Removal of cresols from synthetic wastewater using laccase.

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REMOVAL OF CRESOLS FROM SYNTHETIC WASTEWATER USING LACCASE

by

Amy B. Vermette

A thesis submitted to the
College of Graduate Studies and Research through
Civil and Environmental Engineering
in partial fulfillment of the requirements for the
Degree of Master of Applied Science
at the University of Windsor

Windsor, Ontario, Canada
May, 2000

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ABSTRACT

Laccase, as a developmental preparation from an industrial enzyme producer, catalyzes the oxidation of selected aromatic compounds. The polymers formed from the reaction are insoluble and are readily removed from solution.

Experiments were conducted to evaluate the potential use of laccase in an alternative enzyme-based technology to remove cresols from synthetic wastewater. Reaction parameters were optimized in unbuffered tap water for the removal of o-, m-, and p-cresols. The effects of pH, enzyme dose, PEG addition, dissolved oxygen availability, and hydrogen peroxide addition were investigated. All tests were conducted in continuously stirred batch reactors.

Nearly 90% of o-cresol was removed at optimum conditions of pH and enzyme dose, while p- and m-cresols' removals were 80% and 70%, respectively. The optimum pH for the cresols ranged from 5.6 to 7.0. For each substrate, the optimum enzyme dose varied from 0.3 standardized units of catalytic activity for p-cresol to 0.6 standardized units of catalytic activity for m-cresol. Aeration, at higher concentrations of substrate, increased the initial rate of reaction for substrate removal and improved the efficiency of the reaction. The addition of PEG or hydrogen peroxide did not have significant effects on substrate removal. The results from this study have demonstrated the applicability of laccase for the reduction of cresols from wastewater. This study provides a basis for further investigations into similar enzymatic treatment.
DEDICATION

I dedicate this thesis to my parents, Michael and Charmaine Ferrigan, for the pride they have always shown for me and to my friends, Diane, Doug, Justin, and Mur, who inspired me to do this.

Thank you.
ACKNOWLEDGEMENTS

Sincere thanks are expressed to my advisors and committee members, Dr. J.K. Bewtra, Dr. K.E. Taylor, and Dr. N. Biswas for their guidance, support, and constructive criticisms throughout the course of this research. I am grateful for the time they have taken to actively participate in my efforts. The careful and meticulous reading that the thesis received is very much appreciated.

My thanks are extended to my fellow graduate students, Kaushik Biswas and Ram Mantha, for their direction and suggestions in the laboratory. Thanks also go to Mr. Bill Henderson for his technical help in the laboratory.
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<th>Description</th>
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<tr>
<td>AAP</td>
<td>4-aminoantipyrine</td>
</tr>
<tr>
<td>ARP</td>
<td><em>Arthomyces ramosus</em> peroxidase</td>
</tr>
<tr>
<td>CMP</td>
<td><em>Coprinus macrorhizus</em> peroxidase</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>$K_3Fe(CN)_6$</td>
<td>ferricyanide</td>
</tr>
<tr>
<td>$m$-cresol</td>
<td><em>meta</em>-cresol</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>$o$-cresol</td>
<td><em>ortho</em>-cresol</td>
</tr>
<tr>
<td>$p$-cresol</td>
<td><em>para</em>-cresol</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>SBP</td>
<td>soybean peroxidase</td>
</tr>
</tbody>
</table>
1.0 INTRODUCTION

Cresols are highly toxic compounds that are discharged into the waste streams of various industries. Cresols are isomeric phenols with a methyl substituent at the ortho, meta, or para position relative to the hydroxyl group. Commercially, cresols are produced as by-products in the fractional distillation of crude oil and coal tars and in coal gasification. Cresols have a wide variety of uses including solvents, disinfectants, and in the production of dyes, resins, and pesticides. An estimated annual production of 38.3 million kilograms was reported in the USA for 1990 (WHO, 1996).

The removal of significant concentrations of cresols from industrial wastewater is required to achieve the environmental standards that are placed on industries. The current methods using physical, chemical, and biological techniques suffer from factors such as high cost, hazardous by-product formation, and poor removal efficiency (Kilbanov et al., 1980). Therefore, research is being conducted to develop an alternative approach to the removal of cresols from wastewater by using enzyme-based technology.

1.1 Enzyme Background

Traditionally, it has been customary to evaluate processes for waste treatment in terms of pollution indicators such as biochemical oxygen demand, chemical oxygen demand, and total organic carbon. Today, there is an increased emphasis on the removal of specific pollutants from waste streams. This can be observed from the development of governmental regulations to limit the total toxicity in wastewater discharges (Aitken, 1993).
Existing physical, chemical, and biological treatment processes are limited with respect to the removal of specific chemicals to the levels established by regulation. The particular process may not be highly selective in terms of the range of pollutants removed during treatment or it may have difficulty in removing toxic pollutants consistently to low levels. Enzymes can selectively remove pollutants from waste streams due to the high specificity that enzymes have for their substrates (Aitken, 1993).

Enzymes are biological catalysts that increase the rate of chemical reactions without incurring any change. Most enzymes are proteins made up of amino acid units joined in series. The sequence of amino acids in a protein is specific and this gives each protein unique properties. The reactants of enzyme-catalyzed reactions are called substrates. Each enzyme is specific in character and acts on one or more particular substrates to produce one or more particular products (Palmer, 1981). The nature and specificity of their catalytic activity is primarily due to the three-dimensional structure of the folded protein, which is determined by the sequence of the amino acids that make up the enzyme (Blanch and Clark, 1996). The “substrate-binding” site of the enzyme is the active site where the substrate associates by a variety of non-covalent forces allowing catalytic activity (Palmer, 1981).

Enzymes have a wide range of applications. The pharmaceutical and food and drink industries use enzymes in the production of their products. Enzymes are widely used in clinical chemistry and medical diagnosis. Developing technology may introduce wastewater treatment as another application of enzymes.

Laccase, as a developmental preparation from an industrial enzyme producer, was used in this study. Laccase catalyzes the oxidation of selected aromatic compounds in
the presence of oxygen. This research parallels previous work done using peroxidase enzymes in which hydrogen peroxide was used as the oxidant. Horseradish peroxidase (HRP). Coprinus macrorhizus peroxidase (CMP), Arthromyces ramosus peroxidase (ARP), and soybean peroxidase (SBP) have been shown to be effective in the removal of several phenolic compounds from wastewater (Kilbanov et al., 1980, 1981; Al-Kassim et al., 1993, 1994; McEldoon et al., 1995; Taylor et al., 1996, 1998). This study with laccase was conducted to determine its suitability to be used analogously in wastewater treatment for selected aromatic compounds.

1.2 Objectives

The objectives of this study were to optimize the reaction parameters in unbuffered tap water to achieve at least 90% removal of cresols by using laccase and to determine the stability of laccase activity at room temperature over time.

1.3 Scope

The scope of this study included the following:

1. Aromatic compounds studied were o-, m-, and p-cresols.

2. Reaction parameters optimized were pH and enzyme dose.

3. The effects of PEG and hydrogen peroxide addition on the removal of substrates were determined.

4. The effects of dissolved oxygen availability in the solution were investigated.

5. The stability of laccase activity at room temperature over time was determined.
2.0 LITERATURE REVIEW

2.1 Cresols as Pollutants

2.1.1 Physical and Chemical Properties

Physically, cresols consist of either a white crystalline solid or a yellowish liquid with a strong phenol-like odour. The compounds are highly flammable, moderately soluble in water and soluble in ethanol, ether, acetone, or alkali hydroxides. Cresols undergo electrophilic substitution reactions at the vacant ortho- or para- positions or undergo condensation reactions with aldehydes, ketones, or dienes (WHO, 1996). A summary of the physical and chemical properties of cresols is given in Table 2.1.

2.1.2 Sources and Applications

Cresols and cresol derivatives occur naturally in the oils of various flowering plants and trees such as jasmine, easter lily, yucca, conifers, oaks, and sandalwoods. They are also a product of combustion from natural fires associated with lightning, spontaneous combustion, and volcanic activity.

Cresols are used as solvents, disinfectants, and in the production of fragrances, antioxidants, dyes, pesticides, resins, and as wood preservatives. Commercially, cresols are produced as by-products in the fractional distillation of crude oil and coal tars and in coal gasification. Small amounts of cresols are also produced in petroleum powered vehicle exhaust, municipal waste incinerators and from the combustion of wood, coal, and cigarettes (WHO, 1996).
The three isomers of cresol in pure forms or mixtures of them have a variety of uses. Crude cresols are used as wood preservatives. The isomer o-cresol is used directly as either a solvent or disinfectant. It is also used as a chemical intermediate for a variety

<table>
<thead>
<tr>
<th>Property</th>
<th>o-Cresol</th>
<th>m-Cresol</th>
<th>p-Cresol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical state and colour</td>
<td>white crystalline solid or yellowish liquid</td>
<td>colourless to yellowish liquid</td>
<td>crystalline solid or yellowish liquid</td>
</tr>
<tr>
<td>Odour</td>
<td>phenol-like</td>
<td>phenol-like</td>
<td>phenol-like</td>
</tr>
<tr>
<td>Air odour threshold (mg/m³)</td>
<td>1.4</td>
<td>0.007</td>
<td>0.004</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>30.9</td>
<td>12.2</td>
<td>34.7</td>
</tr>
<tr>
<td>Boiling point at 1 atm (°C)</td>
<td>191.0</td>
<td>202.3</td>
<td>201.9</td>
</tr>
<tr>
<td>Vapour pressure at 25°C (mmHg)</td>
<td>0.31</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>Relative density at 25°C (kg/m³)</td>
<td>1135</td>
<td>1030</td>
<td>1154</td>
</tr>
<tr>
<td>Vapour density (air = 1 at 20°C)</td>
<td>3.7</td>
<td>3.72</td>
<td>3.72</td>
</tr>
<tr>
<td>Solubility in water at 25°C (g/L)</td>
<td>25.95</td>
<td>22.70</td>
<td>21.52</td>
</tr>
<tr>
<td>pK₉₅ (25°C)</td>
<td>10.29</td>
<td>10.09</td>
<td>10.26</td>
</tr>
<tr>
<td>Bioconcentration factors</td>
<td>14.1</td>
<td>19.9</td>
<td>no data</td>
</tr>
<tr>
<td>Odour threshold in water (mg/L)</td>
<td>1.4</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Taste threshold concentration in water (mg/L)</td>
<td>0.003</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Saturation concentration in air at 20°C (g/m³)</td>
<td>1.2</td>
<td>0.24</td>
<td>0.24</td>
</tr>
</tbody>
</table>
of products, including deodorizing and odour-enhancing compounds, pharmaceuticals, fragrances, antioxidants, dyes, pesticides and resins. *para*-Cresol is mainly used in the formulation of antioxidants for lubricating oils, motor fuels, rubber, polymers, elastomers, and food products. It is also used as an intermediate in fragrance and dye production. *meta*-Cresol either pure or mixed with *para*-cresol is necessary in the production of herbicides and insecticides. Many flavour and fragrance compounds and several antioxidants are produced from *m*-cresol. It is also used in the manufacture of explosives. Mixtures of cresols are used as monomers for synthetic resin coatings in wire insulation, as well as solvent components of metal degreasers, cutting oils, and agents to remove carbon deposits from combustion engines (WHO, 1995).

2.1.3 Levels of Exposure

Cresols have been detected in ambient air, surface- and groundwater, and wastewater. They have also been detected in food and beverages. Cresols are rapidly absorbed by inhalation, ingestion, and dermal contact and are readily distributed throughout the body. The primary route of elimination is through the urine. No information is available regarding the effects of chronic exposure to cresols. Based on the results of short-term studies, an acceptable daily intake, ADI, of 0.17 mg/kg body mass per day has been established for cresols as a guidance value (WHO, 1995).

Cresols, at concentrations normally found in the environment, pose no significant risk for the general population. However, the potential for adverse health effects exists for specific sub-populations and under conditions of high exposure (WHO, 1996). Occupational exposure to cresols is likely among workers involved in the production of
cresols or processes that produce cresols as by-products and those who use cresols or products containing cresols. The exposure limit for occupational air in the USA has been set at 22.1 mg/m$^3$ (5ppm) for time-weighted average (TWA) exposure for all isomers of cresol in 1989 (WHO, 1996).

2.2 Wastewater Treatment

Wastewater discharge of cresols is created from the production and use of cresols. Due to the toxicity of cresol to the environment and humans, it is necessary to treat the wastewater streams from those industries that emit cresol in their waste streams.

2.2.1 Conventional Methods

There are several methods of wastewater treatment which have been used effectively. Many factors need to be evaluated when deciding which method to employ. These factors include effluent requirements, the formation of by-products, and economic feasibility. Some examples of current treatment methods which are available for the treatment of wastewaters containing aromatic compounds are listed in Table 2.2 (Nicell, 1991).

These current methods, involving physical, chemical, and biological techniques, suffer from many drawbacks including high cost, hazardous by-product formation, and poor efficiency (Kilbanov et al., 1980). These disadvantages encourage the development of more efficient and cost effective technologies.
<table>
<thead>
<tr>
<th>Recovery Systems</th>
<th>Countercurrent extractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pulsed column extraction</td>
</tr>
<tr>
<td><strong>Physical/Chemical</strong></td>
<td>Chlorine oxidation</td>
</tr>
<tr>
<td><strong>Treatment Systems</strong></td>
<td>Chlorine dioxide oxidation (as sodium chlorite)</td>
</tr>
<tr>
<td></td>
<td>Ozone oxidation</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide oxidation (Fenton’s reagent)</td>
</tr>
<tr>
<td></td>
<td>Potassium permanganate oxidation</td>
</tr>
<tr>
<td></td>
<td>Incineration</td>
</tr>
<tr>
<td></td>
<td>Hydrocarbon stripping and combustion</td>
</tr>
<tr>
<td></td>
<td>Photocatalytic oxidation</td>
</tr>
<tr>
<td></td>
<td>Activated carbon adsorption</td>
</tr>
<tr>
<td></td>
<td>Landfilling</td>
</tr>
<tr>
<td></td>
<td>Coagulation</td>
</tr>
<tr>
<td><strong>Biological Treatment</strong></td>
<td>Bio-oxidation pond</td>
</tr>
<tr>
<td><strong>Systems</strong></td>
<td>Aerated lagoon</td>
</tr>
<tr>
<td></td>
<td>Stabilization pond</td>
</tr>
<tr>
<td></td>
<td>Oxidation ditch</td>
</tr>
<tr>
<td></td>
<td>Trickling filter</td>
</tr>
<tr>
<td></td>
<td>Activated sludge</td>
</tr>
<tr>
<td></td>
<td>Rotating biological contactors</td>
</tr>
</tbody>
</table>
2.2.2 Enzyme-Based Technology

Enzymes that have been isolated from their parent organisms are often preferred over intact organisms containing the enzyme because the isolated enzymes act with greater specificity, which allows specific groups of pollutants to be targeted. In addition, the potency of enzymes can be better standardized, they are easier to handle and store than microorganisms (Vieth and Venkatasubramanian, 1973) and enzyme concentration is not dependent on bacterial growth rates (Bailey and Ollis, 1986). Enzymes also offer a number of advantages over conventional chemical catalysts including a high degree of specificity, operation under mild conditions, and a high reaction velocity which reduces processing costs (Nicell, 1991).

Improvements in the technology to produce, isolate, and purify enzymes are enabling enzymes to be applied in new commercial uses including the development of an enzyme-based wastewater treatment. The potential advantages of an enzyme-based treatment over conventional physical and chemical processes, as noted by Nicell (1991), include:

- operation under milder, less corrosive conditions
- operation in a catalytic manner
- operation on trace level organic compounds and on organics not removed by existing physical / chemical processes
- reduced consumption of oxidants
- reduced amounts of adsorbent materials for disposal.

Also noted by Nicell (1991) are the potential advantages of an enzyme-based treatment over conventional biological treatment which include:

- application to a broad range of compounds
- action on, or in the presence of, many substances which are toxic to microbes
- operation over wide temperature, pH, and salinity ranges
- operation both at high and low concentrations of contaminants
- no shock loading effects
- no delays associated with shutdown/startup (acclimatization of biomass)
- reduction in sludge volume (no biomass generation)
- better defined systems with simpler process control.

An enzyme-based technology is a flexible system capable of treating a broad range of situations that may be encountered. This type of technology is advantageous because wastewater can vary widely in parameters such as temperature, pH, and level of other solutes and particulates.

2.3 Enzyme Kinetics

Enzymes are catalysts that accelerate a chemical reaction, in most cases by reducing the energy of activation. The reactant physically binds to the enzyme at an active site, forming a metastable complex. The reaction is assumed to be rapid and reversible, with no chemical changes occurring to the substrate. The transition state, accessible from this complex, is different from that involved in the uncatalysed reaction in that it is more stable and of lower energy. For catalysed and uncatalysed reactions, the amount of free energy liberated or taken up when a reaction has been completed will be the same, as well as the free energy levels of the initial and final states (Palmer, 1981). After the enzyme and substrate combine, the enzyme-substrate complex undergoes a chemical change, resulting in the formation of product and the release of product from the enzyme, with a first order dependence on the concentration of the enzyme-substrate complex (Blanch and Clark, 1996):

\[ E + S \rightleftharpoons ES \rightleftharpoons E + P \]  

Eq. 2.1
The kinetic feature that most distinguishes enzyme-catalysed reactions from simple chemical reactions is that they show saturation. Nearly all enzyme-catalysed reactions show a first-order dependence of rate on substrate concentration at very low concentrations, but instead of increasing indefinitely as the concentration increases, the rate approaches a limit at which there is no dependence of rate on substrate concentration and the reaction becomes zero-order with respect to substrate. (Cornish-Bowden et al., 1988). This was found to be generally true for all single-substrate enzyme-catalyzed reactions and for multi-substrate reactions where the concentrations of all the substrates but one were kept constant (Palmer, 1981).

Initial velocity studies have been found useful for investigating the kinetics of enzyme-catalyzed reactions. Initial velocity is an important kinetic parameter because it is determined for the reaction in a situation that can be easily specified. The initial velocity of a reaction is usually determined from a plot of either substrate disappearance or product formation against time as the reaction proceeds. If there is a difference between substrate and product in the absorbance of light of a particular wavelength, spectrophotometric or colorimetric techniques may be used. Based on proportionality, the actual concentrations of a substance can be calculated from absorbance readings and be plotted against time (Palmer, 1981). Alternatively, absorbance units may be plotted against time.

Usually, at the start of a reaction, the concentrations of the reactants are known and there are no products present. Therefore, by starting with the reactants and measuring the appearance of products with time, a graph of the form represented in Figure 2.1 is obtained.
Figure 2.1: Graph of Product Concentration Against Time for a Chemical Reaction. (Palmer, 1981)

The initial velocity ($v_0$) of the reaction is the reaction rate at $t = 0$ and may be determined by drawing a tangent to the graph at time zero or by obtaining a derivative with the limit approaching zero. The slope of this tangent, $d[P]/dt$, is $v_0$, where the units of $v_0$ are those used for product concentration divided by the units used for time (Palmer, 1981). Maximum initial velocity is dependent on enzyme concentration. (Palmer, 1981). By restricting investigations to initial velocity determinations, errors caused by loss of enzyme activity with time may be avoided.

A graph of initial velocity ($v_0$) against initial substrate concentration ($[S]_0$) at constant total enzyme concentration ($[E]_0$) was found to be a rectangular hyperbola (Palmer, 1981) as is shown in Figure 2.2. Although some enzymes do not give hyperbolic graphs, the attainment of a maximum initial velocity with increasing substrate concentration at constant total enzyme concentration is characteristic of many enzymes. The initial velocity of enzyme-catalyzed reactions has a limiting value at each total enzyme concentration; this occurs when the enzyme is saturated with substrate. The
overall rate of reaction (formation of P) must be limited by the amount of enzyme available and by the rate of breakdown of the enzyme-substrate complex.

![Diagram](image)

**Figure 2.2: The Initial Rate of Reaction as a Function of Substrate and Enzyme Concentrations**
*(Palmer, 1981)*

The Michaelis-Menten equation is the cornerstone of the understanding of enzyme kinetics (Blanch and Clark, 1996) originally invoked to explain the phenomenon of saturation. The equation relates the initial rate to the initial substrate concentration by the hyperbolic function:

\[ v_0 = \frac{V_{\text{max}}[S]_0}{K_M + [S]_0} \]

Eq. 2.2

where \( V_{\text{max}} \) is the limiting initial velocity at saturation and \( K_M \) is known as the Michaelis constant. The Michaelis constant is the initial substrate concentration giving an initial
velocity equal to $V_{\text{max}}/2$. The form of this equation provides an explanation for the observed hyperbolic dependence of initial rate on substrate concentration and the linear dependence on enzyme concentration.

2.4 **Enzyme Stability**

Enzymes may easily become denatured and lose catalytic activity. In general, temperatures higher than 40°C and extremes of pH, below pH 5 and above pH 9, should be avoided. Enzymes should be stored at low temperatures. At room temperature, most enzymes are denatured by high concentrations of organic solvents such as ethanol or acetone. These factors should be taken into consideration when storing or handling enzymes. During pH adjustment or changes in organic solvent concentration, continuous mixing prevents the localised denaturation of enzymes (Palmer, 1981). Depending on the nature of the enzyme preparation, freezing and thawing may cause loss of activity. Storing frozen enzyme solutions in portions is best to avoid repeated freezing and thawing.

2.5 **Criteria for Successful Enzymatic Watewater Treatment**

To date, most of the work that has been reported on enzyme-catalyzed transformations of pollutants has focused on the disappearance of the pollutant from solution (Taylor et al., 1996, 1998). Minimal research has been done on process design issues or on performance criteria other than target compound removal. These issues are important in developing the technology further. The following criteria, given by Aitken
(1993), need to be met in order for enzymatic waste treatment processes to be considered technically and economically feasible.

1. The reaction products must be less toxic, more biodegradable or otherwise more amenable to subsequent treatment than is the case for the parent compound.

2. The enzymes should selectively attack target compounds in a waste mixture.

3. The enzyme must exhibit a reasonable amount of its native activity under typical treatment conditions since only limited control over the reaction conditions such as temperature, pH, and waste composition can be expected to be economical in waste treatment.

4. Reactors for enzymatic processes must be relatively simple since the acceptance of a technology corresponds to the simplicity of the technology.

5. The enzyme must be relatively stable under the required reaction conditions.

6. The enzyme of interest must be available.

2.6 Candidate Enzymes

Aside from technical feasibility, the development of any commercial application of enzymes requires the consideration of cost. For some enzymes, potential cost due to their properties prevents their development into a feasible treatment. Enzymes that require cofactors such as the adenosine phosphates, the pyridine nucleotides or their reduced forms cannot be candidates for commercial application until economical means of retaining and regenerating these cofactors are developed. Also, intracellular enzymes require more processing to achieve marketable form than do extracellular enzymes.

The most studied enzymes in terms of work directed specifically at waste treatment applications are the organophosphate pesticide hydrolases and phenol oxidizing enzymes including laccase, peroxidases, and polyphenol oxidase.
2.7 Treatment of Wastewater with HRP, ARP, and SBP

The applicability of an enzyme-based treatment technology for the removal of aromatic compounds in an aqueous mixture has been studied by many researchers. Kilbanov et al. (1980) were first to propose a treatment method involving horseradish peroxidase (HRP) which was used to remove over 30 different phenols and aromatic amines from water with removal efficiencies for some pollutants exceeding 99% (Caza et al., 1999). Although HRP is the peroxidase that has been most researched, other peroxidases, such as Coprinus macrorhizus peroxidase (CMP), Arthromyces ramosus peroxidase (ARP), and soybean peroxidase (SBP) have proven to be very effective in removing phenolic compounds from water (Al-Kassim et al., 1993, 1994; McEldoon et al., 1995; Taylor et al., 1996, 1998).

These research findings show a very promising future for the removal of certain aromatic compounds through enzymatic treatment of wastewater.

2.8 Use of Laccase in Wastewater Treatment

Laccase catalyzes the oxidation of selected aromatic compounds in the presence of oxygen. The product of the enzymatic reaction is a free radical which subsequently couples with another radical to form various dimers. The dimers can undergo further enzymatic cycles to eventually form insoluble polymers that are readily removed from solution. The reaction mechanism is similar to that for peroxidases (Yu et al., 1994).

Laccase is an oxidoreductive enzyme that requires bimolecular oxygen but no coenzyme for activity (Bollag, 1992). Although a few laccases have been isolated from plant sources, e.g., lacquer, sycamore, and tobacco, most of the known laccases have
fungal origins, e.g., white-rot fungi. Laccases are believed to participate in various cellular and microbial activities such as conidial pigment formation, lignin biosynthesis and degradation, and fungal pathogenicity (Schneider et al., 1999). Progress has been made in explaining the structure of the copper sites, the catalytic amino acid residues, and the kinetic mechanism of laccases. Laccases are being studied for potential industrial uses such as delignification, wood fiber modification, dye or stain bleaching, chemical/medicinal synthesis, and contaminated water/soil remediation (Schneider et al., 1999).

Laccases from various soil micro-organisms have been shown by Bollag and coworkers (Hoff et al., 1985) to cause oligomerization of phenols and anilines. Oxidoreductive enzymes play a major role in the coupling of pollutants to humic substances. This observation has led to the idea that enzymatic coupling may represent a means for removal or detoxification of certain chemicals in water environments. Once coupled or bound, the compounds become part of the humic organic phase in water and their activity, fate, and toxicity change radically (Sarkar et al., 1988).

Dec and Bollag (1990) showed that laccase from the fungi *Rhizoctonia prunicola* and horseradish peroxidase were effective in polymerizing several phenols producing a mixture of oligomers with average molecular masses of up to 800 for the fraction soluble in dioxane. They also observed dehalogenation of chlorinated phenols by oxidoreductive enzymes, including laccase (Dec and Bollag, 1994, 1995). Laccases may prove to be the most useful of the oxidoreductases because they produce very reactive radicals and because, unlike peroxidases, they do not require the presence of hydrogen peroxide (Bollag, 1992).
3.0  MATERIALS AND METHODS

3.1  Materials

Laccase (SP 850, Batch No. US Enz -142) was received from Novo Nordisk, Franklinton, NC. The activity of this preparation was nominally 1170 LAMU/mL. A laccase unit (LAMU) of activity catalyzes the conversion of 1 μmole of syringaldazine per minute in a 19 μM solution at pH 7.5 and temperature 30°C. The enzyme stock was stored frozen at a temperature of -15°C, and solution in use was stored in a refrigerator at 4°C.

Catalase (EC 1.11.1.6, 15000 units/mg solid) was purchased from Sigma Chemical Co., St. Louis, MO. One unit of catalase decomposes 1.0 μmole of H₂O₂ per minute at pH 7.0 and 25°C. Catalase was stored as dry powder in the freezer at -15°C until needed, while the aqueous stock solution was prepared when needed and stored at 4°C.

Polyethylene glycol (average molecular mass of 3350 g/mol) was purchased from Sigma Chemical Co., St. Louis, MO.

Hydrogen peroxide (30% mass/volume) was purchased from BDH Inc., Toronto, ON. A 100 mM hydrogen peroxide stock solution was prepared as needed and stored at 4°C.

The buffers were prepared according to the method of Gomori (1955). Acetate and phosphate buffers were used and their corresponding pH ranges in which they were applied are listed in Table 3-1.
All cresols had a purity of 99% or greater and were supplied by Aldrich Chemical Co., Milwaukee, WI. For o-, m-, and p-cresols, 1 mM is equivalent to 108 mg/L. Stock solutions were prepared and stored at room temperature.

**Table 3.1: Buffers Used and Their Applied pH Ranges**

<table>
<thead>
<tr>
<th>Buffering Reagents</th>
<th>pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid – Sodium acetate</td>
<td>4.4 – 5.6</td>
</tr>
<tr>
<td>Monobasic sodium phosphate - Dibasic sodium phosphate</td>
<td>5.8 – 7.1</td>
</tr>
</tbody>
</table>

3.2 **Analytical Equipment**

The pH of the samples was measured with an Expandable Ion Analyzer, Model EA 940, manufactured by Orion Research. The standard buffer solutions of pH 4.0, 7.0, and 10.0 were obtained from BDH Inc., Toronto, ON.

Dissolved oxygen in the reactors was measured using a dissolved oxygen probe, Model 57, from Yellow Springs Instrument Co. Inc., Yellow Springs, OH.

An IEC Centra-8 Centrifuge, purchased from International Equipment Company, USA, was used for centrifugation to experimental details.

Colour absorbance of the samples was measured by using a Hewlett Packard, Model 8452A, Diode Array Spectrophotometer, with a wavelength range of 190 to 820 nm and a 2 nm resolution. The spectrophotometer was controlled from a Hewlett Packard Vectra ES/12 computer. Polystyrene disposable semi-micro cuvettes were
purchased from Bio-Rad Laboratories, Hercules, CA. Quartz cells with a 10 mm path length were supplied by Hellma (Canada) Ltd.

3.3 Experimental Procedure

This study was designed to achieve at least 90% removal of the aromatic substrate by optimizing the effects of pH, enzyme dose, PEG addition, dissolved oxygen availability, and hydrogen peroxide addition. The stability of laccase at room temperature over time was also observed.

The experiments were conducted at a room temperature of 21 ± 2°C in batch reactors that contained tap water, laccase, buffer if necessary, and aromatic substrate. All of the reactors were stirred vigorously using a magnetic stirrer and Teflon coated stir bars. Enzyme was added to initiate the reaction. For the purpose of this study, reaction times from 3 to 6 hours were chosen for convenience and were considered to be sufficient times based on previous similar experiments with enzymes (Kilbanov et al., 1980).

Afterwards, for the pH, enzyme dose, and PEG addition experiments, alum was added to a final level of 50 mg/L and the pH of the solutions was adjusted between 6.3 and 8.0. The contents of the reactors were gently stirred for 10 minutes to promote coagulation. Approximately 5 mL of each sample were withdrawn and centrifuged for 30 minutes at 3000 rpm. The supernatant was analyzed for remaining substrate by the colorimetric or direct spectrophotometric method.
For the experiments on dissolved oxygen availability, alum and centrifugation were not used. Samples from the reactors were withdrawn throughout the course of the experiments and were analyzed for remaining cresol concentration.

The following sections describe the procedures used to determine the effects of the parameters studied and enzyme stability.

3.3.1 pH

The first parameter optimized was pH. The batch reactors were 30 mL glass vials that contained a mixture of tap water, buffer, aromatic substrate, and laccase enzyme. The substrate concentration was 1 mM for all the cresol compounds. Laccase dose was approximately 1.0 LAMU/mL of reactor volume. The pH was fixed with 50 mM buffer solution. For the subsequent experiments conducted to study the effects of other parameters, buffer was not used in experiments with o- or m-cresols.

3.3.2 Enzyme Dose

These tests were conducted at the previously determined optimum pH. Concentrated hydrochloric acid or sodium hydroxide was used to adjust pH. Laccase was added in predetermined amounts in order to determine the minimum laccase dose required to achieve the highest removal of substrate in a fixed reaction time.

3.3.3 PEG Addition

A 10 g/L stock solution of PEG was prepared and stored at room temperature.
The optimum pH was used and the laccase enzyme dose was kept at a less than sufficient
doze to determine the effects of the PEG addition. Samples were collected, centrifuged,
and analyzed for aromatic compounds remaining.

3.3.4 Dissolved Oxygen Availability

The effects of oxygen on the reactions were determined in 133 mL glass vials due
to the large size of the dissolved oxygen probe. The reactor contents were scaled-up from
the volumes used in the 30 mL reactors and the optimum pH was used. In time intervals,
the dissolved oxygen in the reactor solution was measured with the probe and samples
were withdrawn and analyzed for remaining cresol concentration.

(a) Diffusion of Atmospheric Oxygen

The effect of the absorption of atmospheric oxygen on the removal of aromatic
substrates was observed by measuring the dissolved oxygen and the presence of substrate
in an open reactor compared with the results obtained in a closed reactor.

(b) Aeration

Air was bubbled into a reactor to increase the dissolved oxygen content of the
reaction solution. A control was used without the air addition and the dissolved oxygen
values and cresol concentrations were compared.
(c) Hydrogen Peroxide Addition

The addition of hydrogen peroxide to the reaction mixture causes the production of dissolved oxygen in the mixture due to a reaction with the laccase enzyme or a contaminating catalase. A control was used that differed from the experimental reactor by the absence of hydrogen peroxide. The dissolved oxygen probe was used to monitor the change in dissolved oxygen concentration while samples were withdrawn for analysis to observe the reduction in cresol concentration.

(d) Hydrogen Peroxide and Catalase Addition

The reaction of hydrogen peroxide with catalase and/or laccase causes the production of dissolved oxygen as stated above. The effects of catalase with hydrogen peroxide were observed by comparing dissolved oxygen and cresol concentrations in the experimental reactor against a catalase-free control and against a control free of both catalase and hydrogen peroxide.

3.3.5 Laccase Stability

The stability of the laccase enzyme at room temperature over time was observed by performing an experiment in a batch reactor containing substrate and enzyme. Samples from the reactor were withdrawn in approximately 2-hour intervals and the activity of the enzyme was measured. This was done for 6.5 hours since the longest experiment conducted in this research was for approximately 6 hours duration.
3.4 Analytical Methods

3.4.1 Laccase Activity Assay

The laccase enzyme activity was measured by using an assay based on syringaldazine as a substrate in which syringaldazine was oxidized to the corresponding quinone under aerobic conditions. All components except enzyme were provided in excess so that the initial rate of reaction became directly proportional to the amount of enzyme present.

The assay mixture consisted of 850 µL of 25 mM Tris buffer (pH 7.5), 50 µL of 0.38 mM syringaldazine solution, and 100 µL of enzyme solution. Immediately following the addition of enzyme, the cuvette was shaken and placed in the spectrophotometer where the change in absorbance with time was monitored at a peak wavelength of 510 nm. A detailed description of the method is given in Appendix A.

3.4.2 Aromatic Compound Concentration Assay

The aromatic compound concentration in the samples was determined by either the colorimetric method or the direct spectrophotometric method. The colorimetric method used ferricyanide reagent and 4-aminoantipyrine (AAP) reagent as colour generating substrates when combined with the cresol compounds. The assay mixture in the plastic cuvette consisted of 100 µL of 83.4 mM ferricyanide solution in 0.25 M NaHCO₃ (aq), 100 µL of 20.8 mM AAP solution in 0.25 M NaHCO₃ (aq), 100 to 800 µL of cresol sample, and deionized water to bring the total volume to 1000 µL. The results with o- and m-cresols showed that absorbance of the colour generated at the peak
wavelength of 510 nm was directly proportional to the concentration of the aromatic substrate. A detailed description for the colorimetric method can be found in Appendix B.

The results showed that the relationship between absorbance and concentration was not linear for p-cresol in the colorimetric method. Consequently, the direct spectrophotometric method was used for this compound. The direct spectrophotometric method was based on the absorbance of ultraviolet (UV) light by phenols. Phenolic compounds, such as p-cresol, absorb ultraviolet light at a maximum wavelength between 270 and 284 nm in a quantity directly proportional to the concentration of the phenolic compound in solution. For p-cresol, the maximum wavelength was 276 nm. The assay mixture in the quartz cuvette consisted of the p-cresol sample and deionized water to a total volume of 1000 μL. The concentrations of the substrates are expressed in terms of molar quantities, where one millimolar is equal to 108 mg/L of cresols. Appendix C contains the colorimetric calibration curves for o- and m- cresols and the direct spectrophotometric calibration curve for p-cresol.

3.5 Sources of Error

To help minimize random errors, some procedures were done more than once to check reproducibility. For instance, calibration curves were repeated and compared to verify accuracy. Also, some experiments were done more than once to check reliability and reproducibility of results.

However, systematic errors due to analytical techniques and instruments may have occurred. One possible systematic error is the analysis of cresol concentration in the samples. The products of the enzymatic reaction are mostly phenolic compounds. If
a portion of these reaction products remained in solution after centrifugation and/or filtering of the sample, they would absorb light at the same wavelength as the cresol being analyzed. This would lead to results showing higher levels of cresol remaining in the sample than what actually was present. This error could occur in both the colorimetric and the direct spectrophotometric methods.
4.0 RESULTS AND DISCUSSION

The objective of this research was to optimize the reaction parameters in unbuffered tap water to achieve at least 90% removal of cresols by using the enzyme laccase. The reaction parameters that were optimized were pH and enzyme dose. The effects of PEG addition, dissolved oxygen availability, hydrogen peroxide addition, and presence of catalase were also studied. In addition, the stability of the activity of the laccase enzyme at room temperature over time was determined.

For the compound \( p \)-cresol, accurate measurements by the direct spectrophotometric technique were possible only when the samples were first filtered and then mixed with 0.2 M sodium hydroxide solution. This procedure removed the turbidity present in the samples and enabled accurate analysis. The calibration curve for \( p \)-cresol was done using the same base.

Removal efficiencies for both \( o \)- and \( m \)-cresols were slightly improved when samples were filtered before analysis. However, the 15% difference that results showed may not be significant to justify incorporation of a filtration unit into a full-sized wastewater treatment plant.

4.1 pH

The optimum pH for each of the cresols tested was determined using a pH range of about 4.5 to 9.5. The initial substrate concentration was 1.0 mM and the laccase enzyme dose was approximately 1.0 LAMU/mL. Substrate and enzyme doses were kept constant in each reactor so that removal efficiency in each reactor depended only on pH.
Different buffers were prepared to obtain each pH tested. The reaction time was kept at 3 hours, which was considered to be sufficient time based on previous similar experiments with enzymes (Kilbanov et al., 1980).

Removal efficiencies as a function of pH are shown in Figures 4.1.1 through 4.1.3. For o- and m-cresols, the optimum pH occurred at near-neutral conditions. The optimal pH for p-cresol was around 5.6. Since the optimum pH range for substrate removal for o- and m-cresols was similar to the pH of the tap water, remaining experiments were performed without using a buffer for these two compounds. A 50 mM buffer solution was used for experiments with p-cresol.

4.2 Enzyme Dose

The optimum enzyme doses for the cresols were determined based on a 6-hour reaction time at optimal pH values. Initial substrate concentrations were 1.0 mM. The results for o-, m-, and p-cresols are presented in Figure 4.2.1. For o-cresol, a minimum dose of 0.5 LAMU/mL of reactor solution was required to remove nearly 90% of the substrate in a six-hour reaction time. For p-cresol, 0.3 LAMU/mL of reactor solution was required to obtain the maximum of 80% substrate removal, while m-cresol showed the maximum of 70% removal with 0.6 LAMU/mL of reactor solution. Additional substrate removal would have been achieved with filtration of the sample prior to analysis. Higher enzyme doses would be required to treat higher substrate concentrations.

Further experiments were conducted to observe the effect of adding enzyme dose in series. An initial m-cresol concentration of 2 mM was reacted at optimal pH in an open reactor. The control reactor received one dose of enzyme at the start of the reaction.
Figure 4.1.1: Effect of pH on the Removal of o-Cresol

Reactor Conditions:
Substrate: 1 mM o-cresol
Enzyme dose: 1.01 LAMU/mL
Buffer: 50 mM
Volume: 30 mL
Reaction time: 3 h
Figure 4.1.2: Effect of pH on the Removal of m-Cresol

**Reactor Conditions:**
- Substrate: 1 mM m-cresol
- Enzyme dose: 1.34 LAMU/mL
- Buffer: 50 mM
- Volume: 30 mL
- Reaction time: 3 h
Figure 4.1.3: Effect of pH on the Removal of p-Cresol

Reactor Conditions:
Substrate: 1 mM p-cresol
Enzyme dose: 0.97 LAMU/mL
Buffer: 50 mM
Volume: 30 mL
Reaction time: 3 h
Figure 4.2.1: Effect of Laccase Dose on the Removal of o-, m-, and p-Cresols

Reactor Conditions:
Substrate: 1 mM
Buffer: 50 mM
o- and m- cresols: pH = 7.0
p-cresol: pH = 5.6
Volume: 30 mL
Reaction time: 6 h
The other reactor received four doses, each dose being one fourth of the dose in the control, at approximately one-hour intervals. Results are shown in Figure 4.2.2. Adding enzyme in series did not improve the removal of the substrate. Maximum substrate removal was achieved by adding the full enzyme dose at the start of the reaction.

4.3 PEG Addition

The addition of PEG to each of the reactors containing different cresols had no effect on the removal efficiencies of the substrates. Concentrations of PEG from 50 mg/L to 400 mg/L were tested. The addition of PEG was investigated because in certain previous studies, PEG was found to be effective in improving removal efficiencies in peroxidase-catalyzed reactions (Wu et al., 1997; Caza et al., 1999).

4.4 Dissolved Oxygen Availability

Since laccase uses oxygen as the oxidant, experiments were conducted to determine the effect of changing the dissolved oxygen concentration in the reactor. This change in dissolved oxygen was accomplished by natural diffusion from the atmosphere in an open reactor and by physically adding oxygen to the solutions by aeration. Reactors that were closed, were briefly opened to withdraw samples. No headspace was present above the reaction solution, otherwise. Natural diffusion into the reactor was assumed negligible. All of the experiments were started with a dissolved oxygen concentration near saturation, that is, about 0.25 mM oxygen. Since the stoichiometric requirement of dissolved oxygen for cresols is 1:4, the saturation concentration in the reactor can theoretically remove up to 1 mM of cresol without requiring additional dissolved oxygen.
**Figure 4.2.2: Effect of Series Addition of Enzyme on Substrate Removal**

**Reactor Conditions:**
Substrate: 2 mM m-cresol  
Enzyme single dose: 3.0 LAMU/mL  
Series = 0.75 LAMU/mL/dose  
Series doses: 4  
pH = 7.0  
Volume: 133 mL
4.4.1 Diffusion of Atmospheric Oxygen

It was observed that cresol concentrations up to 1 mM showed no effect on substrate removal from the addition of dissolved oxygen through diffusion of atmospheric oxygen (Figure 4.4.1). The effect on dissolved oxygen for this experiment is shown in Figure 4.4.2. However, with higher cresol concentrations, better substrate removal was achieved in a reaction where the reactor was kept open to the atmosphere. The continuous absorption of oxygen into the reaction solution increased the efficiency of the reaction. The reactions were carried out with initial substrate concentrations of 2 mM at optimal pH. Figures 4.4.3 and 4.4.4 show the effect of natural diffusion from the atmosphere on substrate removal for \textit{m}- and \textit{o}-cresols, respectively. The effect on dissolved oxygen in these same reactions is shown in Figures 4.4.5 and 4.4.6, respectively for \textit{m}- and \textit{o}-cresols.

It was demonstrated that the rate of substrate removal in both reactors was the same as long as there was dissolved oxygen present. With the complete depletion of dissolved oxygen in the closed reactors, the substrate removal had stopped. Continuous addition of dissolved oxygen into the open reactors resulted in corresponding removal of substrate.

Preliminary results were obtained for an experiment performed on \textit{p}-cresol for the effect of absorption of oxygen from the atmosphere on substrate removal. The reaction was performed with an initial substrate concentration of 2 mM at the optimum pH of 5.6. Due to equipment constraints, the results were incomplete and a plot of substrate removal could not be generated. However, the depletion of dissolved oxygen is shown in Figure 4.4.7 for the first 25 minutes of reaction. The percents of initial concentration of \textit{p}-cresol
**Reactor Conditions:**

Substrate: 1 mM o-cresol  
Enzyme dose: 0.97 LAMU/mL  
\( pH = 7.0 \)  
Volume: 133 mL

**Figure 4.4.1: Effect of Atmospheric Diffusion on the Removal of o-Cresol**
Figure 4.4.2: Effect of Atmospheric Diffusion on Dissolved Oxygen Availability

**Reactor Conditions:**
- same reaction as Fig. 4.4.1
- Substrate: 1 mM o-cresol
- Enzyme dose: 0.97 LAMU/mL
- pH = 7.0
- Volume: 133 mL
Reactor Conditions:
Substrate: 2 mM m-cresol
Enzyme dose: 1.47 LAMU/mL
pH = 7.0
Volume: 133 mL

Figure 4.4.3: Effect of Atmospheric Diffusion on the Removal of m-Cresol
Figure 4.4.4: Effect of Atmospheric Diffusion on the Removal of o-Cresol

Reactor Conditions:
- Substrate: 2 mM o-cresol
- Enzyme dose: 0.74 LAMU/mL
- pH = 7.0
- Volume: 133 mL
**Reactor Conditions:**
- Same reaction as Fig. 4.4.3
- Substrate: 2 mM m-cresol
- Enzyme dose: 1.47 LAMU/mL
- pH = 7.0
- Volume: 133 mL

Figure 4.4.5: Effect of Atmospheric Diffusion on Dissolved Oxygen Availability
Reactor Conditions:
same reaction as Fig. 4.4.4
Substrate: 2 mM o-cresol
Enzyme dose: 0.74 LAMU/mL
pH = 7.0
Volume: 133 mL

Figure 4.4.6: Effect of Atmospheric Diffusion on Dissolved Oxygen Availability
Reactor Conditions:
Substrate: 2 mM p-cresol
Enzyme dose: 1.47 LAMU/mL
pH = 5.6
Volume: 133 mL

Figure 4.4.7: Effect of Atmospheric Diffusion on the Removal of p-Cresol
remaining after 25 minutes of reaction were 98.7% for the closed reactor and 93.6% for the open reactor. These results display the similar pattern demonstrated by the results of \( o \)- and \( m \)-cresols.

Figure 4.4.8 shows the results for the effect of exposed surface areas of the reactors on the removal of \( m \)-cresol substrate. Reactor 1 had an exposed surface area to the atmosphere of approximately 490 mm\(^2\), while that for Reactor 2 was about 710 mm\(^2\). Due to equipment constraints, the dissolved oxygen could not be measured in both of the reactors. However, substrate concentrations were measured. This experiment showed that exposed surface area had no effect on substrate removal for the two sizes of reactors used.

4.4.2 Aeration

Next, the effect of bubbling air into a reactor, as compared against an open reactor acting as a control, was studied. This experiment was conducted using \( m \)-cresol at an initial concentration of 2 mM. The reaction mixture was near the optimum pH of 7.0. Figure 4.4.9 shows that at a concentration of 2 mM, \( m \)-cresol was not removed more efficiently with aeration than with the control. From Figure 4.4.10, it can be seen that dissolved oxygen levels were maintained high during aeration, whereas dissolved oxygen in the control became depleted before increasing again.

However, significant effects of oxygen addition on substrate removal can be seen in Figure 4.4.11 when the concentration of \( m \)-cresol was increased to 5 mM. Here, it was demonstrated that the reaction in which oxygen was added through aeration, had the highest substrate removal. The higher concentration of substrate required a higher
Figure 4.4.8: Effect of Surface Area of the Reactor on the Diffusion of Oxygen From the Atmosphere
Reactor Conditions:
Substrate: 2 mM m-cresol
Enzyme dose: 2.9 LAMU/mL
pH = 7.0
Volume: 133 mL

Figure 4.4.9: Effect of Aeration on Substrate Removal
Reactors Conditions:
same reaction as Fig. 4.4.9
Substrate: 2 mM m-cresol
Enzyme dose: 2.9 LAMU/mL
pH = 7.0
Volume: 133 mL

Figure 4.4.10: Effect of Aeration on Dissolved Oxygen Availability
Figure 4.4.11: Effects of Diffusion and Aeration on Substrate Removal

Reactor Conditions:
Substrate: 5 mM m-cresol
Enzyme dose: 2.5 LAMU/mL
pH = 7.0
Volume: 133 mL
concentration of oxygen for complete reaction, based on the stoichiometry of the reaction. Information from the same experiment, Figure 4.4.12, shows the effects of diffusion and aeration on dissolved oxygen availability. The reaction open to the atmosphere removed substrate better than the closed reaction since the oxidant of the reaction was oxygen.

This experiment also showed that the substrate was not significantly volatile during the reaction time. Figure 4.4.11 shows that the substrate concentration remained constant throughout the experiment when air was bubbled into the reactor but no enzyme was added. From Figure 4.4.12, it can be seen that in a stirred reactor, without enzyme, the dissolved oxygen concentration had initially decreased slightly, possibly from mechanical mixing, and then plateaued at a constant concentration.

It is summarized that when dissolved oxygen was available in the reactor, the reaction took place at its maximum rate. This rate depended on the experimental conditions, such as type of substrate and substrate and enzyme concentrations, but was independent of dissolved oxygen concentration. However, after the dissolved oxygen was completely depleted, the rate of reaction depended on the rate at which oxygen was added into the reactor. Obviously, bubbling air through the reactor provided the highest rate of oxygen addition and maximum rate of reaction that could be achieved under this condition.

4.4.3 Hydrogen Peroxide Addition

Another method used to add oxygen into the solution was through the addition of hydrogen peroxide because the laccase has a peroxide decomposing activity (catalase) to produce oxygen. Results obtained showed that the addition of hydrogen peroxide raised
**Reactor Conditions:**
same reaction as Fig. 4.4.11  
Substrate: 5 mM m-cresol  
Enzyme dose: 2.5 LAMU/mL  
\( p\text{H} = 7.0 \)  
Volume: 133 mL

**Figure 4.4.12:** Effects of Diffusion and Aeration on Dissolved Oxygen Availability
the dissolved oxygen content of the solution. However, the presence of excess dissolved oxygen provided only minor improvements in substrate removal efficiency when compared to the control. This was tested for 1 mM and 2 mM concentrations of o-cresol.

Additional experiments were performed with an initial o-cresol concentration of 2 mM. At optimal pH conditions, hydrogen peroxide was added at different times in three separate reactors. Figures 4.4.13 through 4.4.15 show the cresol removal with the addition of hydrogen peroxide at t=0, t=24 minutes, and t=51 minutes, respectively.

The addition of H₂O₂ at t=0 caused an instantaneous increase in the dissolved oxygen level as is shown in Figure 4.4.16. The higher dissolved oxygen concentration took longer to be consumed in the variable than in the control. The corresponding substrate removal graph, Figure 4.4.13, shows that o-cresol had the same initial rate of reaction in the variable than in the control and after nearly 4.5 hours, both reactions plateaued to a similar final removal. Hydrogen peroxide addition after 24 minutes produced similar results as is shown in Figures 4.4.14 and 4.4.17.

The third reactor had the same addition of hydrogen peroxide after 51 minutes of reaction time. The dissolved oxygen level increased instantaneously as is shown in Figure 4.4.18; however, substrate removal did not improve. Thus, instantaneous increases in dissolved oxygen were observed when hydrogen peroxide was added at different times. Other than providing additional dissolved oxygen in the reaction, hydrogen peroxide addition had no influence on the rate of reaction.
Figure 4.4.13: Effect of Dissolved Oxygen from Hydrogen Peroxide Added at t=0 on Substrate Removal

**Reactor Conditions:**
- Substrate: 2 mM o-cresol
- Enzyme dose: 1.47 LAMU/mL
- pH = 7.0
- H2O2: 1 mM
- Volume: 133 mL
- Reactors: open
Figure 4.4.14: Effect of Dissolved Oxygen from Hydrogen Peroxide Added at t=24 min on Substrate Removal
Figure 4.4.15: Effect of Dissolved Oxygen from Hydrogen Peroxide Added at t=51 min on Substrate Removal
**Reactor Conditions:**
- same reaction as Fig. 4.4.13
- Substrate: 2 mM o-cresol
- Enzyme dose: 1.47 LAMU/mL
- pH = 7.0
- H2O2: 1 mM
- Volume: 133 mL
- Reactors: open

**Figure 4.4.16:** Effect of Hydrogen Peroxide Added at t=0 on Dissolved Oxygen Availability
**Reactor Conditions:**
same reaction as Fig. 4.4.14
Substrate: 2 mM o-cresol
Enzyme dose: 1.47 LAMU/mL
pH = 7.0
H2O2: 1 mM
Volume: 133 mL
Reactors: open

*Figure 4.4.17: Effect of Hydrogen Peroxide Added at t=24 min on Dissolved Oxygen Availability*
Figure 4.4.18: Effect of Hydrogen Peroxide Added at t=51 min on Dissolved Oxygen Availability

Reactor Conditions:
- same reaction as Fig. 4.4.15
- Substrate: 2 mM o-cresol
- Enzyme dose: 1.47 LAMU/mL
- pH = 7.0
- H2O2: 1 mM
- Volume: 133 mL
- Reactors: open
4.4.4 Hydrogen Peroxide and Catalase Addition

The effect of the addition of dissolved oxygen was also studied by adding hydrogen peroxide and catalase to the reaction mixture. This experiment was conducted using o-cresol at an initial concentration of 1 mM. The reaction mixture was near the optimum pH of 7.0. The concentration of hydrogen peroxide used was 1.0 mM and 1500 units of catalase were added. Catalase was added after 100 minutes of reaction time, then hydrogen peroxide was added within seconds later. Results are plotted for substrate removal and dissolved oxygen consumption in Figures 4.4.19 and 4.4.20, respectively. These figures show that the addition of catalase had no effect on the reaction rate and on the quantity of dissolved oxygen added in the solution. Once the hydrogen peroxide was added, the amount of dissolved oxygen had increased instantaneously, as was observed without the addition of catalase.

4.5 Laccase Stability

The stability of the activity of the laccase enzyme at room temperature over time was observed. Figure 4.5.1 shows that enzyme activity was stable at room temperature for the duration of the experiments performed in this research. The longest experimental time was approximately 6 hours. The enzyme was shown to be stable for 6.5 hours.

4.6 Reaction Kinetics

Preliminary calculations were performed for the kinetics of the reaction between cresol substrate and dissolved oxygen. A reaction was carried out in an open batch reactor with an initial substrate concentration of 2 mM of m-cresol at optimal pH.
Figure 4.4.19: Effect of Hydrogen Peroxide and Catalase on the Reduction of Substrate
**Reactor Conditions:**
same reaction as Fig. 4.4.19
Substrate: 1 mM o-cresol
Enzyme dose: 3.0 LAMU/mL
Catalase: 1500 U
H2O2: 1.0 mM
pH = 7.2
Reactor: 1: H2O2
2: H2O2 + catalase
3: control
Volume: 133 mL

**Figure 4.4.20:** Effect of Hydrogen Peroxide and Catalase on Dissolved Oxygen Reduction
Figure 4.5.1: Stability of Laccase Enzyme Activity at Room Temperature, Over Time
Figures 4.6.1 and 4.6.2 show the decrease in substrate and dissolved oxygen concentrations, respectively. The initial velocity ($v_0$) of a reaction is the initial slope of its data line. Trendlines were drawn to obtain the slope of each line. From the results, the initial velocity for substrate removal was 0.0071 mM cresol/min and for dissolved oxygen consumption, it was 0.0026 mM oxygen/min. This resulted in 2.73 mM cresol/mM dissolved oxygen. The stoichiometric requirement of dissolved oxygen for cresols is 1:4. Further experimentation is necessary to obtain reliability.

4.7 Error Analysis

Experiments were conducted to determine the reliability of the results in this study. To test the reliability of the laboratory techniques used, the removal of o-cresol at optimum pH was repeated five times and the standard deviation was calculated. The batch reactors were formulated separately as follows: pH = 7; initial o-cresol concentration = 1.0 mM; Laccase enzyme dose = 0.68 LAMU/mL of reactor volume. The reaction proceeded for three hours and analysis was done using the colour test. The results of the experiment are listed in Table 4.1.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Percent Remaining</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.9</td>
<td>18.8</td>
<td>+/- 0.51</td>
</tr>
<tr>
<td>2</td>
<td>19.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>18.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>19.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Error Analysis of Laboratory Procedures
Reactor Conditions:
Substrate: 2 mM m-cresol
Enzyme: 3.39 LAMU/mL
pH = 7.0
Volume: 133 mL
Reactor: open

Figure 4.6.1: Determining Initial Velocity of Substrate Removal
Figure 4.6.2: Determining Initial Velocity of Dissolved Oxygen Consumption

**Reactor Conditions:**
- same reaction as Fig. 4.6.1
- Substrate: 2 mM m-cresol
- Enzyme: 3.39 LAMU/mL
- pH = 7.0
- Volume: 133 mL
- Reactor: open

\[ y = -6E-07x^2 - 0.0026x + 0.2831 \]

\[ R^2 = 0.9937 \]
These results indicate that the standard deviation was \( \pm 0.51 \), which is within acceptable range. This experiment was also used to determine the reliability of the colour test and the enzyme activity test. To examine the colour test, five samples were withdrawn from a single reactor, after one hour of reaction to test the analysis of high concentrations of substrate, resulting in high absorbance readings. After a reaction time of three hours, five more samples were withdrawn and analysed to test the reliability of analysis of low substrate concentrations, or low absorbance readings. The results are given in Table 4.2. Likewise, for the enzyme activity test, five samples were withdrawn from a single reactor and analysed. These results are listed in Table 4.3.

### Table 4.2: Error Analysis of Colour Test

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Reaction Time (h)</th>
<th>Percent Remaining</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>68.0</td>
<td>68.2</td>
<td>+/- 0.15</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>68.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>68.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>68.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>19.2</td>
<td>18.9</td>
<td>+/- 0.33</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>18.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>18.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>19.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>18.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3: Error Analysis for Enzyme Activity Test

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Enzyme Activity (LAMU/mL)</th>
<th>Mean (LAMU/mL)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.68</td>
<td>0.68</td>
<td>+/- 0.005</td>
</tr>
<tr>
<td>2</td>
<td>0.67</td>
<td>0.68</td>
<td>+/- 0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.69</td>
<td>0.68</td>
<td>+/- 0.005</td>
</tr>
<tr>
<td>4</td>
<td>0.68</td>
<td>0.68</td>
<td>+/- 0.005</td>
</tr>
<tr>
<td>5</td>
<td>0.68</td>
<td>0.68</td>
<td>+/- 0.005</td>
</tr>
</tbody>
</table>

The results for the colour analysis show a low deviation of ± 0.15 at high absorbance readings and a deviation of ± 0.33 at low absorbance readings, which is within acceptable range for reliability. Similarly, enzyme activity results had a low standard deviation of ± 0.005.

An experiment was conducted to determine the reliability of the dissolved oxygen probe. A 1 mM solution of o-cresol at pH 7.0 was prepared in a 133 mL flask. Before enzyme was added, five readings of the dissolved oxygen concentration were taken and the standard deviation was calculated, as shown in Table 4.4.

Table 4.4: Error Analysis of Dissolved Oxygen Probe

<table>
<thead>
<tr>
<th>Sample #</th>
<th>DO Concentration (mg/L)</th>
<th>Mean (mg/L)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>7.01</td>
<td>+/- 0.02</td>
</tr>
<tr>
<td>2</td>
<td>7.1</td>
<td>7.01</td>
<td>+/- 0.02</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>7.01</td>
<td>+/- 0.02</td>
</tr>
<tr>
<td>4</td>
<td>7.0</td>
<td>7.01</td>
<td>+/- 0.02</td>
</tr>
<tr>
<td>5</td>
<td>7.0</td>
<td>7.01</td>
<td>+/- 0.02</td>
</tr>
</tbody>
</table>
The standard deviation in these results was \( \pm 0.02 \), which is within an acceptable range.

In summary, the error analyses performed show that all deviations were within an acceptable range. Therefore, the results obtained in this study are considered to be reliable.
5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The results of this study demonstrate the applicability of using laccase to treat wastewater contaminated with cresols. The reaction parameters optimized to achieve at least 90% removal of cresols were pH and enzyme dose. The effects of aeration, addition of PEG, hydrogen peroxide addition, and the presence of catalase were also investigated. The stability of laccase activity at room temperature over time was determined.

- The optimum pH for o- and m-cresols was determined to be 7.0 while it was 5.6 for p-cresol.
- In a fixed time, excess laccase did not show better removal of cresols; however, lower substrate removal efficiencies resulted when laccase was limited. Maximum substrate removal was achieved by adding the full enzyme dose at the start of the reaction rather than in aliquots.
- Reaction time was an important parameter. The substrate removal efficiency increased and the enzyme dose required to obtain the same substrate removal decreased with an increase in reaction time. Reaction times from 3 to 6 hours were chosen for convenience.
- The effect of aeration on substrate removal was studied by using m-cresol. At lower substrate concentrations, sufficient dissolved oxygen concentration was always available and aeration did not improve substrate removal. However, at higher cresol
concentrations, aeration greatly increased the efficiency of substrate removal by providing the required dissolved oxygen.

- Likewise, the addition of hydrogen peroxide with or without catalase, to provide additional dissolved oxygen, had minimal effect.
- Addition of PEG had no effect on the removal efficiencies of the substrates.
- Laccase activity was stable for the duration of all experiments conducted.

### 5.2 Recommendations

- The results of this study have shown that laccase has the potential to significantly reduce cresol concentrations. Since the enzyme laccase has not been studied as much in depth as HRP, ARP, and SBP, further research must be done to verify the results presented in this study and to continue optimizing the reaction parameters.
- In this study, only \( m \)-cresol was tested for the effects of aeration. Additional experiments should be conducted to verify if \( o \)- and \( p \)-cresols yield similar results. Also, relationships between the reduction in substrate concentration and the oxygen demand for each substrate should be determined. Further studies should be done to optimize the addition of hydrogen peroxide, for example, a continuous addition.
- The effects of temperature and reaction time on the stability of the laccase enzyme should be investigated further.
- In order to implement this enzymatic method of treatment to full-scale industrial applications, several other aspects must be considered. Even though concentrations of toxic cresol compounds are reduced in this process, final products of the reaction
need to be identified as they may be potentially toxic, possibly even more than cresols.

- The coprecipitation of two or more cresols together should be investigated. Mixtures of these substrates could be present in industrial wastewaters and may require different parameters for treatment than their individual substrates. Due to coprecipitation, treating mixtures of these compounds may produce better results in removal efficiencies.

- A real industrial wastewater matrix should be treated with the enzyme to determine laccase’s effectiveness in a real situation. Other components that are present in a real wastewater stream may improve or interfere with the removal of the cresol compounds.

- Finally, a cost analysis of the process should be performed to determine the economic effectiveness of using this enzyme treatment method over current treatment processes as well as alternative treatment methods involving other enzymes.
REFERENCES


APPENDIX A

Enzyme Activity Assay
1. General

The purpose of the enzyme activity assay is to determine the amount of active enzyme that is contained in a solution. Under saturating conditions of syringaldazine, the initial rate is measured by observing the rate of colour formation in a solution. Laccase catalyzes the oxidation of syringaldazine to the corresponding quinone such that the products of the reaction form a purple-coloured solution that absorbs light at a peak wavelength of 530 nm.

2. Reagents

i) Tris Maleate buffer (23 mM, pH 7.5 +/- 0.05)
   - 25 mL of 1.0 M Tris solution
   - 5 mL of 1.0 M maleic acid
   - Distilled water to 1 L

ii) Syringaldazine solution (0.38 mM)
   - 6.8 mg syringaldazine in flask
   - 25 mL of 96% ethanol dissolved for 1.5 hours
   - Distilled water to 50 mL
   - Store in dark

3. Procedure

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

- 850 μL Tris Maleate buffer
- 50 μL Syringaldazine solution
- 100 μL Laccase solution

The sample volume must be 1 mL and the rate of colour formation must be measured before substrate depletion becomes significant. Immediately after the addition of the sample, shake the cuvette and then place it in the spectrophotometer to monitor the
absorbance change with time at 530 nm. The change in absorbance should be measured at 15 s and 75 s.

4. Calculations

i) Calculate the activity in the cuvette:

\[
\text{Activity in the cuvette (LAMU/mL)} = \frac{\Delta A \times 1.0 \text{ mL} \times 10^{-3} \times D}{0.065 \times 0.1 \text{ mL}}
\]

\[
= \Delta A \times 0.1538 \times D
\]

where, \( \Delta A \) : Change in absorbance per minute: \( \Delta A = (A_{755} - A_{155}) \)
The absorbance range should be 0.1 – 0.4 \( \Delta A/\text{min} \).
1.0 : Total volume in cuvette (mL)
0.1 : Sample volume into cuvette from reactor (mL)
0.065 : Micromolar extinction coefficient (\( \mu \text{M/L} \))
10\(^{-3} \) : LAMU/L converted to LAMU/mL
\( D \) : Dilution factor

The activity is in terms of micromoies of syringaldazine converted per minute at 20 °C and pH 7.5.

ii) Calculate the activity of the sample:

\[
\text{Activity in the sample (LAMU/mL)} = \text{Activity in the cuvette (LAMU/mL)} \times \text{enzyme solution added to reactor (mL)} / \text{reactor volume (mL)}
\]
APPENDIX B

Aromatic Substrate Assay
1. **General**

This is a colorimetric assay used to measure the concentration of an aromatic substrate in an aqueous sample. The assay uses ferricyanide and 4-aminoantipyrine as colour-generating substrates when combined with the aromatic sample. The limiting reagent is the amount of aromatic compound in the sample. Therefore, the absorbance of the colour developed at a peak wavelength of 510 nm is proportional to the aromatic concentration present in the sample.

2. **Reagents**

i) **Ferricyanide reagent (83.4 mM of K$_3$Fe(CN)$_6$ in 0.25 M NaHCO$_3$)**
- 2.75 g K$_3$Fe(CN)$_6$
- 2.1 g NaHCO$_3$
- Distilled water to 100 mL

ii) **4-Aminoantipyrine reagent (20.8 mM of AAP in 0.25 M NaHCO$_3$)**
- 0.423 g AAP
- 2.1 g NaHCO$_3$
- Distilled water to 100 mL

3. **Procedure**

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

- 800 µL of aromatic sample (diluted if necessary)
- 100 µL of AAP reagent
- 100 µL of ferricyanide reagent

The final assay sample volume should be 1 mL. After approximately 12 minutes, measure the absorbance at 510 nm against a reagent blank.
4. **Calculations**

Using the appropriate calibration curve from Appendix C, covert the absorbance readings into desired concentration units.
APPENDIX C

Standard Curves for Aromatic Compounds
1. **General**

The following section consists of the standard curves for the aromatic compounds studied, based on a **Beer's Law** fit.

According to **Beer's law**:

\[ A = \varepsilon C L \]

where:

- **A** : Absorbance
- **\varepsilon** : Molar extinction coefficient (M⁻¹ cm⁻¹)
- **C** : Concentration (M)
- **L** : Length of the path of light travelled (cm)

(The semi-micro cuvettes used in this research had a path length of 1 cm.)
$o$-Cresol:

Sample Name: $o$-cresol  
Solvent Name: dist water  
Conc Units: $\mu$M

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

**Bohr's Law Fit**

Concentration = $7.716 \times 10^1 \times$ Absorbance
**m-Cresol:**

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

Analytical Wavelength: 610 nm
Reference Wavelength: None Selected
Confirmation Wavelengths: None Selected
Integration Time: 1 seconds

![Bohr's Law Fit Graph](image)

Concentration = 9.492 E+01 * Absorbance
**p-Cresol:**

Sample Name: p-cresol  
Solvent Name: 0.2MNaOH  
Conc Units: µM

Analytical Wavelength: 285 nm  
Reference Wavelength: None Selected  
Confirmation Wavelength: None Selected  
Integration Time: 1 seconds

---

**Bors' Law Fit**

Concentration = 6.468 E+02 * Absorbance
VITA AUCTORIS

Name: Amy B. Vermette

Place of birth: Leamington, Ontario, Canada

Date of birth: November 8, 1974

Education:

Degree of Master of Applied Science
Environmental Engineering
University of Windsor
Windsor, Ontario, Canada
1998 – 2000

Degree of Bachelor of Engineering Science
Biochemical Engineering
University of Western, Ontario
London, Ontario, Canada
1995 – 1998

Bachelor of Engineering Science
Environmental Engineering
University of Windsor
Windsor, Ontario, Canada
1993 – 1995

Work/Experience:

Research Assistant
Department of Civil and Environmental Engineering
University of Windsor
Windsor, Ontario, Canada
1998 – 2000

Waste Reduction Coordinator
General Motors of Canada Ltd., Windsor Transmission Plant
Windsor, Ontario, Canada
May to August, 1996

Awards: Tuition Scholarship 1998, 1999