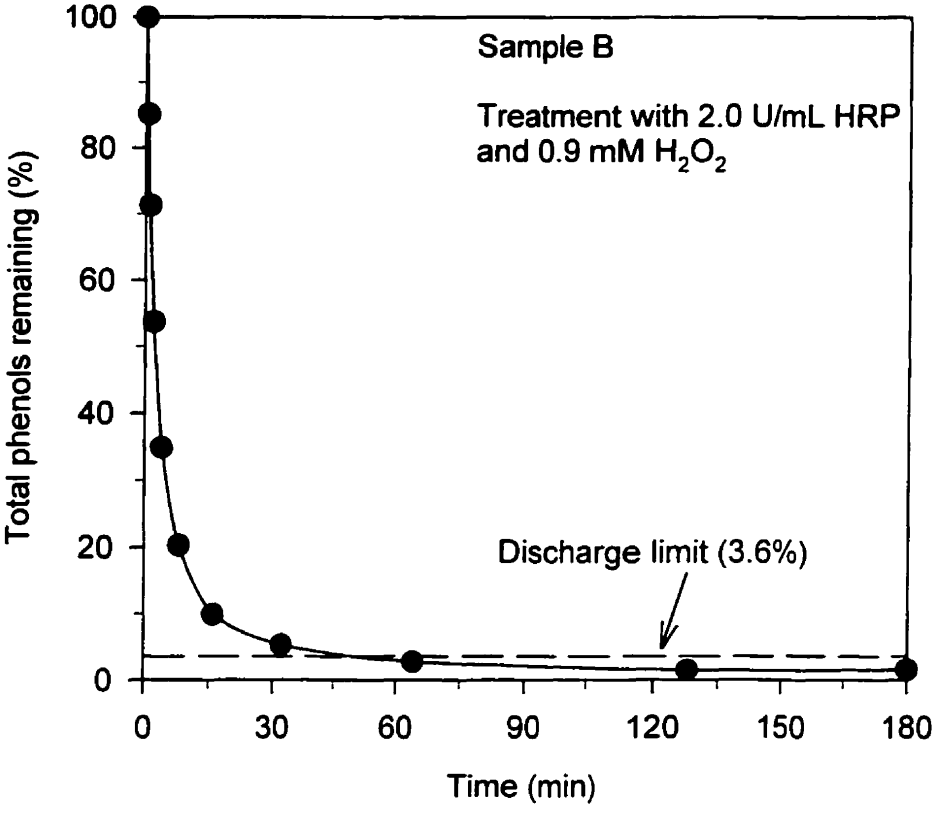


Figure 8.4: Total phenols remaining as a function of the reaction time.

In order to test the impact of the reaction pH on the enzymatic treatment of the petroleum refinery wastewater, the pHs of several aliquots of wastewater B were adjusted to between 4.9 and 9.5 using 0.1 N NaOH or 0.1 N HCl solutions. Subsequently, the samples were treated with 0.8 U/mL HRP and 0.9 mM H_2O_2 for three hours and the residual phenol concentration was recorded. The result of this experiment is presented in Figure 8.5. At least 50 % phenol conversion was achieved at all pH levels tested, while the maximum conversion occurred at pH 8.1. This value closely matches the pH optimum for treatment of phenol as reported by Nicell *et al.* (1993). High phenol removal also occurred at pH 8.6, which was the original pH of the wastewater.

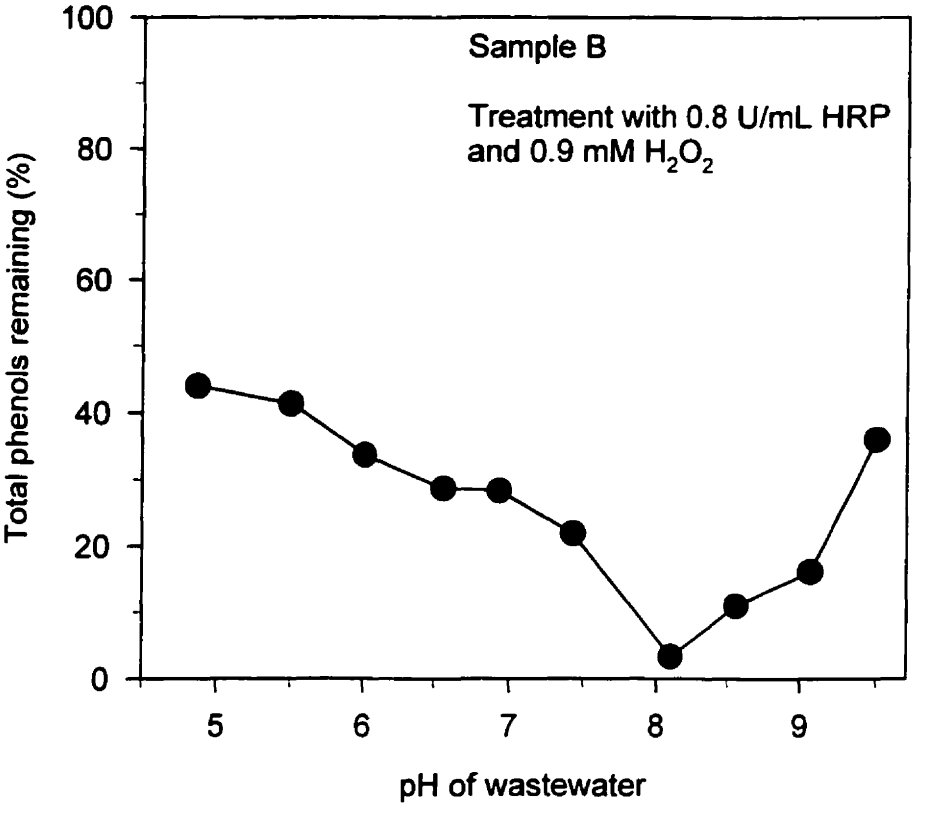


Figure 8.5: Total phenols remaining as a function of the reaction pH.

8.1.3 The use of protective additives

In order to determine whether additives are able to protect the enzyme from inactivation during the treatment of the petroleum refinery wastewater, batch reactions were conducted in the presence of PEG_{35000} and chitosan (420 cps viscosity grade). The concentrations of the additives ranged from 10 to 100 mg/L for chitosan and 5 to 60 mg/L for PEG_{35000}. A smaller range of PEG concentrations was tested because previous research has shown that PEG is effective at lower concentrations than chitosan (Nicell *et al.*, 1995). The addition of chitosan to the petroleum refinery wastewater resulted in a drop of pH from its initial value of 8.6 to about pH 6.7 due to the fact that the chitosan stock solution had been prepared in 1 % acetic acid. Since chitosan tends to precipitate out at pH values higher than 8, the pHs of all samples containing chitosan were raised only to pH 7.0 using 0.1 N NaOH before the start of the reaction. The results of this experiment are presented in Figure 8.6.

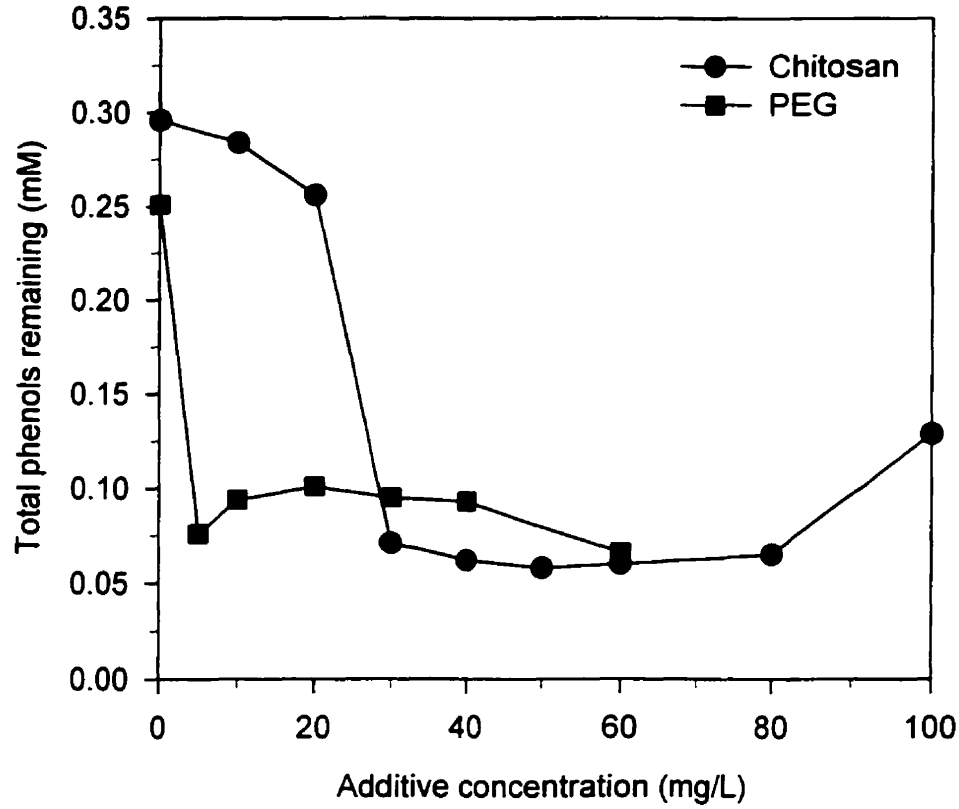


Figure 8.6: Impact of the addition of chitosan and PEG on phenol removal. $[H_2O_2]_0$ = 1.9 mM; HRP_0 = 0.038 U/mL for chitosan and 0.075 U/mL for PEG. $[phenol]_0$ = 0.35 mM (sample B).

The minimum concentration of chitosan that was required to achieve maximum protection of the enzyme was 30 mg/L. Chitosan had an adverse effect on phenol removal in concentrations exceeding 80 mg/L. For PEG, on the other hand, maximum protection was achieved at 5 mg/L with no further improvement or deterioration of treatment at higher PEG doses. In order to ensure maximum protection of the enzyme, 10 mg/L of PEG and 60 mg/L of chitosan were used in further experiments.

Figure 8.7 illustrates the extent of phenol removal as a function of the enzyme dose in the presence or absence of additives. The minimum enzyme requirement to achieve complete treatment ([total phenols] < 0.013 mM as phenol) was reduced to 0.05-0.1 U/mL in the presence of chitosan and to 0.4 U/mL when PEG was used as an additive. Compared to the case when no additive was present (in which 1.6 U/mL were required),

this corresponds to a 25-fold reduction in enzyme requirements in the case of chitosan and 4-fold reduction in the case of PEG.

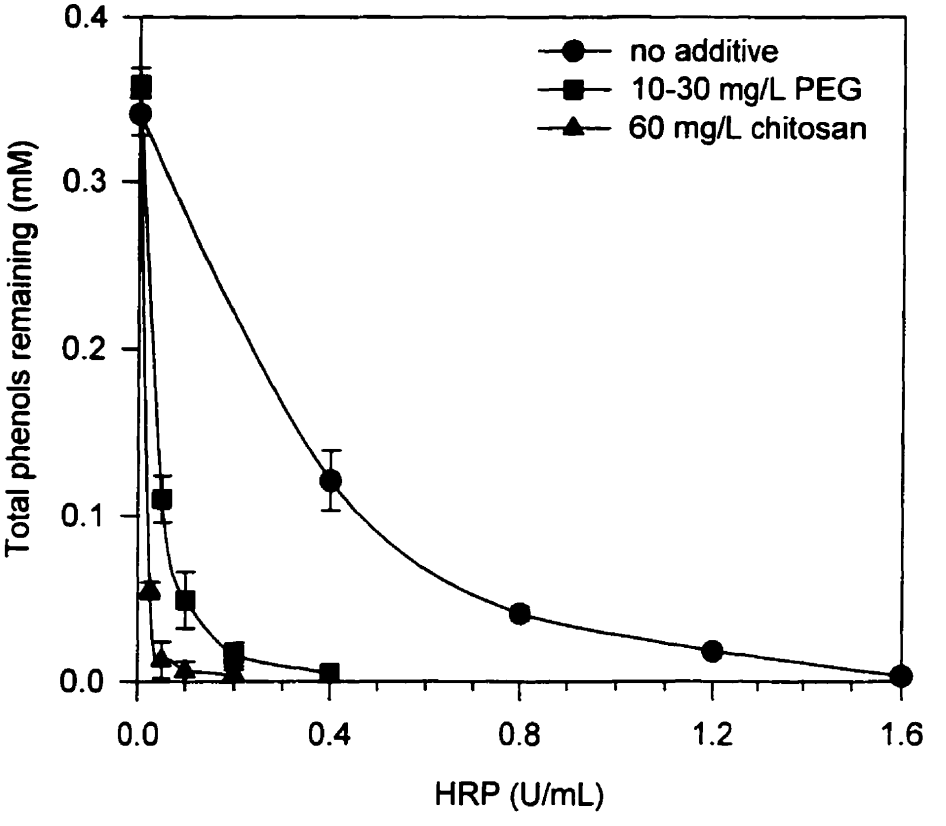


Figure 8.7: Total phenols remaining as a function of the HRP dose. $[H_2O_2]_0 = 0.9\text{-}1.4$ mM for treatment with no additives and 1.3-1.9 mM with chitosan or PEG. $[phenol]_0 = 0.35$ mM (sample B). Error bars represent the standard deviation of 3 independent experiments.

Furthermore, a precipitate spontaneously formed in samples that were treated in the presence of chitosan; thus they did not require coagulation to remove the phenolic polymer products. Note that the pH difference could not account for the better performance of chitosan since the optimum pH for the enzymatic treatment of this wastewater was found to be 8.1 (Figure 8.5). A higher H_2O_2 dose was required for treatments conducted in the presence of additives (at least 1.3 mM) because of the increased significance of spontaneous decomposition of H_2O_2 when lower enzyme doses were employed.

8.1.4 Impact of treatment on toxicity

The impact of peroxidase treatment on the acute toxicity of the refinery wastewater was assessed using the Microtox™ assay. Wastewater samples were treated to varying degrees by limiting the enzyme or the H_2O_2 concentrations. Experiments were conducted with wastewater sample B, both with and without additives. Sample A, which had a higher phenol concentration, was also treated for comparison. Samples were not coagulated prior to toxicity testing since preliminary experiments showed that coagulation slightly increased the toxicity. The following conclusions can be drawn from the results of these experiments, which are summarized in Figure 8.8.

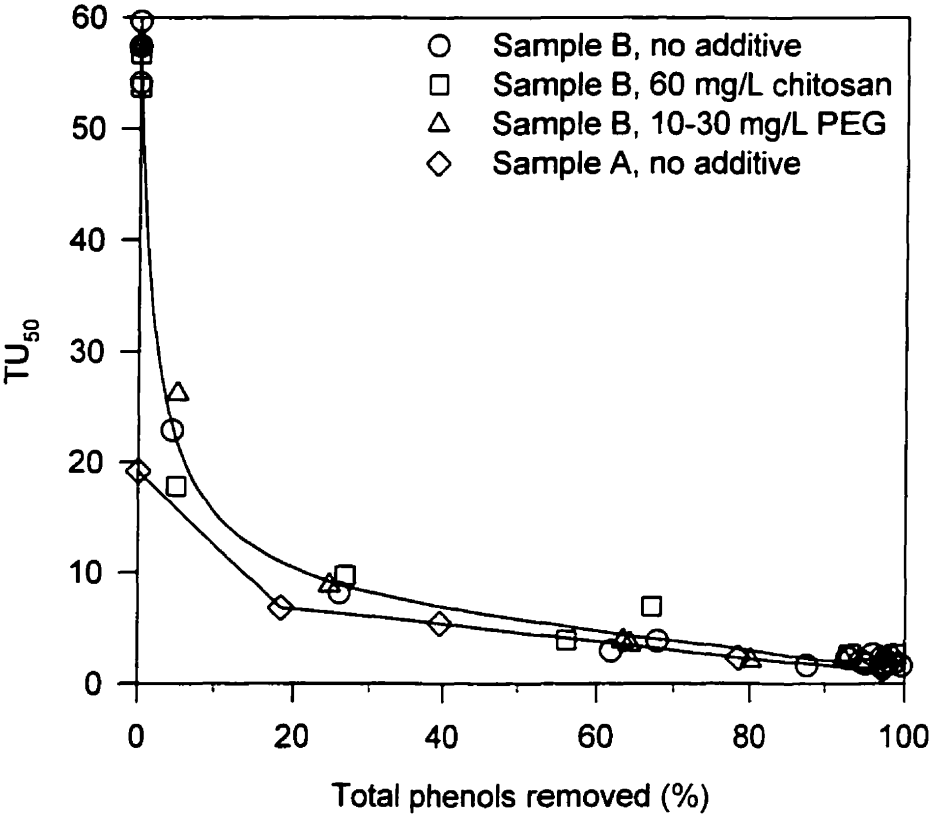


Figure 8.8: Acute toxicity as a function of the % total phenols removed. Sample B was treated with and without additives. Sample A was treated without additives.

Firstly, wastewater sample B (TU_{50} = 57) was significantly more toxic that sample A (TU_{50} = 19) even though sample B contained less total phenols. Secondly, very effective detoxification was achieved through enzymatic treatment, with 93% and 96% toxicity

reductions for samples A and B, respectively. Thirdly, enzymatic treatment of samples A and B led to a disproportionate reduction in toxicity compared to total phenols. For example, for 5% transformation of phenol, there was a corresponding 60% decrease in toxicity for sample B. Fourthly, the TU_{50}s of samples A and B dropped to their minima of 1.4 ± 0.3 and 2.1 ± 0.1, respectively, once a residual total phenols concentration of 0.1 mM was reached. No further toxicity reduction was observed for higher levels of phenol conversion. Fifthly, additives had no impact on the toxicity when added to untreated samples, nor at any level of treatment. Note that treatment with H_2O_2 or HRP alone did not lead to a toxicity reduction. Therefore, the toxicity drop resulted from the enzymatic oxidation of wastewater constituents.

8.1.5 Impact of treatment on chemical and biochemical oxygen demands

The COD of the wastewater was determined before and after treatment (residual [total phenols] < 0.013 mM as phenol) for treatments conducted with and without the presence of additives. The results of these experiments are presented in Figure 8.9.

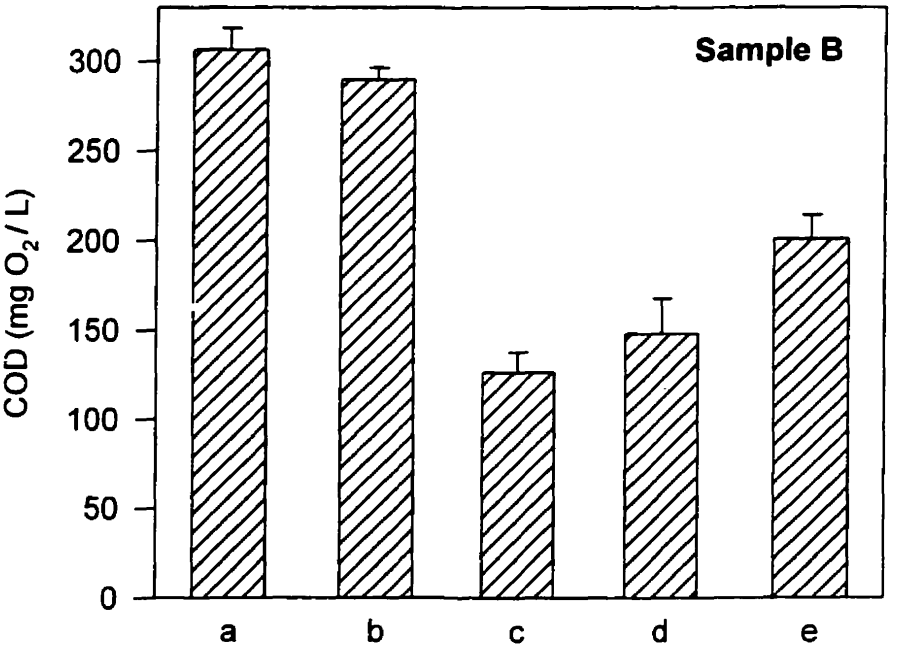


Figure 8.9: COD of (a) original sample, (b) after coagulation, (c)-(e) after HRP-treatment followed by the removal of reaction products. Treatment with (c) no additives, (d) 10 mg/L PEG and (e) 60 mg/L chitosan. Error bars stand for the standard error of the mean of 2 independent experiments.

For samples treated using either no additive or PEG, colloidal products were removed through alum coagulation prior to COD measurement. Samples treated with chitosan did not require coagulation since a precipitate formed spontaneously during the reaction.

As shown in Figure 8.9, coagulation alone had no significant effect on the COD of the untreated sample. A COD reduction of almost 58% was achieved when treatment was conducted without additives. The COD removal decreased slightly to 50% when 10 mg/L PEG was employed and dropped to 33% when chitosan was used as an additive. A mass balance conducted with the aid of control samples revealed that 86% of the additional COD in the chitosan-treated samples came from the acetic acid contained in the chitosan stock solution. Based on these calculations it was estimated that 80% of the chitosan precipitated during treatment. The resolution of the COD measurements was insufficient to state whether PEG precipitated or remained in the treated solutions. Note that the enzyme itself contributes about 14 mg/L to the final COD of the treated samples at the maximum enzyme dose of 2 U/mL.

Further experiments were carried out to determine if the products of enzymatic oxidation would be amenable to aerobic biodegradation. The wastewater was treated to the target residual total phenols concentration of 0.013 mM (as phenol) and the COD and the BOD_5 values of the treated sample were determined before and after the colloidal reaction products were removed by coagulation. No additives were used in these experiments. The results are displayed in Figure 8.10. No COD reduction in the HRP-treated sample was accomplished without coagulation, while the BOD_5 dropped from 94 mg/L to approximately 20 mg/L after enzymatic treatment regardless of whether or not reaction products were removed by coagulation. Thus, upon enzymatic treatment most of the compounds that constituted the BOD_5 were converted to materials that were less amenable to biodegradation, probably reflecting the conversion of monomeric substrates into polymeric products. Furthermore, the results in Figure 8.10 indicate that about one third of the COD was associated with the BOD_5. Of this fraction, 78% was removed through enzymatic treatment and subsequent coagulation. Additionally, the less biodegradable fraction of the COD, i.e. the other two-thirds, was reduced by 49%.

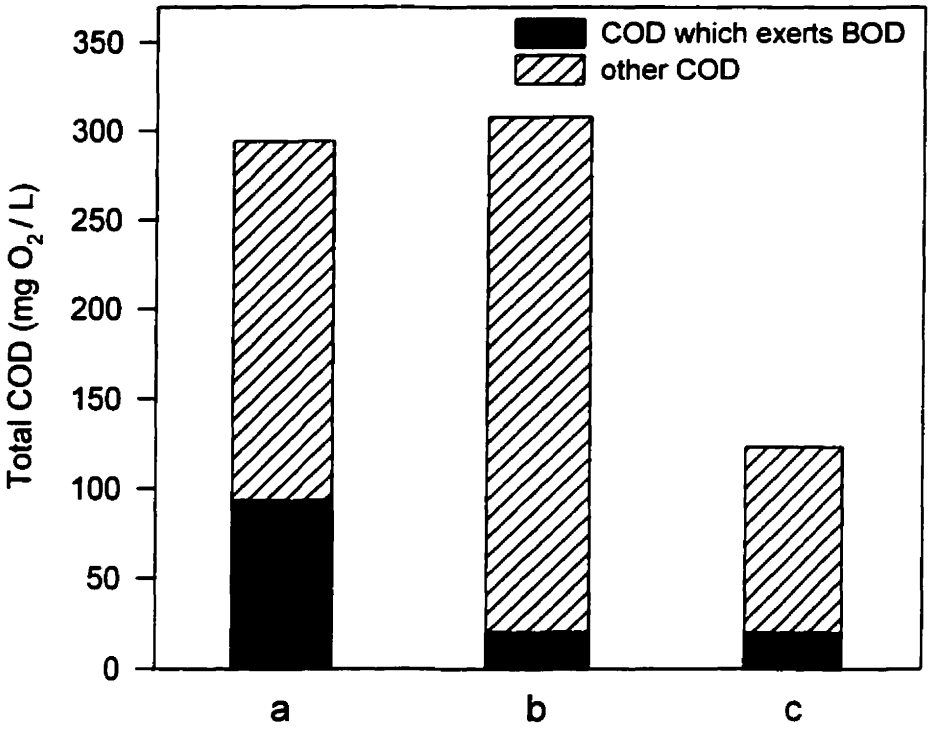


Figure 8.10: COD and BOD of sample B (a) before treatment, (b) after HRP treatment and (c) after HRP treatment followed by coagulation.

Interestingly, the 74-mg/L reduction in BOD_5 compares well to the theoretical oxygen demand (78 mg/L) of the removed phenols assuming that the predominant phenolic species in the wastewater is phenol. If, in fact, the COD fraction associated with the BOD_5 is mainly due to phenolic compounds, the reduction of the less biodegradable fraction suggests that considerable co-precipitation of other organic contaminants took place.

8.1.6 Impact of organic compounds on phenol removal efficiency

Refinery wastewaters contain a variety of organic contaminants including aromatic, polyaromatic and aliphatic hydrocarbons whose concentrations can vary over time in a given facility. In order to examine the impact that these classes of compounds might have on phenol removal efficiency, toluene, phenanthrene and a mixture of hexanes were added separately and in different concentrations to the refinery wastewater prior to enzymatic treatment. The highest concentrations used (140 mg/L for toluene and hexanes

and 7.7 mg/L for phenanthrene) were about 10 times the maximum values typically found in refinery wastewaters according to Dyer and Mignone (1983). Subsequently, the samples were treated with 0.8 U/mL HRP and 0.9 mM H_2O_2 for three hours and the residual phenol concentration was measured. Twelve samples, to which no organics had been added, were treated in an identical manner and served as controls. As illustrated in Figure 8.11, phenol concentrations remaining in the treated control samples ranged from 0.047 to 0.072 mM with an average of 0.060 mM.

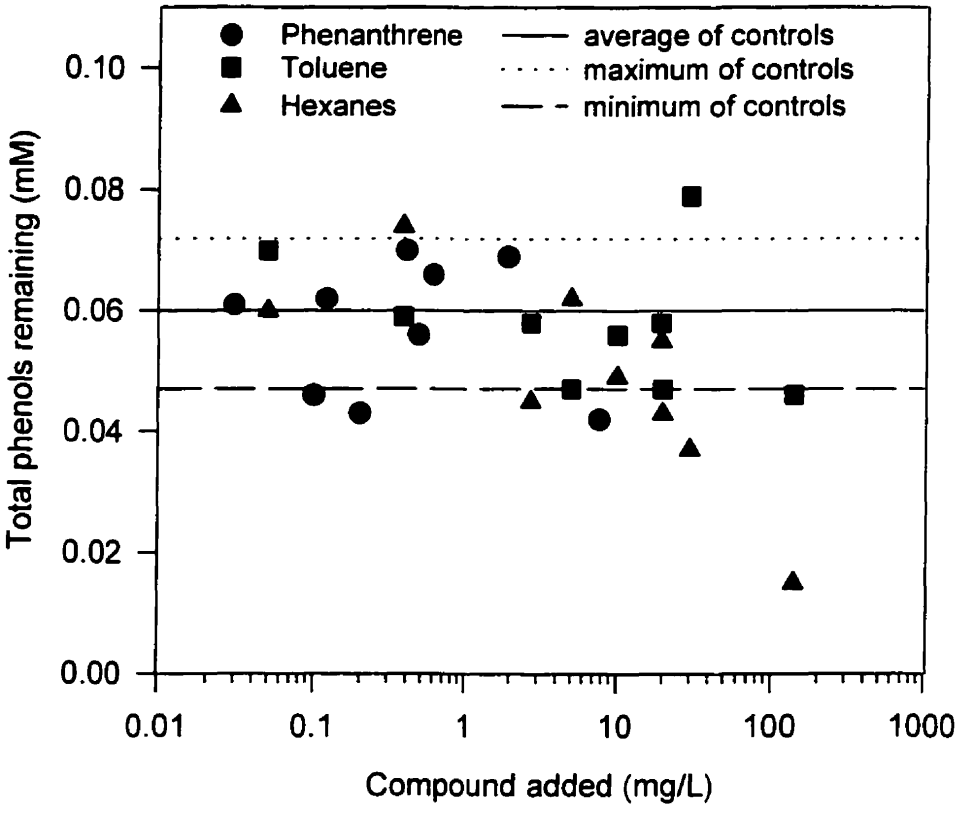


Figure 8.11: Impact of added organics on removal of total phenols from Sample B. Control samples did not contain additional organics. Treatment with 0.8 U/mL HRP and 0.9 mM H_2O_2.

No significant difference in the residual total phenols was found between the controls and the samples that contained the additional organics ($p = 0.21$ in ANOVA single factor test). Also, no trend towards higher or lower residual phenol concentrations could be discerned as the concentrations of toluene and phenanthrene increased; however, phenol

removal efficiency improved slightly with an increasing concentration of hexanes ($p = 0.005$).

8.1.7 Co-precipitation of phenanthrene

In order to determine whether co-precipitation of organic wastewater constituents occurred upon HRP-treatment, phenanthrene was added to the wastewater samples at concentrations ranging from 100 to 600 µg/L. Subsequently, the samples were treated with 0.9 mM H_2O_2 and 2 U/mL HRP for one hour and the reaction products were removed by alum coagulation. Tests were also conducted to determine whether a reduction of the phenanthrene concentration resulted from a reaction of the phenolic radicals with the phenanthrene molecules or rather from an adsorption of the hydrophobic phenanthrene to the polymeric reaction products. To this end, phenanthrene was added to the treated wastewater one hour after the start of the reaction, at which point of time the phenol conversion was essentially completed (see Figure 8.4). This was followed by coagulation of the phenolic reaction products. After the centrifugation of the samples, the phenanthrene remaining in solution was measured using spectrofluorescence. The results of these experiments are presented in Figure 8.12.

Regression analysis revealed that for all initial concentrations tested, on average 92.6% of phenanthrene was removed through HRP-treatment followed by coagulation. However, it was not required that phenanthrene be present during the enzymatic reaction in order for the removal to take place, since it was almost as effectively removed when added after the phenol conversion has gone to completion, but before coagulation (Figure 8.12). Tests were also conducted to determine if alum coagulation alone without prior HRP-treatment would cause a reduction in the phenanthrene concentration. However, the results showed no decrease in the phenanthrene concentration by coagulation alone. Thus, the presence of phenolic reaction products was required for phenanthrene removal to take place; thereby suggesting that adsorption to the colloidal reaction products was the main co-precipitation mechanism.

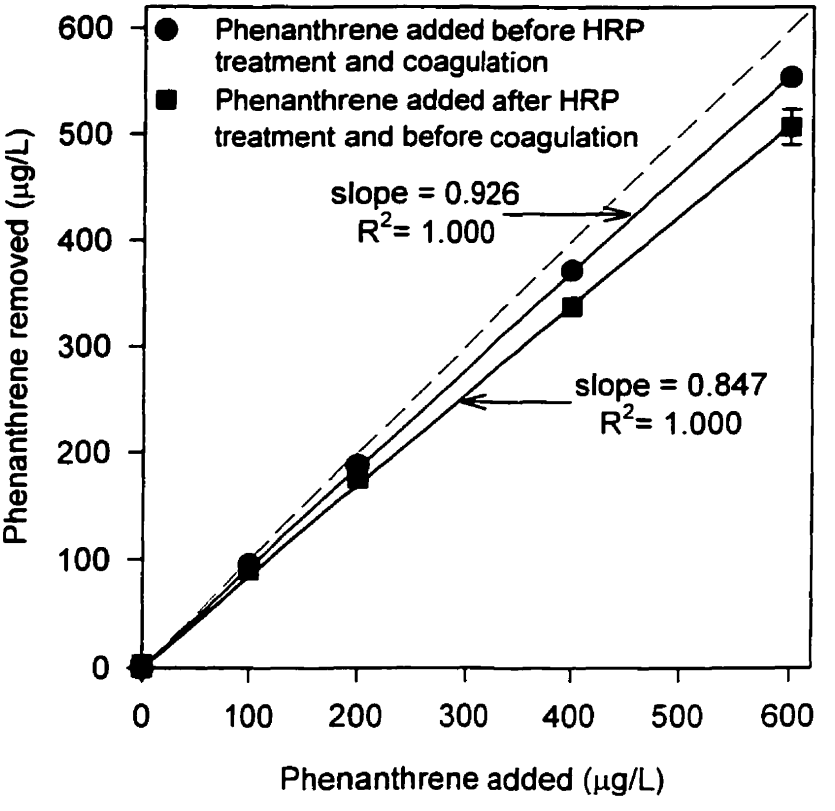


Figure 8.12: Removal of phenanthrene upon HRP treatment and coagulation. Sample B was treated with 2.0 U/mL HRP and 0.9 mM H_2O_2.

8.2 Discussion

This study has demonstrated that HRP-treatment can reduce the total phenol concentration in a petroleum refinery wastewater to below the discharge limit. Along with phenol removal, the quality of the wastewater improved significantly. This was reflected by a substantial reduction of the COD and the BOD_5 combined with extensive detoxification. Interestingly, significant detoxification was achieved even at low levels of treatment when most of the total phenols were still present (Figure 8.8). Given that some phenolic compounds are more easily removed than others (Klibanov, 1982), one can speculate that one or more highly toxic phenol species might be those first removed at low levels of treatment. Also, different phenols produce different quantities of color when reacting in the phenol assay and, thus, are reflected to different degrees in the total phenol

measurement. These two factors could explain the observation of a significant toxicity drop even though the bulk of the phenols was apparently still present. Alternatively, it is also possible that other, non-phenolic compounds might have been mainly responsible for the high toxicity and these were removed along with the phenols through co-precipitation.

When excess amounts of HRP were used (7 U/mL), approximately 1.35 moles H_2O_2 were required to remove one mole of total phenols (Figure 8.1). This value is higher than the 1:1 stoichiometry found for the treatment of a number of phenols in synthetic wastewaters (Nicell *et al.*, 1992). One reason for this discrepancy could be that the summary parameter "total phenols" does not truly reflect the molar concentration of the sum of the phenols present in the sample. However, it is also possible that even at very high HRP-doses a part of the H_2O_2 was very quickly consumed in side reactions with reduced wastewater components thus increasing the apparent phenol-H_2O_2 stoichiometry. In fact, hydrogen peroxide decomposed to H_2O and O_2 in the petroleum refinery wastewater, which led to increased H_2O_2 demands, particularly when lower enzyme doses were applied. This effect could be compensated by (1) providing higher enzyme doses in order to increase the rate of the enzymatic peroxide utilization; (2) providing higher peroxide doses to satisfy both the demands of the spontaneous peroxide decomposition and the enzymatic phenol oxidation; or (3) decreasing the instantaneous peroxide concentration through slow addition of the peroxide to the reaction mixture. The first approach is economically unfavorable since the enzyme is the most expensive component of the treatment system. In comparison to the first approach, the second approach resulted in a 100 % increase of the required peroxide dose but led to 20% enzyme savings. Moreover, it was demonstrated that the peroxide was used more efficiently when it was added to the reaction mixture over an extended period of time. Thus, the third approach results in a savings of peroxide but it comes at the cost of a longer retention time.

Despite the problems associated with H_2O_2 decomposition, HRP requirements were comparable with those needed for the treatment of synthetic wastewaters containing pure phenol. However, a number of substituted phenols required less HRP for their conversion (Aitken and Heck, 1998) than did phenol. Therefore, a direct comparison between synthetic and real wastewaters only provides limited information of the effect of the

waste matrix on removal efficiency as long as the identities of the phenolic species in the wastewater are unknown.

HRP-requirements were reduced by using PEG_{35000} or chitosan as protective additives. Interestingly, chitosan proved to be substantially more effective than PEG for the treatment of this wastewater. However, when treating synthetic phenol solutions, both additives showed nearly the same effectiveness (see Chapter 4). This indicates that the wastewater matrix had an impact on the protective action of the additives. Similarly, Cooper and Nicell (1996) report that PEG_{3350} exhibited a reduced efficiency when used for the treatment of a foundry wastewater, which was presumably due to an interaction between the polymer and organic components in the waste matrix.

The COD was not as effectively removed when chitosan was used, due mainly to the acetic acid that was used as a solvent for the chitosan stock solution. It is possible that better COD removals could be obtained using chitosan dissolved in dilute mineral acids such as hydrochloric acid.

The oxygen demand experiments indicate that the phenolic substrates were transformed into less biodegradable polymeric compounds which were removed by subsequent coagulation. The resulting solids need to be characterized in further research in order to find adequate methods for their treatment or disposal. The oxygen demand experiments together with the toxicity analyses also suggest that other compounds apart from phenols were removed upon enzymatic treatment. The possibility that this indeed could have occurred was confirmed by the experiments involving the addition of phenanthrene to the wastewater sample and its subsequent removal through HRP treatment followed by coagulation. Similar experiments were carried out by Klibanov *et al.* (1983) who observed that the concentration of PCBs, which they had added to a coal conversion wastewater, was significantly reduced upon HRP-treatment. However, no PCB removal occurred when a pure solution of the PCBs in water was treated, confirming that these types of compounds are not substrates of HRP.

Interestingly, phenanthrene was almost as efficiently removed when it was added after the completion of phenol conversion but prior to coagulation. Thus, the removal of phenanthrene was not due to its reaction with phenolic radicals for these have a very short lifetime and therefore would not likely be present after the completion of phenol

conversion. It seems rather that the hydrophobic phenanthrene adsorbed onto the phenolic polymer, and was subsequently removed from solution through coagulation. Importantly, the fraction of phenanthrene that was removed was approximately the same at all initial concentrations tested (Figure 8.12). This could be interpreted as resulting from an equilibrium that arose between the phenanthrene remaining in solution and the phenanthrene adsorbed to the phenolic polymer.

9. CONCLUSIONS

This investigation has led to the conclusions outlined below.

9.1 The Use of Chitosan as a Protective Additive

The presence of chitosan during the enzymatic reaction substantially increased the level of phenol transformation. As shown previously for PEG_{3350}, the amount of chitosan required to attain maximum phenol conversion was proportional to the initial phenol concentration. When limiting HRP-doses were used, the quantity of phenol removed increased linearly with increasing chitosan concentrations suggesting that chitosan and phenolic reaction products combined at a constant mass ratio. The effectiveness of chitosan was comparable to that of PEG_{35000} and significantly higher than that of PEG_{3350}. Although, when compared to PEG_{3350} on a mass basis higher chitosan doses were required, chitosan may be a more suitable additive for wastewater treatment applications due to its biodegradability.

9.2 Toxicity Reduction

This study has shown that toxic compounds formed upon HRP-catalyzed oxidation of 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2-methylphenol and the non-substituted phenol, which is in agreement with results reported by other researchers. However, results of this study also indicate for all phenols tested except for 2-methylphenol that these reaction products are quite unstable, since toxicity decreased within a 21 hours period.

Upon the introduction of an additional dose of H_2O_2 to reaction mixtures from which phenol had been removed through enzymatic treatment, detoxification was accelerated and the toxicity was virtually eliminated except in the cases of the *ortho*-substituted phenols. HRP-treated samples can also be detoxified upon UV light illumination at a comparatively low energy input.

The presence of the protective additive PEG during the transformation of phenol led to higher toxicities, while the presence of chitosan did not have an impact when toxicities

were measured 3 hours after the start of the enzymatic reaction. However, a small, but significant decrease in toxicity compared to the controls was observed when samples containing chitosan were allowed to incubate for an extended period of time. While the effect of chitosan may not be very strong, these results indicate that chitosan may be a better choice as an additive when treating phenolic solutions that give rise to toxic reaction products.

The formation of toxic reaction products was significantly reduced when H_2O_2 was added slowly during the enzymatic reaction, whereas slow addition of the peroxidase yielded an opposite effect. Although the underlying mechanisms for these effects are unknown, this observation establishes the dependency of resulting toxicity levels on the instantaneous concentrations of the reagents H_2O_2 and/or HRP and thus demonstrates the potential for minimizing toxicity levels through manipulation of the mode of reagent addition.

9.3 Impact of Wastewater Parameters

The impact of various wastewater constituents on the enzymatic treatment of synthetic phenol solutions was investigated under a variety of reaction conditions. The constituents studied included suspended solids, inorganic anions, organic compounds, metals and inorganic salts.

Silica gel (up to 10000 mg/L), cellulose (up to 10000 mg/L) and powdered activated carbon (up to 100 mg/L) had only a minor impact on phenol conversion. Bentonite, kaolin and peat moss increased phenol transformation at pH 5 and 7, but had a negative impact at pH 9 when present at 10000 mg/L (in the case of bentonite and kaolin) or at or above 1000 mg/L (in the case of peat moss). In the case of bentonite and peat moss, the enhancing effects were associated with the dissolved or colloidal constituents, while in the case of kaolin, the enhancing effects were due to the solid material. Freshly made bentonite suspensions inactivated the peroxidase enzyme. H_2O_2 was unstable in solutions containing peat moss constituents, which may have limited the extent of phenol transformation. Phenolic solutions treated in the presence of bentonite, kaolin and peat moss were significantly less toxic than the controls indicating that these materials were able to interact with and partially neutralize precursors of toxic reaction products.

Iodide and nitrite increased phenol transformation under enzyme-limited conditions. However, under conditions that lead to high levels of phenol conversion, these compounds successfully competed for the available H_2O_2, particularly at a low pH. This resulted in lower phenol transformations, but could be partially overcome by providing higher H_2O_2 doses.

The reduced sulfuric compounds sulfite, thiosulfate and sulfide increased H_2O_2 requirements. In the case of sulfite and sulfide, this was due to a direct oxidation with H_2O_2; however, observations suggest that in the case of thiosulfate other mechanisms may have been responsible. The presence of the oxidation products of sulfite and sulfide, and in particular the presence of thiosulfate led to higher levels of phenol conversion provided sufficient H_2O_2 was supplied. However, these compounds seemed also to increase the amount of soluble or colloidal reaction products as indicated by the elevated UV absorbance of treated solutions.

The presence of thiocyanate up to a concentration of 400 mg/L only had a minor effect on phenol conversion.

Sulfide and cyanide inhibited phenol transformation. In the case of sulfide, this could be overcome by supplying an additional equimolar amount of H_2O_2 to oxidize the sulfide to sulfur. However, increasing the H_2O_2 dose was ineffective when attempting to overcome the strong inhibiting effect of cyanide even at concentrations at or below 4 mg/L.

Among the metal ions tested, only Mn(II) substantially inhibited phenol removal at a relatively high concentration of 1 mM (corresponding to 54.9 mg/L) but not at 0.01 mM.

The inorganic salts NaCl, $CaCl_2$, $MgCl_2$, NH_4Cl and $(NH_4)_2SO_4$ reduced phenol conversion compared to the treatment in distilled-deionized water. This effect was limited and non-specific and therefore can probably be attributed to the increased ionic strength of the solution.

9.4 Treatment of Industrial Wastewaters

This study has demonstrated that phenolic compounds present in a foul condensate from Kraft pulping and a petroleum refinery wastewater can be treated with HRP and H_2O_2.

In the case of the foul condensate, HRP treatment resulted in the reduction of total phenols to levels below 1 mg/L. Treatment was only moderately influenced by the pH in the range of 6 to 9.2. Even though COD removal was only marginal, a 46% reduction in Microtox™ toxicity was achieved, thereby indicating that treatment selectively removed a large fraction of the toxic components of the wastewater. Experiments with spiked wastewaters demonstrated that compounds found in the condensate protect the enzyme from inactivation during the reaction. Analytical results and observations of the treatment of real and synthetic wastewaters suggest that these compounds are lignin derivatives.

In the case of the petroleum refinery wastewater, enzymatic treatment reduced the total phenol concentration below the discharge limit. Along with phenol removal, the quality of the wastewater improved significantly. This was reflected by a 58% and 78% reduction of the COD and the BOD_5 values, respectively, and a reduction in toxicity of approximately 95%. Optimization of peroxide concentration resulted in 20% enzyme savings. Moreover, it was demonstrated that the peroxide was used more efficiently when it was added to the reaction mixture over an extended period of time. The application of PEG and chitosan effectively protected the enzyme from inactivation resulting in 4-fold and 25-fold reductions in enzyme requirements, respectively. The COD was not removed as effectively when chitosan was used. The phenolic substrates were transformed into less biodegradable polymeric compounds which could be removed by subsequent coagulation. Oxygen demand experiments and toxicity analyses suggest that other compounds apart from phenols were also removed upon enzymatic treatment. This was confirmed by the addition and subsequent observation of the removal of phenanthrene from the wastewater sample. Finally, the addition of different hydrocarbons in concentrations exceeding those that are typically found in petroleum refinery wastewaters did not impair phenol removal, thereby suggesting that fluctuations in the concentrations of these wastewater constituents will not affect the phenol removal efficiency.

10. RECOMMENDATIONS

This study has shown that the HRP-based phenol removal process can be carried out under a variety of conditions that may be encountered in industrial wastewaters. Furthermore, HRP-treatment of real industrial wastewaters did not only result in phenol transformation, but also substantially improved the wastewater quality. This was accomplished with HRP and H_2O_2 doses which were comparable with the amounts required for the treatment of synthetic phenolic solutions. However, further research is needed before this process can be used in industrial applications.

10.1 Enzyme Source

The main operating cost of the HRP-based treatment process will be due to the cost of the peroxidase catalyst. In this investigation, an expensive and highly purified enzyme formulation was used, which is usually employed for analytical applications. However, previous research has shown that treatment with crude enzyme extracts such as horseradish juice (Cooper and Nicell, 1996) or minced horseradish (Dec and Bollag, 1994) gives equivalent results in terms of phenol removal, although the COD reduction was compromised due to the presence of additional organic material in the crude enzyme preparation (Cooper and Nicell, 1996). Nevertheless, the use of crude enzyme preparations would substantially reduce treatment costs and therefore deserves further investigation.

Other possibilities to reduce the cost of the catalyst include:

- industrial scale biotechnological production of HRP in hairy roots (Taya *et al.*, 1989);
- production of HRP in other organisms (e.g., tobacco plants, bacteria) using transgenic technology;
- replacement of HRP by peroxidases from a cheaper source, for example by the peroxidase from soybean hulls or peroxidases from microorganisms that could be produced by large scale fermentation.

10.2 Additives

This research has demonstrated that high molecular weight organic polymers, such as PEG and chitosan can substantially reduce enzyme requirements in real wastewater applications. However, the use of these additives always carries with it the potential of increasing the organic load of the treated effluent and, therefore, should be considered very carefully. On the other hand, it was observed in this study that colloidal or dissolved materials derived from the inorganic clay solid bentonite are able to significantly enhance phenol transformation. Further research should be carried out to identify the components that are responsible for this effect and to determine the underlying mechanisms. This research could possibly lead to the development of an additive that combines effectiveness with low pollution potential and low costs.

10.3 Pilot Scale Studies

Pilot scale studies are required before the HRP-based treatment process can be implemented in any particular facility. These studies should focus on identifying the reactor configuration that would optimize treatment costs and wastewater quality in terms of phenol removal and toxicity reduction. The kinetic model developed by Buchanan and Nicell (1997) would aid this work. Furthermore, the operational stability of the process should be studied carefully in order to develop strategies for effective process control. Also, coagulation procedures should be optimized if no spontaneous precipitation of reaction products occurs.

10.4 Environmental Impact Assessment

An environmental impact assessment is required in order to compare the overall benefit of the HRP-based process with other treatment options. This assessment should include an investigation of the impact of the production of HRP and H_2O_2, the toxicity and genotoxicity of the treated effluent, and the disposal of the precipitated or coagulated reaction products. Toxicity testing should include tests based on diverse species such as fish, algae or the water flea (*Daphnia magna*) in order to obtain broader information on the effect of HRP-treated effluents on aquatic life.

Statement of Original Contributions to Knowledge

This research has demonstrated that (1) horseradish peroxidase enzyme (HRP) can accomplish the transformation of phenols and a reduction in toxicity in real wastewaters; and (2) certain compounds that are present in the waste matrix can have positive and negative effects on treatment efficiency. Specifically, it was demonstrated that:

(1) The toxicity of HRP-treated phenol solutions declines with time and is dependent on the presence of protective additives, the mode of reagent addition, and the presence of wastewater constituents.

(2) Many phenolic solutions can be completely detoxified by providing an additional dose of hydrogen peroxide after the completion of the enzymatic reaction. It has also been demonstrated that ultraviolet light can be used as a polishing step to reduce effluent toxicity.

(3) The enzymatic phenol removal process can be carried out in the presence of a wide range of concentrations of suspended solids, salts, metals, and other inorganic and organic species that are frequently present in industrial wastewaters.

(4) In certain instances, treatment is more effective in a real waste matrix as compared to the treatment of pure solutions of phenol due to the presence of particular waste components.

(5) Actual industrial wastewaters can be treated with HRP and H_2O_2 to meet regulatory discharge limits for phenol. It was demonstrated that the enzymatic treatment process can selectively target phenols, which are a major source of toxicity in the wastes, for treatment.

(6) Sulfide, manganese(II) and low quantities of cyanide negatively impact upon the enzymatic transformation of phenol.

(7) Treatment of real wastewaters may require higher than stoichiometric doses of hydrogen peroxide due to the inherent peroxide demand of reduced wastewater constituents or catalytic decomposition of peroxide.

(8) Chitosan can be used as a protective additive to enhance the enzymatic treatment of industrial wastewaters.

(9) HRP-treatment improves wastewater quality as reflected in a significant toxicity reduction and can result in a substantial decrease in the biochemical and chemical oxygen demands.

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Appendix A: Analytical Methods

A.1 Horseradish Peroxidase Activity Assays

A.1.1 HRP Assay based on Phenol and 4-Aminoantipyrene

This assay is used to determine the amount of active peroxidase in a sample. Peroxidases utilize H_2O_2 to oxidize phenol to phenolic radicals. These radicals react with 4-aminoantipyrene (4-AAP), which is present in the assay solution, to form a non-precipitating red anti-pyrilquinoneimine dye. The overall color formation reaction is represented by:

$$\text{Phenol} + \text{4-AAP} + 2\ H_2O_2 \longrightarrow \text{Dye} + 4\ H_2O$$

Phenol, 4-aminoantipyrene and hydrogen peroxide are present at sufficiently high concentrations such that their decrease during the reaction time is small enough to be neglected. Thus, the initial reaction rate, as reflected through the rate of color formation, will be proportional to the amount of active peroxidase present. The progression of the reaction is followed by monitoring the formation of the dye, which adsorbs light at a wavelength of 510 nm with an extinction coefficient of 7100 $M^{-1}cm^{-1}$ based on H_2O_2. One unit of activity (U) is defined as the number of micromoles of peroxide utilized in one minute at pH 7.4 and 25°C in an assay mixture containing 10 mM phenol, 2.4 mM 4-AAP, and 0.2 mM H_2O_2. It should be noted that this assay is subject to interferences associated with the presence of phenolic reaction products. Therefore, it should not be used to monitor the activity during the enzymatic phenol oxidation.

A.1.1.1 Preparation of Reagents

a) 0.1 M Sodium Phosphate Buffer (pH 7.4)

Add 95 mL of 0.2 M monobasic sodium phosphate to 405 mL of 0.2 M dibasic sodium phosphate and dilute to 1 L with distilled-deionized water.

b) 200 mM Phenol

Dissolve 941 mg of phenol in 50 mL of 0.1 M NaPP buffer (pH 7.4).

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

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

d) 2.0 mM H_2O_2

Dilute 487 µL of 31% (w/w) hydrogen peroxide to 50 mL in distilled water to make a 100 mM solution. Mix well. Withdraw 1 mL and dilute to 50 mL with distilled water to make a 2 mM solution. This reagent is to be prepared daily.

A.1.1.2 Procedure

The total assay volume is 1 mL. First, blank the spectrophotometer at 510 nm with the following solution using a semi-micro cuvette (1 cm path length):

- 600 µL of 0.1 M sodium phosphate buffer (pH 7.4)
- 50 µL of 200 mM phenol
- 250 µL of 9.6 mM 4-AAP
- 100 µL of 2.0 mM H_2O_2

For the assay, place in a semi-micro cuvette (1 cm path length):

- 550 µL of 0.1 M sodium phosphate buffer (pH 7.4)
- 50 µL of 200 mM phenol
- 250 µL of 9.6 mM 4-AAP
- 100 µL of 2.0 mM H_2O_2
- 50 µL of enzyme sample

The volume of the enzyme sample may be increased up to 150 µL for less concentrated enzyme samples. The buffer volume must then be decreased accordingly.

As soon as the peroxidase has been added, cap and invert the cuvette three times to ensure complete mixing of the reagents. Place the cuvette into the spectrophotometer and monitor the increase of the absorbance at 510 nm for 1 minute.

The activity of the peroxidase in the cuvette must not exceed 0.05 U/mL for the color development to remain linear over the 1-minute duration of the assay. Therefore, more concentrated solutions need to be pre-diluted.

A.1.1.3 Calculations

One unit of activity is defined as the number of micromoles of peroxide converted per minute at pH 7.4 and 25°C according to:

$$\text{Activity} = -\frac{d[H_2O_2]}{dt} = \frac{dA_{510}}{dt}\frac{1}{\varepsilon \cdot d}$$

in which dA_{510}/dt is the change in the absorbance at 510 nm, ε is the extinction coefficient of the red dye per mol of H_2O_2 converted (ε = 7100 au $M^{-1}cm^{-1}$), and d is the path length of the light in the reaction solution (d = 1 cm).

The change of the absorbance at 510 nm is the slope of the plot of the absorbance at 510 nm versus time. The activity in the cuvette, in units of μmol H_2O_2 $min^{-1}mL^{-1}$ (i.e. U mL^{-1}), is then calculated according to:

$$\text{Cuvette Activity (U mL}^{-1}) = \frac{\text{slope (au} \cdot \text{s}^{-1})}{7100 \text{ au L mol}^{-1}}\frac{60\text{s}}{\text{min}}\frac{10^6 \mu\text{mol}}{\text{mol}}\frac{\text{L}}{10^3 \text{mL}}$$

The activity of the sample can then be determined according to:

$$\text{Sample Activity (U mL}^{-1}) = \text{Cuvette Activity (U mL}^{-1})\frac{1.0 \text{ mL}}{\text{Sample Volume in Cuvette (mL)}}$$

A.1.2 HRP Assay based on ABTS

This assay is used to determine the amount of active peroxidase in a sample. Peroxidases catalyze the oxidation of ABTS to a resonance-stabilized radical (ABTS·) with hydrogen peroxide according to:

$$2 \text{ ABTS-H} + H_2O_2 \longrightarrow 2 \text{ ABTS}\cdot + 2 H_2O$$

The concentrations of both substrates in the assay are sufficiently high to ensure that the decrease in their concentration during the reaction time is small enough to be neglected. Thus, the initial reaction rate will be proportional to the amount of active peroxidase present. The progression of the reaction is followed by monitoring the

formation of ABTS·, which absorbs light at the wavelength of 405 nm with an extinction coefficient of 1.86×10^4 $M^{-1}cm^{-1}$. One unit of activity (U) is defined as the number of micromoles of peroxide utilized in one minute at pH 6.0 and 25°C in an assay mixture containing 1.7 mM ABTS and 0.83 mM H_2O_2. This assay is not subject to the same interferences as the 4-AAP assay (described above) and may be used to monitor the activity of peroxidase over the course of phenol oxidation.

A.1.1.1 Preparation of Reagents

a) 0.067 M Sodium Phosphate Buffer (pH 6.0)

Add 294 mL of 0.2 M monobasic sodium phosphate to 41 mL of 0.2 M dibasic sodium phosphate and dilute to 1 L with distilled-deionized water.

b) 20 mM ABTS

Dissolve 0.1 g of ABTS in 10 mL of 0.067 M sodium phosphate buffer (pH 6.0). Store in refrigerator for no longer than 3 days.

c) 10 mM H_2O_2

Dilute 487 µL of 31% (w/w) hydrogen peroxide to 50 mL in distilled water to make a 100 mM solution. Mix well. Withdraw 1 mL and dilute to 10 mL with distilled water to make a 10 mM solution. This reagent is to be prepared daily.

A.1.1.2 Procedure

The total assay volume is 1.2 mL. First, blank the spectrophotometer at 405 nm with the following solution using a semi-micro cuvette (1 cm path length):

- 1000 µL of 0.067 M sodium phosphate buffer (pH 6.0)
- 100 µL of 20 mM ABTS
- 100 µL of 10 mM H_2O_2

For the assay, pipette into a semi-micro cuvette (1 cm path length):

- 950 µL of 0.067 M sodium phosphate buffer (pH 6.0)
- 100 µL of 20 mM ABTS
- 100 µL of 10 mM H_2O_2
- 50 µL of enzyme sample

The volume of the enzyme sample may be increased up to 150 µL for less concentrated enzyme samples. The buffer volume must then be decreased accordingly.

As soon as the peroxidase has been added, cap and invert the cuvette three times to ensure complete mixing of the reagents. Place the cuvette into the spectrophotometer and monitor the increase of the absorbance at 405 nm for 1 minute.

A.1.1.3 Calculations

One unit of activity is defined as the number of micromoles of peroxide converted per minute at pH 6.0 and 25°C according to:

$$\text{Activity} = -\frac{d[H_2O_2]}{dt} = \frac{1}{2} \times \frac{d[\text{ABTS}\cdot]}{dt} = \frac{1}{2} \times \frac{dA_{405}}{dt} \frac{1}{\varepsilon \cdot d}$$

in which dA_{405}/dt is the change in the absorbance at 405 nm, ε is the extinction coefficient of the ABTS· radical ($\varepsilon = 1.86 \times 10^4$ au $M^{-1}cm^{-1}$), and d is the path length of the light in the reaction solution (d = 1 cm).

The change of the absorbance at 405 nm is the slope of the plot of the absorbance at 405 nm versus time. The activity in the cuvette, in units of µmol H_2O_2 $min^{-1}mL^{-1}$ (i.e. U mL^{-1}), is then calculated according to:

$$\text{Cuvette Activity (U mL}^{-1}) = \frac{1}{2} \times \frac{\text{slope (au} \cdot \text{s}^{-1})}{1.86 \times 10^4 \text{ au L mol}^{-1}} \frac{60\text{s}}{\text{min}} \frac{10^6 \mu\text{mol}}{\text{mol}} \frac{\text{L}}{10^3 \text{mL}}$$

The activity of the sample can then be determined according to:

$$\text{Sample Activity (U mL}^{-1}) = \text{Cuvette Activity (U mL}^{-1}) \frac{1.2 \text{ mL}}{\text{Sample Volume in Cuvette (mL)}}$$

A.2 Hydrogen Peroxide (H_2O_2) Assays

A.2.1 H_2O_2 Assay Based on Phenol and 4-Aminoantipyrene

This assay is a modified version of the peroxidase activity assay that is based on phenol and 4-aminoantipyrene. The modified assay mixture contains excess amounts of HRP and initially no H_2O_2. The absorbance at 510 nm is recorded 6 minutes after the addition of a sample containing H_2O_2 to the assay mixture. The absorbance at 510 nm is converted to hydrogen peroxide concentrations in the cuvette by the means of a calibration line. For hydrogen peroxide to remain the limiting substrate, its concentration in the cuvette should not exceed 0.07 mM.

A.2.1.1 Preparation of Reagents

a) 0.1 M Sodium Phosphate Buffer (pH 7.4)

This is the same reagent as used for the activity assay (see Section A.1.1.1).

b) 200 mM Phenol

This is the same reagent as used for the activity assay (see Section A.1.1.1).

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

This is the same reagent as used for the activity assay (see Section A.1.1.1).

d) HRP Solution (1 mg/mL)

Dilute 2 mL of a 5 mg/mL HRP stock solution to 10 mL in phosphate buffer. Store in refrigerator.

A.2.1.2 Procedure

The total assay volume is 1 mL. First, blank the spectrophotometer at 510 nm with the following solution using a semi-micro cuvette (1 cm path length):

- 600 µL of 0.1 M sodium phosphate buffer (pH 7.4)
- 50 µL of 200 mM phenol
- 250 µL of 9.6 mM 4-AAP
- 100 µL of 1 mg/mL HRP solution

For the assay, place these reactants in a reaction tube (3 to 5 mL capacity) in the following order:

- 400 to 590 µL of 0.1 M sodium phosphate buffer (pH 7.4)
- 50 µL of 200 mM phenol
- 250 µL of 9.6 mM 4-AAP
- 100 µL of 1 mg/mL HRP solution
- 10 to 200 µL of sample

Wait for the color development, transfer the assay solution into the same cuvette that was used for the blank and record the absorbance at 510 nm exactly 6 minutes after the addition of the samples containing H_2O_2.

Perform a calibration by introducing 10 to 70 µL of a 1 mM H_2O_2 solution into the assay, recoding the resulting absorbance and plotting the absorbance versus the phenol concentration. Determine the slope of this calibration line in units of (au mM^{-1}). To obtain a linear calibration line the H_2O_2 concentration in the cuvette should not exceed 0.07 mM.

A.2.1.3 Calculations

The H_2O_2 concentration in the cuvette is determined from the calibration line according to:

$$[H_2O_2]_{cuvette} = \frac{A_{510}(au)}{slope\,(au\ mM^{-1})}$$

If the H_2O_2 concentration in the cuvette exceeds 0.07 mM dilute the sample or use smaller sample volumes and repeat the assay.

The H_2O_2 concentration in the sample then is calculated according to:

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \frac{1.0\,mL}{Sample\ Volume\ in\ Cuvette\,(mL)}$$

A.2.2 H_2O_2 Assay Based on ABTS

This assay is a modified version of the peroxidase activity assay that is based on ABTS. The modified assay mixture contains excess amounts of HRP and initially no H_2O_2. The

absorbance at 405 nm is recorded 6 minutes after the addition of a sample containing H_2O_2 to the assay mixture. The absorbance at 405 nm is converted to hydrogen peroxide concentrations in the cuvette by the means of a calibration line. For hydrogen peroxide to remain the limiting substrate, its concentration in the cuvette should not exceed 0.02 mM. This assay can be used to measure the H_2O_2 concentration over the course of a HRP-catalyzed phenol oxidation reaction.

A.2.2.1 Preparation of Reagents

a) 0.067 M Sodium Phosphate Buffer (pH 6.0)

This is the same reagent as used for the activity assay (see Section A.1.2.1).

b) 20 mM ABTS

This is the same reagent as used for the activity assay (see Section A.1.2.1).

c) HRP solution (0.05 mg/mL)

Dilute 0.1 mL of a 5 mg/mL HRP stock solution to 10 mL in distilled water. This reagent is to be prepared daily.

A.2.2.2 Procedure

The total assay volume is 1.2 mL. First, blank the spectrophotometer at 405 nm with the following solution using a semi-micro cuvette (1 cm path length):

- 1000 μL of 0.067 M sodium phosphate buffer (pH 6.0)
- 100 μL of 20 mM ABTS
- 100 μL of HRP solution

For the assay, place these reactants in a reaction tube (3 to 5 mL capacity) in the following order:

- 800 to 990 μL of 0.067 M sodium phosphate buffer (pH 6.0)
- 100 μL of 20 mM ABTS
- 100 μL of 10 mM H_2O_2
- 10 to 200 μL of enzyme sample

Wait for the color development, transfer the assay solution into the same cuvette that was used for the blank and record the absorbance at 405 nm exactly 6 minutes after the addition of the samples containing H_2O_2.

Perform a calibration by introducing 10 to 120 µL of a 0.2 mM H_2O_2 solution into the assay, recoding the resulting absorbance and plotting the absorbance versus the H_2O_2 concentration. Determine the slope of this calibration line in units of (au mM^{-1}). To obtain a linear calibration line the H_2O_2 concentration in the cuvette should not exceed 0.02 mM.

A.2.2.3 Calculations

The H_2O_2 concentration in the cuvette is determined from the calibration line according to:

$$[H_2O_2]_{cuvette} = \frac{A_{405}(au)}{slope\,(au\ mM^{-1})}$$

If the H_2O_2 concentration in the cuvette exceeds 0.02 mM dilute the sample or use smaller sample volumes and repeat the assay.

The H_2O_2 concentration in the sample is then calculated according to:

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \frac{1.2\,mL}{Sample\ Volume\ in\ Cuvette\,(mL)}$$

A.3 Total Phenol Assay

Phenol concentrations are determined using a colorimetric assay in which the phenolic compounds react with 4-aminiantipyrene (4-AAP) in the presence of ferricyanide to form a red dye, which absorbs light at a peak wavelength of 510 nm. The absorbance at 510 nm is converted to total phenol concentrations using a calibration line based on phenol. This assay represents a modification of the direct photometric method, which is a standard analytical procedure for phenols (APHA, 1998). The modified assay employs higher concentrations of 4-AAP and potassium ferricyanide, allowing the measurement of higher phenol concentrations than under the standard method, while also using smaller sample volumes. It should be noted that this assay is not very sensitive to the presence of para-substituted phenols.

A.3.1 Preparation of Reagents

a) 0.25 Sodium Bicarbonate

Dissolve 21.0 g of $NaHCO_3$ in 1 L of distilled water.

b) 83.4 mM Potassium Ferricyanide

Dissolve 13.73 g of $K_3Fe(CN)_6$ in 500 mL of 0.25 M $NaHCO_3$. Store in refrigerator.

c) 20.8 mM 4-Aminoantipyrene

Dissolve 2.115 g of 4-AAP in 500 mL of 0.25 M $NaHCO_3$. Store in refrigerator.

A.3.2 Assay Procedure

The total assay volume is 1 mL. First, blank the spectrophotometer at 510 nm with the following solution using a cuvette with the path length of 1 cm:

- 800 µL of 0.25 M sodium bicarbonate
- 100 µL of 4-AAP reagent
- 100 µL of ferricyanide reagent

For the assay, place these reactants in a reaction tube (3 to 5 mL capacity) in the following order:

- 300 to 790 µL of 0.25 M sodium bicarbonate
- 10 to 500 µL of sample
- 100 µL 4-AAP reagent
- 100 µL ferricyanide reagent

Wait for the color development, transfer the assay solution into the same cuvette that was used for the blank and record the absorbance at 510 nm exactly 6 minutes after the addition of ferricyanide.

Perform a calibration by introducing 10 to 100 µL a 1 mM phenol solution into the assay, recoding the resulting absorbance and plotting the absorbance versus the phenol concentration. Determine the slope of this calibration line in units of (au mM^{-1}). To obtain a linear calibration line the total phenol concentration in the cuvette should not exceed 0.1 mM (as phenol).

A.3.3 Calculations

The concentration of total phenols in the cuvette is determined from the calibration line based on phenol according to:

$$[\text{phenol}]_{\text{cuvette}} = \frac{A_{510}\,(\text{au})}{\text{slope}\,(\text{au mM}^{-1})}$$

If the concentration in the cuvette exceeds 0.1 mM (as phenol) dilute the sample or use smaller sample volumes and repeat the assay.

The total phenol concentration in the sample is calculated according to:

$$[\text{phenol}]_{\text{sample}} = [\text{phenol}]_{\text{cuvette}} \frac{1.0\,\text{mL}}{\text{Sample Volume in Cuvette (mL)}}$$

Appendix B: Results of the Experiments with Chitosan

B.1 Determination of the optimum chitosan dose

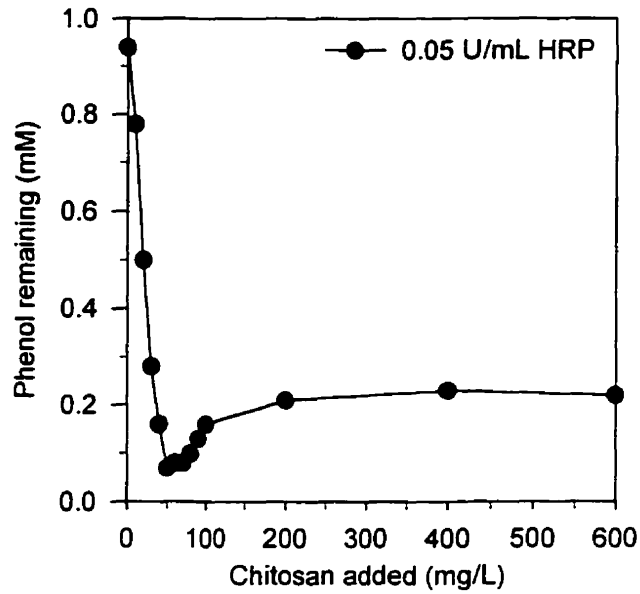

Figure B.1: Phenol remaining as a function of the amount of chitosan added. $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.2$ mM.

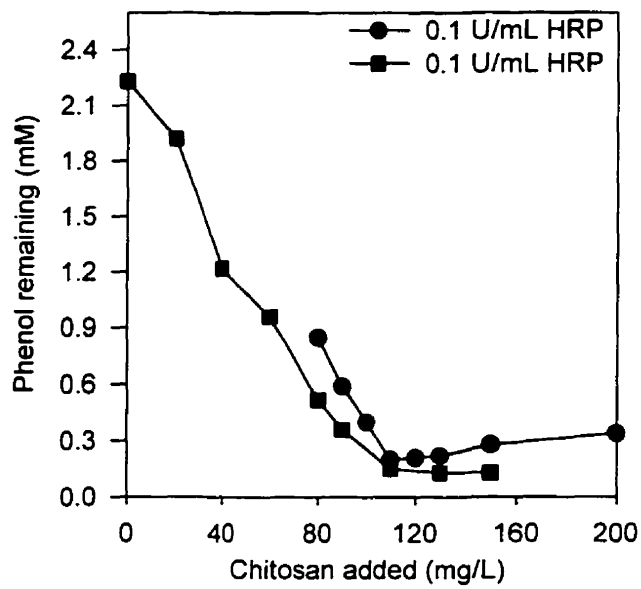

Figure B.2: Phenol remaining as a function of the amount of chitosan added. $[phenol]_0 = 2.7$ mM; $[H_2O_2]_0 = 3.0$ mM.

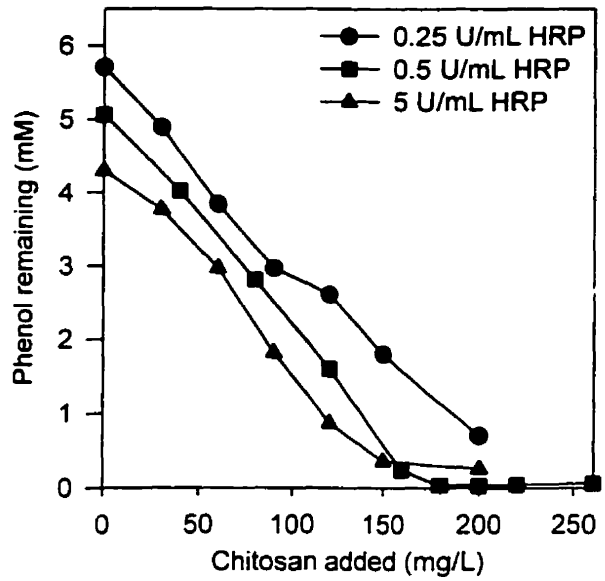

Figure B.3: Phenol remaining as a function of the amount of chitosan added. $[phenol]_0 = 5.9$ mM; $[H_2O_2]_0 = 7.2$ mM.

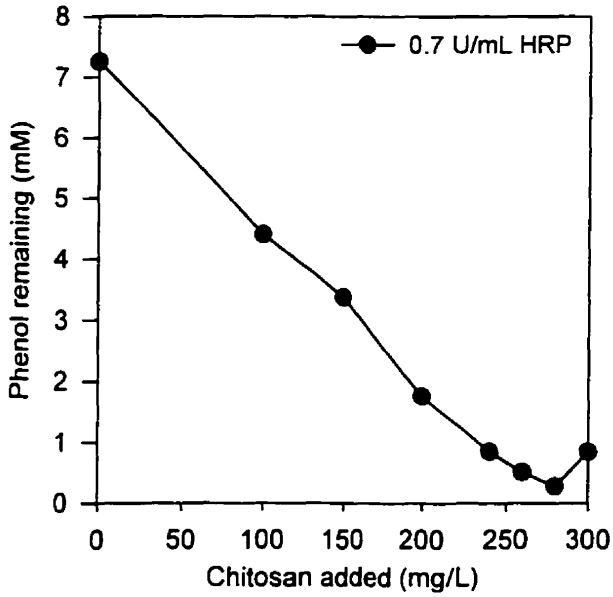

Figure B.4: Phenol remaining as a function of the amount of chitosan added. $[phenol]_0 = 7.6$ mM; $[H_2O_2]_0 = 9.0$ mM.

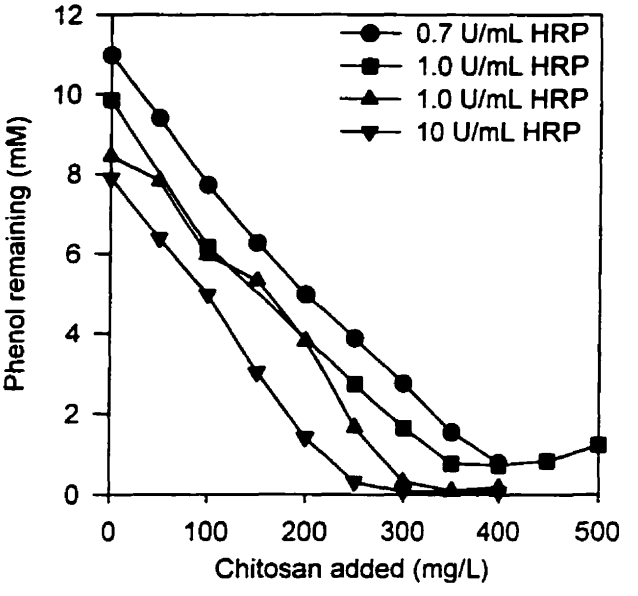

Figure B.5: Phenol remaining as a function of the amount of chitosan added. $[phenol]_0 = 10.7$ mM; $[H_2O_2]_0 = 12.5$ mM.

B.2 Determination of HRP requirements in the presence of chitosan

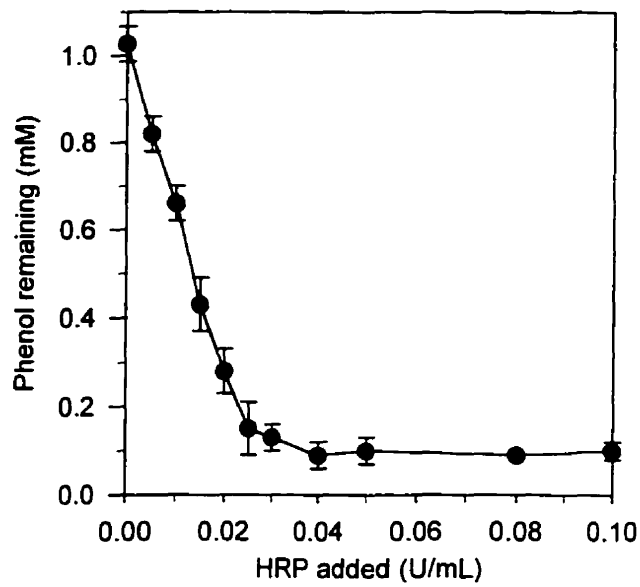

Figure B.6: Phenol remaining as a function of the HRP dose in the presence of chitosan. $[phenol]_0 = 1.0$ mM; $[H_2O_2]_0 = 1.2$ mM; chitosan = 50 mg/L.

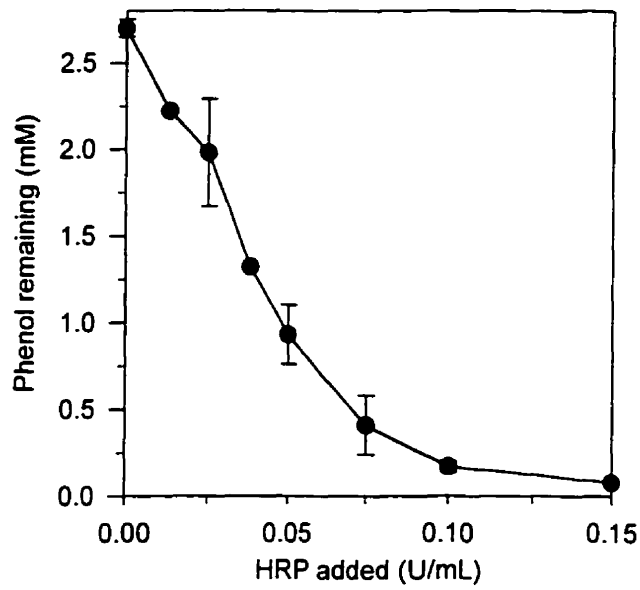

Figure B.7: Phenol remaining as a function of the HRP dose in the presence of chitosan. $[phenol]_0 = 2.7$ mM; $[H_2O_2]_0 = 3.0$ mM; chitosan = 120 mg/L.

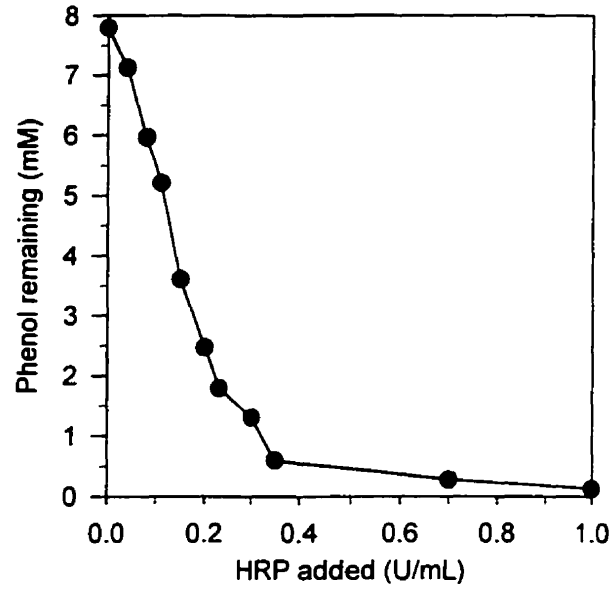

Figure B.8: Phenol remaining as a function of the HRP dose in the presence of chitosan. $[phenol]_0 = 7.8$ mM; $[H_2O_2]_0 = 9.0$ mM; chitosan = 280 mg/L.

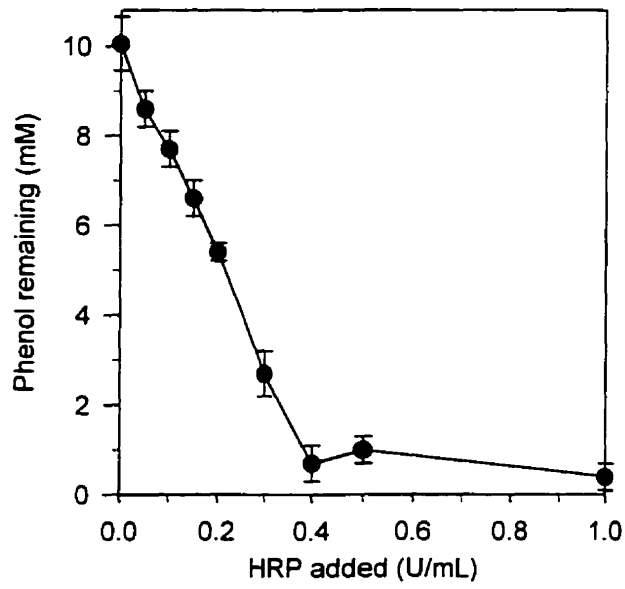

Figure B.9: Phenol remaining as a function of the HRP dose in the presence of chitosan. $[phenol]_0 = 10.1$ mM; $[H_2O_2]_0 = 12.0$ mM; chitosan = 375 mg/L.

Appendix C: Determination of the Molar Concentration of Peroxidase

C.1 Introduction

Native HRP (HRP_N) absorbs light in the Soret region, which is the wavelength region in which the absorbance of the heme prosthetic group occurs at a peak wavelength of 403 nm. This feature can be used to determine the molar concentration of HRP_N, since the molar extinction coefficient of HRP_N is known (Dunford and Stillman, 1976). In this study, this method was routinely used to measure the molar concentration of HRP_N in the stock solutions.

However, this method has several drawbacks. First, no distinction can be made between active or inactive HRP molecules, as long as the heme absorbance is not affected. Second, this method can only be applied for relatively freshly made HRP solutions, since the solutions become cloudy upon storage, leading to a continuous increase in the absorbance at 403 nm. And finally, this method can only be used with peroxidase enzymes of known extinction coefficients.

A new method was developed to measure the molar content of active heme peroxidase molecules in a sample. The basic premise for this method was proposed by Mr. Harold Wright, but was first developed and applied in this study.

When H_2O_2 is added to a solution containing HRP_N, the enzyme will be oxidized to Compound I, which will result in a decrease of its absorbance at 403 nm. Since one molecule of HRP_N reacts with one molecule of H_2O_2 the decrease in the absorbance will be proportional to the number of moles of H_2O_2 added up to a point when the number of moles of H_2O_2 introduced into the solution becomes equal to the number of moles of active HRP_N present. At this point the absorbance will reach a plateau value, which will remain constant when more H_2O_2 is introduced. When the absorbance at 403 nm is plotted versus the quantity of H_2O_2 added, the quantity of active HRP_N can be determined from the intersection of the plateau value with the linear slope of absorbance versus the amount of H_2O_2.

C.2 Method Procedure

50 μL of a HRP stock solution (~5 mg dry powder/mL water) was added to a cuvette of 3 mL capacity containing 1950 μL distilled-deionized water and a magnetic stirring bar. The cuvette was placed into the cuvette holder of the spectrophotometer (model HP 8453), which features a magnetic stirrer base. Stirring was initiated by turning on a pump, which pumped water that powered the magnetic stir base and the absorbance at 403 nm was recorded. This value was used to calculate the quantity of HRP_N present based on the extinction coefficient at 403 nm of 102 $mM^{-1}cm^{-1}$.

Subsequently, 1 μL of a concentrated H_2O_2 solution (580 nmol/mL in distilled-deionized water) was introduced into this solution by the means of a microsyringe and the absorbance at 403 nm was recorded at intervals of 1 second for a period of approximately 2 minutes. (Note that the absorbance decreases to a minimum and then rises again because, due the presence of indigenous reducing substrates, Compound I is reduced to Compound II, which is then reduced back to the native enzyme.) The lowest absorbance value that was reached during this time period was recorded. This procedure was repeated with fresh HRP solutions and larger volumes of the H_2O_2 solution until the minimum recorded absorbance values at 403 nm remained constant with increasing addition of H_2O_2.

C.3 Results and Discussion

A typical plot that was used to determine the molar amount of peroxidase in the solution is shown in Figure C.1.

The molar concentration of HRP_N in stock solutions containing approximately 5 mg/L solid was determined using the H_2O_2 addition method as well as the method based on the extinction coefficient at 403 nm. Also, the activity of the stock solutions was measured using the assay based on phenol and 4-AAP. For these experiments, horseradish peroxidase preparations of three different purities were employed. The degree of purity is reflected in their purity number (RZ-value), which is the ratio of the absorbance at 403 to 280 nm. High RZ values (3.0 to 3.4) signify a low content of non-peroxidase proteins in the enzyme preparation.

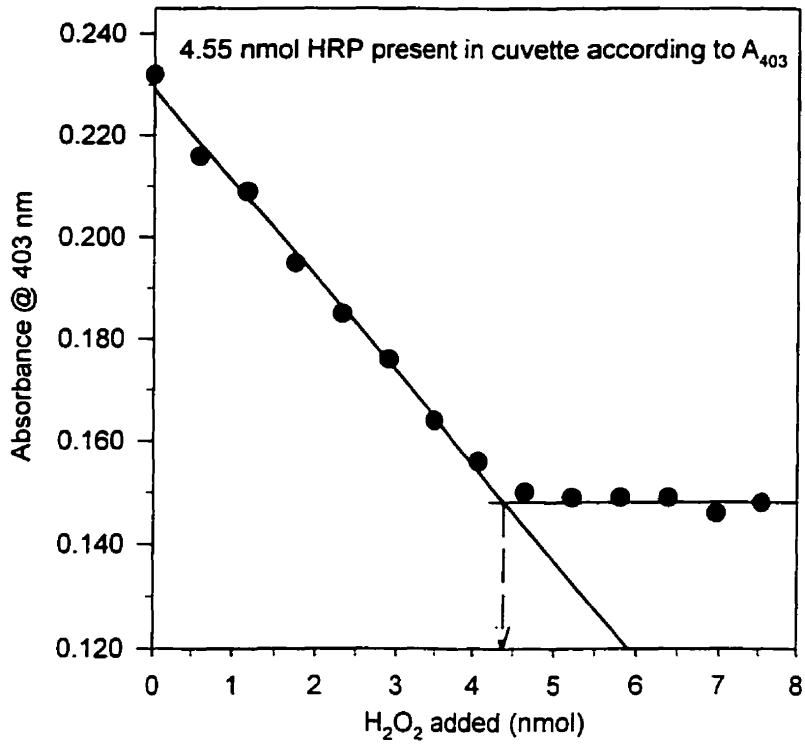

Figure C.1: Determination of the molar amount of HRP_N using the H_2O_2 addition method. (HRP type: RZ =1.3; 4.8 mg solids/mL)

Additionally, a soybean peroxidase preparation was characterized in the same fashion. Since the molar extinction coefficient of SBP_N at 403 nm is not yet available, the same extinction coefficient as for HRP_N was employed. The results of these experiments are summarized in Table C.1.

Table C.1: Results of the characterization of various peroxidase preparations.

Peroxidase type	**Solids concentration (mg/L)**	**Molar content (nmol/mg)**		**Specific activity (U/nmol)**	
		according to A_{403}	**according to assay**	**according to A_{403}**	**according to assay**
HRP-RZ 1.1	4.7	6.4	6.5	18.7	18.7
HRP-RZ 1.3	5.3	9.3	9.9	18.2	17.2
	5.1	9.2	9.6	19.1	18.5
	4.8	9.4	9.1	18.7	19.4
HRP-RZ 3.1	1.1	35.6	33.2	22.5	24.2
SBP	5.0	5.9	5.1	2.7	3.1

As can be seen from Table C.1, in the case of the HRP-preparations both methods yielded very similar results indicating that virtually all heme containing HRP molecules are active. In the case of SBP, however, an approximately 13 % lower value for the molar content was determined using the H_2O_2 addition method. This could be due to either the presence of some inactive peroxidase molecules, which also adsorb light in the Soret region, or to a slightly lower extinction coefficient for SBP_N as compared to HRP_N.

Overall, these results show that the H_2O_2 addition method may be used to measure the molar concentration of native heme peroxidases. Further research is required to establish whether this method can be applied to peroxidases from other sources and/or to less purified preparations.